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Preliminary DNA fingerprinting of the turf grass *Cynodon dactylon* (Poaceae: Chloridoideae)

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ABSTRACT

Identification of different cultivars of turf grasses is often very difficult. In a preliminary attempt to identify different cultivars of *Cynodon dactylon* (L.) Pers., random amplified polymorphic DNA (RAPD) analyses of some well-known cultivars used in South Africa, i.e. Bayview, Cape Royal, Florida, Harrismith, Silverton Blue, Skaapplaas and Tifdwarf, as well as 10 potential new cultivars, were done. These results were used to determine the genetic distances among cultivars. Only five primers were needed to obtain a specific fragment pattern for each cultivar. The degree of amplification was used as an additional criterion by including all visible fragments, excluding very faint fragments and only including the brightest fragments. The neighbour-joining trees of *C. dactylon* showed best resolution from the data set with all visible fragments included, although fragment intensity did not affect the tree topology. The cultivars Silverton Blue and Bayview exhibited the greatest genetic variation and two potential new cultivars were identified. RAPD analyses can, therefore, be used to distinguish between different *C. dactylon* cultivars and to determine the genetic variation between them by calculating genetic distances.

INTRODUCTION

The genus *Cynodon* Rich. comprises six species indigenous to South Africa: *C. bradleyi* Stent, *C. dactylon* (L.) Pers., *C. hirsutus* Stent, *C. incompletus* Nees, *C. polevansii* Stent and *C. transvaalensis* Burtt Davy, as well as two naturalized species, *C. aethiopicus* Clayton & Harlan and *C. nlemfuensis* Vanderyst (Gibbs Russell *et al.* 1990). These species are morphologically very similar. *Cynodon bradleyi*, *C. dactylon* and *C. transvaalensis* are cultivated as turf grasses. Often potential new cultivars are introduced, but are these really new cultivars or are some just variable morphological forms of existing cultivars?

In an attempt to find an easy, inexpensive and efficient method to distinguish between the different cultivars, and potential new cultivars, random amplified polymorphic DNA (RAPD) fingerprinting was used. Welsh & McClelland (1990), as well as Williams *et al.* (1990) first described this method. The technique has proven to be a powerful tool for investigating genetic variation in various plant groups (Williams *et al.* 1990; Carlson *et al.* 1991; Klein-Lankhorst *et al.* 1991; Michelmore *et al.* 1991; Welsh *et al.* 1991; Vierling & Nguyen 1992; Harada *et al.* 1993; Huff *et al.* 1993; Howell *et al.* 1994; Van Buren *et al.* 1994; Brummer *et al.* 1995; Hilu 1995; Multani & Lyon 1995; Wachira *et al.* 1995; Marillia & Scoles 1996; Bai *et al.* 1997; Parani *et al.* 1997; Swoboda & Bhalla 1997; Barker *et al.* 1999; Sun *et al.* 1999; Baranek *et al.* 2000; Gwanama *et al.* 2000; Lanteri *et al.* 2001).

RAPD analysis has also been widely applied to turfgrass and related grass profiling at molecular level. These include studies of perennial ryegrass (Sweeney & Danneberger 1994, 1997; Huff 1997); *Agrostis stolonifera* L. (Golembiewski *et al.* 1997); *Agrostis* spp. (Ohmura *et al.* 1997); *Poa pratensis* L. (Huff & Bara 1993; Barcaccia *et al.* 1997); *P. annua* L. (Sweeney & Danneberger 1995, 1996); as well as *Cynodon* (Busey *et al.* 1996).

Amplification conditions for RAPD analysis are similar to those used in a normal polymerase chain reaction, except that only one primer is used instead of two primers with specific sequences (Williams *et al.* 1990). As a result, amplification in RAPD analysis occurs everywhere in a genome, where it contains two complementary sequences to the primer that are within the length-limits of the polymerase chain reaction (PCR), which is ± 3 kb. The PCR patterns obtained from RAPDs are dependent on both the template and the specific PCR primer. Yu *et al.* (1993) observed the fragment size range to be from 0.5 to 2.5 kb and the fragment numbers from 1–10.

Polymorphisms detected by the RAPD technique are inherited as dominant markers in a Mendelian fashion and can be generated in any species without prior DNA sequence information (Williams *et al.* 1990; Welsh *et al.* 1991). Marsan *et al.* (1993) showed that DNA fragments, from inbred maize lines, were always present in one or both of the respective parental lines, thus suggesting that RAPD fragments were stably transmitted from generation to generation.

A general characteristic of the RAPD profile is the difference in fragment intensities. These differences in fragment intensities were therefore, also used as criteria in determining genetic variation within and between known cultivars and unknown specimens.

The aim of this preliminary study is to use DNA profiles generated by the RAPD method to identify various known *Cynodon* cultivars from vegetative material, and to identify potential new cultivars, by comparing them with some well-known cultivars currently used in the industry. A further purpose is to use the RAPD data to calculate the genetic distances between the different cul-

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tivars, thereby investigating the variation within and between the studied specimens.

MATERIALS AND METHODS

Plant material

Material from some of the most important commercially available cultivars were received from Top Crop Nursery and planted in a greenhouse at the Department of Plant Sciences: Genetics at the University of the Free State, under controlled environmental conditions. The plants were cultivated from vegetative material. Voucher herbarium specimens (Table 1) are housed in the Geo Potts Herbarium, University of the Free State, Bloemfontein (BLFU). The cultivars used were Bayview, Cape Royal, Florida, Harrismith, Silverton Blue, Skaapplaas and Tifdwarf, as well as 10 potential new cultivars (SAG.01–06 & 09–13).

TABLE 1.—Cultivar names and voucher numbers of *Cynodon dactylon* specimens

Cultivar*/code	Voucher no.
Cape Royal	*Spies 5821, 5822*
Tifdwarf	*Spies 5823, 5824*
Florida	*Spies 5825, 5826*
Bayview	*Spies 5827, 5828*
Silverton Blue	*Spies 5829, 5830*
Harrismith	*Spies 5831*
Skaapplaas	*Spies 5832*
SAG.01	*Spies 5833*
SAG.02	*Spies 5834*
SAG.03	*Spies 5835*
SAG.04	*Spies 5836*
SAG.05	*Spies 5837*
SAG.06	*Spies 5838*
SAG.09	*Spies 5840*
SAG.10	*Spies 5841*
SAG.11	*Spies 5842*
SAG.13	*Spies 5843*

*Duplicate samples of the first five cultivars were received, representing two different geographical areas. This was done to determine the variability within cultivars.

DNA extraction and RAPD amplification

DNA was extracted from ground leaves according to the method described by Edwards *et al.* (1991). The polymerase chain reaction (PCR) was carried out in a total volume of 25 µl, containing ± 25 ng of genomic DNA, 5–12pmol primer, 5 µl 5X Buffer [500 µl 10X Taq Polymerase buffer (500 mM potassium chloride, 100 mM Tris-HCl {pH 9.0}, 1% triton X-100), 1 mg gelatine, 2.25 µl triton X-100, 100 µl of each 100 mM deoxynucleotidephosphate, 457 µl sterilized water], 1.5 mM magnesium chloride and 1.25U of Taq polymerase. Five primers were used that showed clear reproducible banding patterns, i.e. OPA11 (5'-CAATCGCCGT-3'), OPA16 (5'-AGCCAGCGAA-3'), OPA20 (5'-GTTGCGATCC-3'), OPB03 (5'-CATCCCCCTG-3') and OPB06 (5'-TGCTCTGCCC-3') (Operon Technologies, Alameda, California). These primers were chosen because they provided excellent resolution with a large range of unrelated grasses in our laboratory (results not shown). Amplification cycles were as follows: initial denaturation at 94°C for 2 minutes, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 34°C and 90 seconds at 72°C with a final elongation step of 5 minutes at 72°C.

The reproducibility of the technique was tested by duplicating each reaction (44 reactions for 22 specimens per primer). This was done by performing amplifications on identical DNA samples in two different reactions. These findings confirmed that the fragment pattern for a particular combination of primer and DNA was reproducible for replicates, both in and between experiments.

Between 5–10 µl of the amplification product was mixed with gel loading buffer and separated on a 1% (m/v) agarose gel in TBE containing ethidium bromide (0.4 mg/ml). The gel was run in 0.3X TBE (1X TBE = 0.089 M Tris-HCl, 0.089 M boric acid, 0.002 M EDTA) or 0.5X TAE (1X TAE = 0.04 M Tris-HCl, 1.142 ml acetic acid, 0.001 M EDTA) buffer at 120 V for ± 60 minutes. DNA lambda molecular weight markers VI or X were included in each gel. The fragments were viewed under UV light and documented with a 35 mm photograph.

TABLE 2.—Comparison between five primers used with respect to no. of fragments observed, repeatability, fragment intensity and range of fragments

Primers	Fragments							
	Total no. scored	% without replication	% Faint	% Medium	% Bright	Min. no. per plant	Max. no. per plant	± Range of sizes (bp)
OPA-11	282	1.46	40.07	26.60	33.33	2	14	394–2300
OPA-16	374	2.82	49.73	25.67	24.60	4	15	394–2300
OPA-20	314	1.91	41.40	38.85	19.75	1	13	394–2300
OPB-03	302	1.33	40.07	28.80	31.13	3	10	350–2300
OPB-06	302	2.65	56.30	21.85	21.85	3	13	394–2176
Total/	1574	-	-	-	-	-	-	-
Average	-	2.03	45.52	28.35	26.13	2.6	13	-

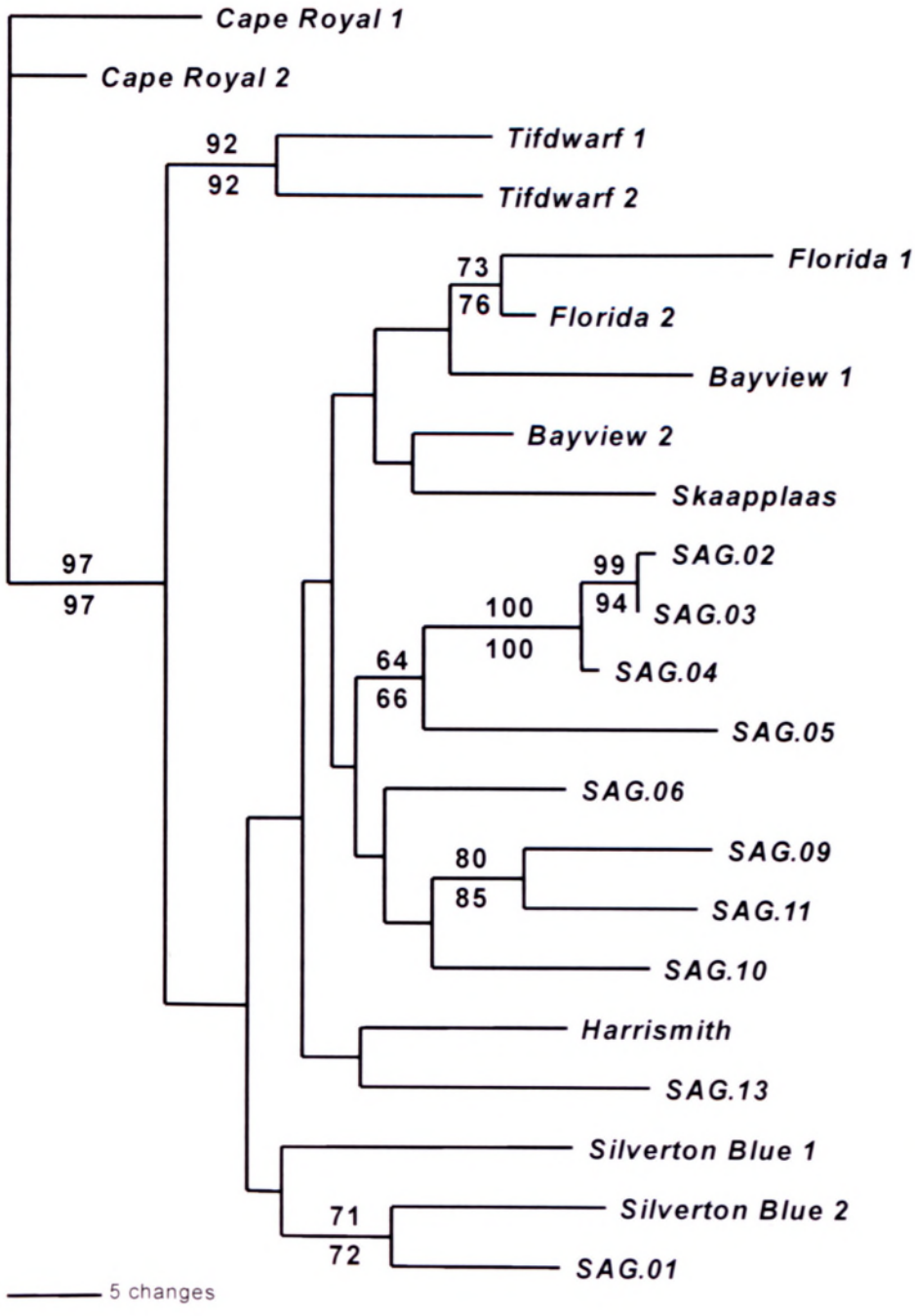

FIGURE 1.—Neighbour-joining tree constructed from the data matrix that included all visible RAPD fragments for *Cynodon*. Bootstrap values are indicated on branches and jackknife values below branches.

Fragment and phylogenetic analysis

The fragments were manually scored for each primer as present (1) or absent (0), for all the cultivars studied. Furthermore, the fragments were divided into three categories according to intensity of the fragments: bright, medium and faint fragments. The data were classified in three different sets, namely (a) including all visible fragments, (b) excluding faint fragments, and (c) using only bright fragments (Table 2).

The different fragment intensities observed with the amplification products were also scored, by comparing the fragments within a specific specimen, for each primer. This was done due to different specimens amplifying at different intensities.

The data were analysed with PAUP* (phylogenetic analysis using parsimony *and other methods) 4.0b8a (Swofford 1998). Cluster analysis was performed by using the neighbour-joining method (NJ) as implemented in this software and neighbour-joining trees were constructed, using total character difference as distance measure. Cape Royal was used as the outgroup in this study, being the morphologically distinct cultivar. Bootstrap values were calculated from 500 replicates (Felsenstein 1985) with resampling of all 96 characters. Jackknife values were calculated from 500 replicates, with 50% deletion and the emulate Jac resampling option in effect (Lanyon 1985).

RESULTS AND DISCUSSION

The 22 specimens of *C. dactylon* came from seven known cultivars, duplicate specimens (collected from different localities) of five of these cultivars and ten

potential new cultivars (Table 1). Table 2 contains information on the total number of fragments scored, the percentage of fragments that showed no replication, the percentage of faint, medium and bright fragments, the minimum and maximum number of fragments per specimen, and the range of fragment sizes.

For primer OPA11, Cape Royal 2 specimen and for primer OPA16, Cape Royal 1 and Silverton Blue 1, the duplicated reactions failed, as a result of total PCR failure (and thus not failure of repeatability). This information was not used in the calculations of the percentage of fragments that showed no repetition (Table 2).

The percentage of fragments, which showed no repetition in the duplicates of a reaction, varied from 1.33% in OPB03 to 2.82% in OPA16, with an average of 2.03% (Table 2). This indicates that fragment reproducibility was high with all the primers used, OPB03 being the most reproducible. Most of the fragments that showed no repetition were of faint intensity.

A series of tests, done on different DNA extractions from the same plant and different amplification of the same DNA sample, indicated that RAPD results are reliable. Well-amplified regions corresponded in all repeats from the same sample. The only differences observed were in faint fragments found in certain repeats.

A general characteristic of the RAPD profile is the difference in fragment intensities. Many speculations for the reason of this phenomenon have been given. One explanation is that the difference may be linked to the degree of homology between primer and template DNA (Thormann *et al.* 1994). Caetano-Anollés *et al.* (1991) speculated that it might be the result of amplification of multiple copies in the genome.

All five primers exhibited differences in the duplicate specimens of the cultivars Cape Royal, Tifdwarf, Florida, Bayview and Silverton Blue, which ranged from faint to bright fragment differences. These results indicate varying degrees of variability within these cultivars, especially Silverton Blue and Bayview. One fragment was consistent throughout all the specimens for two primers, namely a ± 700 bp fragment with primer OPA-16 and a ± 570 bp fragment with primer OPB-06. All the primers exhibited a few other prominent fragments in most of the specimens. Very few unique cultivar-specific fragments were found, which could be linked to the small sample size.

For the neighbour-joining analysis, the three different data sets for *Cynodon* (according to fragment intensity) were used separately. Though their intensities differ, the three data sets gave neighbour-joining trees with the same topology. The resolution decreased with fewer parameters (number of fragments), therefore, the neighbour-joining tree using all visible fragments was the best resolved and will be discussed further (Figure 1): SAG.01 groups with the Silverton Blue clade, with relatively high bootstrap and jackknife support. It is probably not a new cultivar, but a morphological variant of this cultivar. SAG.13 and Harrismith seem to follow the same pattern. There is, however, no substantial support for this grouping. Of the other potential new cultivars, SAG. 02–SAG.05 form a monophyletic clade and SAG.06 + SAG.09–SAG.11 form another monophyletic cluster. When comparing the fingerprinting patterns for the different specimens, the close affinities between specimens SAG.02, SAG.03, SAG.04 and SAG.05 (Figure 1; Table 2) were also evident. The groupings SAG.02–SAG.04 and SAG.09 + SAG.11 probably represent two potential new cultivars, with the variation within the clades being so small as to indicate that the specimens in each cluster are probably the same cultivar. These close affinities are corroborated by the bootstrap and jackknife support values within these groups, which are 100% and 80–85% respectively. The other members of these clades, SAG.05 and SAG.10 + SAG.06, are probably closely related variant forms. The distances within some of the existing cultivars are very large, which indicate large levels of genetic variation within these cultivars. This is especially true for the two cultivars Bayview and Silverton Blue. This is corroborated by the variability in fragment patterning observed in the different specimens for these cultivars. These results, in which these two cultivars are clearly non-monophyletic, might indicate that either the taxonomy of these cultivars are confused or the samples are in fact not purebred cultivars any more. The last possibility is very feasible in this group of grasses that constitute a heterogeneous group of varieties with considerable genotypic as well as phenotypic variation and in which outcrossing is frequent. The other duplicated cultivars form distinct groupings with high support bootstrap values for Cape Royal and Tifdwarf (97% and 92% respectively). Florida also exhibits some variability but not to the same extent as Silverton Blue and Bayview.

The number of specimens studied per cultivar was only two, due to the preliminary nature of the study. By increasing this number, the variability within cultivars can be investigated in more detail.

Very small genetic differences can be detected with RAPDs. In some cases these differences may include only a single DNA change. A single difference in the fragmenting patterns of different specimens does, therefore, not indicate separate cultivar status. It was, however, possible to distinguish between the different *Cynodon* cultivars with the RAPD fingerprinting patterns. More primers included and more samples per cultivar, would help to further resolve relationships, especially where the status of a cultivar is uncertain.

Although the reproducibility of this RAPD technique can be influenced by factors that may vary, such as template quantity and primer structure (Kernodle *et al.* 1993; Multani & Lyon 1995), the use of a standardized RAPD protocol and sufficient replication can ensure reproducible RAPD patterns (Multani & Lyon 1995). Furthermore, all reactions were always amplified simultaneously, and found to be repeatable across different amplification times.

These markers have the potential to be employed as genetic fingerprints for future identification.

CONCLUSIONS

This study indicated that different *Cynodon* cultivars differ genetically, and these variations can be determined by RAPDs.

The only two specimens with a similar fragmenting pattern, irrespective of the primer used, were SAG.03 and SAG.04. However, these specimens show similar patterns to SAG.02 and SAG.05 with most primers. This indicates that these four specimens are genetically very similar and could well be the same cultivar. This was reflected by the neighbour-joining analysis where SAG.02–SAG.05 and SAG.06 + SAG.09–SAG.11 form definite monophyletic groups with the clusters SAG.02–SAG.04 and SAG.09 + SAG.11, which appear to be new cultivars. This is supported by bootstrap and jackknife values and very little variance within these clusters. SAG.13 appears to be related to the Harrismith cultivar and SAG.01 to the Silverton Blue cultivar.

Furthermore, the variability within existing cultivars was very high in some instances, questioning their status as true cultivars. Due to the variable nature of the species it is very difficult to recognize the different cultivars of these turf grasses vegetatively, especially when they are frequently cut on lawns, bowling greens or golfing greens. This complicates the unequivocal identification of these cultivars.

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