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Evaluation of Diversity Among *Cynodon dactylon* (L.) Pers. Using RAPD Markers

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ABSTRACT

Cynodon dactylon (L.) Pers. is a wide geographic distributed species, which is known to be originated from Middle East. Out of 18 tested decamer arbitrary sequenced primers, 7 primers with high polymorphic bands were applied on DNA samples of 75 accessions of *Cynodon dactylon* from different parts of Iran for profiling the diversity between them. A total of 68 polymorphic amplified DNA fragments were obtained. A minimum similarity of 11% was obtained between 24 - Is and 30 - Ir, and maximum similarity of 80% was observed between 39 - IL and 41 - IL accessions using Jaccard's coefficient. The UPGMA cluster generated by similarity coefficient grouped the accessions in to 7 major clusters consistent with their geographical locations, chromosome number, and some morphological characteristics. This study showed clearly the potential of RAPD markers for identification and management of *Cynodon dactylon* for breeding programs. Extensive germplasm collection and more primers are required to gain more precise results.

Key Words: Accessions; Bermuda-grass; Germ plasm; Similarity

INTRODUCTION

The genus *Cynodon* contains grasses of economic importance for livestock grazing, turf and soil conservation (Assefa *et al.*, 1999). Bermuda-grass [*Cynodon dactylon* (L.) Pers] is one of the most widely used warm season turf-grass, because of its wide range of texture, high sod density, high wear, drought and salt tolerance and multi purpose use (Beard, 1973; Ackerson & Yongner, 1975; Dudeck *et al.*, 1983; Kim, 1987).

The center of diversity for some races of *Cynodon dactylon* L. was reported to be Turkey, Iran, Afghanistan and west part of Pakistan, which can be rich of desirable genes and genotypes (Harlan & de Wet, 1969; Harlan, 1970). Therefore, characterizing its germplasms for genetic diversity is an essential step in selection and breeding of this grass. DNA molecular markers can be used to evaluate genetic variation with in and among plant taxa, which have gained wide spread popularity (Paterson *et al*., 1991; Yang *et al*., 1996). Several DNA marker techniques have been used to assess nucleotide sequence variation in different grasses. Randomly amplified polymorphic DNA (RAPD) has been used as a tool to evaluate diversity among plants with in populations of several grasses including switch-grass (Gunter *et al*., 1996), perennial rye-grass (Huff, 1997), creeping bent-grass (Golembiewsk *et al.*, 1997), zoysia-grass (Caetano-Anolles *et al*., 1991) and buffalo-grass (Wu & Line, 1994). The ability of DNA profiling to discriminate between genetically different *Cynodon* plants is documented (Caetano-Anolles *et al*., 1995; Caetano-Anolles, 1998; Assefa *et al*., 1999; Roodt *et al*., 2002; Wu *et al*., 2004). In the present study, RAPD markers were used to evaluate diversity of 75 bermuda-grass accessions collected from different regions of Iran.

MATERIALS AND METHODS

Plant materials and chromosome counting. Stolones of seventy-five accessions of *Cynodon dactylon* (L.) Pers. were collected from Isfahan, Charmahal and Bakhtiari, Gilan and Mazandaran provinces, Iran (Table I). Stolons of each accessions and Tifdwarf (*Cynodon dactylon* × *Cynodon transvaalensis*) were grown in 20 cm diameter pots under green-house conditions. After establishment, the accessions were identified as *Cynodon dactylon* at the herbarium of Department of Botany, University of Isfahan. Somatic chromosome counting was conducted from meristemic root tips according to Agayev (1998), using at least 5 cells (Assefa *et al*., 1999).

DNA extraction and RAPD conditions. DNA was extracted from fresh leaf tissues of 75 *Cynodon dactylon* and Tifdwarf according to Dellaporta *et al*. (1983). Eighteen single oligonucleotide primers from sets A, B and C (Operon technologies, Inc, USA), were used to amplify DNA fragments of the genotypes. PCR reaction was performed in a Biometra (T - 1 Theroblock, Germany) Thermal Cycler, using 25 µL PCR mixture containing 2.5 µL of 1X reaction buffer (100 mm Tris - HCl, 15 mm $MgCl_2$, 500 mm KCl, pH 8.3), 0.5 mm $MgCl_2$, 200 µm each of dATP, dGTP, dCTP and dTTP, one unit of *Taq* DNA polymerase (Roche, Co. Germany) 0.4 µm of 10 mer primer, and 100 ng of template DNA, overlaid with 25 µL

of sterile mineral oil. The amplification condition was: initial step of denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 92°C for 1 min, primer annealing at 35°C for 1 min and extension at 72°C for 2 min, followed by an extended elongation step at 72°C for 5 min. Samples of 25 μL PCR products were mixed with 5 μL of 6X loading buffer (0.25% Bromo-phenol blue, 0.25% Xylene Cyanol & 40% sucrose) and spin briefly in a micro-centrifuge before loading (Sambrook & Russell, 2001). The amplification products were analyzed on 1.2% MP agarose gel (Roche, Co. Germany) in 1X TBE buffer running at 60 volts for three hours followed by staining in ethidium bromide (0.5 μgmL^{-1}), visualizing under UV light and photographing (Vilber Lourmat, France).

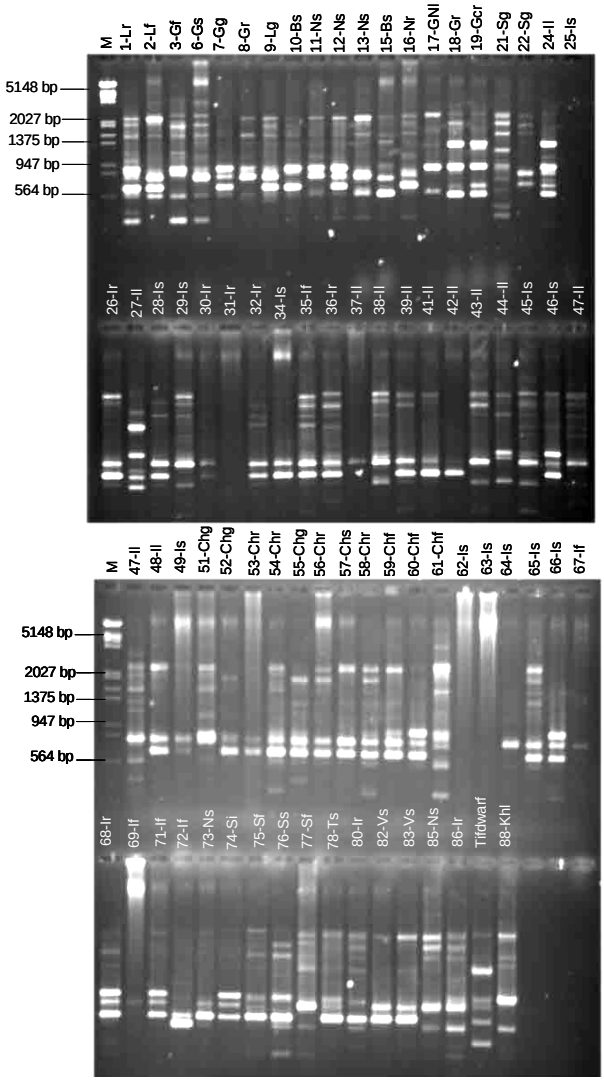
Data analysis. Each amplificated product was considered as RAPD marker and gels were scored on the basis of the present (1) absent (0) or ambiguous (9) of each band for all accessions. All amplifications were repeated at least twice and only reproducible and scorable bands were considered for analysis. Jaccard's similarity coefficient values for each pairwise comparison between accessions were calculated and a similarity coefficient matrix was constructed. This matrix was subjected to un-weighted paired group method with arithmetic averages (UPGMA) to generate a dendrogram using average linkage procedure by software NTSYS-pc version 2.02 (Raholf, 1998).

RESULTS AND DISCUSSION

Chromosome counting. The majority of the tested accessions had 2n = 4x = 36 chromosome. However, two accessions (17 - GNi & 88 - Khl) with 2n = 2x = 18 and one accession (44 - Il) with 2n = 3x = 27 were exceptions (Table I). The observed basic chromosome number of *Cynodon dactylon* was x = 9, which was in agreement with that of reported by Harlan and de Wet (1969) and Silva and Snaydon (1995). Most of *Cynodon dactylon* accessions were reported to be tetraploid (Harlan *et al*., 1970; Taliaferro, 1995), some of which diploid (Harlan *et al*., 1970; Taliaferro, 1995) and seldom triploid (Harlan & de Wet, 1969).

RAPD analysis. Based on preliminary screening, primers producing a higher level of polymorphism and reproducible fragment patterns were selected and used. Among the 18 primers, 7 revealed polymorphism in the examined accessions. A total of 68 readable and reproducible polymorphic fragments were produced from the selected seven primers. The number of amplified fragments varied from 7 - 13 with an average of 9.71 fragments per primer having a range of 380 bp to 3 kb fragment size (Table II & Fig. 1). The UPGMA cluster tree generated by similarity coefficients grouped the 75 accessions and Tifdwarf in to 7 major clusters designated A, B, C, D, E, F and G in similarity coefficient of 0.39 (Fig. 2). Using Jaccard's coefficient the highest genetic distance of 11% was obtained

Fig. 1. A RAPD gel profile of *Cynodon dactylon* L. accessions generated by primer OPB08

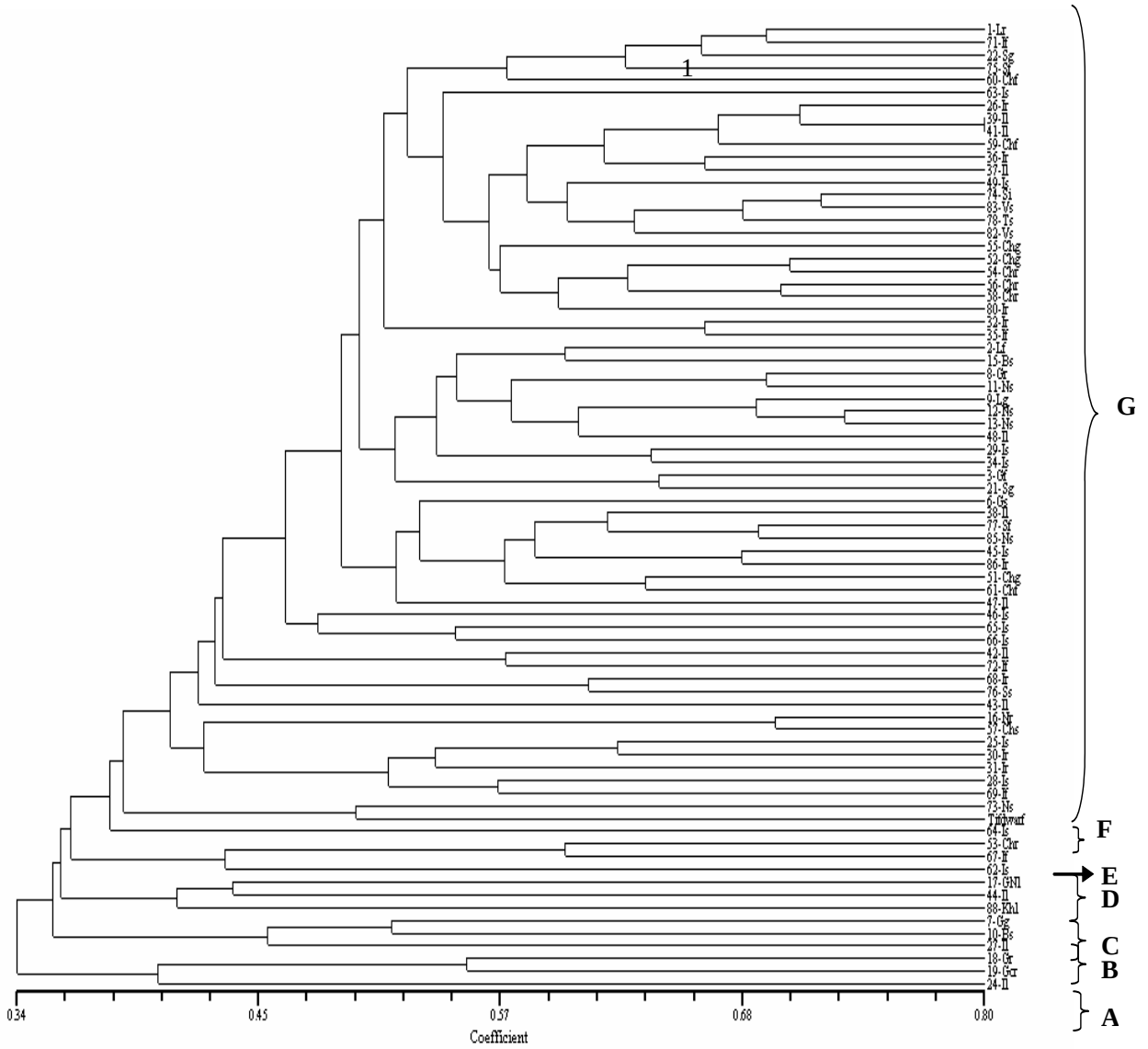


for 24 - Is and 30 - Ir accessions and the highest similarity of 80% was observed for 39 - Il and 41 - I1 accessions. These results indicated a high genetic diversity among the studied accessions of *Cynodon dactylon*, which is consistent with that observed by Caetano-Anolles *et al*. (1995), Assefa *et al*. (1999), Roodt *et al*. (2002) and Wu *et al*. (2004). Cluster A included 3 tetraploid accessions, two of which (18 - Gr & 19 - Gcr) were from subtropical and semi-humid (Annual rainfall 1800 mm) regions and 24 - Il collected in Isfahah, it may be transferred from subtropical region by seed. These accessions have also some similar morphological characteristics such as width of leaf and stolon color. Genetic similarity coefficients (SC) values for accessions in cluster A ranged from 0.39 to 0.55. Accessions in other clusters collected from temperate zone except 17 - GNl that had diploid chromosome and Tifdwarf that was a hybrid cultivar. Cluster B comprised 3 tetraploid accessions (7 -

Table I. List of *Cynodon dactylon* L. accessions used for RAPD analysis.

Accessions	Collected Site (Province - City)	Location	Altitude (m)	Annual Rainfall (mm)	Mean Daily Temperature (°C)	Chromosome No.
1- Lr	Charmahal and Bakhtiari- Lordgan	Road side	1900	551	17.5	36
2- Lf	Charmahal and Bakhtiari- Lordgan	Field	1900	551	17.5	36
3- Gf	Charmahal and Bakhtiari- Borujen	Field	2200	245	12.8	36
6- Gs	Charmahal and Bakhtiari- Borujen	Riverside	1930	245	12.8	36
7- Gg	Charmahal and Bakhtiari- Borujen	Grassland	1930	245	12.8	36
8- Gr	Charmahal and Bakhtiari- Borujen	Roadside	1930	245	12.8	36
9- Lg	Charmahal and Bakhtiari- Lordegan	Grassland	1930	551	17.5	36
10- Bs	Charmahal and Bakhtiari- Lordegan	Riverside	1900	551	17.5	36
11- Ns	Isfahan- Natanz	Riverside	1800	198	16.4	36
12- Ns	Isfahan- Natanz	Riverside	1800	198	16.4	36
13- Ns	Isfahan- Natanz	Riverside	1800	198	16.4	36
15- Bs	Isfahan- Natanz	Riverside	1300	198	16.4	36
16- Nr	Isfahan- Natanz	Roadside	1780	198	16.4	36
17- GNl	Mazandaran - Neka	Lawn	15	895	16.8	18
18- Gr	Gilan- Chaboksar	Roadside	-15	1220	15.9	36
19- Gcr	Gilan- Chaboksar	Roadside	-15	1220	15.9	36
21- Sg	Charmahal and Bakhtiari- Shahrecord	Grassland	2060	317	12.5	36
22- Sg	Charmahal and Bakhtiari- Shahrecord	Grassland	2060	317	12.5	36
24- Il	Isfahan- Isfahan	lawn	2060	122	16.2	36
25- Is	Isfahan- Isfahan	Riverside	1577	122	16.2	36
26- Ir	Isfahan- Isfahan	Roadside	1577	122	16.2	36
27- Il	Isfahan- Isfahan	lawn	1577	122	16.2	36
28- Is	Isfahan- Isfahan	Riverside	1577	122	16.2	36
29- Is	Isfahan- Isfahan	Roadside	1577	122	16.2	36
30- Ir	Isfahan- Isfahan	Roadside	1577	122	16.2	36
31- Ir	Isfahan- Isfahan	Roadside	1577	122	16.2	36
32- Ir	Isfahan- Isfahan	Field	1580	122	16.2	36
34- Is	Isfahan- Isfahan	Roadside	1580	122	16.2	36
35- If	Isfahan- Isfahan	Field	1580	122	16.2	36
36- Ir	Isfahan- Isfahan	Roadside	1580	122	16.2	36
37- Il	Isfahan- Isfahan	Lawn	1573	122	16.2	36
38- Il	Isfahan- Isfahan	Lawn	1573	122	16.2	36
39- Il	Isfahan- Isfahan	Lawn	1573	122	16.2	36
41- Il	Isfahan- Isfahan	Lawn	1573	122	16.2	36
42- Il	Isfahan- Isfahan	Lawn	1573	122	16.2	36
43- Il	Isfahan- Isfahan	Lawn	1570	122	16.2	36
44- Il	Isfahan- Isfahan	Lawn	1570	122	16.2	27
45- Is	Isfahan- Isfahan	Riverside	1570	122	16.2	36
46- Is	Isfahan- Isfahan	Riverside	1570	122	16.2	36
47- Il	Isfahan- Isfahan	Lawn	1570	122	16.2	36
48- Il	Isfahan- Isfahan	Lawn	1570	122	16.2	36
49- Is	Isfahan- Isfahan	Riverside	1570	122	16.2	36
51- Chg	Isfahan- Chadegan	Grassland	2150	317	10.2	36
52- Chg	Isfahan- Chadegan	Grassland	2150	317	10.2	36
53- Chr	Isfahan- Chadegan	Roadside	2150	317	10.2	36
54- Chr	Isfahan- Chadegan	Roadside	2160	317	10.2	36
55- Chg	Isfahan- Chadegan	Grassland	2180	317	10.2	36
56- Chr	Isfahan- Chadegan	Roadside	2180	317	10.2	36
57- Chs	Isfahan- Chadegan	Riverside	2170	317	10.2	36
58- Chr	Isfahan- Chadegan	Roadside	2170	317	10.2	36
59- Chf	Isfahan- Chadegan	Field	2160	317	10.2	36
60- Chf	Isfahan- Chadegan	Field	2160	317	10.2	36
61- Chf	Isfahan- Chadegan	Field	2160	317	10.2	36
62- Is	Isfahan- Isfahan	Riverside	1565	122	16.2	36
63-Is	Isfahan- Isfahan	Riverside	1565	122	16.2	36
64- Is	Isfahan- Isfahan	Riverside	1565	122	16.2	36
65- Is	Isfahan- Isfahan	Riverside	1565	122	16.2	36
66- Is	Isfahan- Isfahan	Riverside	1565	122	16.2	36
67- If	Isfahan- Isfahan	Field	1550	122	16.2	36
68- Ir	Isfahan- Isfahan	Roadside	1500	122	16.2	36
69- If	Isfahan- Isfahan	Field	1550	122	16.2	36
71- If	Isfahan- Isfahan	Field	1470	122	16.2	36
72-If	Isfahan- Isfahan	Field	1470	122	16.2	36
73- Ns	Isfahan- Najafabad	Riverside	1600	151	15.2	36
74- Sl	Isfahan- Shareza	Lawn	1600	140	16.1	36
75- Sf	Isfahan- Semirom	Field	1800	371	11.5	36
76- Ss	Isfahan- Semirom	Riverside	2400	371	11.5	36
77- Sf	Charmahal and Bakhtiari- Saman	Field	1900	337	13.5	36
78- Ts	Isfahan- Tiran	Riverside	1900	14.5	175	36
80- Ir	Isfahan- Isfahan	Roadside	1500	122	16.2	36
82- Vs	Isfahan- Varzane	Riverside	1400	73.3	15.4	36
83- Vs	Isfahan- Varzane	Field	1420	73.3	15.4	36
85- Ns	Isfahan- Naien	Riverside	2400	103	18.6	36
86- Ir	Isfahan- Isfahan	Riverside	1550	122	16.2	36
88- Khl	Isfahan- Khomeinishahr	Lawn	1560	130	15.8	18

Fig. 2. UPGMA dendrogram depicting patterns of genetic diversity for 75 *Cynodon dactylon* L. accessions and Tifdwarf by RAPD marker



Gg, 10 - Bs & 27 - Il) that have the same number of spicklets and leaf width, Genetic SC values among the accessions with in the cluster B were very similar, ranging from 0.45 to 0.52. Cluster C consisted of 17 - GNl and 88 – Khl, which are diploids and 44 - Il (triploid) accessions. This showed the ability of DNA profiling discriminated between genetically different chromosome number of *Cynodon dactylon* plants, as reported by Assefa *et al.* (1999) and Anderson *et al.* (2001). Wu and Lin (1994) also succeeded in distinguishing diploid and hexaploid lines of buffalograss using RAPD analysis. These accessions had similar ligule length. Genetic SC values for accessions in cluster C ranged from 0.40 to 0.44. Accessions in other clusters were tetraploid, except Tifdwarf. Cluster D consisted of 53 - Chr, 62 - Is and 67 - If accessions that had some similar morphological characteristics, such as leaf width, length of ligule and flower stem and stolon color. The genetic SC values for these accessions ranged from 0.38 to 0.66. Accessions in this cluster had longer ligule length than other clusters. Cluster E consisted of one accession (64 - Is). This accession was tetraploid with especial morphological characteristics such as, long and wide leaf, long stolon length and average of 6 spickelet number that differentiates this accession from others. Cluster E comprised Tifdwarf and 73 - Ns. Genetic SC value was 0.50. There is no explanation for similarity of these accessions. Tifdwarf is a hybrid cultivar (*Cynodon dactylon* × *Cynodon trasvaalensis*) with triploid chromosome and 73 - Ns have

Table II. List of selected 10-mer random primers used for molecular analysis of *Cynodon dactylon* L. accessions.

Primer	Nucleotide sequence	No. of fragments amplified	Fragment size
OPA 11	5′- CAATCGCCGT- 3′	12	540bp-2.7kb
OPA 16	5′- AGCCAGCGAA-3′	11	380 bp- 3.0 kb
OPA 18	5′- AGGTGACCGT- 3′	13	420 bp- 2.8 kb
OPA 20	5′- GTTGCGATCC-3′	7	720 bp- 2.2 kb
OpB 08	5′- GTCCAC ACGG-3′	11	400bp-2.8 kb
OPB15	5′- GGAGGGTGTT- 3′	7	650bp- 1.9 kb
OPC 12	5′- TGTCATCCCC-3′	7	450bp- 2.5 kb

tetraploid chromosome, in spite of having the same ligule length. The major cluster G included the largest number of accessions (61 tetraploids). These accessions collected from temperate regions that had altitude above 1300 m and rainfall below 500 mm. Genetic SC values in this group was high ranging from a low 0.26 between 30 - Ir and 75 - Sf to high 0.80 between 39 - IL and 41 - Il. One of the sub-groups of cluster G consisted of one accession (63 - Is), which separated from other accessions because of absence of hairs on stems and leaves.

The results obtained in this study demonstrated that RAPD analysis could be used to detect genetic diversity and certain ploidy levels of bermuda grass accessions. Little correlation was found between morphological characteristics and molecular analysis. Similar phenomena have been also reported by Roland-Ruiz *et al*. (2001) and Martinez *et al*. (2003) in ryegrass and grape, respectively. In *Cynodon dactylon*, there is much environmental influence accounting for the morphological variability. Compared with DNA fingerprinting techniques, morphological traits are relatively less reliable and efficient for precise discrimination of closely related genotypes and analysis of their genetic similarities. Therefore, it appears that larger numbers of accessions and primers are required to detect a significant correlation between genetic traits and morphological characteristics.

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