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Application of Sequence-related Amplified Polymorphism Markers for Characterization of Turfgrass Species

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***Abstract*. A simple marker technique called sequence-related amplified polymorphism (SRAP) provides a useful tool for estimation of genetic diversity and phenetic relationships in natural and domesticated populations. Previous studies and our initial screen showed SRAP is highly polymorphic and more informative when compared to AFLP, RAPD and SSR markers. In this study, applicability of the SRAP markers to obtain an overview of genetic diversity and phenetic relationships present among cool-season (C3) and warm-season (C4) turfgrass species and their relationship with other Gramineae species were tested. Phenetic trees based on genetic similarities (UPGMA, N-J) were consistent with known taxonomic relationships. In some cases, well-supported relationships as well as evidence by genetic reticulation could be inferred. There was widespread genetic variation among C3 and C4 turfgrass species. In Dice based cophenetic matrix, genetic similarities among all species studied ranged from 0.08 to 0.94, whereas in Jaccard based cophenetic matrix, genetic similarities ranged from 0.05 to 0.85. C3 and C4 species were clearly distinguishable and a close relationship between italian ryegrass and tall fescue were obtained based on SRAP. Genome structures of turfgrasses are comparable to other Gramineae species. This research indicates that the SRAP markers are useful for estimating genetic relationships in a wide range of turfgrass species. The SRAP markers identified in this study can provide a useful reference for future turfgrass breeding efforts.**

Grasses are one of the most complex genetic systems in nature, while grass family is defined as a single genetic system which evolved ≈55 to 70 million years ago (Kellogg, 2000). Though there have been many years and levels of investigation, the exact composition, organization and evolution of its genome is still unknown. Grass genomes differ tremendously in nuclear DNA content, chromosome number, and repetitive DNA content, but are significantly conserved in both gene content and gene order (Van Deynze et al., 1998). This extensive conservation of gene content order among grass chromosomes has led to the proposal of a progenitor genome structure for all grasses (Bennetzen and Freeling, 1993).

Turfgrasses constitute a heterogeneous group of species, which differs in reproductive strategy, genome organization and evolutionary history (Caetano-Anolles, 1998). Domestication of turfgrass is recent compared to any other grasses, and is classified into more than 30 species belonging to 20 genera and 3 subfamilies of Gramineae (Gould and Shaw, 1983). Turfgrass species, dispersed throughout the world, are divided into two major groups: warm-season (C4) and cool-season (C3) species based on their physiological aspects and geographic origins. Current knowledge of the phenetic relationships among turfgrass species, their genetic relatedness to the other grasses, the basic patterns of sequence organization and their relationship to genome function are only poorly understood due to sexual incompatibility and a limited number of molecular markers (Pitman et al., 1987). In molecular improvement of turfgrass species, there is still a knowledge gap compared to the agronomically important cereal crops (Gresshoff et al., 1998). Therefore, it is important to close this gap and accurately identify turfgrasses through molecular approaches that can answer a broad range of genetic, evolutionary relationships and ecological questions (Huff, 1998).

A number of molecular techniques have been introduced to help traditional methods for the estimation of biodiversity (Karp et al., 1997). Eleven turfgrass species have been evaluated for their chloroplast DNAs (*cp*DNAs) (Yaneshita et al., 1993), restriction fragment length polymorphism (RFLP) analysis of the nuclear ribosomal DNA (*r*DNA) (Ohmura et al., 1993), and conventional systematic studies. PCR-based co-dominant DNA markers such as SRAP have not been used to evaluate the diversity and phenetic relationships among a broad range of cool and warm-season turfgrass species. SRAP has proven informative to study mapping and gene tagging (Ferriol et al., 2003; Li and Quiros, 2001) and genetic relationship among subpopulations of single species (Budak et al., 2004). The SRAP is based on two primer amplifications and primers are 17 or 18 nucleotides long and targets coding sequences in the genome. Previous studies (Budak et al., 2004; Ferriol et al., 2003) and our initial screen showed SRAP was relatively polymorphic and more informative when compared to AFLP, RAPD, and SSR markers. SRAP would be a useful tool for estimating genetic diversity, the identifying unique genotypes as potentially important new sources of alleles for enhancing turfgrass characteristics, and analyzing the evolutionary and historical development of genotypes at the genomic level.

The objectives of this study were to a) determine phylogenetic relationships among 21 cool and warm-season turfgrasses and b) identify the SRAP markers that can be used in future turfgrass breeding efforts.

Materials and Methods

Plant materials. Twenty-one turfgrass species representing both vegetative and seeded types, diverse C3 and C4 adaptive types, and six other grass species including wheat (*Triticum aestivum* L.), triticale (×*Triticosecale* Wittmack), rice (*Oryza sativa* L.), maize (*Zea mays* L.), sorghum [*Sorghum bicolor* (L.) Moench.], and pearl millet [*Pennisetum glaucum* (L.) R. Br.] were investigated in this study (Table 1).

DNA extraction and SRAP reaction. Genomic DNA from 20 plants of each species was extracted using CTAB method outlined in Wagner et al., (1987), and quantified by Hoefer TKO 100 Fluorometer (Hoefer Scientific Instruments, San Francisco). In total, 21 SRAP primer pairs were assayed with 21 species, chosen to represent a C3 and C4 turfgrass species, and 6 species to represent the other Graminae species studied. A number of combinations of the forward and reverse primers, designed for open reading frame (ORF) regions (Li and Quiros, 2001) were used (Table 2). Primers were excluded from the study if banding patterns were difficult to score or if the primers failed to amplify consistently in all lines. 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 per μL of *Taq* DNA polymerase (Promega Corp., Madison, Wis.) (Budak et al., 2003, 2004). Amplifications were performed 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 (Budak et al., 2004).

The PCR products (25 μL) were fractionated on 12% polyacrylamide on a vertical-gel apparatus (SE600; Hoefer). Gels consisted of acrylamide (37.5 acrylamide : 1 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. Electrophoresis conditions were held at 300 V for 3 h at room temperature. Circulating water bath temperature was set to 20 °C to maintain the room temperature condition. Gels were stained in ethidium bromide (1 $\mu g \cdot mL^{-1}$) for 20 min, destained in deionized water for 1 h, and photographed under the Gel Doc 2000 (Bio-Rad) (Budak et al., 2003, 2004).

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Table 1. Description of tufgrass species and other grasses used in this study.

Common name	Species	Cultivar
Cool-season species (type C3)		
Alpine bluegrass	*Poa alpina* L.	
Annual bluegrass	*Poa annua* L.	
Chewing fescue	*Festuca rubra ssp. commutate* (Thuill.)	SR 5100
Creeping bentgrass	*Agrostis stolonifera* L.	Penncross
Hard fescue	*F. trachyphylla* (Hackel) Krajina	SR 3100
Italian ryegrasses	*Lolium multifolium* Lam.	
Kentucky bluegrass	*Poa pratensis* L.	Midnight
Quackgrass	*Agropyron repens* L.	
Red fescue	*F. rubra* L. ssp. *rubra* Gaudin	Flyer I
Supina bluegrass	*Poa supina* Schrad.	
Tall fescue	*F. arundinacea* Schreb.	Bonsai
Warm-season species (type C4)		
Buffalograss	*Bucholoe dactyloides* (Nutt.) Engelm.	Bowie
Bahiagrass	*Paspalum notatum* Flugge	
Centipedegrass	*Eremechloa ophiuroides* (Munro) Hack.	
Common bermudagrass	*Cynodon dactylon* (L.) Pers.	
Hybrid bermudagrass	*C. dactylon* x *C. transvaalensis* Burtt-Davy	419
Kikuyugrass	*Pennisetum clandestinum (Hochst. Ex Chiov.)*	
Manilagrass	*Zoysia matrella* Willd.	
Seashore paspalum	*Paspalum vaginatum* Swartz	
St. augustinegrass	*Stenotaphrum secundatum* (Walt.) Kuntze	Raleigh
Zoysiagrass	*Z. japonica* Steud.	Zenith
Other Gramineae species		
Maize	*Zea mays* L.	Mo17
Pearl millet	*Pennisetum glaucum* (L.) R. Br.	9Rm/4Rm
Rice	*Oryza sativa* L.	Nova
Sorghum	*Sorghum bicolor* (L.) Moench.	Wheatland
Triticale	x *Triticosecale* Wittmack	Newcale
Wheat	*Triticum aestivum L.*	Cheyenne

Table 2. The forward and reverse SRAP primer information and the 19 primer combinations used in this study.

Primer information	
Forward primer	
Me1	TGA GTC CAA ACC GGA TA
Me2	TGA GTC CAA ACC GGA GC
Me3	TGA GTC CAA ACC GGA AT
Me4	TGA GTC CAA ACC GGA CC
Me5	TGA GTC CAA ACC GGA AG
Me6	TGA GTC CAA ACC GGA CA
Me7	TGA GTC CAA ACC GGA CG
Me8	TGA GTC CAA ACC GGA CT
Reverse primer	
Em6	GAC TGC GTA CGA ATT GCA
Em7	GAC TGC GTA CGA ATT CAA
Em8	GAC TGC GTA CGA ATT CAC
Primer combination	
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) Em7 and Me1	
8) Em7 and Me2	
9) Em7 and Me3	
10) Em7 and Me4	
11) Em7 and Me5	
12) Em8 and Me1	
13) Em8 and Me2	
14) Em8 and Me3	
15) Em8 and Me4	
16) Em8 and Me5	
17) Em8 and Me6	
18) Em8 and Me7	
19) Em8 and Me8	

Data analysis. Presence or absence of each SRAP fragment was coded as 1 and 0, where 1 = the presence of a specific allele, and 0 = its absence. The distance matrix and dendrogram were constructed using the Numerical Taxonomy Multivariate Analysis System (NT-SYS-pc) version 2.1 (Exeter Software, Setauket, N.Y.) software package (Rohlf, 2000). Among the various similarity indices, those of Jaccard (1901) and Dice (1945) were chosen as the most appropriate for this marker. Dice index differs from the Jaccard index for the higher weight that gives to the coincidences of the band presence with respect to the non-coincidences. The similarities are calculated as follows:

Dice = $2N_{AB}/(2N_{AB} + 2N_A + N_B)$

Jaccard = $N_{AB}/(N_{AB} + N_A + N_B)$

where N_{AB} is the number of bands shared by samples, N_A represents amplified fragments in sample A and N_B represents fragments in sample B. *Nei's* standard genetic distance coefficients (Nei and Li, 1979) and a UPGMA dendrogram (Sneath and Sokal, 1973) were obtained. The FIND module was used to identify all trees and the trees were compiled by CONSEN module.

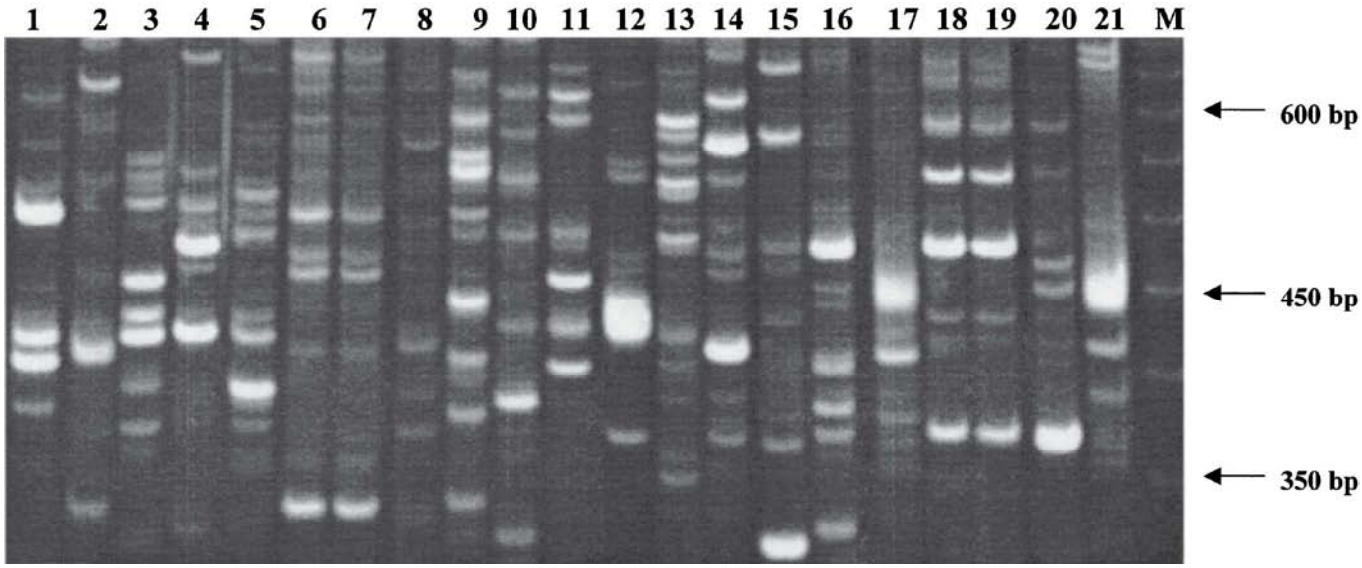


Fig. 1. Amplification of genomic DNA from 21 trufgrass species. Lanes: 1= SR3100 (*Festuca trachyphylla*), 2 = Flyer I (*F. rubra*), 3 = italian ryegrass (*Lolium multifolium*), 4 = manilagrass (*Zoysia matrella*), 5 = Penncross (*Agrostis stolonifera*), 6 = Midnight (*Poa pratensis*), 7 = alpine bluegrass (*P. alpina*), 8 = annual bluegrass (*Poa annua*), 9 = supina bluegrass (*Poa supina*), 10 = quackgrass (*Agropyron repens)*, 11 = Bowie (*Buchloe dacytloides*), 12 = Bonsai (*F. arundinaceae)*, 13 = SR5100 (*F. rubra)*, 14 = kikuyugrass (*Pennisetum clandestinum)*, 15 = seashore paspalum (*Paspalum vaginatum)*, 16 = bahiagrass (*Paspalum notatum*), 17 = centipedegrass (*Eremechloa ophiurioides)*, 18 = common bermudagrass (*Cynodon dactylon*), 19 = hybrid bermudagrass (*C. dactylon X C. transvaalensis)*, 20 = st. augustinegrass (*Stenotaphrum secundatum)*, 21 = zenith (*Z. japonica)*, and lane M contains a 50-bp size marker (Promega Corp.). The first SRAP combinations were assayed. The DNA samples were fractionated on 12% nondenaturing acrylamide gels stained with ethidum bromide.

Results and Discussion

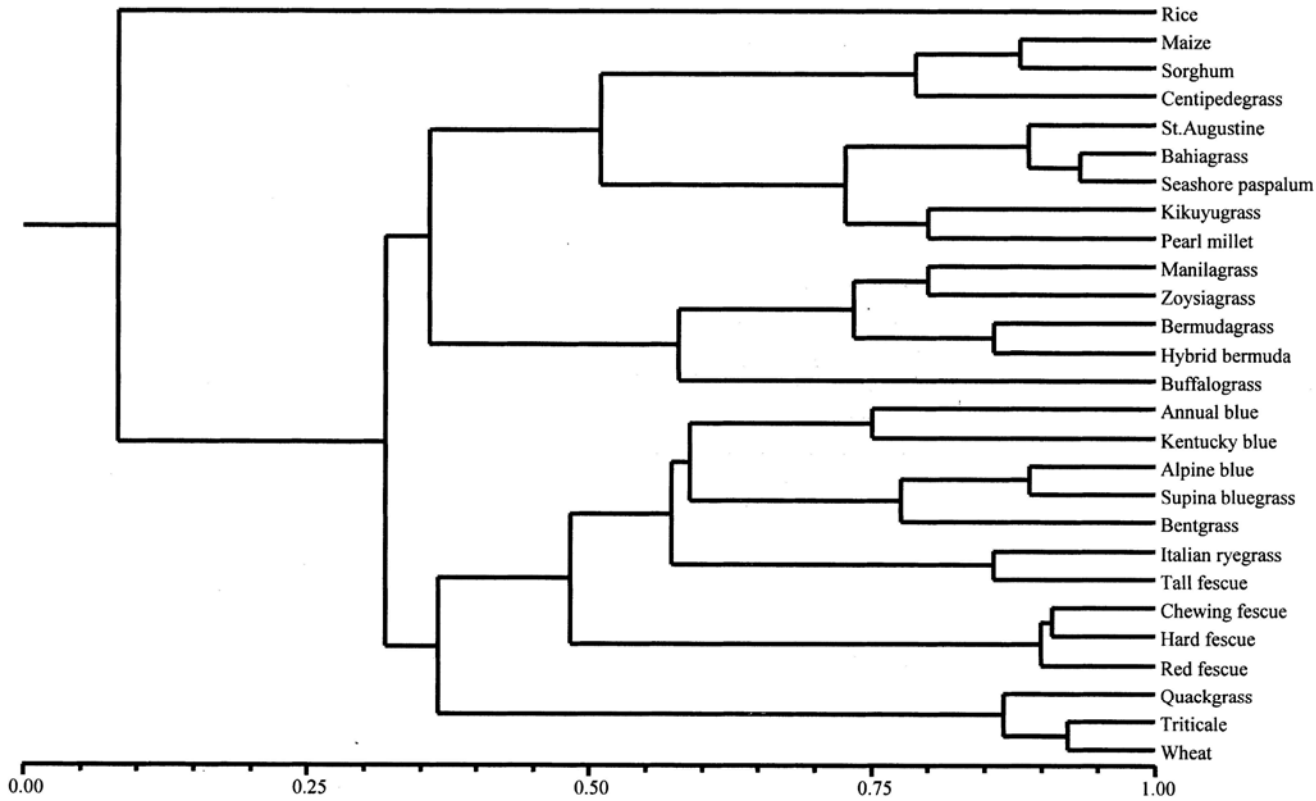
The 19 SRAP primer combinations, which demonstrated high level of polymorphism and consistent band patterns in our initial screen, were tested to estimate genetic diversity and phenetic relationships among C3 and C4 turfgrass species. About 32% of the samples were repeated to test for reproducibility, and the reproducible and unambiguous bands were used for the analysis. The first SRAP primer combinations were examined among 21 species (Fig. 1) to present an estimate of range associated with each locus. The 19 primer combinations with a range of 2 to 4 reproducible bands per primer combinations were tested in each set of 21 turfgrass species.

Among the similarity indices, those of Jaccard and Dice were chosen as the most appropriate for this marker. The UPGMA clustering algorithm based on Dice's and Jaccard's similarity matrix gave similar results, with the identification of 5 clusters corresponding to the 21 turfgrass species (Fig. 2 and 3). SRAP analysis indicated a wide variation among all grass species. In both dendrograms, cool-season turfgrasses were clearly different from warm season turfgrasses. In Dice-based cophenetic matrix, genetic similarities among all species ranged from 0.08 to 0.94, with a mean similarity of 0.51. In Jaccard-based cophenetic matrix, genetic similarities ranged from 0.05 to 0.85. In both dendrograms, grouping of species revealed by the present analysis generally agreed with the traditional taxonomic species with one discrepancy: 11 species fell into a C3 cluster, 10 species into a C4 cluster. The overall robustness of 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 generated from the same data. These two criteria demonstrated that the topologies of all species were equally robust in both Jaccard and Dice based matrices. Despite the great and similar discriminating power of both Jaccard and Dice based cophenetic matrices, genetic similarity values were lower for Jaccard than for Dice. This result is expected because the Dice index differs from the Jaccard index for the higher weight that gives to the coincidences of the band presence with respect to the non-coincidences.

Only the Dice-based matrix was discussed in this study. The turfgrass species were divided into five major clusters, which agree with the subfamilies of Gramineae. Maize (*Zea mays*), sorghum (*Sorghum bicolor*) and centipedegrass (*Eremechloa ophiuroides*) belonging the Andropagoneae tribe were relatively close to each other at 78% level of similarity. St. augustinegrass (*Stenotaphrum secundatum)*, bahiagrass (*Paspalum notatum*), and seashore paspalum (*Paspalum vaginatum*) belonging the Setoriinaes subtribe grouped together at the 89% level. Pearl millet (*Pennisteum glaucum*) grouped with kikuyugrass (*Pennisetum clandestinum*) at the 80% level of similarity, which is expected because both species belong to the Cenchriae subtribe. The five warm-season turfgrass species (centipedegrass, st. augustinegrass, bahiagrass, seashore paspalum, and kikuyugrass) belonging to the Panicoideae subfamily were relatively close to each other at the 55% level of similarity. On the other hand, manilagrass (*Zoysia matrella*), zoysiagrass (*Z. japonica*), bermudagrass (*Cynodon dactylon*), hybrid bermudagrass (*C. dactylon* x *C. transvaalensis*), and buffalograss (*Buchloe dactyloides*), belonging to the Eragrostoideae subfamily grouped at the 55% level of similarity. Yaneshita et al. (1993) reported similar results. Their results are not unexpected. They used *cp*DNA, which is known to be highly conserved among the plants (Palmer et al., 1989). In addition, this study used five species from Eragrostoideae subfamily rather than four, which increased the potential diversity. The cool-season turfgrass species belonging to the Festucoideae subfamily clustered at a 48% level of similarity. Chewing fescue (*Festuca rubra spp. commumata*), creeping red fescue (*Festuca rubra* spp. *rubra*), and hard fescue (*Festuca trachyphylla*) belonging to the Loliinae subtribe clustered at a high level of similarity (i.e., 90%). The two cereals (wheat and triticale) grouped with one of the cool season turfgrass species, quackgrass, at an 86% level of similarity. This might be because these two cereals and quackgrass (*Agropyron repens*) belong to the Triticeae subtribe. Rice (*Oryza sativa*) grouped with the turfgrass species at a very low level of similarity (0.08%). This response may have been anticipated as well, since rice belongs to the Ehrhartoideae subfamily and none of species were associated with this subfamily. Interestingly, creeping bentgrass (*Agrostis stolonifera*), which belongs to the Agrostidinae subtribe, grouped with alpine bluegrass (*Poa alpine*) and supina bluegrass (*Poa supine*), which are members of the Loliinae subtribe at a 77% level. The bluegrasses and creeping bentgrass belong to the Poeae tribe, which clustered at a 59% level of similarity.

In the Festucodieae subfamily, high level of relatedness of tall fescue (*Festuca arundinacea*) with italian ryegrass (*Lolium multifolium*) (87%) was observed based on SRAP. This relationship

Fig. 2. Dendrogram of 27 grass species derived from a UPGMA analysis using the Dice genetic index.



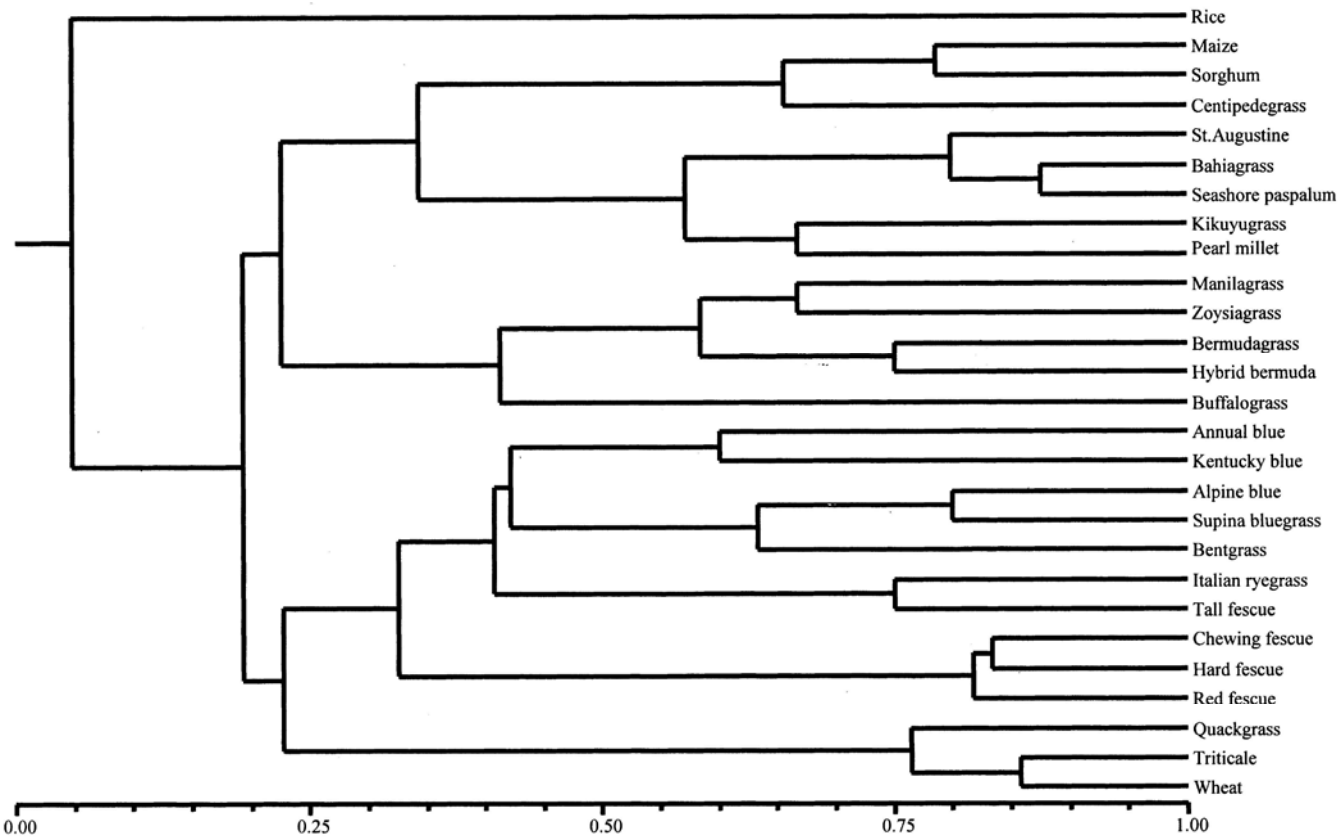


Fig. 3. Dendrogram of 27 grass species derived from a UPGMA analysis using the Jaccard genetic index.

was also noted by Yaneshita et al. (1993) and Lehvaslaiho et al. (1987). They both used *cp*DNA to evaluate the phenetic relationship among the grasses belonging Festucodieae. Our results might also be due to a meiotic chromosome pairing between *Lolium* and *Festuca* (Humphreys, 1989). It should be noted that SRAP markers in grasses should be reliable for the evaluation of their diversity and phenetic relationships.

The SRAP loci identified in this study can readily be placed onto linkage maps derived from intraspecific and interspecific crosses. Results from this study also demonstrated the use of the SRAP markers for tagging a particular genomic region and identifying homoeologous region in other taxa at least over short evolutionary distances. The SRAP markers can be used for fingerprinting and the development of a comprehensive picture of the genome, which leads to the development of improved turfgrass cultivars.

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