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Comparisons of DNA marker-based genetic diversity with phenotypic estimates in maize grown in Pakistan

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ABSTRACT. We compared DNA-based genetic diversity estimates with conventional estimates by investigating agronomically important traits in maize grown in the northwestern region of Pakistan. RAPD markers were used to characterize 10 commonly cultivated maize genotypes. The same material was tested for phenotypic variation of quantitative traits using replicated field trials. The genetic distances between pairs of genotypes using RAPD data were used to generate a similarity matrix and to construct a phenogram. Statistical analyses were carried out on the data obtained from field trials of all maize genotypes for days to 50% tasseling, days to 50% silking, plant height, ear height, grain yield, grain weight per cob, and ear length. Analysis of variance and single degree of freedom contrasts were

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performed on morphological data to examine the relationship between molecular-based clusters and agronomic traits. A molecular marker-based phenogram led to the grouping of all genotypes into four major clusters, some of which were distantly related. These clusters contained one to four genotypes. Analysis of variance showed significant variations among all genotypes for agronomic traits. The single degree of freedom contrasts between groups of genotypes indicated significant differences for most traits. Pair-wise comparisons between clusters were also significant. The two types of data correlated well, providing an opportunity for better choices for selection.

Key words: RAPD markers; Genetic variation; Quantitative traits; Maize; *Zea mays*

INTRODUCTION

Maize (*Zea maize* L.) is one of the most diverse crop species, containing tremendous variation in morphological and physiological traits and extensive polymorphism in its DNA sequences. This exceptional diversity allows maize to be cultivated in a range of environments from temperate to tropical regions including parts of Africa, the Amazonian rainforest, Arizona deserts, the Gaspe Peninsula in Canada, and the Andes Mountains in Latin America. Internationally, maize-breeding programs have focused on the development of high-yielding cultivars that can meet the challenges of biotic and abiotic stresses and resistance against pests and diseases. Hybrid seed production and distribution in maize has been the main focus of private sectors around the world. Genetically, maize is a well-characterized crop species that has attained the highest genetic gain relative to any other crop over the past several decades. Much of the genetic gain in maize yield was obtained by exploitation of the phenomenon of hybrid vigor or heterosis. In order to further exploit heterosis and other morphological parameters, precise knowledge of germplasm diversity is essential, which has a significant impact on the improvement of crop plant species. The morphological and molecular characterization of maize genetic material would be important for crop improvement, including hybrid seed production and identification of heterotic groups in an indigenous germplasm. There are, however, serious challenges in identifying and describing the genes that control diverse phenotypes and adaptation of maize at these loci.

By and large, 3 methods have been most commonly used in genetic diversity studies in maize germplasm: pedigree records, field trials and molecular markers (Iva et al., 2005). With the advent of molecular marker techniques, germplasm characterization and genome structure analyses have been greatly facilitated (Williams et al., 1990; Melchinger et al., 1991; Lübberstedt et al., 2000; Popi et al., 2000; Shah et al., 2000; Xia and Achar, 2001). DNA fingerprinting technology opened the possibility to explore and characterize within cultivar heterogeneity (McCouch et al., 1988; Jarne and Lagoda, 1996; Shah et al., 2000) with a greater precision. Molecular markers have several advantages over conventional techniques, in view of their high precision in detecting variation with greater potential to explore genetic relationship among populations. The use of molecular markers is an invaluable tool in the arsenal of traditional plant breeding techniques, as they are useful in clarifying the number, chromosomal locations and genetic contribution of genes controlling complex (Zhang C et al., 1998; Franco et al., 2001; Iva et al., 2005; Zhang Y et al., 2006; Stevens, 2008). In the past, restriction-based and amplification-

based DNA markers (such as RFLPs, RAPD, VNTR, SSR, AFLP, SNP) were developed and used in almost every crop species including maize (Smith, 1984; McCouch et al., 1988; Williams et al., 1990; Melchinger et al., 1991; Mukhtar et al., 2002; Shah et al., 2000, 2006; Ghafoor et al., 2007; Kafkas et al., 2008). The use of these markers facilitated greatly the process of genome analysis, systematic mapping of agriculturally important traits, and marker-assisted selection in maize and other crop plant species (Williams et al., 1990; Jarne and Lagoda, 1996; Xia and Achar, 2001; Troggio et al., 2007). It is now possible to precisely estimate genetic distances between genotypes and to select an individual with consistent performance across environments.

DNA-based fingerprinting technologies have been proven useful in genetic diversity studies. Nonetheless, mere marker-based fingerprinting may lead to erroneous results if not confirmed with alternative data collection and analysis system. Therefore, the objective of the current study was to compare genetic diversity estimates using RAPD marker data with those of phenotypic estimates using field trials analyzed with conventional statistical procedures.

MATERIAL AND METHODS

Plant material

Ten maize genotypes cultivated in the northwestern part of Pakistan and Kashmir region were used in this study. Of these 10 genotypes, 7 were commonly cultivated maize varieties and 3 hybrid genotypes (as described by Shah et al., 2006).

All genotypes were provided by the Maize Genetics and Breeding Division of the Cereal Crop Research Institute (CCRI), Pirsabak, Nowshehra, Pakistan. This material was selected based on its geographical distribution and performance in diverse locations of Northwestern Pakistan. The cultivars included Kisan 90, Pahari, Jalal 2003, Sarhad Yellow, Ghauri, Babar, and Sarhad White, while hybrid genotypes were 3 candidate lines, namely CSCY (candidate single cross yellow), C3WY (candidate three way yellow), and CSCW (candidate single cross white). It is important to note that these candidate lines and crosses were not released (at least at the completion of the current study) as varieties and were included in this study to see the variability in potentially new germplasm with the collaboration of CCRI, Pirsabak, Pakistan.

Field trials

All maize genotypes were subjected to field trials using standard field plot techniques. The planting was completed in the 1st week of April, and the final harvesting was done in July. The trial was conducted using a randomized complete block design with 5 replicates per maize genotype. A plot consisting of 3 rows of 5 m long was used as an experimental unit. Rows were spaced 75 cm apart with a plant to plant distance of 20 cm. The soil was silty loam with clay and the climatic conditions were mild spring followed by hot summer. Standard irrigation and morphological practices were regularly applied to the growing plots. The recommended dose of 120 kg nitrogen and 50 kg phosphorus per hectare was applied in the form of urea and diammonium phosphate. All phosphorous fertilizer and half of the nitrogen was applied during soil preparation for planting, whereas the remaining nitrogen was applied at the tassel emerging stage. No herbicide was applied, while fungicides and insecticides were applied as needed.

Several traits of economic importance were considered and scored in this study. Par-

ticularly, the data were recorded for days to 50% tasseling (DTT), days to 50% silking (DTS), plant height (PH), ear height (EH), grain yield (GYLD), grain weight per cob (GWPC), and ear length (EL). Data on DTT were measured by counting the days from planting to mid tasseling when approximately 50% plants had tassels in the plots. This character can easily be scored by careful observation in the field. The data on DTS was recorded when more than 50% plants in the plot showed silks from the ears. PH was measured in centimeters on five randomly selected plants from each plot at the time of maturity by recording the distance from ground level to the flag leaf and then by averaging the individual plant distances. EH was recorded in centimeters on five plants from each plot by measuring the distance from ground to the base of upper most developed ear. GYLD in kg per hectare was measured by determining the yield per plot. For this determination two rows of the three-row plot were harvested. The yield for each plot was adjusted to 12% moisture based on the number of plants harvested and the grain moisture percentage of ears at harvest. GYLD per hectare at 12% moisture was determined by the formula:

GYLD/ha = field weight x (100 - moisture %) x 0.8 (S) x 10,000 m^2 / ha / (100 - 12) x harvested area

DNA analysis

DNA was prepared according to Weining and Langridge (1991) as described in detail elsewhere (Shah et al., 2006). The DNA concentration of all genotypes was estimated by relative comparison of DNA bands of each genotype by gel electrophoresis (running on a 0.8% agarose gel). Polymerase chain reaction analysis was performed (Shah et al., 2000) on the DNA of all maize genotypes using random amplified polymorphic DNA (RAPD) primers (GL series) synthesized by Gene Link Technology (USA). DNA amplification was carried out in a 25-µL reaction mixture, on a Perkin Elmer 9700 thermocycler (ABI, Foster City, CA, USA). The amplification products were resolved on a 2% agarose gel stained with ethidium bromide under UV light. The gels were photographed using the UVitec gel documentation system (Habib et al., 2006; Shah et al., 2006; Ghafoor et al., 2007), and the data were scored based on the presence and absence of DNA fragment bands.

Data analysis

Bivariate data matrix (I 0) was used to estimate similarity or dissimilarity on the basis of the number of shared amplification products (Nei and Li, 1979). The data were adjusted and standardized, and a cluster analysis was performed to generate a phenogram using SAHN command in the NTSys v.2.1 software. The genetic distance between genotypes was estimated using the unweighted pair-group method with arithmetic average (UPGMA) algorithm (Nei and Li, 1979).

Analysis of variance (ANOVA) was carried out using the general linear model procedure of the SAS program on all phenotypic traits. Single degree of freedom (SDF) contrasts were run between pair of traits in groups defined by the phenogram using RAPD primers. The comparisons were made between markers based on similarity or dissimilarity group (phenogram) and the SDF contrasts of all groups.

RESULTS AND DISCUSSION

The polymorphic pattern of an RAPD primer (A-02) used to amplify DNA of 10

maize genotypes is shown in Figure 1. Multiple loci were detected and can be visualized by the segregating bands (Figure 1) among genotypes. Almost the same pattern was observed for all RAPD primers used. The data from the combined analysis of all RAPD markers on 10 maize genotypes used in this study are presented in Table 1 as a genetic coefficient matrix indicating percent similarities or dissimilarities between and among maize cultivars.

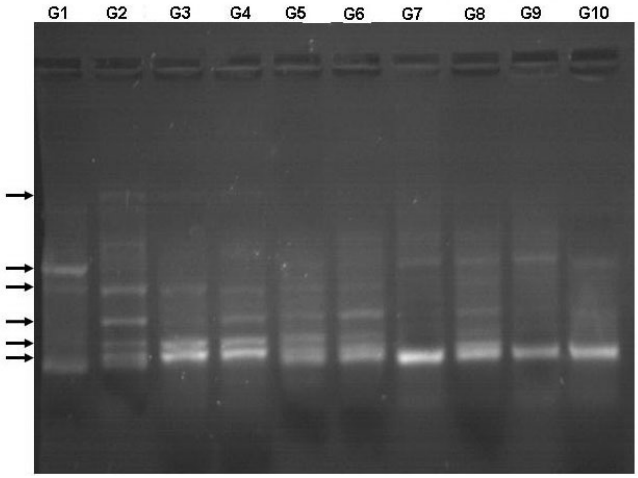

Figure 1. Polymerase chain reaction amplified products by primer A-02 of Pahari (G1), C3WY (G2), Babar (G3), CSCW (G4), Kisan 90 (G5), CSCY (G6), Jalal 2003 (G7), Sarhad Yellow (G8), Ghauri (G9), Sarhad White (G10). Arrows show the presence of DNA fragment bands among genotypes.

Table 1. Description of genetic similarity for maize genotypes in terms of genetic coefficients for all primers used.

	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10
G1	1									
G2	0.698	1								
G3	0.6012	0.85	1							
G4	0.6167	0.92	0.784	1						
G5	0.6947	0.84	0.760	0.88	1					
G6	0.639	0.88	0.8015	0.893	0.926	1				
G7	0.638	0.64	0.607	0.7334	0.683	0.6734	1			
G8	0.6501	0.747	0.705	0.9167	0.90	0.8667	0.7875	1		
G9	0.75	0.7292	0.6125	0.7223	0.8	0.7334	0.875	0.8	1	
G10	0.75	0.7292	0.6125	0.7223	0.8	0.7334	0.875	0.8	1	1

G1 = Pahari; G2 = C3WY; G3 = Babar; G4 = CSCW; G5 = Kisan 90; G6 = CSCY; G7 = Jalal 2003; G8 = Sarhad Yellow; G9 = Ghauri; G10 = Sarhad White.

The phenogram (Figure 2) generated using a similarity matrix grouped all genotypes into four major clusters (Shah et al., 2006). The clusters showed a range containing from a single to multiple genotypes in a cluster (Figure 2). As reported, a range of genetic similarities was observed among the maize genotypes by the use of RAPD primers, which ranged from 100 to 60% (Table 1; Figure 2). The maximum distance (60%) as translated by the similarity coefficient was observed between genotypes Babar and Pahari followed by Babar and Jalal 2003 (60.7%),

whereas minimum distance was observed between Sarhad White and Ghauri (similary coefficient = 100%). The major clusters in the phenogram were subdivided into several sub-groups, each containing from a single to multiple genotypes. The genetic distance estimates varied among groups and sub-groups. In general, the phenogram corresponds to the genetic distance estimates and indicated variations that may be attributed due to various breeding programs at different breeding stations and timing of releases of the cultivars. The major groups were named alphabetically from A-D (Figure 2). Group 'A' contained Pahari, Ghauri and Sarhad White, group 'B' contained C3WY and Babar, group 'C' contained CSCW, Kisan 90, CSCY, and Sarhad Yellow, and group 'D' contained Jalal 2003.

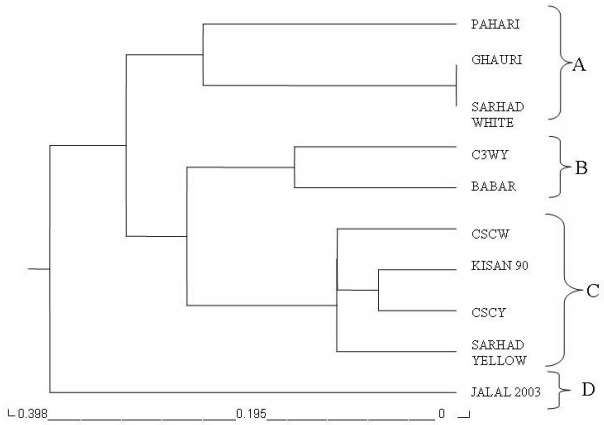

Figure 2. Phenogram of 10 maize genotypes generated using RAPD primers (Shah et al., 2006) following the UPGMA procedure with the NTSYS PC 2.1 software.

The largest cluster or group appeared to be cluster "C" comprising cultivars Kisan 90, CSCY, CSCW, and Sarhad Yellow, followed by cluster "A" and "B" both containing 3 and 2 cultivars, respectively (Figure 2). Placing these genotypes in the same cluster showed the narrow genetic background of these genotypes (Shah et al., 2006). Interestingly, cultivar Jalal 2003 was distinct from all other groups, showing diverse genetic background. The reason for genotype clustering could be due to the selection of elite lines from a single base population or a single institute/center where released, as has been reported in cotton by Iqbal et al. (1997) and in rice by Bligh et al. (1999).

All cultivars used in the marker study were used to perform field trials and an analysis between and among possible combinations of the groups generated in marker-based studies. The overall ANOVA followed by SDF contrasts between groups was conducted to provide a comparison between both data. The analysis of variance and the comparison for SDF contrasts between groups are presented in Table 2. From the field trials, all morphological traits, i.e., DTT, DTS, PH, EH, GWPC, EL, and GYLD, showed significant variation when a combined ANOVA was performed (Table 2). The group contrasts A *vs* B and A *vs* D were significant for all traits except GWPC. The contrast between A and C was significant only for GWPC and GYLD. The difference between groups B and C was significant for all traits except GYLD, while the contrast B *vs* D was significant only for PH and GWPC. Group C and D showed significant difference for all traits except DTT, GWPC and GYLD. Comparing an individual group with a combination of 2 or more groups, revealed that there

Table 2. Mean squares and degrees of freedom (d.f.) of agronomic traits; days to 50% tassling (DTT), days to 50% silking (DTS), plant height (PH), ear height (EH), grain weight per cob (GWPC), ear length (EL), and grain yield (GYLD) of maize genotypes.

Source	Bloc	Entry	Error	A *vs* B	A *vs* C	A *vs* D	B *vs* C	B *vs* D	C *vs* D	A *vs* B+C	D *vs* A+B+C
d.f.	4	8	30	1	1	1	1	1	1	1	1
DTT	7.44	69.47**	8.73	119.16**	0.034	145.8**	114.06**	4.28	4.05	2.1	92.54**
DTS	1.93	52.27**	3.415	38.25**	0.0001	30.94**	45.21**	0.58	16.2*	39.28**	16.45*
PH	716.09	2147.11**	202.74	3542.25**	44.196	7311.49**	2504.15**	1052.46*	2055.58**	309.29	5472.49**
EH	194.62	839.178**	88.01	1236.97**	52.81	1950.31**	740.37**	174.42	439.45*	105.05	1248.135**
GWPC	89.06	782.96*	292.07	107.39	2542.64**	645.53	4050.34**	1359.17*	221.16	193.11	317.39
EL	19.19	16.226**	3.65	46.19**	0.46	52.03**	60.58**	2.17	22.78*	4.15	35.45**
GYLD	4266585.38	4674728.65**	1254432.69	13974656.42**	5057695.48*	13059590.98**	1624828.27	48601.08	108025.89	3208578.67	3540854.09

**Significant at $P < 0.01$, *significant at $P < 0.05$; the letters A, B, C, and D represent clusters of maize cultivars developed using UPGMA: A = Pahari and Sarhad White; B = C3WY and Babar; C = CSCW, Kisan 90, CSCY, and Sarhad Yellow; D = Jalal 2003.

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was a significant difference only for DTS between group A and a combination of B and C (Table 2). All traits showed a significant difference, except for GWPC and GYLD between group D and a combination of groups A, B, and C.

RAPD primer analyses generated genotype-specific amplification products for all maize cultivars and lines under investigation. Hence, RAPD proved to be a promising DNA fingerprinting technique for the identification of varieties. This would be of immense use in establishing maize breeders' intellectual property rights in the country. The determination of varietal purity in both cultivars and hybrids by DNA fingerprinting is relatively easier and highly informative. RAPD analysis has been found to be a valuable diagnostic DNA tool to evaluate and estimate genetic diversity (Melchinger et al., 1991; Joshi and Nguyen, 1993; Iqbal and Rayburn, 1994; Iqbal et al., 1997; Franco et al., 2001; Mukhtar et al., 2002; Shah et al., 2006; Ghafoor et al., 2007).

In the past, germplasm diversity studies in maize and other species were conducted using various statistical and biochemical principles and procedures (Wrigley and Shephered, 1977; Melchinger et al., 1991; Joshi and Nguyen, 1993; Iqbal et al., 1997; Popi et al., 2000; Franco et al., 2001), including pedigree data (Nei, 1972; Cox et al., 1985; Lübberstedt et al., 2000), calculations of genetic distance measured by inter-varietal heterosis (Troyer et al., 1983), and biochemical data such as isozymes and zein chromatographic profiles (Smith, 1984). Pedigree data alone can be subjective, and do not account for the effects of selection, mutation, mistaken or uncontrolled pollination, and error (Wrigley and Shepherd, 1977). Biochemical and genetic data can thus provide a more objective and accurate appraisal of genetic diversity, because they allow direct comparisons of genotypes. Nevertheless, there is a huge environmental effect of protein expression profiles, and thus, the marker system is not stable. Random amplified polymorphic DNA (Williams et al., 1990) is a stable marker system and has been widely used in genetic diversity studies in almost all crop species including maize (Lübberstedt et al., 2000; Popy et al., 2000; Xia and Achar, 2001; Iva et al., 2005). It is obviously clear that any single system alone may not be satisfactory in providing answers, and therefore, we chose to analyze germplasm diversity using molecular markers along with morphological traits and statistical analyses.

In the current study, the inter-data comparisons showed that the genetic base of most of the genotypes used was narrow, meaning that inter- and intra-varietal polymorphism was not significant, resulting in genetic homogeneity among the cultivars. A notable result was comparison between varieties; Ghauri and Sarhad White were not discriminated using both analyses. The result could be attributed to the autogamous nature of maize crop, having identical alleles at different loci. Other genotypes such as Pahari, Sarhad White, Sarhad Yellow, C3WY, Ghauri, CSCY, and C3CW showed enough similarities relative to Babar and Jalal 2003. Polymorphism in amplification profiles of individual plants has been earlier reported in rye (Iqbal and Rayburn, 1994), cotton (Iqbal et al., 1997) and wheat (Mukhtar et al., 2002). Likewise testing of the two genotypes Pahari and Babar showed maximum polymorphism for each using RAPD markers. A maximum genetic distance (39.88% polymorphism) was shown between them. Thus, these varieties could be a useful source for future breeding and a genome mapping program.

A similar trend was reported by Mukhtar et al. (2002) in wheat genotypes of different origin using the RAPD technique. The reason for narrow genetic background in this material may be due to the breeders' selection and/or sharing of material among research stations and

centers. Consequently, identical breeding material results at different breeding stations, creating a problem of close kinship, which leads to confused grouping. Thus, the new cultivars will be different at some loci if any.

The genetic diversity estimates of parental populations at a molecular level will enhance our understanding to make better crosses between diverse genotypes to obtain heterosis from indigenous maize germplasm. The information on genetic diversity deduced from this study will be helpful in avoiding any chance of elite germplasm becoming genetically uniform and endangering long-term productivity. RAPD marker data were enhanced with field trials and statistical analyses to maximize information on the genetic diversity of maize cultivars that will be used in the future breeding programs.

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