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Molecular characterization of Buffalograss germplasm using sequence-related amplified polymorphism markers

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Abstract Buffalograss [*Buchloe dactyloides* (Nutt.) Englem] germplasm has a broad resource of genetic diversity that can be used for turfgrass, forage and conservation. Buffalograss is the only native grass that is presently used as a turfgrass in the Great Plains region of North America. Its low growth habit, drought tolerance and reduced requirement for fertilizer and pesticides contribute to interest in its use. The objectives of this study were to use sequence-related amplified polymorphism (SRAP) markers in the evaluation of genetic diversity and phenetic relationships in a diverse collection of 53 buffalograss germplasms, and to identify buffalograss ploidy levels using flow cytometry. Based on their DNA contents, buffalograss genotypes were grouped into four sets, corresponding to their ploidy levels. Thirty-four SRAP primer combinations were used. This is the first report of the detection of differentiating diploid, tetraploid, pentaploid and hexaploid buffalograss genotypes, representing diverse locations of origin, using SRAP markers. Cluster analysis by the unweighted pair-group method with arithmetic averages based on genetic similarity matrices indicated that there were eight clusters. The coefficients of genetic distance among the genotypes ranged from 0.33 up to 0.99 and averaged D=0.66. The genetic diversity estimate, *He*, averaged 0.35. These results demonstrated that genotypes with potential traits for turfgrass improvement could readily be distinguished, based on SRAP. The use of PCR-based technologies such as SRAP is an effective tool for estimating genetic diversity, identifying unique genotypes as new sources of alleles for enhancing turf characteristics, and for analyzing the evolutionary and historical development of cultivars at the genomic level in a buffalograss breeding program.

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Introduction

Buffalograss [*Buchloe dactyloides* (Nutt.) Englem.] is of interest for its potential drought and heat tolerance, low growth habit, and low maintenance requirements. It is native to the Great Plains of North America, ranging from Mexico to Canada (Beetle 1950; Reeder 1971). Development of turf-type buffalograss cultivars to partially replace currently used species may reduce the amount of water used for turf maintenance in the United States (Riordan et al. 1993). Buffalograss is also used in other regions of North America as a forage and conservation species.

Buffalograss is an open pollinated dioecious turfgrass species and is highly heterogeneous. It has a series of ploidy levels. The base chromosome number is x = 10 with diploid ($2n$=$2x$=20), tetraploid ($2n$=$4x$=40), pentaploid ($2n$=$5x$=50) and hexaploid ($2n$=$6x$=60) genotypes (Johnson et al. 1998). However, the genome relationship with these ploidy levels is unknown. The linear correlation between the DNA contents of ploidy levels and their chromosome numbers indicates the ploidy series in buffalograss is probably due to autoploidy and multiplication of one genome. While diploid and tetraploid genotypes are mainly adapted to the southern portions of the Great Plains, hexaploid genotypes are found throughout the Great Plains region (Johnson et al. 1998). Previous research (Huff et al. 1993) has indicated diploid, tetraploid, and hexaploid types of buffalograss in the North American Great Plains, but in many cases they were indistinguishable, especially between tetraploids and hexaploids. This has led to the mislabeling of some cultivars.

In evolutionary biology and plant breeding programs, one of the major objectives is the molecular characterization of genetic variation within and among genotypes. Current knowledge of the genetic basis of buffalograss

agronomic characteristics and molecular markers that are applicable to its genetic improvement are limited. Molecular marker studies have been used to measure genetic relationships among and within diploid buffalograss genotypes, using random amplified polymorphic DNA (RAPD) technology (Huff et al. 1993), and allozymes (Peakall et al. 1995). Vegetatively propagated buffalograss lines were also differentiated using RAPD markers (Wu and Lin 1994). However, PCR-based marker development, identification and use in buffalograss lag behind other major crop plants. Identification and use of PCR-based molecular markers linked to agronomically important genes in buffalograss germplasm would enhance the effectiveness of the breeding program. Such markers can be used in marker assisted selection (MAS) and genetic resource conservation. The identification of genotypes with good combining ability for hybrid development, the enhancement of overall genetic diversity used in the breeder's selection program, and the development of disease and insect-resistant genotypes would be greatly enhanced by genotype fingerprinting with PCR-based molecular markers.

Each PCR-based marker technique has its own advantages and disadvantages. For instance, RAPD provides a simple PCR-based molecular tool for the evaluation of genetic variation, but its poor consistency and low reproducibility limit its use (Welsh and McClelland 1990; Roodt et al. 2002). Amplified fragment length polymorphism (AFLP) technology is now widely used for genomic fingerprinting (Zhang et al. 1999; Karaca et al. 2002) due to its high multiplexing ratio (Vos et al. 1995). However, AFLP is complex, requires multiple steps, and has pseudo-polymorphism when methylation-sensitive restriction enzymes are used (Li and Quiros 2001).

Sequence-related amplified polymorphism (SRAP) technology has been recognized as a new and useful molecular marker system for mapping and gene tagging in *Brassica* (Li and Quiros 2001) and *Cucurbita* (Ferriol et al. 2003). It targets coding sequences and results in the identification of a number of codominant markers. SRAP is based on two-primer amplifications where the primers are 17 or 18 nucleotides long. Primers consist of a core sequence of 13 or 14 bases, where the 5′-most 10 or 11 bases are non-specific, followed by the sequence CCGG in the forward primer and AATT in the reverse primer. The core sequence is followed by three selective nucleotides at the 3' end of each primer (Li and Quiros 2001). SRAP is more reproducible than RAPD and less complicated than AFLP. Ferriol et al. (2003) reported that the information obtained from SRAP markers was more concordant with the morphological variations and the evolutionary history of the morphotypes than that found with AFLP markers.

The objectives of this research were to utilize SRAP markers that can be used for buffalograss improvement to enhance our knowledge of the genetic basis of its agronomic characteristics, and to estimate the genetic diversity of buffalograss germplasm. We also aimed to determine buffalograss ploidy levels using flow cytometry.

Materials and methods

Plant materials

Fifty-three genotypes representing diverse ploidy levels and adaptation ranges were propagated in the Department of Agronomy and Horticulture greenhouse at Lincoln, Neb. (Table 1).

Flow cytometry

Flow cytometry as described by Arumuganathan and Earle (1991) was used to identify the ploidy levels of the accessions used in this study. A flow cytometry investigation had previously been done by Johnson et al. (1998) on approximately half of the genotypes used. Ploidy levels of the remaining genotypes were identified in this study (Table 1). Mean DNA content was based on analysis of 1,000 nuclei. Each genotype was analyzed by three separate extractions and flow cytometric runs. Base pair composition calculations were done as outlined by Godelle et al. (1993).

Primer selection, amplification and detection

A previous study by Ferriol et al. (2003) and our initial screening demonstrated that SRAP provided highly polymorphic and more informative markers than AFLP, RAPD and SSR. A total of 45 SRAP primer pairs were assayed on the 53 accessions (Table 2). Primers were excluded from the study if their banding patterns were difficult to score or if they failed to amplify consistently in all lines. Of these 45 primer pairs, 34 combinations (Table 3) were selected for their consistent amplifications and clear banding patterns.

Genomic DNA was extracted from greenhouse-grown fresh leaf material of each buffalograss genotype, using the CTAB method outlined by Wagner et al. (1987), and quantified using a TKO 100 Fluorometer (Hoefer Scientific Instruments, San Francisco). The PCR reaction mixtures (25 μl total volume) consisted of 10 mM Tris-HCl, pH 8.8 at 25°C, 50 mM KCl, 2.0 mM $MgCl_2$, nucleotides dATP, dTTP, dCTP, and dGTP (200 μM each), 0.2 μM primer, 30 ng template DNA, and 1.5 units/μl of *Taq* DNA polymerase (Promega). Amplifications were carried out in a MJ Research PTC-100 thermocycler programmed for 32 cycles of 1 min at 94°C, 1 min at 47°C, 1 min at 72°C, and ending with 5 min at 72°C.

The PCR products (25 μl) were fractionated on 12% polyacrylamide gels using a Hoefer vertical-gel apparatus (SE600). Gels consisted of acrylamide (37.5:1 acrylamide: bisacrylamide) in TAE buffer (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA; pH 7.7). Gels were 0.75 mm in thickness and 16x18 cm dimension. Electrophoresis conditions were held at 300 V for 3 h at room temperature, with a circulating water bath set at 20°C to maintain the gel temperature conditions. Gels were stained with ethidium bromide (1 ng/ml) for 20 min, destained in deionized water for 1 h, and photographed under the Gel Doc 2000 apparatus (Bio-Rad).

Data analysis

Presence or absence of each SRAP fragment was coded as "1" and "0", where "1" indicated the presence of a specific allele, and "0" indicated its absence. The distance matrix and dendrogram were constructed using the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) version 2.1 (Exeter Software, Setauket, N.Y.) software package. Genetic polymorphism (P-5%), Nei's gene diversity (*He*), and Shannon's information index were used to compute Nei's standard genetic distance coefficients (Nei and Li 1979), and to construct an unweighted pair-group method with arithmetic averages (UPGMA) dendrogram (Sneath and Sokal

Table 1 Buffalograss genotypes, their ploidy levels, DNA content, and origins

Name	Ploidy level	DNA content	Origin
Cody[a,c]	Hexaploid	2.54	Maternal parent from Arizona, Nebraska and Oklahoma
Texoka[a,c]	Hexaploid	2.47	Maternal parent from Oklahoma
Legacy[a,c]	Hexaploid	2.57	Maternal parent from Kansas
NE 98–039[b,c]	Hexaploid	2.53	Nebraska
NE 91–181[c]	Hexaploid	2.58	Nebraska
NE 92–502[b,c]	Hexaploid	2.52	Nebraska
NE 93–164	Hexaploid	2.52	Kansas
NE 93–166[c]	Hexaploid	2.62	Kansas
NE 93–167[c]	Hexaploid	2.62	Kansas
NE 93–168[c]	Hexaploid	2.62	Kansas
NE 93–170[c]	Hexaploid	2.40	Nebraska
Syn3–1[c]	Hexaploid	2.62	Nebraska
Syn3–2	Hexaploid	2.72	Nebraska
Syn3–3[c]	Hexaploid	2.59	Nebraska
Syn3–4[c]	Hexaploid	2.74	Nebraska
NE 86–120[a]	Hexaploid	2.59	Kansas
NE 98–023[b,c]	Hexaploid	2.58	Nebraska
NE 93–181[c]	Hexaploid	2.53	Colorado
A22–266[c]	Hexaploid	2.77	Colorado
NE 95–2[b,c]	Hexaploid	2.54	South Dakota
315[a,c]	Pentaploid	2.15	Maternal Parent from Colorado
378[a,c]	Pentaploid	2.30	Nebraska
NE 98–002[b,c]	Pentaploid	2.44	Nebraska
NE 98–003[b,c]	Pentaploid	2.36	Nebraska
NE 98–006[b,c]	Pentaploid	2.25	Nebraska
NE 98–015[b]	Pentaploid	2.15	Nebraska
NE 98–025[b,c]	Pentaploid	2.16	Nebraska
NE 98–037[b]	Pentaploid	2.14	Nebraska
NE 98–040[b,c]	Pentaploid	2.20	Nebraska
NE 98–046[b]	Pentaploid	2.14	Nebraska
NE 93–184[c]	Pentaploid	2.20	Unknown origin
NE 91–118[a,c]	Tetraploid	1.81	Maternal parent from Texas
609[a,c]	Tetraploid	1.87	Texas
NE 98–014[b]	Tetraploid	2.12	Nebraska
NE 98–022[b,c]	Tetraploid	2.20	Nebraska
NE 98–032[b,c]	Tetraploid	1.79	Nebraska
NE 98–035[b,c]	Tetraploid	1.69	Nebraska
NE 98–036[b,c]	Tetraploid	1.67	Nebraska
NE 98–038[b]	Tetraploid	1.72	Nebraska
NE 98–043[b,c]	Tetraploid	2.19	Nebraska
NE 98–047[b]	Tetraploid	2.12	Nebraska
NE 98–048[b,c]	Tetraploid	1.72	Nebraska
NE 85–436[c]	Tetraploid	2.44	Nebraska
A27–1[b]	Tetraploid	2.19	Nebraska
NE 84–45–3[c]	Tetraploid	1.78	Colorado
UCR95[a,c]	Diploid	0.94	Unknown
NE 95–65[b,c]	Diploid	0.93	Texas
NE 95–66[b,c]	Diploid	0.93	Texas
NE 95–67[b]	Diploid	0.93	Texas
NE 95–68[b,c]	Diploid	0.93	Texas
NE 95–81	Diploid	0.93	Texas
NE 95–82[c]	Diploid	0.93	Texas
NE 95–83[c]	Diploid	0.93	Texas

[a] Released cultivars
[b] Genotypes with their DNA content and ploidy levels determined in this study. Flow cytometric analysis of the remaining genotypes was performed by Johnson et al. 1998
[c] Base collection assembled from the 53 buffalograss accessions

1973). FIND module (part of the NTSYS package) were used to identify all trees that could result from different choices of tied similarity or dissimilarity values. To test the robustness of the tree topology, the trees were compiled by CONSEN (part of the NTSYS package). A Principal Coordinate Analysis (PCA) was performed based on the variance covariance matrix calculated from marker data using PCA (part of the NTSYS package).

Results and discussion

The DNA contents of the buffalograss genotypes were grouped into four distinct ploidy levels, based on the relationships of their DNA contents and inferred chromosome number (Table 1). DNA contents of the clones within each genotype group were significantly different. The base genome size of hexaploids was significantly different from tetraploids and diploids. This finding concurs with the work done by Johnson et al. (1998, 2001). Variations due to ploidy level range of within each group were found. There was a strong linear correlation (r =0.973, P<0.01) between the DNA contents of four ploidy levels and chromosome numbers. Flow cytometry results indicated that the buffalograss populations within the Great Plains can be a mixture of ploidy levels (Table 1). Hence, DNA contents of the germplasm should be identified before they are utilized in breeding programs.

Forty-five primer combinations were first tested for selective amplification of DNA fragments from a diverse collection of 53 genotypes representing diploid, tetraploid, pentaploid and hexaploid buffalograsses. About 40% of the samples were repeated to test for reproducibility, and only reproducible and unambiguous bands were used for the analysis. Eleven SRAP primer combinations amplified inconsistent band patterns per line. This inconsistency may have been the results of residual heterozygosity or the amplification of similar sequences in two separate genomic regions. It may also result from the use of polyacrylamide gels, which have a higher resolution power than most agarose gels (Fig. 1). Hence, out of the original 45 primer pairs, only 34 combinations were used. A total of 243 bands were observed; 231 of these (95%) were polymorphic within the collection, ranging in size from 150 bp to 1,000 bp, and were shared among at least two individuals.

Genetic similarities among all individuals ranged from 0.33 to 0.99, with a mean similarity of 0.66. Cody (hexaploid) and NE 95–83 (diploid) were found to span the extremes of the dendrogram, with all other germplasm distributed in between (Fig. 2). The UPGMA clustering algorithm grouped the genotypes into eight clusters. Genotype groupings generally agreed with the ploidy levels of the genotypes with few discrepancies. Germplasm from different geographical regions grouped together. This response might be due to germplasm exchange and ecotype selection.

The overall robustness of the phenetic and putative phylogenetic topologies was evaluated by bootstrap analysis of UPGMA trees generated from the total data set, and comparison of UPGMA trees with N-J trees

Table 2 The forward and reverse sequence-related amplified polymorphism (SRAP) primer information for this study

Forward primers		Reverse primers	
Me1	TGA GTC CAA ACC GGA TA	Em1	GAC TGC GTA CGA ATT AAT
Me2	TGA GTC CAA ACC GGA GC	Em2	GAC TGC GTA CGA ATT TGC
Me3	TGA GTC CAA ACC GGA AT	Em3	GAC TGC GTA CGA ATT GAC
Me4	TGA GTC CAA ACC GGA CC	Em4	GAC TGC GTA CGA ATT TGA
Me5	TGA GTC CAA ACC GGA AG	Em5	GAC TGC GTA CGA ATT AAC
Me6	TGA GTC CAA ACC GGA CA	Em6	GAC TGC GTA CGA ATT GCA
Me7	TGA GTC CAA ACC GGA CG	Em7	GAC TGC GTA CGA ATT CAA
Me8	TGA GTC CAA ACC GGA CT	Em8	GAC TGC GTA CGA ATT CAC
Me9	TGA GTC CAA ACC GGA GG	Em9	GAC TGC GTA CGA ATT CAG
Me10	TGA GTC CAA ACC GGA AA	Em10	GAC TGC GTA CGA ATT CAT
Me11	TGA GTC CAA ACC GGA AC	Em11	GAC TGC GTA CGA ATT CTA
Me12	TGA GTC CAA ACC GGA GA	Em12	GAC TGC GTA CGA ATT CTC
Me13	TGA GTC CAA ACC GGA AG	Em13	GAC TGC GTA CGA ATT CTG
–	–	Em14	GAC TGC GTA CGA ATT CTT
–	–	Em15	GAC TGC GTA CGA ATT GAT
–	–	Em16	GAC TGC GTA CGA ATT GTC

Table 3 The 34 SRAP primer combinations used in this study

	Primer combinations.
1	Em6 and Me1
2	Em6 and Me2
3	Em6 and Me3
4	Em6 and Me4
5	Em6 and Me5
6	Em6 and Me6
7	Em6 and Me7
8	Em6 and Me8
9	Em6 and Me9
10	Em6 and Me10
11	Em6 and Me11
12	Em6 and Me12
13	Em6 and Me13
14	Em7 and Me1
15	Em7 and Me2
16	Em7 and Me3
17	Em7 and Me4
18	Em7 and Me5
19	Em7 and Me6
20	Em7 and Me7
21	Em7 and Me8
22	Em7 and Me9
23	Em7 and Me10
24	Em7 and Me11
25	Em7 and Me12
26	Em7 and Me13
27	Em8 and Me1
28	Em8 and Me2
29	Em8 and Me3
30	Em8 and Me4
31	Em8 and Me5
32	Em8 and Me6
33	Em8 and Me7
34	Em8 and Me8

generated from the same data. These results demonstrated that the topologies of the hexaploid group were more robust than those of the diploid group. The hexaploid group had two major clusters. The first group consisted of Syn3–3, NE 98–0039, NE 98–0047, Syn3–1, NE 95–2, NE 98–0023, NE 93–167, NE 93–168, and clustered with 60% similarity. The four hexaploid cultivars, Cody, Texoka, Legacy and NE 86–120 were also in this group. This result was expected and served as an internal control for our analysis. Indigenous lines originating from one location mostly clustered together with small variation being detected. The second hexaploid group did not have any cultivars within the cluster. A clear pattern in geographical distribution of hexaploid buffalograss was not observed, which confirmed the work done by Johnson et al. (2001). For instance, NE 95–2, originated from South Dakota, grouped with genotypes originating in Oklahoma, Nebraska, and Kansas. The tetraploid genotypes from Nebraska, NE 98–0038, NE 98–0014, NE 98–0022, and NE 98–0048, clustered in one cluster with 62% similarity. This cluster separated two hexaploid groups. These results reflect the genetic diversity of this species. The pentaploid groups NE 98–0015, NE 98–0025, NE 98–0037, NE 98–0040, NE 98–0046, and NE 93–184 grouped at a 66% similarity level. The two Nebraska pentaploid cultivars, 315 and 378 were also in this group. The two tetraploid Nebraska cultivars (609 and NE 91–118) clustered with the pentaploid group with 51% and 43% similarity levels. Wu and Lin (1994) found that a tetraploid cultivar, Prairie, clustered with the hexaploid group. These results also show there is a large genetic diversity of this species. Tetraploid genotypes from Nebraska (NE 85–436, NE 84–45–3, NE 98–0043, A27–1, NE 98–0032, NE 98–0035, and NE 98–0036) grouped with 66% similarity. There were two major diploid groups in this study, and all these originated from Texas except UCR-95 which is of unknown of origin. The first consisted of UCR-95, NE 95–65, NE 95–66, and NE 95–67. The level of similarity for this group was very high (80%). They clustered with the tetraploid genotypes from Nebraska. On the other hand, similarity of the second diploid group, NE 95–81, NE 95–82, and NE 95–83, was relatively low (48%). These results demonstrated significant genetic diversity among the diploid genotypes within the same geographic region. These results agreed with the results of Peakall et al. (1995), who observed remarkable variations within and among regional diploid genotypes.

Principal coordinate analysis (PCA) was performed based on the genetic similarity matrix to better understand relationships within ploidy levels and ecological distri-

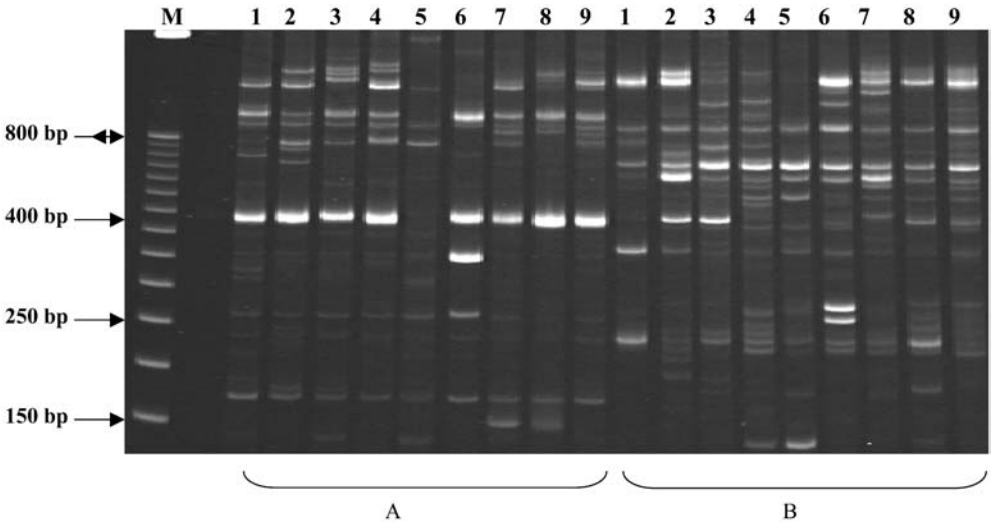

Fig. 1 PCR amplification of buffalograss genomic DNA from 9 genotypes. *Lanes: 1* Cody, *2* Texoka, *3* NE 98–032, *4* NE 85–436, *5* NE 93–184, *6* NE 95–2, *7* NE 98–043, *8* NE 98–0015, *9* Syn3–1, *M* 50 bp marker (Promega). Two sequence-related amplified polymorphism (SRAP) primer combinations, Em7+Me6 (*A*) and Em7+Me5 (*B*) were assayed. The DNA samples were fractionated in 12% non-denaturing acrylamide gels stained with ethidium bromide

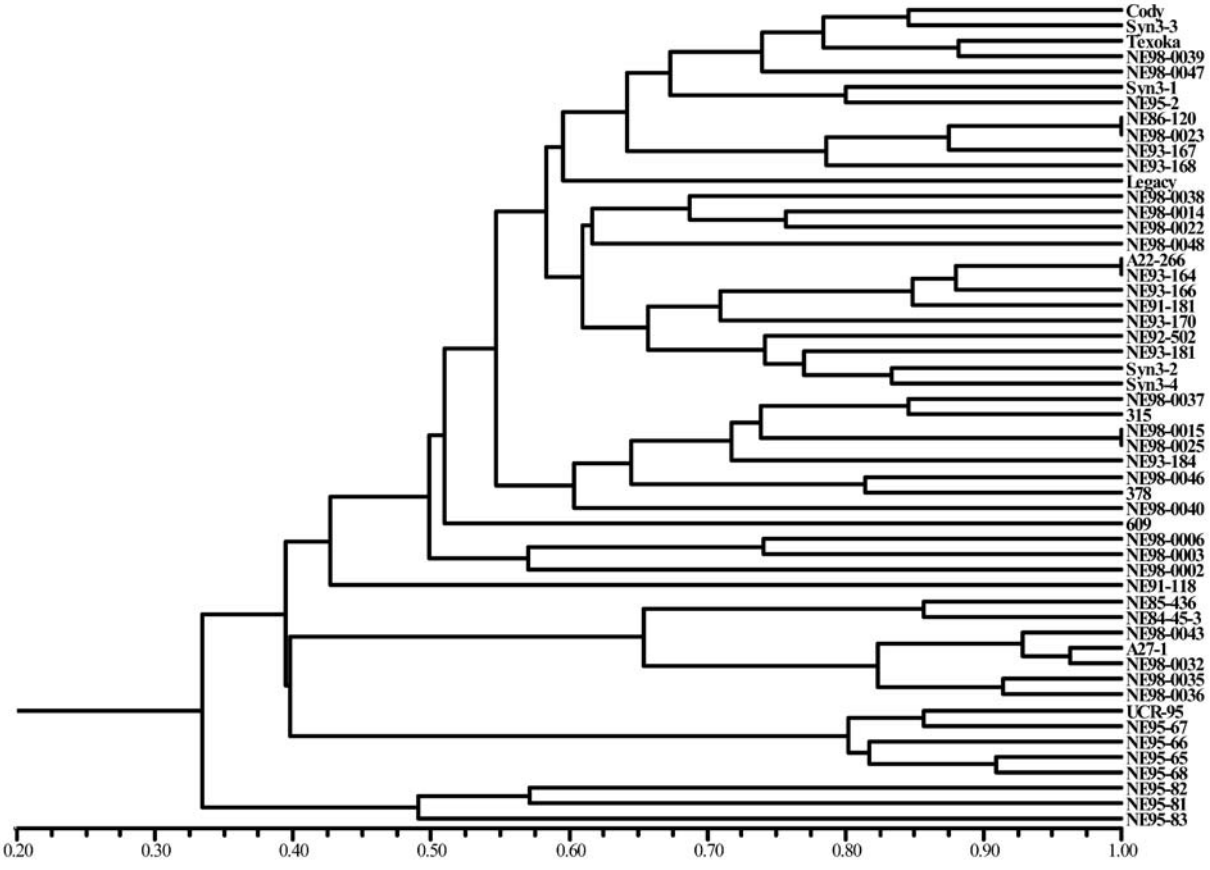

Fig. 2 An unweighted pair-group method with arithmetic averages (UPGMA) dendrogram of genetic relationships among 53 buffalograss genotypes calculated on the basis of genetic similarity analysis by means of 34 SRAP primer combinations

butions (Fig. 3). The PCA revealed similar groupings as the UPGMA analysis. The first three eigen vectors accounted for 61% of the variation observed. Although different ploidy levels were not always geographically separated, there was a tendency for diploids and tetraploids to be located in southern, and hexaploids in northern locations. Most genotypes originating from Nebraska, Colorado and Kansas (hexaploids and tetraploid buffalograss are most dominant) grouped together. Most hexaploid and pentaploid genotypes were relatively close to each other. Diploid genotypes, UCR-95, NE 95–65, NE 95–66, and NE 95–67, grouped together, which

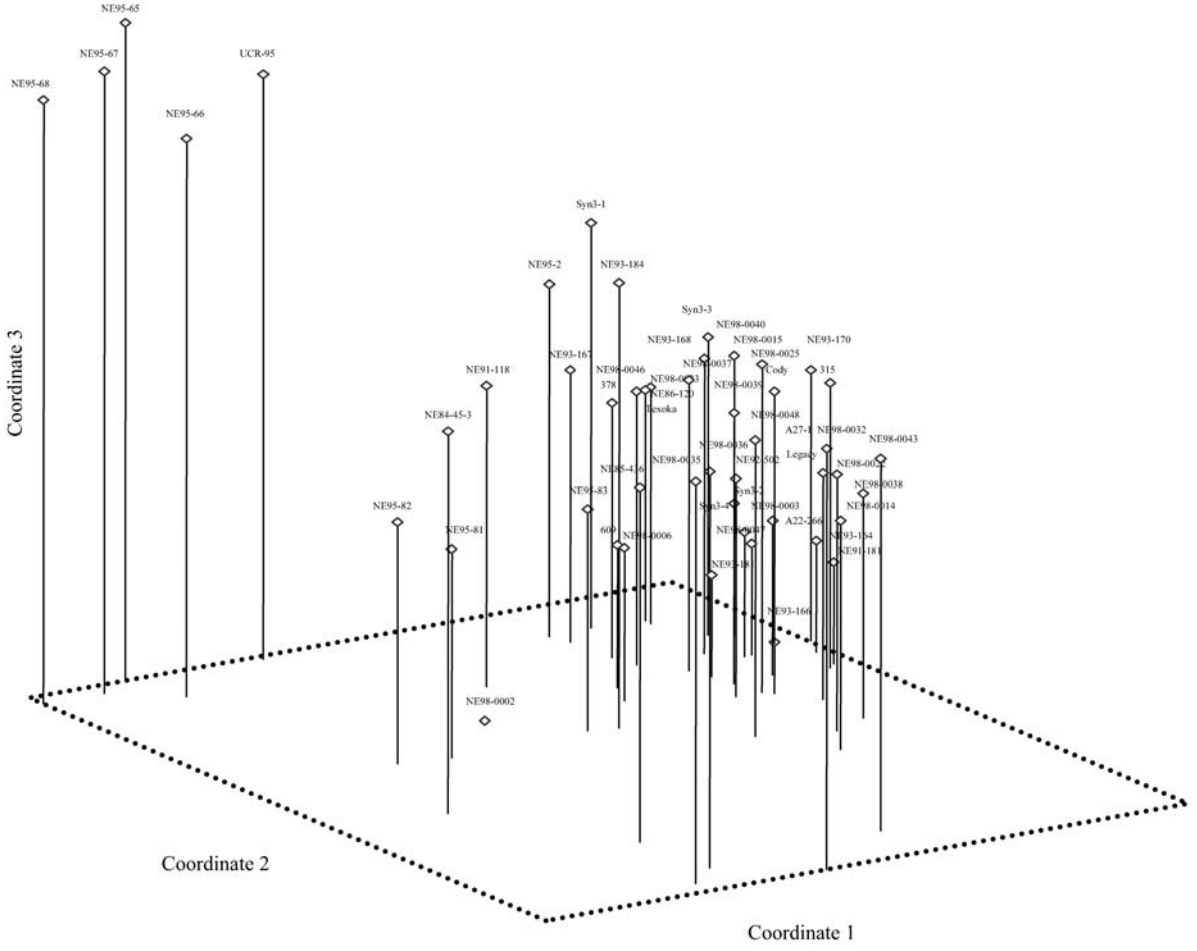

Fig. 3 Relationships among the 53 buffalograss genotypes representing different ploidy levels visualized by principal coordinate analysis (PCA) of SRAP-based genetic similarities. *Coordinates 1*, *2*, and *3* are indicated

was similar to the dendrogram. The PCA results indicated there was a clear separation between diploid and hexaploid genotypes used in this study. Two tetraploid genotypes, NE 91–118 and NE 84–45–3, clearly separated from hexaploid genotypes. One pentaploid genotype, NE 98–002, separated from the tetraploids and hexaploids. Adaptation patterns among the remaining tetraploids, pentaploids and hexaploids were not clear but the pentaploid genotypes grouped with those that came from the western part of the Great Plains.

These results demonstrate the adaptation range and the ecological and genetic relatedness of a number of buffalograss genotypes. Such results could be useful for ecotype selection in a breeding program. Both PCA and UPGMA analyses indicated that there were ploidy level distributions. These findings agree with the results of Johnson et al. (2001). Ploidy distribution might be due to environmental effects (Johnson et al. 1998), or the evolutionary and historical development of genotypes. There are two main strategies that may give a clear indication of ploidy level distributions; firstly, a better understanding of the evolutionary and historical development of buffalograss genotypes, and secondly, elucidating environmental covariates to explain genotype by environment interactions with the emphasis on physiological characteristics.

From these analyses, it is evident that many genotypes overlap, showing redundancy in these accessions. A base collection of 41 buffalograss accessions was identified by eliminating about one-fifth of the overlapping individuals and those in close proximity within each quadrant in the PCA (Table 1). Out of the eight released cultivars included in the study, seven were part of the base collection. These cultivars did not cluster together in the dendrogram with few exceptions. Buffalograss has many agronomically important traits that are governed by quantitative traits. Hence large base samples are needed if the primary objective is to evaluate the diversity of quantitatively inherited traits.

The SRAP loci identified can readily be placed onto buffalograss linkage maps. An integrated approach that combines linkage mapping with genetic fingerprinting can provide detailed phylogenetic relationships of individual chromosomal segments among and within buffalograss germplasm. These results provide a useful reference for appropriate parent selection that can be used in

crossing schemes, sampling strategies and managing germplasm repositories. This process could be the first step toward efficient perennial germplasm management. The SRAP markers preferentially amplify open reading frames (ORF), can be used for estimating genetic diversity and phenetic relationships and for genetic fingerprinting. This technique equals or exceeds that of the more labor intensive processes involved in the use of restriction fragment length polymorphism (RFLP) markers, and can provide a valuable tool for turfgrass breeding programs.

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