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Evaluation of bermudagrass [*Cynodon* (L.) Rich] accessions with different ploidy levels

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Abstract: Eastern Mediterranean is considered as a center of diversity of *Cynodon* spp. (Bermudagrass). Phylogenetic analysis from diploids to hexaploids can help our understanding underlying mechanism of polyploidization. Inter-primer binding site (iPBS) markers amplify highly polymorphic long terminal repeat retrotransposons. The objectives were to (1) investigate associations between ploidy level and genetic diversity based on iPBS markers and (2) correlate between similarity matrices of iPBS retrotransposon marker and four nuclear molecular marker systems for *Cynodon* accessions' genetic analyses. The samples included 40 bermudagrass genotypes with ploidy series ranging from diploids to hexaploids. The iPBS fragments were highly similar to the retrotransposons according to the BLAST search. The simple matching-based UPGMA analysis clustered the 44 genotypes into two subclusters with a mean similarity value of 0.57 but failed to produce a clear ploidy-based grouping. The similarity matrix of iPBS markers poorly associated ($r < 0.35$) with those of the four other nuclear marker systems. However, iPBS markers resulted in a higher discrimination power and PIC (0.78). Although disomic inheritance of simple sequence repeats (SSR) markers strongly indicated segmental allo-tetraploidy in previous studies, the iPBS-based phylogeny hinted that bermudagrass may have independently followed both auto- and allo-polyploidization, involving C. *transvaalensis* and C. *dactylon*.

Key words: Inter-primer binding site, iPBS, *Cynodon dactylon, C. transvaalensis*, polyploidy

1. Introduction

Bermudagrass [*Cynodon* (L.) Rich.] evolved in Africa, and an area from West Pakistan to Turkey is believed to be center of evolutionary activity (Harlan and De Wet, 1969). The genus involves members used for turfgrass, pasture, forage, soil stabilization, and soil remediation (Burton, 1947). It comprises polyploidy series of diploids, triploids, tetraploids, pentaploids, and hexaploids with a base chromosome number of $x = 9$ (Wu et al., 2006). In the genus *Cynodon,* early studies reported autoploidy (Zeven, 1980), while recent studies verified segmental alloploidy (Jewell et al., 2012; Zhi-Yun et al., 2013; Guo et al., 2015; Khanal et al., 2017; Silva et al., 2018; Chaves et al., 2019).

Although taxonomic classification of *Cynodon* remains unclear, ten species are consistently agreed upon (Harlan and de Wet, 1969; Nightingale et al., 2005). Knowing ploidy level of individuals is particularly important, because, except for the triploids and all other odd cytotypes, intra- and interploidy level crosses can produce viable seeds (Taliaferro, 2003). *C. dactylon* (L.) Pers. and *C. transvaalensis* Burtt Davy readily hybridize and are extremely morphologically diverse. Therefore, clear taxonomic separation of some of these species is difficult. *C. dactylon* is the most widely distributed and morphologically and genetically variable species, with numerous subspecies (Harlan and de Wet, 1969; Nightingale et al., 2005; Wu, 2011). Analyses of *Cynodon* accessions originating from China and Korea indicated that all ploidy levels but diploids existed whereas Turkish accessions possessed diploids as well with a frequency of 2.5% (Wu et al., 2006; Kang et al., 2008; Gulsen et al., 2009). In general, genetic diversity was abundant and ploidy- and geography-based clustering was weak among bermudagrasses. In addition, marker systems such as intersimple sequence repeats (ISSR) and sequence related amplified polymorphism (SRAP) differ in estimating relationships and rare alleles among bermudagrasses. This is expected because genomic regions that marker systems amplify may be subject to differential mutation frequencies and/or evolutionary history. Thus, Gulsen et al. (2009) suggested a combined data generated from different genomic targets for genetic analyses of bermudagrasses.

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The structural changes in the nuclear genome at both ploidy and individual chromosome level are important aspects of speciation. Polyploidy is common among (De Storme and Mason, 2014) and probably the major driving force behind the divergence and biodiversity of the angiosperms (Leitch and Leitch, 2008). Duplicated genes/genomes in polyploids may lead to increased polymorphism detected by molecular markers. Polyploidy as assessed by SRAP, ISSR, peroxidase gene polymorphism (POGP), and random amplified polymorphic DNA (RAPD) markers represented increased polymorphism rate among bermudagrass accessions (Gulsen et al., 2009). A similar trend was reported for buffalograsses [*Buchloe dactyloides* (Nutt.) Engelm.] where higher molecular marker diversity was evident among higher ploidy levels (Gulsen et al., 2005). In addition to polyploidy, plant genomes also expand due to the copy-and-paste proliferation of a few long terminal repeat retrotransposons (LTRs) (Lee and Kim, 2014). LTRs retrotranspose via an mRNA intermediate in a copy-and-paste process leading to extremely high copy numbers in the genome. Transposable element fractions in plant genomes vary from as low as ~3% in small genomes to as high as ~85% in maize, indicating that genome size is a linear function of transposable element content (Schnable et al., 2009; Lee and Kim, 2014). In sorghum and rice, with around 500 Mbp genomes, transposable element fraction is about 30% (Michael, 2014). PCR-based molecular marker methods were developed to target insertional polymorphisms of LTRs, including retrotransposon-based insertion polymorphism (RBIP), inter-retrotransposon amplified polymorphism (IRAP), retrotransposon-microsatellite and amplified polymorphism (REMAP) (Schulman et al., 2004; Alzohairy et al., 2014; Kalendar et al., 2011; Kalendar and Schulman, 2014). Kalendar et al. (2010) described the PCR-based iPBS marker system. The method is based on the virtually universal presence of a tRNA complement as a reverse transcriptase primer binding site (PBS) in LTR retrotransposons. It has been successfully employed in flax (Smykal et al., 2011), apricot (Baránek et al., 2012), grape (Guo et al., 2014), okra (Yıldız et al., 2015; Kantar et al., 2021), and pea (Baloch et al., 2015; Solberg et al., 2015) for genetic diversity evaluation. As dispersed, ubiquitous, and very rapidly evolving nature of transposable elements, markers that target LTRs may have more discriminating power than the other marker systems and draw alternative relationships among samples. Genetic analyses with retrotransposon markers may extend our understanding of the evolutionary history of bermudagrass. The objectives of this study were to (1) investigate associations between ploidy level and genetic diversity as assessed by iPBS retrotransposon markers, and (2) correlate between similarity matrices of iPBS markers and four nuclear molecular marker systems (SRAP, ISSR, POGP and RAPD) derived from *Cynodon* accessions representing all known ploidy levels.

2. Material and methods

2.1. Plant materials

Forty-four different *Cynodon* genotypes were used in the study. It involved *Cynodon* accessions, previously collected in Turkey from an area south of the Taurus Mountains along the Mediterranean coast, including 5 diploids, 3 triploids, 10 tetraploids, 10 pentaploids, and 7 hexaploids, in addition to a diploid *C. transvaalensis* (TR97ZF09), and commercially available cultivars; triploid Tifway, tetraploid Riviera, Princes, and SWI-1045 (Contessa). The four triploid progenies (T1-A1, T4-A35, T4-G10, T6-D2) derived from crosses between diploid *C. transvaalensis* and tetraploid *C. dactylon* were included as control. The vegetative propagules of the accessions were previously collected from a region bounded by 37°23′27″ to the north, 35°53′51″ to the south, 27°18′42″ to the east, and 36°34′50″ to the west, ranging from 0 to 1350 m from the sea level. The collection was previously evaluated using SRAP, ISSR, POGP and RAPD markers (Gulsen et al., 2009).

2.2. Production of molecular markers

Initially, the iPBS primers reported by Kalendar et al. (2010) were screened in two DNA bulks, representing tetraploid and hexaploid germplasms with different geographic origins. Promising primers, generating multiple strong and clear amplified bands, were chosen for genetic diversity evaluation (Table 1). Conditions for iPBS-PCR amplification were conducted as described by Kalendar et al. (2010) with modifications. The PCR was performed in a 15 µL reaction mixture containing 25–50 ng DNA, 1x Taq PCR buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA), 1.5 mM $MgCl_2$ (Thermo Fisher Scientific Inc.), 1 µM of primer, 0.2 µM dNTPs (Thermo Fisher Scientific Inc.), and 1 unit Taq DNA polymerase (Thermo Fisher Scientific Inc.). The PCR program consisted of: 1 cycle at 94 °C for 3 min; 35 cycles of 95 °C for 30 s, 45–56°C (Table 1) for 59 s, and 72 °C for 59 s; a final extension step of 72 °C for 10 min by using a PTC-225 Programmable Thermal Controller (MJ Research Inc., Waltham, MA, USA) as reported by Kalendar et al. (2010). Products were analyzed by gel electrophoresis in a 2.0% (w/v) agarose gels with 1X TBE electrophoresis buffer (0.09 M Tris, 0.09 M boric acid, 0.02 M EDTA, pH: 8.5) at 95 V for 4.5 h and visualized by staining with ethidium bromide and photographing with gel documentation system (DNR Bio Imaging System, Neve Yamin, Isreal).

2.3. Cloning PCR products

PCR was set up using the template DNA of plant materials and the iPBS primers. Fragments that belong to different genotypes were excised from the agarose

Table 1. Interprimer binding site (iPBS) primer sequences and annealing temperatures, number of fragments and polymorphic bands, percent polymorphism, product size ranges of the fragments.

iPBS primers	Sequence (5'-3')	Annealing Temp. (°C)	Number of fragments	Number of polymorphic bands	Percent polymorphism (%)	Fragment size (bp)
2074	GCTCTGATACCA	50	9	7	77.7	300-1250
2076	GCTCCGATGCCA	51	12	12	100	260-1250
2077	CTCACGATGCCA	46	8	6	75.0	400-1100
2375	TCGCATCAACCA	50	14	10	71.4	350-1200
2376	TAGATGGCACCA	50	12	10	83.3	300-1250
2383	GCATGGCCTCCA	46	12	8	66.7	550-1250
2384	GTAATGGGTCCA	45	16	12	75.0	340-1245
2387	GCGCAATACCCA	46	9	7	77.7	450-1200
2391	ATCTGTCAGCCA	48	13	11	84.6	250-1200
2394	GAGCCTAGGCCA	48	10	10	100	300-1550
2220	ACCTGGCTCATGATGCCA	52	2	1	50.0	745
2232	AGAGAGGCTCGGATACCA	56	11	9	81.8	400-1750
2237	CCCCTACCTGGCGTGCCA	56	10	6	60.0	450-1050
2251	GAACAGGCGATGATACCA	56	9	8	88.9	550-1500
TOTAL			147	117	79.6	

gels, purified according to the manufacturer's protocol (Invitrogen, PureLink PCR Purification Kit), and cloned into pJET1.2 PCR cloning kit (Thermo Fisher Scientific Inc.). Transformation into *E. coli* DH5α strain was performed, the colonies were selected and grown under antibiotic selection. Plasmids were isolated with the aid of GeneJET Plasmid Miniprep Kit. The selected colonies were sequenced from both ends.

2.4. Data analysis

Data were scored based on the presence (1) and absence (0) of fragments and then formatted for Numerical Taxonomy Multivariate Analysis System software package (NTSYS-pc version 2.1) (Rohlf, 2000). Five different data files analyzed in this study are as follows: iPBS, SRAP, ISSR, RAPD and POGP markers. Six similarity matrices were constructed for each and combined data file based on markers and simple matching's coefficient by using SIMQUAL module nested in the program. Simple matching coefficient: $m = n$; where m is total number of 1–1 and 0–0 matches between two individuals; n is total number of loci scored. The similarity matrix was used to construct a dendrogram using the unweighted pair-group method arithmetic average (UPGMA) to determine genetic relationships among the accessions studied. The neighbor-joining (NJ) analysis was used to construct a dendrogram based on the NJ, a dissimilarity matrix calculated based on average taxonomic distance coefficient. It is based on the idea of parsimony and yield relatively short estimated evolutionary trees (Saitou and Nei, 1987). It produces an unrooted graph and estimates the length of each branch of the graph. Approach of MIDPOINT for rooting was used to draw the trees. These results in a tree that may not have equal branch length or path length may not be ultrametric.

Mantel's test (Mantel, 1967) was used to estimate correlation coefficients between each pair of similarity matrices using MXCOMP option. Comparisons were made between iPBS marker-based file and those of the others. It computes the product–moment correlation, r, and the Mantel test statistic, Z, to measure the degree of relationship between the two matrices. The number of permutations for each run was 10.000 and normalized Mantel test was used for comparisons in this study. The degree of fit can be interpreted subjectively as follows: $0.9 < r$, very good fit; $0.8 < r < 0.9$, good fit; $0.7 < r < 0.8$, poor fit; and $r < 0.7$, very poor fit (NTSYS PC 2.1 Manual). For iPBS primers, the mean polymorphism information contents (PIC) of each primer was calculated by using the following formula in excel: $PIC=\sum(1-Pi^2)/n$, where Pi is the frequency of presence (1) for each band, n is the number of bands for each primer. Mean number of bands obtained with iPBS primers among ploidy series was calculated as described by Gulsen et al. (2009).

The sequences of cloned PCR fragments were blasted against nucleotide database using National Center for Biotechnology Information (NCBI) (Altschul et al., 1990).

3. Results

The 14 iPBS primers reported by Kalendar et al. (2010) were used to screen the bermudagrass genotypes with known origins and ploidy series of diploids to hexaploids. Ten of eighteen 12-mers and four of twenty-three 18-mers primers yielded the highest polymorphism between the bulks made up of 6 to 13 individuals representing different ploidy levels (Table 1). The selected ten 12-mer and four 18-mer iPBS primers yielded an average bands of 7.9, 7.1, 7.5, 5.6, and 6.4 for diploids, triploids, tetraploids, pentaploids, and hexaploids. Contrary to the results obtained with SRAP, POGP, ISSR and RAPD markers (Gulsen et al., 2009), the mean number of bands obtained with iPBS primers among ploidy series did not increase linearly with ploidy level. This clearly shows that genome duplication via polyploidization does not correlate with the level of iPBS polymorphism or number of loci, probably due to their relatively recent expansion in bermudagrass genome. Band sizes ranged from 200 to 2000 bp. Fourteen iPBS primers produced 147 bands, 117 (80%) of which were polymorphic, with an average of 8.4 markers per primer among bermudagrasses. Among all accessions, both percentage of polymorphic bands (80%) and average band per amplification (8.4) in bermudagrasses exceeded those (76% and 6.75) of field pea (Baloch et al., 2015). iPBS marker analysis in bermudagrasses, on average, also produced more markers (8.4) per amplification than SRAP (5.4), POGP (6.5), and ISSR (7.6), but less than RAPD (10.6) (Gulsen et al., 2009). The average PIC of the iPBS was the highest and the mean PICs of iPBS, SRAP, POGP, ISSR and RAPD markers were 0.78, 0.64, 0.68, 0.51 and 0.76, respectively.

The iPBS-based UPGMA analysis clustered the 44 genotypes into two subclusters at similarity value of 0.57 (Figure 1). The cluster I included 3 of 6 diploids, 2 of 8 triploids, 7 of 13 tetraploids, 9 of 10 pentaploids and 2 of 7 hexaploids. The rest of were placed in cluster II along with *C. transvaalensis*. We observed a pattern of ploidy-based grouping for pentaploids and hexaploids. Besides, the four half-sib triploid hybrids (T1-A1, T4-A35, T4-G10, T6-D2) were closely placed in cluster II, pointing the efficiency of the analysis. In our previous studies, we have genotyped these 40 accessions with four other nuclear and two chloroplast markers, and none indicated this level of separation with such a low similarity value (0.57) (Gulsen et al., 2009; Gulsen and Ceylan, 2011). Overall, this analysis indicated that retrotransposons differently evolved among bermudagrasses presented in subclusters I and II, and they may better define history of polyploidization among bermudagrass than other markers. Karyotype asymmetry

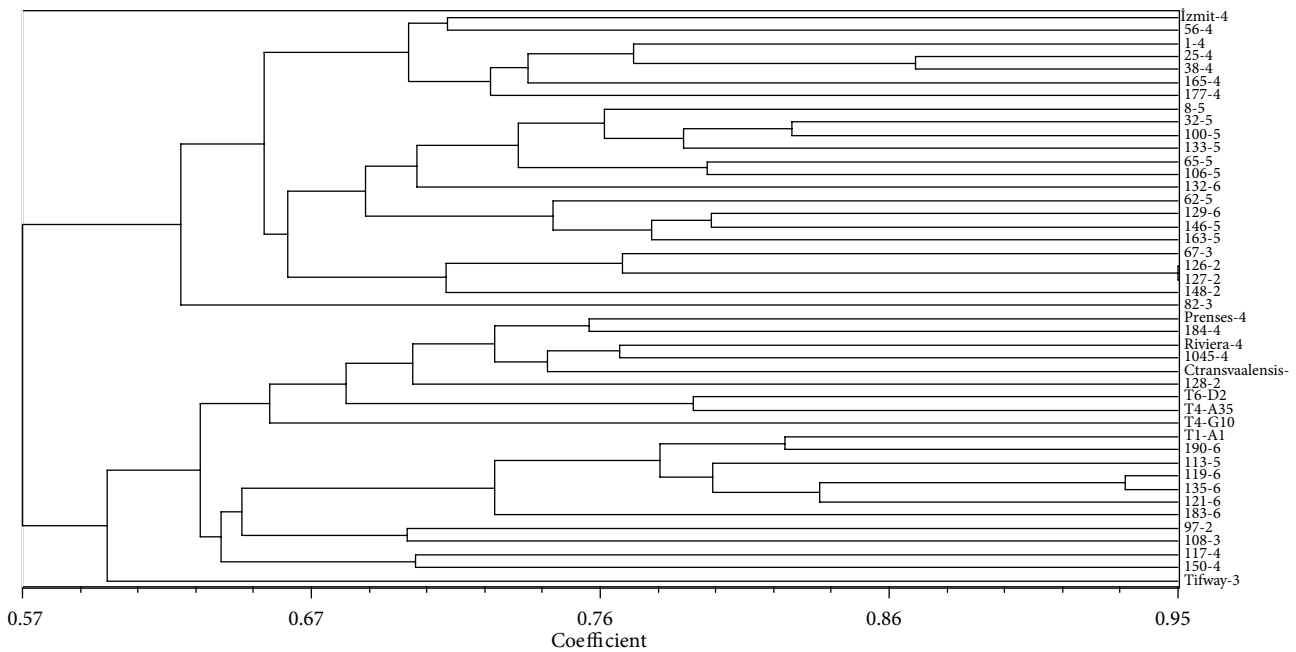

Figure 1. The UPGMA tree of the 44 *Cynodon* genotypes constructed by using 117 polymorphic retrotransposon iPBS markers. The genotypes included four triploid hybrids derived from crosses involving C. *transvaalensis* and tetraploid genotypes (T1-A1, T4-A35, T4-G10, T6-D2), four commercial cultivars (Prenses-4, Riviera-4, Tifway-3, 1045-4) and 35 accessions collected from eastern Mediterranean coast of Turkey (1-4, 8-5, 25-4, 32-5, 38-4, 56-4, 62-5, 65-5, 67-3, 82-3, 97-2, 100-5, 106-5, 108-3, 113-5, 117-4, 119-6, 121-6, 126-2, 127-2, 128-2, 129-6, 132-6, 133-5, 135-6, 146-5, 148-2, 150-4, 163-5, 165-4, 177-4, 183-6, 184-4, 190-6, İzmit-4) and *C. transvalensis*-2. The numbers after dash (-) indicate ploidy level of that genotype.

also divided *Cynodon* into two groups, one involving C. *dactylon* and C. *transvaalensis*, and the other C. *incompletus* (Chiavegatto et al., 2016).

For confirmation, iPBS fragments representing four different primer products were cloned and sequenced (Table 2). The two sequenced fragments belong to a diploid *C. transvaalensis*, one each of fragments to tetraploid (4X) and hexaploid (6X) genotypes, amplified using bulk DNA samples within ploidy groups. The BLAST results showed that the fragments derived from *C. transvaalensis* belonged to retroelement "Cassandra", 4X to "Copia Maximus", and 6X to "Gypsy" as well as "Copia Maximus" (Table 2). Three and one of the fragments were products of 12-mer and 18-mer iPBS primers, respectively. Although number of cloned and sequenced fragments is limited, the results indicated that iPBS primers targeted the retrotransposons as expected.

The NJ approach was also used to estimate relationships among the 44 *Cynodon* accessions using iPBS markers (Figure 2). Similar to the results of the UPGMA analysis, the NJ tree failed to discriminate ploidy series among the *Cynodon* accessions. Conversely to the UPGMA tree discussed above, it is difficult to place the genotypes into groups and the four triploid hybrids were scattered throughout the tree (Figure 2). For the UPGMA and the NJ analyses, we used simple matching and correlation option to calculate between pairwise distances, respectively. This may have caused differences between the results of two approaches. Seemingly, the results of the UPGMA analysis better explain our hypothesis compared to that of the NJ analysis. We also analyzed combined data of five marker systems (iPBS, SRAP, ISSR, RAPD and POGP markers) by using SM and UPGMA approaches to estimate whether this set of data would produce a similar output. The combined data included a total of 524 markers, of which 117, 182, 62, 85, 77 were iPBS, SRAP, ISSR, RAPD and POGP, respectively. Analysis of iPBS markers resulted in different clustering of diploids and pentaploids from the analyses carried out separately (Figure 3). Five of six diploids and ten pentaploids were closely clustered. The rest of the genotypes with triploids, tetraploids and hexaploids did not indicate any clustering pattern. This may verify stronger resolution of retrotransposon-based iPBS markers than other four nuclear genome based markers (SRAP, ISSR, RAPD and POGP). Discrimination power among marker systems can vary due to differences in evolutionary rates among genomic targets of marker systems. The findings reported above suggested that the results of iPBS markers considerably differed from those of four other nuclear genome markers. LTRs retrotranspose via an mRNA intermediate in a "copy-and-paste" process that can lead to extremely high copy numbers in the genome. Results suggest that various LTRs may have independently evolved within different genomes of *Cynodon* spp.

Correlation coefficients of so-called Mantel test among the similarity matrices were calculated to detect whether diversification of five marker systems among the bermudagrasses studied followed a similar pattern (Table 3). The iPBS marker-based similarity matrix was relatively loosely correlated with the matrices based on POGP (r = 0.29), RAPD (0.32), SRAP (0.33) and ISSR markers (0.38) (Table 3). However, the similarity matrices of the other four marker systems correlated significantly (r > 0.70). Garcia et al. (2004) and Gulsen et al. (2009) found the similar results in maize (*Zea mays* L.) and *Cynodon* accessions, respectively. Thus, the result strongly justifies inclusion of iPBS markers into phylogeny studies.

4. Discussion

Both autoploidy (Zeven, 1980) and segmental/complete alloploidy (Harris-Shultz et al., 2010; Jewell et al., 2012; Guo et al., 2015) were suggested for bermudagrasses. Genetic evidence on the polyploidization history of bermudagrass has been accumulating. Four previously evaluated nuclear genome marker systems largely failed to distinguish ploidy series among bermudagrasses (Gulsen et al., 2009). Gou et al. (2015) used over five-hundred first-generation selfed (S1) populations derived from selfing two tetraploid (2n = 4x = 36) bermudagrass, and run segregation analysis using 33 SSR markers. Because the SSR primer pairs amplified 34 loci with only two alleles, the results strongly suggested that the tetraploid bermudagrass parents possessed an allotetraploid genome with two distinct subgenomes (Guo et al., 2015). Furthermore, in a triploid mapping population, Harris-Shultz et al. (2010) showed that tetraploid parent had two alleles segregating at each locus for 15 SSR loci, suggesting the parent displayed disomic inheritance and cannot be an autotetraploid. Jewell et al. (2012) suggested that diploid *C. transvaalensis* may be a parent of tetraploid *C. dactylon*, supported by haplotypes obtained from chloroplast and mitochondrial genes where several *Cynodon* hybrids derived from crosses involving *C. transvaalensis* as maternal parent that grouped within the C. *dactylon* clade. Harlan and De Wet (1969) also noted that *C. transvaalensis* is very closely related to, and may even represent a variety of, C. *dactylon*. Gulsen and Ceylan (2011), based on the chloroplast and mitochondrial haplotypes of the accessions used in this study, indicated that the diploids had different organelle genome from the rest of the ploidy series, suggesting alloploidization and not autoploidization of bermudagrass, because tetraploid, pentaploid and hexaploid accessions did not share organelle genome of the diploids. This may suggest polyphyly origin for most bermudagrasses, and that the diploid accessions defined in this study and in earlier

Table 2. Interprimer binding site (iPBS) primer sequences, and BLAST results of sequences derived from cloned iPBS fragments.

Sample ID	Primers	Sample source	Sequence ID	Retrotransposon familiy	Sequence (5' - 3')	Identity	Query	Clone name
Brmd1	2074 (12-mer) F 5' GCTCTGATACCA 3' R 5' GCTCTGATACCA 3'	Transvaalensis	KM262797.1	TRIM LTR-Retroelemenets "Cassandra" has origin from 5s rRNA gene	**GCTCTGATACCA**TCTATCATACCCCTCCCGCGGAGG AGTACCGTGCATCCTGTCCCAATCCCTGGATCAGTT GTTGGTACGCACAATCAGGTAGTCTCAAATTCATAC AGGAACTAGAATACGAGGAAAAGCGGAAGACTCA CCAATCAATACTAGGATACAATCCAGAAGAGGCAG CGAAACTGGGCATGATCAACACTTCAATTCACAT GTAAACTTGAGCATACGACGAAACCTGTGACATC CCAGGGATGTCCATCCGGCCCGTAACCTAGAATC GGCCCGTGTTCAGCCCGGTATTGGGCGCGGCAC TGTTCACCAAGCACCGTACACGAGGTGTAATGTA GCGTCGGGGGGGTACTGTAGCTGCGGAACACTGT AGCATCGCGGCGCACTGTAGCATCAGTGTTAGT CCAATACTGGAAATCGACTTGGAGCTCGACTAG CTTAAATACTCGGTCCGTGGTAGCAGTAGTAACC ATGTTAACCCTTTTCCGCGAAGCGAGGACGAAA CCGTAAGAGTTGCTACTGCCAGGTAAGGCTTGCA CTGCAGGCGTACGGGCCTGATGCCGTGCCCTGG GCCGGGACGTTACAGAA**TGGTATCAGAGC**	27/27 (100%)	582-608	Panicum virgatum (Switchgrass) clone

Table 2. (Continued).

Brmd13	2375 (12-mer) 5' TCGCATCAACCA 3' 5' TCGCATCAACCA 3'	6x Bulk	KX608914.1	LTR Gypsy and LTR Copia Maximus	**TCGCATCAACCA**TTTAAATTCCCCGATTCACGG AACCGATTCCATTCCCTTGCAACTTACTAGAA ATAATTCCCTTGTCATTCATAATCTTGAGGAA GCACCCATTGAGAGTATCCCTGTGGTCTCTG AATTTTTGGATGTGTTTCCGGAAGATCTCCC GGGAATGCCACCCGACCGGGAAGTCGAATT TAAAATCGAGTTGAAACCGGGAACTTCACC CGTCTATCGCCCACCATACAAGATGCTTCTC AATGAATTGACCGAGCTGAAAATCCAATTAA ATGAATTATTGGAGAAGGGGTTCATTAGGCC AAGCTCATCATCTTGGGACTGCCCCGCTATG TTTGTGAAAAAGAAGGACAAGTCGCTATGC CTTGTTGTCGACTATCGGCCTTTAAATGAAG TGACCATCAAAAATAAATACCTGCTGCCCC GGATTGATATATTATTTGATCAAT**TGGTTGATGCGA**	230-318 (72%)	143-382	Saccharum hybrid cultivar R570 isolate
Brmd13		6x Bulk	JN800040.1	RLG_scDEL_1.4 retrotransposon (Gypsy subfamiliy?)	‘’	176-241 (73%)	127-367	‘’
Brmd13		6x Bulk	JN800042.1	RLG_scDEL_1.6 retrotransposon	‘’	175-241 (73%)	127-367	‘’
Brmd13		6x Bulk	JN800038.1	RLG_scDEL_1.2 retrotransposon	‘’	230-325 (71%)	136-460	‘’
Brmd13		6x Bulk	HQ116788.1	BAC SHCRBa_201_A23 transposon mutator	‘’	230-325 (71%)	136-460	‘’
Brmd13		6x Bulk	KX608907.1	LTR Gypsy scDEL_1.1 /DNA transposon, Retrotransposons LTR Copia scMaximus 1.1	‘’	243-345 (70%)	112-434	‘’
Brmd13		6x Bulk	JN800044.1	RLG_scDEL_2.2 retrotransposon	‘’	243-346 (70%)	112-457	‘’
Brmd13		6x Bulk	DQ3810634.1	Ty3 Gypsy retrotransposon reverse transcriptase gene	‘’	66-82 (80%)	379-460	Phelipanche bungeana clone pPbugy8
Brmd13		6x Bulk	DQ381013.1	Ty3 Gypsy retrotransposon reverse transcriptase gene	‘’	66-82 (80%)	379-460	Oronbanche crenata clone p0crGgy7
Brmd13		6x Bulk	EF562447.1	Retrotransposon danelle polyprotein gene	‘’	78-101 (77%)	113-213	Zea mays clone
Brmd13		6x Bulk	GQ252887.1	Putative retrotransposon proteins	‘’	57-69 (83%)	296-364	Phyllostachys edulis clone

Table 2. (Continued).

Brmd13		6x Bulk	EU098296.1	Retrotransposon Gorge 3 reverse transcriptase (Pol) gene	**TCGCATCAACCA**ATTGATCAAATAATATATCA ATCCGGGGCAGCAGGTATTTATTTTTGATGG TCACTTCATTTAAAGGCCGATAGTCGACAAC AAGGCATAGCGACTTGTCCTTCTTTTTCACA AACATAGCGGGGCAGTCCCAAGATGATGAGC TTGGCCTAATGAACCCCTTCTCCAATAATTCA TTTAATTGGATTTTCAGCTCGGTCAATTCATT GAGAAGCATCTTGTATGGTGGGCGATAGACG GGTGAAGTTCCCGGTTTCAACTCGATTTTAA ATTCGACTTCCCGGTCGGGTGGCATTCCCGG GAGATCTTCCGGAAACACATCCAAAAATTCA GAGACCACAGGGATACTCTCAATGGGTGCTT CCTCAAGATTATGAATGACAAGGGAATTATT TCTAGTAAGTTGCAAGGGAATGGAATCGGTT CCGTGAATCGGGGAATTTAAA**TGGTTGATGCGA**	52-63 (83%)	14-76	Gossypiodes kirkii isolate
Brmd22 (F)	2251 (18-mer) 5'GAACAGGCGATGATACCA3' 5'GAACAGGCGATGATACCA3'	Transvaalensis	KM262797.1	Retrotransposon "Cassandra"	**GAACAGGCGATGATACCA**TCTATCATACCC CTCCCGCGGAGGAGTACCGTGCATCCTGT CCCGATCCCTGGATCAGTTGTTGGTACGC ACAATCAGGTAGTCTCAAATTCATACAGG AACTAGAATACGAGGAAAAGCGGAAGACT CACCAATCAATACTAGGATACAATCCAGAA GAGGCAGCGAAACTGGGCATGATCAACAC TTCAATTCACATGTAAACTTGAGCATACGA CGAAACCTGTGACATCCCAGGGATGTCCA TCCGGCCCGTAACCTAGAATCGGCCCGTG TTCAGCCCGGTATTGGGCGCGGCACTGTT CACCAAGCACCGTACACGAGGTGTAATGT AGCGTCGGGGGGTACTGTAGCTGCGGAA CACTGTAGCATCGCGGCGCACTGTAGCA TCAGTGTTAGTCCAATACTGGAAATCGAC TGGGAGCTCGACTAGCTTAAATACTCGGT CCGTGGTAGCAGTAGTAACCATGTTAACC CTTTTCCGCGAAGCGAGGACGAAACCGT AAGAGTTGCTACTGGCAGGTAAGGCTTGC ACTGCAGGCGTACGGGCCCTGATGCCGTG CCCTGGGGCGGGACGTTACAGAA**TGGTAT CATCGCCTGTTC**	25-25 (100%)	587-611	Panicum virgatum clone

Table 2. (Continued).

Brmd28 (F+R)	2076 (12-mer) 5' GCTCCGATGCCA 3' 5'GCTCCGATGCCA 3'	4x Bulk	KX608895.1	Retrotransposons LTR Copia Maximus Sh029N14_te00005, LTR Gypsy Tat Sh029N14_te00007, and LTR Copia Maximus Sh029N14_te00005, probable calcium-activated outward-rectifying potassium channel 5 (Sh029N14_g0100) pseudogene, and retrotransposon LTR Gypsy Sh029N14_te00010, complete sequence; Phytochrome C (Sh029N14_g0130) gene, complete cds; retrotransposons LTR Copia scMaximus 3.1 Sh029N14_te00015 SOLO and LTR Gypsy Sh029N14_te00020, complete sequence; and hypothetical protein (Sh029N14_g0290) gene, complete cds	**GCTCCGATGCCA**TTGCTTGCTGTTTTCCAA ATT AACTTCCGCTGCGCACTCATGAGAAAG CTTGC CTAGAATCTGAACAAAAATTTGGAG AACTTGT ATCTTGTTTAGGCTATGTTTAAAT TACTGGAAC TGCTGCCCCGTCCCCTTGCCG TTAATTCCAGAAACTTGTAACCTGGGAGAA TATTGTTGGAAACTTTTAATGTACCTTTATG TAACCTTAATATTTGTATATTGTGGTCATTT GTGATGAGAAAATGTTGTGACCGATGTGCT GTGTTCCTGGGCTTACTTGGAAGCATGTCG GGGGACTACCGGAATTATATTCCATTTAGTC GGGTTGGGCTTGCAATTCCCGTTCATGGAC AGCGAGCTAACTTGACTAAGTTGAATGTAAT TTGGACGGTTCCTTACAGCG**TGGCATCGGA GC** (F)**GCTCCGATGCCA**CGCTGTAAGGAACC GTCCAAATTACATTCAACTTAGTCAAGTTAG CTCGCTGTCCATGAACGGGAATTGCAAGCC CAACCCGACTAAATGGAATATAATTCCGGTA GTCCCCCGACATGCTTCCAAGTAAGCCCAG GAACACAGCACATCGGTCACAACATTTTCT CATCACAAATGACCACAATATACAAATATTA AGGTTACATAAAGGTACATTAAAAGTTTCCA ACAATATTCTCCCAGGTTACAAGTTTCTGGA ATTAACGGCAAGGGGACGGGGCAGCAGTTC CAGTAATTTAAACATAGCCTAAACAAGATAC AAGTTCTCCAAATTTTTGTTCAGATTCTAGG CAAGCTTTCTCATGAGTGCGCAGCGGAAGT TAATTTGGAAAACAGCAAGCAA**TGGCATCG GAGC** (R)	95%	8% (1-36)	Saccharum hybrid cultivar R570 isolate

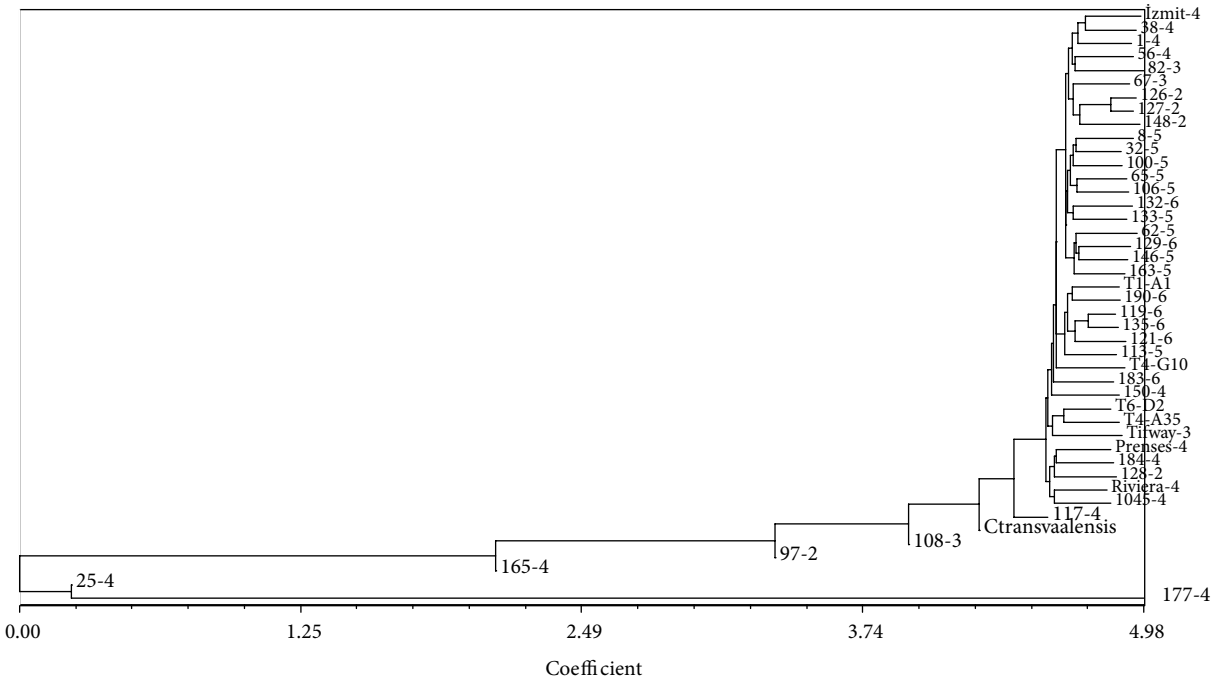

Figure 2. Neighbor-joining tree of the 44 *Cynodon* genotypes constructed by using iPBS markers. The genotypes included four triploid hybrids derived from crosses involving C. *transvaalensis* and tetraploid genotypes (T1-A1, T4-A35, T4-G10, T6-D2), four commercial cultivars (Prenses-4, Riviera-4, Tifway-3, 1045-4) and 35 accessions collected from eastern Mediterranean coast of Turkey (1-4, 8-5, 25-4, 32-5, 38-4, 56-4, 62-5, 65-5, 67-3, 82-3, 97-2, 100-5, 106-5, 108-3, 113-5, 117-4, 119-6, 121-6, 126-2, 127-2, 128-2, 129-6, 132-6, 133-5, 135-6, 146-5, 148-2, 150-4, 163-5, 165-4, 177-4, 183-6, 184-4, 190-6, İzmit-4) and *C. transvalensis*-2. The numbers after dash (-) indicate ploidy level of that genotype.

studies (Gulsen et al., 2009; Gulsen and Ceylan, 2011) may be paternal parents of accessions with higher ploidy levels, and that diploid maternal parent has either been lost or not identified within Turkish accessions, sampled from the Mediterraneaen coastal region spanning more than 1000 km.

From evolutionary perspective, retrotransposons of bermudagrasses probably evolved before polyploidization occurred among the progenitors of bermudagrasses and diversification does not follow distinctive pattern. Alternatively, retrotransposons might have evolved more rapidly than the genomic regions that the four marker systems targeted. In addition, correlation between RAPD and the other markers (SRAP, ISSR and POGP) was lower (0.71) whereas the results of the SRAP, POGP and ISSR markers were higher ($r > 0.85$), which was consistent with the results of Garcia et al. (2004) in maize (*Zea mays* L.).

Here we studied retrotransposon-based iPBS markers for genetic analyses of bermudagrasses with a series of ploidy levels. Transposable elements, present abundantly up to 85% of plant genomes, may generate more genomic diversity due to variable conservations among genomic regions and are thus an excellent source of DNA polymorphism. They can be informative source of genetic variation. The iPBS of retrotransposons could reflect more variations because of their higher evolutionary rate compared to those of the other marker types used in this study. Combined analysis of SRAP, POGP, ISSR and RAPD markers placed all diploids in the same cluster (Gulsen et al., 2009) whereas iPBS markers tented to group pentaploids and hexaploids in addition to inserting all four triploid hybrids in the same cluster. This may mean more discriminatory power of iPBS markers than four other marker systems. Plastid DNA markers concluded similar findings as the four nuclear markers (Gulsen and Ceylan, 2011).

Copy-and-paste proliferation of a few long terminal repeat retrotransposons (LTRs) contributes to significant part of plant genomes (Schnable et al., 2009; Lee and Kim, 2014). As dispersed, ubiquitous, and very rapidly evolving nature of transposable elements, LTRs may have a high potential in phylogenetic analysis. The iPBS markers targeting retroposons have been already employed in flax (Smykal et al., 2011), apricot (Baránek et al., 2012), grape (Guo et al., 2014), and okra (Yıldız et al., 2015; Kantar et al., 2021) for genetic analyses. We evaluated and compared the selected germplasm of bermudagrasses (with known origins and ploidy levels) with iPBS markers.

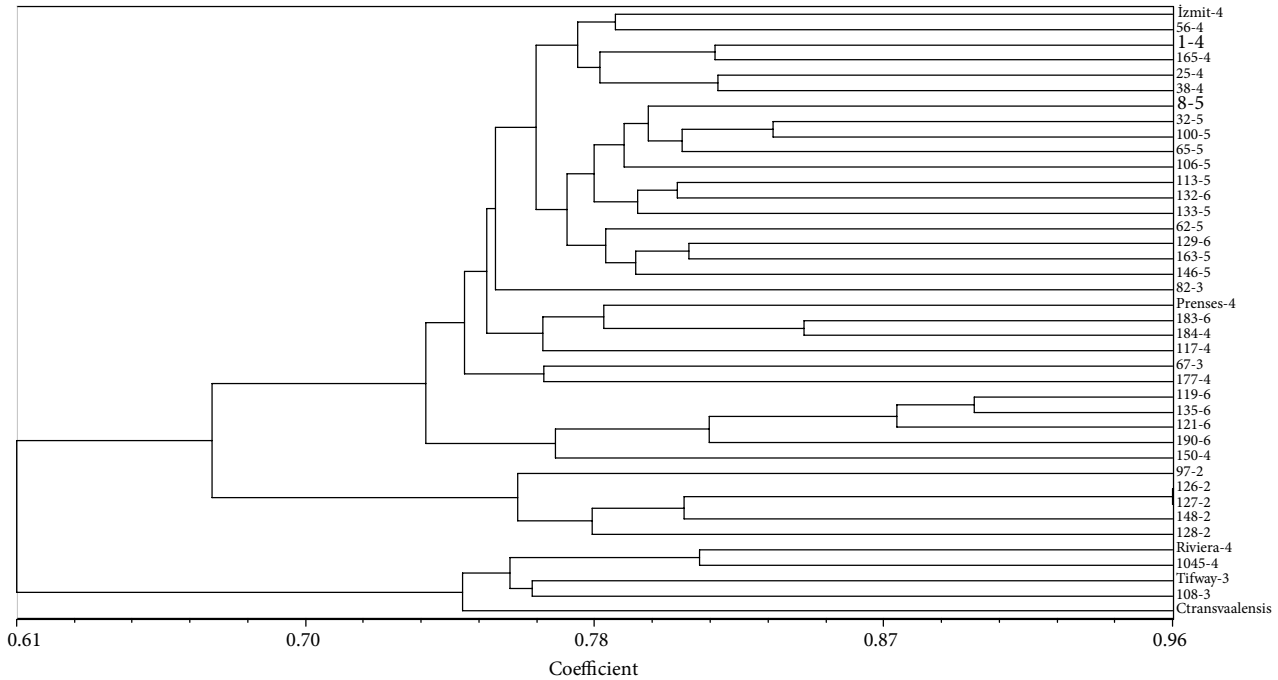

Figure 3. The UPGMA tree of the 40 *Cynodon* genotypes constructed by using data of five marker systems (iPBS, SRAP, ISSR, RAPD and POGP markers) including retrotransposon markers obtained in this study. The genotypes included four commercial cultivars (Prenses-4, Riviera-4, Tifway-3, 1045-4) and 35 accessions collected from eastern Mediterranean coast of Turkey (1-4, 8-5, 25-4, 32-5, 38-4, 56-4, 62-5, 65-5, 67-3, 82-3, 97-2, 100-5, 106-5, 108-3, 113-5, 117-4, 119-6, 121-6, 126-2, 127-2, 128-2, 129-6, 132-6, 133-5, 135-6, 146-5, 148-2, 150-4, 163-5, 165-4, 177-4, 183-6, 184-4, 190-6, İzmit-4) and *C. transvalensis*-2. The numbers after dash (-) indicate ploidy level of that genotype.

5. Conclusion

The genus *Cynodon* includes a series of ploidy levels ranging from diploids to hexaploids. In this study, the selected bermudagrasses with known origins and ploidy levels were evaluated with iPBS markers in comparison to four other molecular markers. The analyses and comparisons in this study concluded that 1) retrotransposon-based iPBS markers have more resolution power than the four nuclear (SRAP, ISSR, RAPD and POGP) and two chloroplast genome-based markers (cpDNA RFLP and cpDNA SSR) as revealed by UPGMA analysis, 2) retrotransposon-based iPBS markers draw different relationships from the other nuclear genome markers as verified by Mantel tests, 3) iPBS marker analyses based on UPGMA clearly separated bermudagrasses into two subclusters, possibly suggesting the presence of two distinct subgenomes as shown by karyotype asymmetry indices. Retrotransposons probably evolved differently among diploid progenitors of bermudagrass.

Taxonomic classification of *Cynodon* remains unclear despite agreement on ten species. The most prominent finding in this study was different evolutionary relationships as verified by Mantel test and the UPGMA analyses. This may help our understanding of bermudagrass evolution. Seemingly, retrotransposon-based iPBS markers used in this study for genetic analyses of bermudagrasses have advantages because they target genomic region covering up to 85% of plant genomes and possibly generate more genomic diversity due to less conservation among genomic regions and are thus an excellent source of DNA polymorphism.

Even though pattern of disomic inheritance has been related to allopolyploidy in C. *dactylon* polyploids, genomic and chromosomal evidence has yet to lead identification and differentiation of progenitor diploid species. Elucidation of genomic organization via gene families may better aid in understanding the evolutionary history of speciation and polyploidization. New studies on chloroplast/mitochondrial genomes may shed lights to maternal origins of polyploids while karyotyping and gene family based genotyping may aid in predicting the paternal ones.

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Conflict of interest

The authors declare that no competing interests exist.

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