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K. Renganayaki · J.C. Read · A.K. Fritz

Genetic diversity among Texas bluegrass genotypes (*Poa arachnifera* Torr.) revealed by AFLP and RAPD markers

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Abstract Texas bluegrass *Poa arachnifera* Torr., is a vigorous sod-forming perennial, dioecious grass, tolerant to heat. It is native to the Southern Great Plains. Genetic relationships existing among 28 Texas bluegrass genotypes were investigated using amplified fragment length polymorphism (AFLP) and randomly amplified polymorphic DNA (RAPD). A total of 3756 AFLP markers were generated on the 28 genotypes of Texas bluegrass. A wide range of polymorphism (23.08–85.33%) was observed among primer combinations with a mean of 64.11%. Among 441 RAPDs assayed, 335 were polymorphic with a mean polymorphic rate of 73.71%. Unweighted pair-group method using an arithmetic average (UPGMA) cluster analysis using AFLP and RAPD data separated the 28 Texas bluegrass accessions into two broad groups. With a few exceptions, the females clustered with females and males with males. These results indicate that, it may be possible to discriminate between males and females using molecular markers. Principal coordinate analysis of AFLP and RAPD data also indicated two distinct groups and revealed genetic variability among and within the groups. Based on their genetic similarity indices, high correlation was observed between AFLP and RAPD markers.

Keywords *Poa arachnifera* · AFLP · RAPD · Genetic diversity

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K. Renganayaki (✉) · A.K. Fritz
Crop Biotechnology Center, Texas A&M University,
College Station, TX-77843, USA
e-mail: renga@tamu.edu
Fax: +1-979-8624790

J.C. Read
Research and Extension Center, Texas A&M University, Dallas,
TX 75252-6599, USA

Present address:
A.K. Fritz, Department of Agronomy, Throckmorton Hall,
Kansas State University, Manhattan, KS 66506-5501, USA

Introduction

The genus *Poa* includes more than 200 species, including 69 species reported in the United States (Hitchcock 1950). Texas bluegrass, *Poa arachnifera* Torr., is a rhizomatous, dioecious grass adapted to the southern USA. It produces an abundance of quality forage and a high-quality turf (Read et al. 1997). Texas bluegrass has heat tolerance and a deep-green color during all seasons of the year if adequate moisture is available. It is native to most of Texas, Oklahoma, Southern Kansas and Western Arkansas (Hitchcock 1950).

Diversity among and within breeding populations and elite germplasm is central to successful breeding programs. The diversity is important for broadening the genetic base and may be exploited via heterosis (Melchinger 1999). Numerous methods have been used to estimate genetic similarities among genotypes. These include multivariate analyses of large numbers of traits (Gizlice et al. 1993; Good and Bird 1977), agronomic performance (Brown 1991; Garcia et al. 1998) and isozymes (Wendel et al. 1992; Hamrick and Godt 1997; Pasquet 1999; Bartsch and Ellstrand 1999). Lin Wu et al. (1984) employed isozyme markers in the identification of Kentucky bluegrass cultivars. Isozymes often fail in the classification of elite breeding materials due to the limited number of marker loci available and the level of polymorphism.

DNA markers are considered to be the most suitable means for estimating genetic diversity because of their abundant polymorphism and the fact that they are independent of environment (Gepts 1993). DNA-based molecular markers such as restriction fragment length polymorphisms (RFLPs, Federici et al. 1998; Desplanque et al. 1999), randomly amplified polymorphic DNA (RAPD, Moeller and Schaal 1999; Rodriquez et al. 1999), simple sequence repeats or microsatellites (Dje et al. 1999; Gilbert et al. 1999), sequence tagged sites (Liu 1999; Vander Stappen et al. 1999) and single nucleotide polymorphisms (Germano and Klein 1999) have been used for fingerprinting varieties, cultivars and clones of plants.

More recently, amplified fragment length polymorphisms (AFLPs) have emerged as a powerful tool for DNA fingerprinting and genome mapping (Zabeau and Vos 1993). This technique combines both classical, restriction enzyme-based (restriction site recognition) and polymerase chain reaction (PCR)-based (stringent PCR conditions for primer annealing) fingerprinting and has been successfully used to study genetic diversity and phylogenetic relationships in many plant species (Vipa Hongtrakul 1997; Barrett and Kidwell 1998; Subudhi et al. 1998; Breyne et al. 1999; Hansen et al. 1999; Vendrame et al. 1999; Zhang et al. 1999).

The genetic diversity of Texas bluegrass has not been previously studied by DNA-based methods. The study reported here was designed to (1) estimate the genetic diversity existing among a set of Texas bluegrass types, (2) compare the utility of AFLP and RAPD markers for estimating genetic diversity in Texas bluegrass and (3) examine the genetic relatedness between male and female Texas bluegrass types. The identification of the gene(s) responsible for the dioecious trait in Texas bluegrass could facilitate the production of hybrids in difficult-to-cross species.

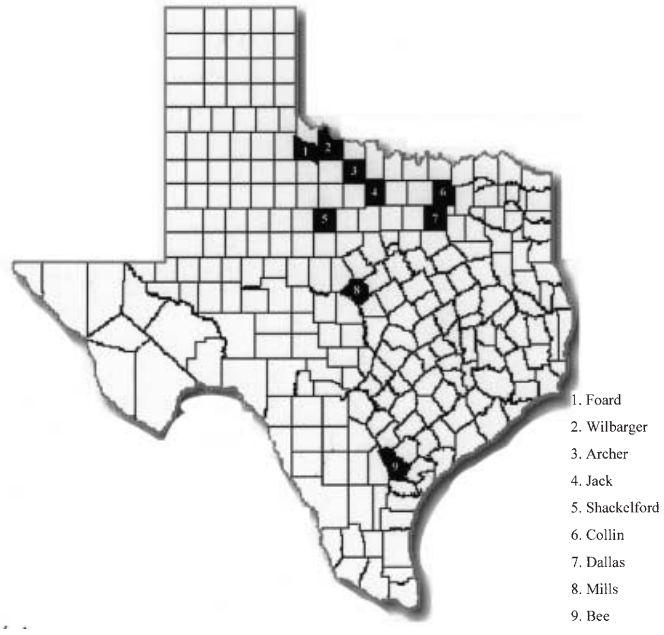

Fig. 1 Texas map highlighting the counties from which the bluegrass genotypes were collected

Materials and methods

Plant materials

Thirty-nine different accessions of Texas bluegrass were collected by seed from distinctly different sites. These accessions were used to establish an introduction nursery for evaluation and use in the forage and/or turf grass-breeding program. The 28 Texas bluegrass plants (16 females and 12 males) used in this study (Table 1) were selected as being superior for either forage or turf potential without regard to sex. The selected plants were from nine different locations (Fig. 1). The plants were removed from the field, divided into ramets and planted in pots and moved to a greenhouse for maintenance. Total genomic DNA was extracted from fresh leaf tissue using the protocol of Gill et al. (1991).

AFLP analysis

AFLP reactions were conducted following the manufacturer's instructions with minor modifications (Life technologies, Gaithersburg, Md.). A kit optimized for a genome size of more than 500 Mb was used, and reaction volumes were reduced by one-half. AFLP templates were prepared by simultaneous digestion of 200 ng of genomic DNA with *Eco*RI and *Mse*I. Restricted genomic DNA fragments were ligated to *Eco*RI and *Mse*I adapters. A 1:1.5 dilution of restricted and adapter-ligated DNA was prepared using 1× TE supplied with the kit. This constituted the template DNA for further amplification. Template DNAs were amplified using preamplification primers having one selective nucleotide. Pre-amplification products were diluted (1:10) using 1× TE and used as templates for selective amplification. All 64 combinations of *Eco*RI and *Mse*I AFLP primers containing three selective nucleotides that were supplied by the manufacturer were used for selective amplification. Following amplification, the PCR products were completely dried in a speed vac (Savant, Holbrook, N.Y.) and resuspended in 5 µl of ddH2O and 5 µl of manual sequencing dye (Sambrook et al. 1989). The products were separated on 5% denaturing polyacrylamide gels, and the DNA bands were visualized using silver staining (Fritz et al.1999).

Table 1 Plant ID and source of Texas bluegrass genotypes included in the study

Plant No.[a]	Plant ID[b]	Source[c]
BG 1	♀TXBlue28–8(21–88)	Shackelford
BG 2	♀TXBlue10–30(3–88)	Dallas
BG 3	♀TXBlue30–7(3–88)	Dallas
BG 4	♀TXBlue20–11(3–88)	Dallas
BG 5	♀TXBlue7–10(3–88)	Dallas
BG 6	♀TXBlue10–24(3–88)	Dallas
BG 7	♀TXBlue20–16(3–88)	Dallas
BG 8	♀TXBlue30–1(3–88)	Dallas
BG 9	♀TXBlue25–11(25–88)	Jack
BG 10	♀TXBlue15–10(34–88)	Jack
BG 11	♀TXBlue25–16(25–88)	Jack
BG 12	♀TXBlue4–20(24–88)	Jack
BG 13	♀TXblue14–3(26–88)	Jack
BG 14	♀TXBlue27–7(35–88)	Archer
BG 15	♀TXBlue25–21(31–88)	Wilbarger
BG 16	♀TXBlue3–13(20–88)	Collin
BG 17	♂Beeville#3[d]	Bee
BG 18	♂Beeville#4[d]	Bee
BG 19	♂TXBlue10–25(3–88)	Dallas
BG 20	♂TXBlue10–26(3–88)	Dallas
BG 21	♂Turfsel.3e	Dallas
BG 22	♂Turfsel.4e	Dallas
BG 23	♂TXBlue5–8(26–88)	Jack
BG 24	♂TXBlue12–28(32–88)	Jack
BG 25	♂TXBlue9–20(39–88)	Foard
BG 26	♂TXBlue9–17(39–88)	Foard
BG 27	♂TXBlue14–16(31–88)	Wilbarger
BG 28	♂TXBlue2–18(16–88)	Mills

[a] Random number given to each genotype for easier identification
[b] Accession number given to each genotype based on their collection
[c] Counties from where the genotypes were collected
[d] Genotypes selected at Bee county for rust resistance
[e] Genotypes selected at Dallas county for turf

RAPD protocol

DNA from 28 plant samples of Texas bluegrass was subjected to RAPD analysis following the conditions of Pammi et al. (1994), with minor modifications. Amplification reactions were performed in 15-µl volumes containing 200 µ*M* of each dNTP, 30 pMoles of primer, 30 ng genomic DNA, 1.67 m*M* $MgCl_2$, 1× PCR buffer and 0.016 U of *ampliTaq* DNA polymerase (Stoffel fragment) (Perkin Elmer, Foster City, Calif.). The amplifications were carried out in a Perkin Elmer Cetus 9700 thermocycler using the following amplification profile: 1 cycle at 94°C for 3 min and 30 s, followed by 28 cycles of 94°C for 30 s, 48°C for 35 s and 72°C for 1 min with a final extension at 72°C for 1 min. PCR products were denatured and resolved by electrophoresis on 5% polyacrylamide gels and visualized by silver staining (Fritz et al.1999)

Data analysis

Only clear and unambiguous DNA bands were included in the analyses. Markers were scored for presence (1) and absence (0) of the band in both AFLP and RAPD analyses. Bands of different electrophoretic mobilities were assumed to be non-allelic, while bands of the same mobility were assumed allelic. A pairwise similarity matrix was calculated using the Jaccard Coefficient. UPGMA cluster analysis was used to identify genetic variation patterns among the Texas bluegrass genotypes. Ordination analysis was performed to study the relatedness within a matrix by converting the pairwise distance into Eigen vectors and values. Correlation between the AFLP and RAPD similarity matrices were assessed using the Mantel test (Mantel 1967), which assumes that the two matrices were obtained independently. Cluster analyses, ordination analyses and the Mantel test were performed using NTSYS-PC (NTSYS – for Numerical Taxonomy SYStems) software version 2.0 (Rohlf 1997).

Results and discussion

Characteristics of AFLP and RAPD markers

In the AFLP analysis, all 64 primer combinations available in the manufacturer's kit were used. A total of 54 primer combinations produced clear and scorable banding patterns that could be used for determining genetic diversity among the Texas bluegrass genotypes. A total of 3756 amplifiable DNA fragments were generated across 28 genotypes of Texas bluegrass, with 2541 fragments being polymorphic (67.65% polymorphism). The number of bands and the degree of polymorphism revealed by each primer combination is given in Table 2. A representative example of the amplification products obtained with 28 Texas bluegrass genotypes using the primer combinations *Eco*RI-AGC/*Mse*I-CAC and *Eco*RI-ACA/*Mse*I-CAT is shown in Fig. 2. The number of bands from each primer combination ranged from 13 (*Eco*RI-ACT/*Mse*I-CTC) to 145 (*Eco*RI-AAC/*Mse*I-CTA). Zhang et al. (1999) detected 48–74 bands per primer combination in 17 bermudagrass genotypes. A similar study on sugar beets showed 11–116 bands per primer combination (Hansen et al. 1999). With the 28 Texas bluegrass genotypes in the present study, a wide variation in the number of polymorphic bands (3–116) and the percentage of polymorphic bands within a primer combination (23–85%) was observed. The mean polymorphic rate was 64.11%. In wheat cultivars, Barrett and Kidwell (1998) detected 2–31 polymorphic bands per primer pair, with a mean polymorphic rate of 11.8%. Studies on rice cytoplasmic male-sterile (CMS) lines showed 8–23 polymorphic bands per primer pair, with an average of 16 polymorphisms (Subudhi et al.1998).

Among the 15 RAPD primers tested on the 28 Texas bluegrass samples, clear and scorable products were ob-

Table 2 The number of bands and degree of polymorphism revealed by AFLP primer combinations

Primer combinations	Total bands	Polymorphic bands	Polymorphism rate (%)
E–ACG+M–CAA	139	113	81.29
E–AAC+M–CTA	145	116	80.00
E–AGC+M–CAC	114	81	71.05
E–AGG+M–CTC	69	44	63.77
E–ACA+M–CTA	127	97	76.38
E–AAG+M–CTG	124	85	68.55
E–AGC+M–CAA	79	48	60.76
E–AGG+M–CTT	67	36	53.73
E–AGG+M–CTA	71	49	69.01
E–ACT+M–CAC	89	52	58.43
E–AAC+M–CTG	56	26	46.43
E–ACA+M–CAC	27	8	29.63
E–ACA+M–CTT	32	8	25.00
E–AAG+M–CTC	32	10	31.25
E–ACT+M–CTC	30	15	50.00
E–AAG+M–CAG	91	68	74.73
E–ACG+M–CTA	78	62	79.49
E–ACG+M–CAG	62	44	70.97
E–AGC+M–CTA	44	30	68.18
E–AGC+M–CAG	67	42	62.69
E–AAG+M–CAC	105	70	66.67
E–ACT+M–CAA	100	63	63.00
E–ACT+M–CTT	50	26	52.00
E–ACT+M–CTG	27	13	48.15
E–ACT+M–CAG	50	30	60.00
E–ACC+M–CTG	45	27	60.00
E–ACC+M–CAT	47	27	57.45
E–ACC+M–CAG	37	25	67.57
E–ACC+M–CAC	57	33	57.89
E–ACC+M–CTA	61	46	75.41
E–ACC+M–CTC	55	35	63.64
E–ACA+M–CAT	111	82	73.87
E–AGC+M–CTG	83	39	46.99
E–AAG+M–CTT	70	43	61.43
E–AAC+M–CAT	75	53	70.67
E–AAC+M–CAC	72	49	68.06
E–ACG+M–CTG	76	55	72.37
E–AAC+M–CTC	54	37	68.52
E–ACG+M–CTC	62	49	79.03
E–ACG+M–CAC	46	37	80.43
E–AGG+M–CAC	97	76	78.35
E–ACT+M–CTC	13	3	23.08
E–AGG+M–CAT	81	57	70.37
E–AGG+M–CTG	104	68	65.38
E–ACG+M–CTG	79	59	74.68
E–ACC+M–CAA	19	10	52.63
E–ACC+M–CTT	44	29	65.91
E–AAC+M–CAA	43	27	62.79
E–AAC+M–CAG	97	77	79.38
E–ACT+M–CTA	77	59	76.62
E–ACG+M–CTG	66	48	72.73
E–ACC+M–CAT	49	39	79.59
E–AAC+M–CAT	86	52	60.47
E–AGG+M–CAG	75	64	85.33

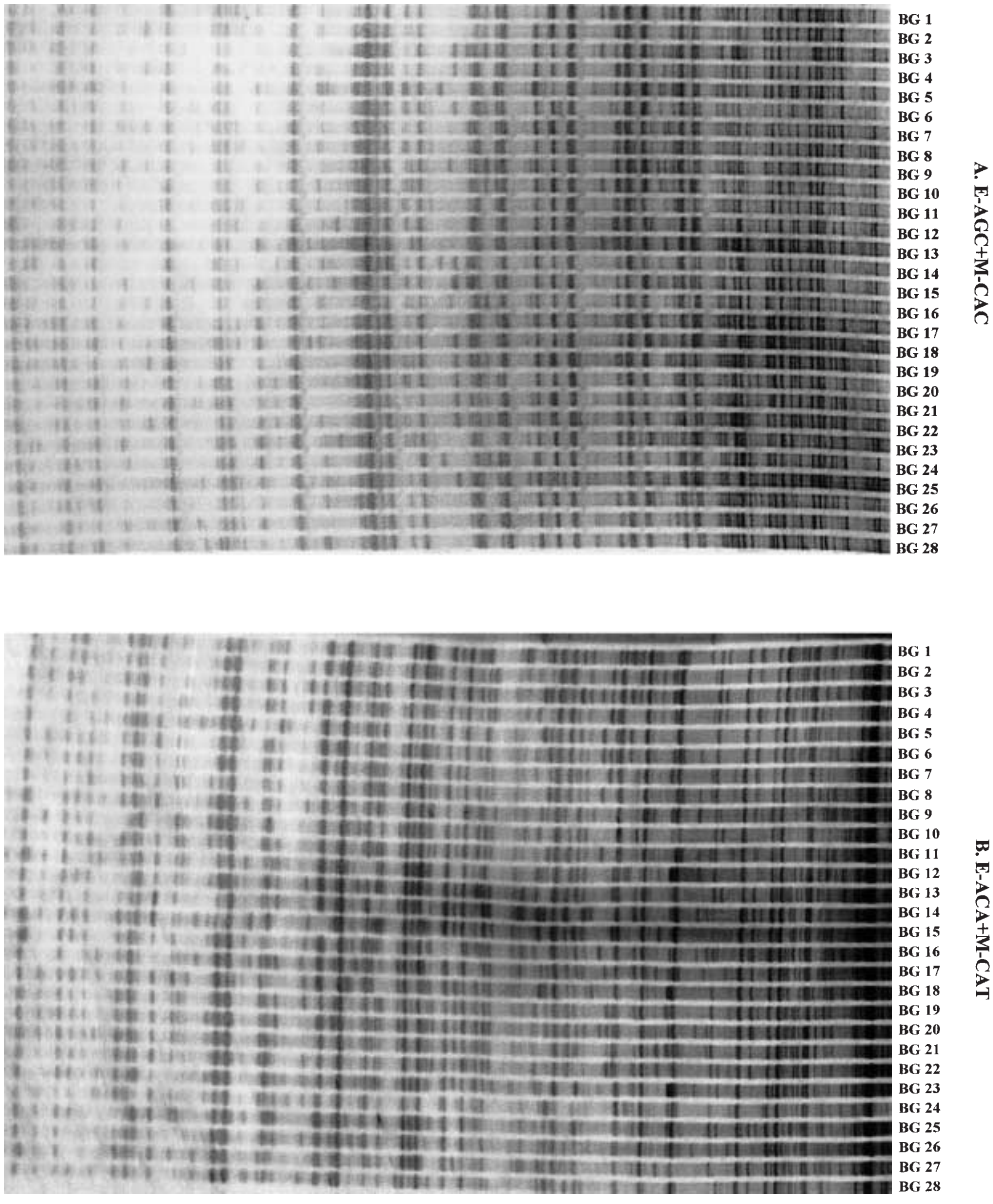

Fig. 2 Silver stained polyacrylamide gel visualizing AFLPs generated using E-AGC+M-CAC (**A**) and E-ACA+M-CAT (**B**) primer combinations

tained from 13 primers that produced 441 DNA fragments, of which 335 were polymorphic. The mean polymorphism rate was 75.96%. The total number of bands per primer ranged from 12 to 50 (Table 3), while the number of polymorphic bands per primer ranged between 6 and 35. The total number of DNA bands generated in this study was greater than that observed in maize accessions which revealed 7–21 bands per primer (Moeller and Schaal 1999).

The level of polymorphism observed using different AFLP primer combinations and RAPD primers indicates a remarkable amount of intraspecific genetic variation among the Texas bluegrass samples. High levels of polymorphism allow for the selection of parents and the identification of true hybrids in breeding programs (Garcia et al. 1998). The large amount of variation may be due to the fact that Texas bluegrass is a dioecious species. The marker data may be a valuable tool for germplasm evalu-

Table 3 The number and degree of polymorphism revealed by RAPD primers

Primers[a] bands	Total bands	Polymorphic rate (%)	Polymorphism
BB10	44	35	79.55
BB18	41	35	85.37
AH04	31	28	90.32
C02	38	30	78.95
F20	17	12	70.59
C10	50	41	82.00
F02	28	6	21.43
AM15	41	34	82.93
V14	33	27	81.82
V16	31	25	80.65
C11	12	7	58.33
C19	46	34	73.91
BA06	29	21	72.41

[a] Random 10-mers obtained from Operon Technologies, Alameda, Calif.

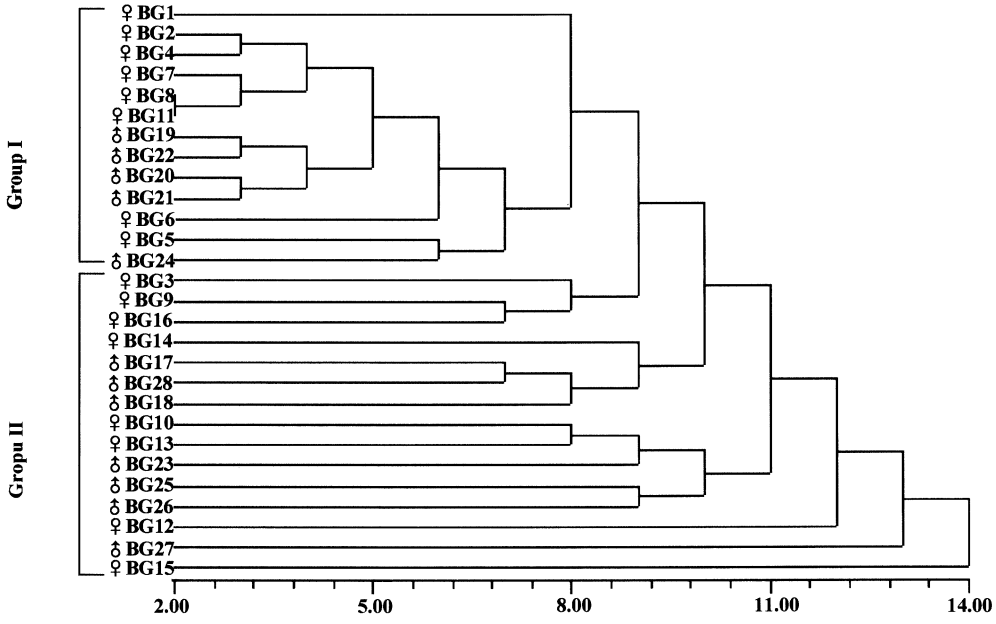

Fig. 3 Genetic similarity among Texas bluegrass genotypes revealed by UPGMA cluster analysis based on AFLP data

ation and parental selection and for exploitation of heterosis in breeding programs. Moreover, the polymorphism information obtained through AFLP and RAPD analyses may also help identify polymorphic primers and primer combinations for further studies in Texas bluegrass.

AFLP analysis

A presence (1) or absence (0) binary data matrix containing 2541 polymorphic AFLP fragments was used to generate the genetic similarity estimates. The similarity coefficient ranged from 0.46 to 0.87, indicating considerable genetic distance among the genotypes.

UPGMA clustering analysis was carried out in order to graphically demonstrate the genetic similarities among the bluegrass genotypes (Fig. 3). The dendrogram separated the 28 Texas bluegrass genotypes into two broad groups. Group-I was primarily comprised of entries from Dallas County, whereas Group-II had six clusters consisting of entries from the remaining counties. Texas bluegrass genotype BG11, obtained from Jack County, was included with the Dallas county material (Group-I), indicating a greater similarity to those accessions. BG11 could not be distinguished from BG8, an accession from Dallas County. An entry from Shackelford County (BG1) remained distinct from the others (Fig. 3). The genotypes from Bee, Jack and Foard counties clustered according to the location from which they were collected. A phenogram of maize accessions revealed a closer relationship between maize accession identities and accessions obtained from the same tribe (Moeller and Schaal 1999). Similarly, a cluster analysis of cotton by Iqbal et al. (1997) unveiled that the inter-varietal genetic relationships are related to their center of origin. The two genotypes obtained from Wilbarger county remained in a different cluster, indicating that they are genetically distinct from the other accessions. The three entries from Jack County, namely BG11, BG9 and BG12, clustered into three different clusters. Information regarding the genetic diversity existing among genotypes from the same geographical location will help in the selection of diverse parents in bluegrass breeding programs.

An interesting phenomenon observed in the study was the tendency for females to cluster with females and males with males, suggesting that female and male genotypes may have some distinct loci that can be used for their identification. This tendency was apparent even when only accessions from Dallas and Jack counties were considered. There were, however, obvious exceptions; specifically, BG5 (female) and BG 24 (male) were found to be closely related. Based on our observations, we are currently in the process of identifying markers associated with the dioecious trait in a controlled segregating population of Texas bluegrass. Parasnis et al. (1999) reported that the microsatellites probes $(GATA)_4$ were able to differentiate males from females in papaya.

Principal coordinate analysis (PCA) was also performed to display the relationship among the Texas bluegrass types on two coordinate axes (Fig. 4). Like the UPGMA clustering dendrograms, the PCA analysis placed the 28 genotypes into two distinct groups. Group-I had entries that were closer to each other than those in Group-II. This suggests that hybridization between entries of the two groups may yield desirable genetic variability for further breeding and selection. There are numerous studies that support the existence of a positive correlation between the genetic divergence of parental lines and hybrid performance across a number of species (Lee et al. 1989; Smith et al. 1990; Zhang et al. 1995). Entries in Group-II remained scattered, showing greater variability among the genotypes than the accessions in Group-I. The Group-I entries were primarily from Dallas

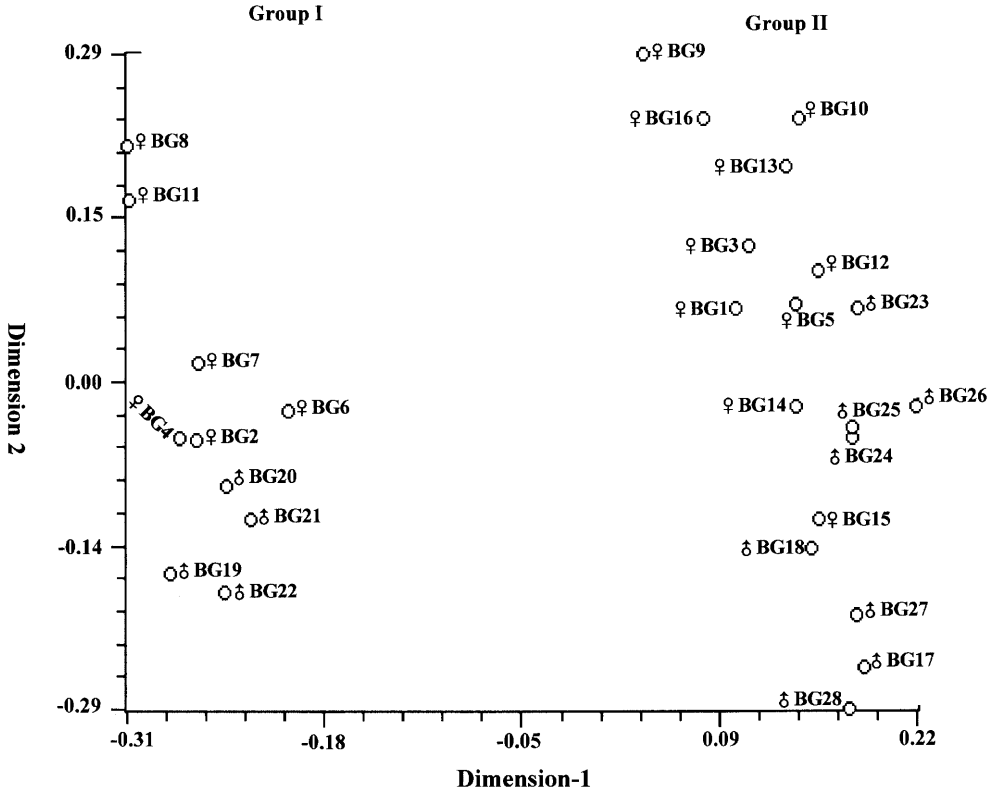

Fig. 4 Two-dimensional picture of PCA analysis estimated using AFLP genetic similarity matrix

County and corresponded to the entries of Group-I identified by UPGMA analysis. The entries in Group-II were from different geographical locations, providing a possible explanation for being a less compact cluster than Group-I. Similar results have been obtained in other crops. Principal coordinate and cluster analysis of sunflower revealed two groups, one comprised of A-lines and the other of R-lines (Vipa Hongtrakul et al. 1997). Dendrograms drawn using melon breeding lines also showed two main groups, namely Piel de Sapo and Galia, based on their botanical origin (Garcia et al. 1998).

RAPD analysis

Genetic similarities among the 28 Texas bluegrass genotypes ranged between 0.39 and 0.89, a distance that was higher than the one reported by Paran et al. (1998) and Rodriquez et al. (1999) in *Capsicum* species. Similar to the AFLP results, the RAPD dendrogram also separated the 28 entries into two broad groups (Fig. 5). The RAPD dendrogram displayed six clusters. As seen in AFLP, the entries from Dallas County, except for BG22, grouped in the same cluster. BG2 and BG4 could not be distinguished from each other based on genetic similarity estimates. Unlike the AFLP results, the entries from Ford County remained in a different cluster. Like the AFLP dendrogram, the entries from Wilbarger County remained in a different cluster, indicating greater genetic diversity. The principal coordinate analysis (PCA) on the RAPD data revealed two distinct groups. Unlike the AFLP analysis, the entries in both groups were widely scattered (Fig. 6), suggesting variability between and among the genotypes in the two groups. In contrast, the multidimensional scaling analysis of *Capsicum* species displayed six discrete clusters corresponding to the six *Capsicum* species included in their study (Rodriquez et al. 1999).

Comparison of AFLP and RAPD genetic similarities

The genetic similarity indices obtained through AFLP and RAPD analyses were compared using regression analysis performed by means of a Mantel test (Fig. 7). A high correlation was observed between AFLP and RAPD markers, in terms of their genetic similarity assessment, among the Texas bluegrass genotypes (r=0.66). The Pearson product-moment correlation coefficient (rp) between the AFLP and RFLP genetic distance matrices of *Lactuca* spp. was 0.86 (Hill et al.1996). Higher correlations between RAPD and morphological characters were also observed in cotton (Tatineni et al. 1996) and melon (Garcia et al. 1998). A comparison of genetic similarity matrices in soybean revealed that, if the comparison was between cultivated and wild soybean accessions, estimates based on RFLPs, AFLPs and simple-sequence repeats (SSRs) were highly correlated. However, correlations between RAPD marker data and RFLPs, AFLPs and SSRs were lower. RAPDs produced higher estimates of interspecific similarities. If comparisons are restricted

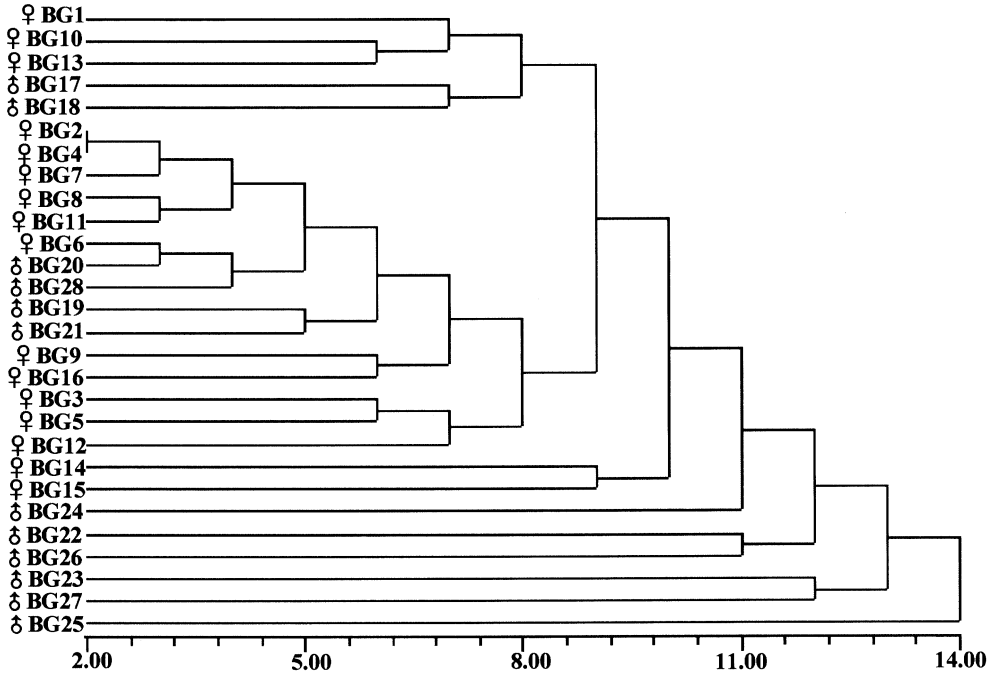

Fig. 5 Genetic similarity among Texas bluegrass genotypes revealed by UPGMA cluster analysis based on RAPD data

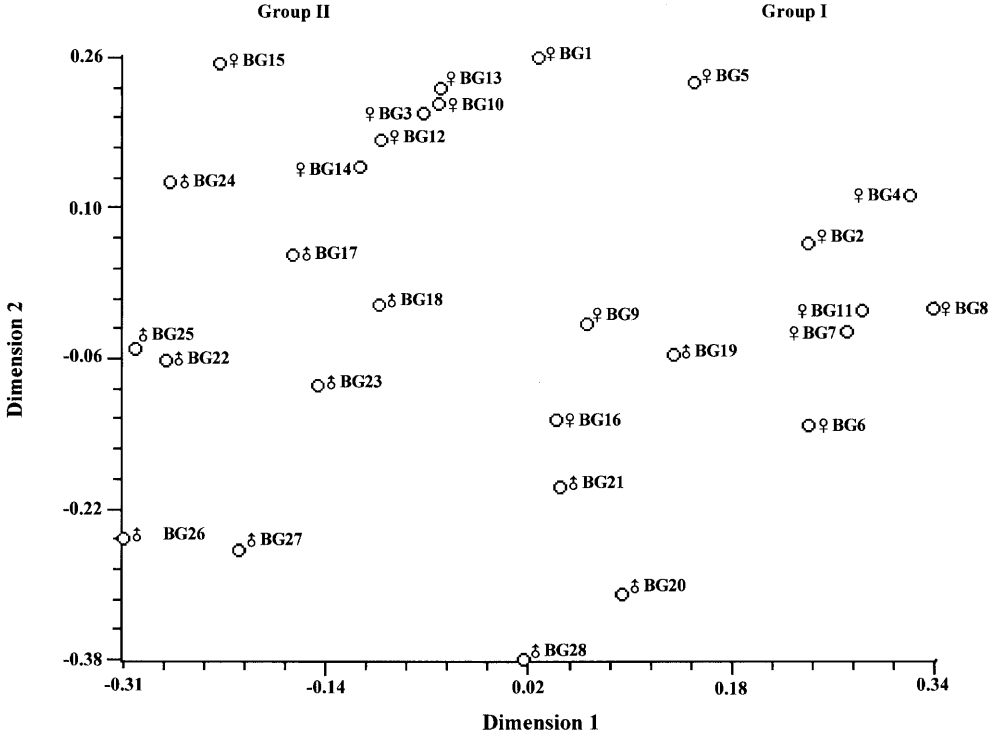

Fig. 6 Two-dimensional picture of PCA analysis estimated using RAPD genetic similarity matrix

within a species, then overall correlations between marker systems are significantly lower. Powell et al. (1996) found similarity estimates between RAPD and AFLPs to be more closely correlated than RFLP and SSR. In the present study, two broad groupings of genotypes based on AFLP (Fig. 4) and RAPD (Fig. 6) data produced consistent clustering of the genotypes, with few exceptions. Clustering among the genotypes within the two broad groups varied to some extent. While the number of RAPD primers used in this study was relatively small, we did score more than 400 bands, including 335 that were polymorphic. Given the random nature of RAPD markers and the number of loci analyzed, we believe our comparison of the two systems is valid. Though the amount of RAPD data is less than we collected for AFLPs the more than 400 loci examined for this study

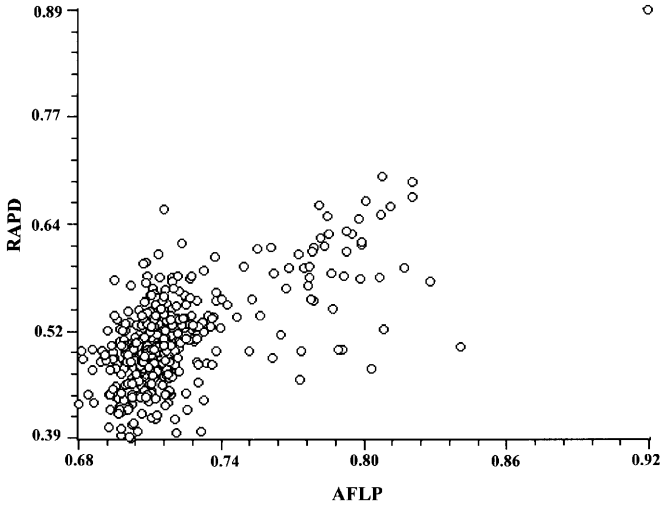

Fig. 7 Comparison of genetic similarity estimates from AFLP and RAPD using Mantel test

far exceeds the number of RFLP or SSR loci used in similar research (Rodriguez et al. 1999; Kong et al. 2000). Subudhi et al. (1998) compared cluster analysis using morphological and AFLP markers and concluded that AFLPs have distinct advantages for fingerprinting rice CMS lines. AFLPs generally showed a lower level of polymorphism per band than RFLPs, but due to the larger number of bands simultaneously analyzed per primer combination, they usually had the highest marker index among all available marker systems (Russell et al. 1997). Further studies comparing AFLP- and RFLP-based genetic similarities revealed that AFLP analysis is less time-consuming and laborious (Hill et al.1996). Automated AFLP systems are also available for further enhancing their advantages for genetic distance and genetic mapping studies.

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