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Full Length Research Paper

Comparative analysis of diversity based on morpho-agronomic traits and molecular markers in durum wheat under heat stress

Abdullah Abdlulaziz Al-Doss[1], Adel Ahmed Elshafei[2], Khaled Ahmed Moustafa[1], Mohamed Saleh[1] and Mohamed Najeb Barakat[2]*

[1]Plant Production Department, College of Food Sciences and Agriculture, King Saud University, Riyadh, Saudi Arabia.
[2]Plant Genetic Manipulation and Genomic Breeding Group, Center of Excellence in Biotechnology Research, King Saud University, Riyadh, Saudi Arabia.

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The objectives of this study were to compare the application and utility of sequence-related amplified polymorphism (SRAP) and target region amplification polymorphism (TRAP) techniques for the analysis of genetic diversity among durum wheat genotypes under heat stress and to compare genetic diversity estimated using molecular markers with morpho-agronomic performance under heat stress. Six durum wheat genotypes were used in this study. They were evaluated phenotypically for heat tolerance. The dendrogram generated from standardized morpho-agronomic data separated the six durum wheat genotypes into three main groups. The dendrogram generated from the standardized morpho-agronomic data separated the six durum wheat genotypes into three clusters, which diverged at similarity index of 0.72. The dendrogram based on SRAP markers differed from that based on TRAP markers. The combined dendrogram (SRAP, TRAP and morpho-agronomic data) agrees better with the grouping of these durum wheat genotypes depending on pedigree and the dendrogram generated by morpho-agronomic data alone.

Key words: Durum wheat, genetic diversity, heat tolerance, morpho-agronomic, sequence-related amplified polymorphism (SRAP) markers, target region amplification polymorphism (TRAP) markers.

INTRODUCTION

Durum wheat currently represents 8 to 10% of the wheat grown and produced worldwide (FAO STAT data, 2006). It is however, concentrated in relatively small geographical areas where it often plays a major role in the food security of urban populations and in the livelihood and nutrition of urban communities. More than 80% of the spring durum cultivars released in the developing world, covering more than 50% of the area planted to this crop, are semi dwarf types, either from The International Maize and Wheat Improvement Center (CIMMYT) crosses or from crosses involving at least one CIMMYT parent (Lantican et al., 2005).

The productivity of durum wheat is often limited by an array of abiotic stresses that avoid a successful growth and a complete grain filling. Heat stress due to increased temperature is an agricultural problem in many areas of the world (Wahid et al., 2007). Post-anthesis high temperature stress in wheat is a major cause of yield reduction in some regions in Saudi Arabia as well as in many wheat-growing regions of the world. Some attempts to develop heat-tolerant genotypes via conventional plant breeding protocols have been successful (Ehlers and Hall, 1998; Camejo et al., 2005) and via molecular breeding have provided additional tools to develop crops with improved heat tolerance (Al-Doss et al., 2009).

In a breeding program, knowledge of the degree of genetic diversity among parental materials for key selection traits will facilitate the development of high yielding stress tolerant durum wheat cultivars. Thus, the

*Corresponding author .E-mail: mnrbarakat@yahoo.com. Tel: 00966595262365.

correct choice of parents employed in the development of the basic population can influence the final result of the artificial selection and promote a better allocation of financial resources during the whole process of adjusting genotypes to a given environment (Bohan et al., 1999). However, to confirm such expectations, it is necessary that the parents combine high means with an increase in variability for the characters under selection.

Molecular and morphological analysis is among the most used tools for the estimation of genetic distances within a group of genotypes. Molecular markers provide an excellent tool for obtaining genetic information and their use in the assessment of genetic diversity in wheat (*Triticum aestivum* L.) has increased in the last few years (Manifesto et al., 2001; Roy et al., 2004; Barakat et al., 2010). Molecular markers are a useful complement to morphological and physiological characterization of cultivars because they are plentiful, independent of tissue or environmental effects and allow cultivar identification early in plant development. Molecular characterization of cultivars is also useful to evaluate potential genetic erosion, defined here as a reduction of genetic diversity in time (Manifesto et al., 2001). Better understanding of the genetic basis of phenotypic variability will improve the efficiency of durum wheat improvement for heat tolerance. Recently, new types of molecular markers, sequence-related amplified polymorphism (SRAP) and target region amplification polymorphism (TRAP), were developed and used in genetic mapping (Li and Quiros, 2001; Hu and Vick, 2003; Liu et al., 2005; Wang et al., 2005).

The objectives of the present study were; (1) to compare the application and utility of TRAP and SRAP marker techniques for analysis of genetic diversity among six genetically diverse durum wheat genotypes under heat stress; (2) to the compare genetic diversity estimated using molecular markers with agronomic performance under heat stress to establish the degree of association between these techniques.

MATERIALS AND METHODS

Field trails and traits evaluation

Six genetically diverse durum wheat genotypes were used in this study. These included the two check cultivars (Kronos and Benysowef) as well as four advanced lines (F_9) (Table 1) selected from the wheat breeding program at the Plant Production Department, College of Food and Agriculture Sciences, King Saud University, Saudi Arabia. The six durum wheat genotypes were evaluated phenotypically for heat tolerance under four sowing dates (20th October, November, December and January), over two season (2005/2006 and 2006/2007) to expose genotypes to different levels of heat stress during the grain-filling period. The first two dates represent the normal conditions (the temperature is around 22 to 25°C at flowering stage) where the other are considered stress condition the temperature is more than 28°C at flowering stage in Saudi Arabia. The seeding rates were 160 kg/ha. The fertilizers were applied at the rate of 120 kg N and 80 kg P_2O_5 per hectare. The cultural practices were carried out according to the recommended practices followed in Riyadh area.

The layout of the experiment was a split-plot design with four replications. The four sowing dates were assigned to the main plots, while the six durum genotypes were allocated to the sub plots. Twelve agronomic traits were scored for the durum wheat genotypes. These were flowering date (DH), maturity date (DM), grain filling period (FP), plant height (PH), grain yield (GY), harvest index (HI), spike number per m^2 (NS/m^2), kernels per spike (NG/S), 1000-kernel weight (KW), number of tillers (NT/m^2), grain color concentration and protein content. Grain yield was determined from the central rows and converted to grain yield per hectare. Spike number was determined by counting the number of grains bearing tiller in an area of 50×50 cm. The count was expressed as the number of spike m^2. Kernel per spike was determined in heads of 10 randomly tillers which were hand threshed and the number of kernels was counted as the average number of kernels per spike. Filling period was calculated by subtracting the number of days to heading from the number of days to maturity.

Molecular characterization

DNA extraction

Frozen young leaves (500 mg) were grounded to powder in a mortar with liquid nitrogen. The powder was poured into tubes containing 9.0 ml of warm (65°C) CTAB extraction buffer. The tubes were incubated at 65°C for 60 to 90 min, 4.5 ml chloroform/octanol (24:1) were added and tubes were rocked to mix for 10 min and centrifuged for 10 min at 3200 rpm. The supernatants were pipetted off into new tubes and 6 ml isopropanol was added. After 60 min, the tubes were centrifuged for 10 min and the pellets obtained were put in the sterile Eppendorf tubes, containing 400 µl of TE buffer of a pH 8.0 (10 mM Tris-HCl, pH 8.0 + 1.0 mM EDTA, pH 8.0).

SRAP and TRAP analysis

A total of 19 primers (Table 3) were used in SRAP analysis, and 9 primers were used in TRAP analysis (Table 4), from Pharmacia Biotech. (Amersham Pharmacia Biotech UK Limited, England HP79 NA). PCR amplification for SRAP and TRAP was carried out in a 20 L^{-1} reaction mixture containing 1 x buffer, 1.5 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} dNTPs, 500 nmol l^{-1} primer, 1U Taq polymerase and 50 to 60 ng template DNA. After 5 min at 94°C, 5 cycles were performed with 1 min at 94°C, 1 min at 35°C, 1 min 40 s at 72°C, then 35 cycles the same as previous except for the annealing temperature at 50°C and a final 7 min at 72°C. Amplification products were electrophoretically resolved on 1.5% agarose gels containing 0.1 µg/ml ethidium bromides and photographed on a UV trans-illuminator.

Statistical analysis

Analysis of variance was performed for all measured traits (agronomic traits) in order to test the significance of variance among genotypes (Steel and Torrie, 1980). To determine a data matrix of pairwise similarities between genotypes, the standardized traits mean values (mean of each traits was subtracted from the data values and the result divided by the standard division) were used, according to Jaccard coefficient (Jaccard, 1908).

SRAP and TRAP data were scored for the presence (1), absence (0) or as a missing observation and each band was regarded as a locus. Two matrices, one for each marker, were generated. Pairwise comparisons of genotypes, based on the presence or

Table 1. Name and origin of the six durum wheat genotypes used in the study.

Number	Name	Pedigree	Origin
1	KSUDW 101	L14\Benyswef-7-17-1	Plant Production Department
2	KSUDW 102	L18\Benyswef- 3-22-2	Plant Production Department
3	KSUDW 103	Stork\Benyswef-34-2-3	Plant Production Department
4	KSUDW 104	Sham1\Benyswef-57-9-5	Plant Production Department
5	Benysowef	Cultivar	Egypt
6	Kronos	Cultivar	USA

Table 2. The interaction between sowing dates and genotypes for grain yield (ton/ha) over two seasons.

Genotype	Sowing date				Overall mean
	October 20th	November 20th	December 20th	January 20th	
KSUDW101	4.69	6.01	6.56	2.68	4.99b
KSUDW102	5.53	5.91	6.65	2.78	5.22ab
KSUDW103	4.00	5.91	5.59	2.60	4.53c
KSUDW104	5.13	7.10	6.61	3.26	5.53a
Benysowef	6.34	5.74	6.78	2.21	5.27ab
Kronous	4.06	4.06	5.39	2.41	4.38c
Overall mean	4.96b	6.05a	6.26a	2.66c	

$LSD_{0.05}$ for sowing date treatment x genotype interaction = 0.87.

absence of unique and shared polymorphic products, were used to determine a data matrix of pairwise similarities between cultivars, according to Jaccard coefficient.

All matrices (based on agronomic traits and molecular markers) were used to obtain the respective dendrograms using the algorithm UPGMA (Unweighed Pair Group Method with Arithmetic Average) (Sokal and Michene,1958) employed the SAHN (aequential, agglomerative, hierarchical and nested clustering) from the software NTSYS.pc (numerical taxonomy and multivariate analysis system, version 1.80 (applied biostatics program (Rohlf,1993)).The correlation coefficients between the Jaccard distance matrix based on agronomic traits and genetic distance matrix obtained with molecular markers were analyzed according to Mantel (Mantel, 1967) using NTSYS-pc.

RESULTS

Diversity analysis based on morpho-agronomic traits

The analysis of variance indicated that, for all the characters evaluated there were statistically significant differences (p = 0.05) among the durum wheat genotypes studied and for most of the characters evaluated for years and the genotype× year interaction. The interaction between sowing dates treatment and genotypes for grain yield (ton\ha) over two seasons is presented in Table 2. Discussion was focused on grain yield because of its importance as the main objective in the breeding program. The highest grain yield was achieved from the durum wheat genotype KSUDW 104 (5.53 ton/ha) across the four sowing dates which was not significantly differencet from the two durum wheat genotypes, KSUDW102 and Benysowef. The KSUDW104 genotype had the highest yield at heat stress condition (January 20th which is considered stress condition in Saudi Arabia), yielding 3.26 ton/ha over the two seasons, out-yielding the recommended cultivar Kronous, as well as the parent Benysowef (2.41 and 2.21 ton/ha, respectively). KSUWD104 should be recognized as heat tolerant potential line.

A dendrogram generated from the standardized morpho-agronomic data is presented in Figure 1. The UPGMA dendrogram separated the six durum wheat genotypes into three clusters, which diverged at similarity index of 0.72. The first cluster contained 4 durum wheat lines (KSUD101, KSUDW102, KSUDW103 and KSUDW104). These lines had one parent in common (Benysowef, Table 1), a cultivar obtained from Egypt and characterized as heat tolerant. The other parent for KSUDW104 was Sham1, a line obtained from ICARDA and characterized as high yielding cultivars (Table 1). The second cluster consisted of the commercial Egyptian durum wheat Benysowef. The commercial adapted Saudi durum wheat variety Kronos clustered separately into the third group (Figure 1). The average genetic similarity among the six durum wheat genotypes was 0.84, with value ranging from 0.77 to 0.90. The KSUDW101 and KSUDW104 genotypes showed a very high degree of similarity (0.90) indicating that, these two genotypes had

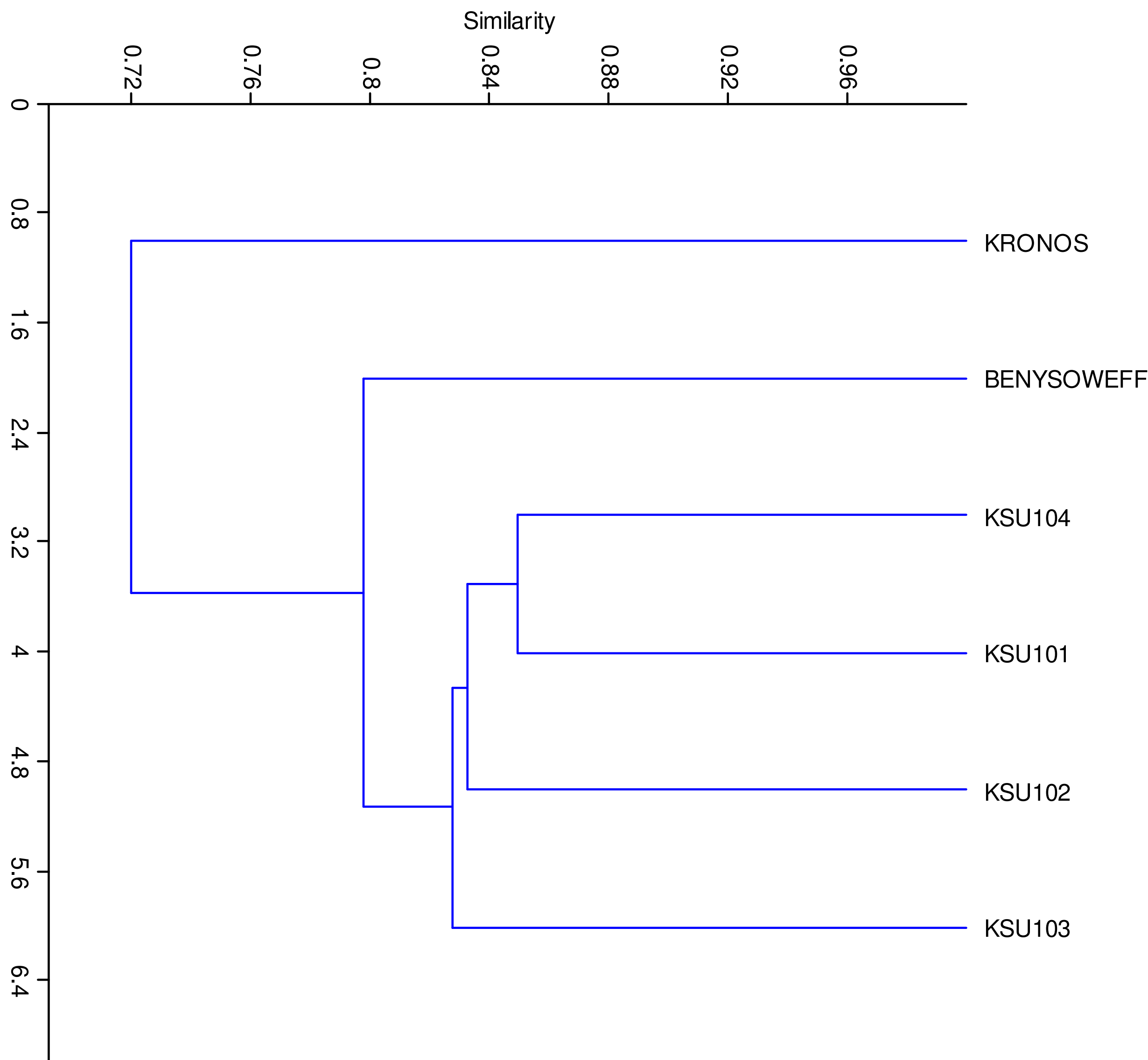

Figure 1. Dendrogram based on Jaccard similarity coefficient of six durum wheat genotypes, generated by twelve agronomic traits over two seasons under heat stress condition.

similar agronomic traits under heat stress. On the other hand, Kronos and KSUDW101 genotypes showed a low degree of similarity (0.77) which indicated that this pair is not closely related genotypes and had different agronomic traits under heat stress.

Molecular characterization

Identification and evaluation of SRAP and TRAP markers for diversity estimates

Nineteen (19) primers were screened for their ability to amplify the genomic DNA from 6 durum wheat genotypes. The number of amplified DNA fragments ranged from 0.0 to 17.0 depending on the primer and the DNA sample with a mean value of 6.7 bands per primer (Table 3). In the present investigation, the size of fragments ranged from 100 to 1300 bp. A total of 128 fragments were produced by the 19 primers. Of these 128 amplified fragments, 65.0% were not polymorphic, while 35.0% were polymorphic among the 6 durum wheat genotypes. Primer SRAP-4 generated the greatest polymorphism (75.0%), while the lowest level of polymorphism (0.0 %) was obtained by primers SRRP-7, SRAP-14 and SRAP-17. Out of the 19 primers, 4 revealed more than 50% polymorphism (Table 3). Figure 2 shows the amplification profiles, generated by primer SRAP-5 across the 6 durum wheat genotypes, all of which had distinguishable banding patterns.

Table 3. Number of amplification and polymorphic products, using 19 SRAP primers in durum wheat genotypes.

Primer number	Nucleotide sequence (5'-3')		Number of amplification a	Number of polymorphic b	Polymorphism b/a (%)
	Forward primer	Reverse primer			
1	TGAGTCCAAACCGGTAG	GACTGCGTACGAATTCTG	6	1	16.66
2	TGAGTCCAAACCGGTCC	GACTGCGTACGAATTGTC	8	1	12.5
3	TGAGTCCAAACCGGTCA	GACTGCGTACGAATTAAT	5	1	20.0
4	TGAGTCCAAACCGGTAG	GACTGCGTACGAATTCGA	4	3	75.o
5	TGAGTCCAAACCGGTAG	GACTGCGTACGAATTCAG	11	5	45.45
6	TGAGTCCAAACCGGTAG	GACTGCGTACGAATTTGA	11	5	45.45
7	TGAGTCCAAACCGGTAG	GACTGCGTACGAATTGCA	0	0	0.0
8	TGAGTCCAAACCGGTTG	GACTGCGTACGAATTGGT	8	2	25.0
9	TGAGTCCAAACCGGTCA	GACTGCGTACGAATTCGA	7	2	28.57
10	TGAGTCCAAACCGGTGC	GACTGCGTACGAATTCAA	17	10	58.82
11	TGAGTCCAAACCGGTGC	GACTGCGTACGAATTTGC	2	1	50.0
12	TGAGTCCAAACCGGTGC	GACTGCGTACGAATTGGT	14	7	50.0
13	TGAGTCCAAACCGGTAG	GACTGCGTACGAATTGAC	5	2	40.0
14	TGAGTCCAAACCGGACC	GACTGCGTACGAATTTAG	6	0	0.0
15	TGAGTCCAAACCGGACC	GACTGCGTACGAATTCAG	5	1	20.0
16	TGAGTCCAAACCGGACC	GACTGCGTACGAATTAGC	5	1	20.0
17	TGAGTCCAAACCGGACC	GACTGCGTACGAATTTAG	5	1	0.0
18	TGAGTCCAAACCGGAAG	GACTGCGTACGAATTTGA	4	1	25.0
19	TGAGTCCAAACCGGAAG	GACTGCGTACGAATTTCG	5	1	40.0

Nine TRAP primers were used to amplify DNA segments from 6 durum wheat genotypes. The number of amplified bands per primer varied between 3 and 10 (Table 4). A total of 55 bands were observed, with 6.11 bands per primer. 22 out of 55 bands (40%) were polymorphic. An example of polymorphism is shown in Figure 2, which shows the amplification profiles, generated by primer TRAP-5 across the 6 durum wheat genotypes, all of which had distinguishable banding patterns.

DISCUSSION

Genetic diversity, relatedness and structure of parental germplasm are important for breeders to design strategy in breeding programme. Diversity analysis is important for deciphering genetic relationship including parentage and for the efficient management of germplasm and thereby, use in breeding of improved varieties. Establishing the identity of crop variety using diversity study has assumed

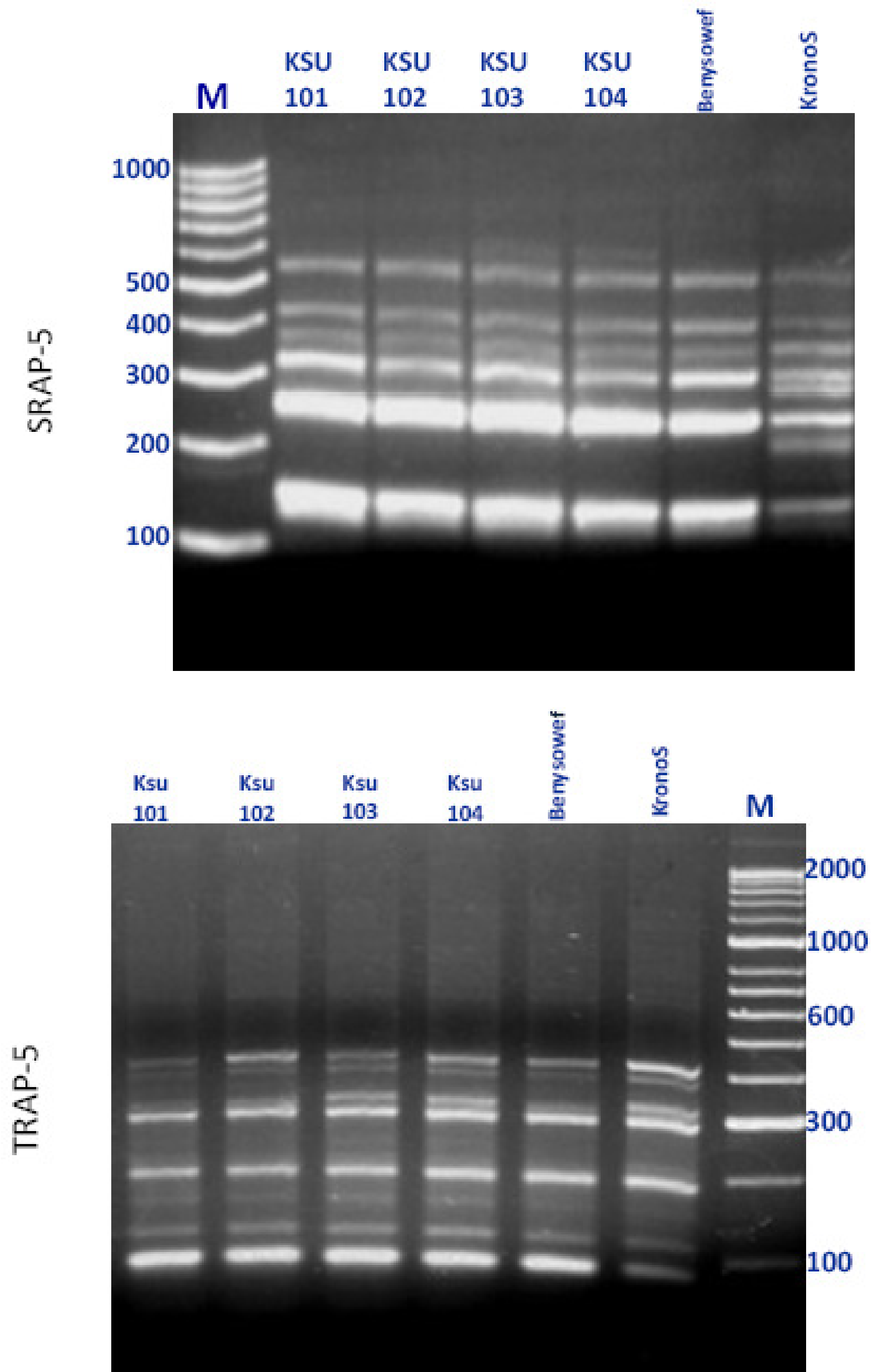

Figure 2. Polymorphism revealed using primer SRAP-5 and primer TRAP-5 to amplify genomic DNA purified from durum wheat genotypes.

greater importance for protecting plant breeders' and farmers' rights. In the present study, SRAP and TRAP markers and phenotypic diversity were analyzed in the six durum wheat genotypes which were planted on four

Table 4. Number of amplification and polymorphic products, using nine TRAP primers in durum wheat genotypes.

Primer number	Nucleotide sequence (5'-3')		Number of amplification a	Number of polymorphic b	Polymorphism b/a (%)
	Fixed primer	Arbitrary primer			
1	TGAGTCCAAACCGGT	TCACCCGCACCTTCTTCC	5	0	0.0
2	TGAGTCCAAACCGGC	CGGACAGTGGCGGAGTTA	6	2	33.33
3	TGAGTCCAAACCGGC	GGCGAACTCCGACATCTT	5	4	80.0
4	TGAGTCCAAACCGGC	GAGGAAGACGACGAGGT	10	8	80.0
5	TGAGTCCAAACCGGA	TTCTTCCTCCCGCTCATT	7	3	42.85
6	TGAGTCCAAACCGGT	CCCTCCACCAATCACAAT	6	1	16.66
7	AGTAACCCACCGCTTC	TCCTACAAACATTGCCTT	3	0	0.0
8	TGCCGCTTCCAACAAA	TCACCCGCACCTTCTTCC	8	3	37.5
9	TGAGTCCAAACCGAT	CAGGCAAGACGCAAGGG	5	1	20.0

sowing dates over two seasons to expose genotypes to different levels of heat stress during the grain-filling period.

Nineteen (19) SRAP primers and nine TRAP primers were used to amplify DNA segments from 6 durum wheat genotypes. Several primers had distinguishable banding patterns between durum wheat genotypes. Polymorphism between genotypes can arise through nucleotide changes that prevent amplification by introducing a mismatch at one priming site, deletion of a priming site, insertions that render priming sites too distant to support amplification and insertions or deletions that change the size of the amplified product (Williams et al., 1990). SRAP is a PCR-based DNA marker system that generates multiple fragments in a single PCR reaction (Li and Quiros, 2001). SRAPs amplify several reproducible and polymorphic loci and alleles and they may amplify functional genes since they are sequence related. SRAP markers possess multiloci and multiallelic features, which make them potentially more efficient for genetic diversity analysis, gene mapping and fingerprinting genotypes. However, SRAP markers may not be randomly distributed across the genome (Li and Quiros, 2001). Previously, Hu and Vick (2003) developed a new marker technique known as target region amplified polymorphism (TRAP), which is a rapid and efficient PCR-based technique that employs two 18-mer primers. One "fixed" primer is designed from a known expressed sequence tag (EST), while the other primer is arbitrary with either an AT- or GC- rich core to anneal with an intron or exon, respectively. Xu et al. (2003) used TRAPs to characterize genetic stocks of tetraploid wheat (*Triticum turgidum* L., 2n = 4, x = 2 8, AABB genomes) and found that a large number of chromosome- specific markers could be generated with this technique. The results indicated that, TRAPs might be suitable for rapidly mapping the wheat genome. Recently, Liu et al. (2005) reported that, TRAP markers were very efficient for rapidly generating a large number of markers scattered across the genome, which allowed linkage groups to be joined and many gaps to be filled. TRAPs also showed the same ability as SSRs to assign linkage groups to chromosomes.

In the present study, the dendrogram generated from SRAP and TRAP data clearly indicated two main clusters. However, the first cluster in SRAP included the commercial cultivars 'Benysowef' and Kronos, while the second cluster included the new durum wheat lines which are more closely related with each other. While in TRAP, the first cluster included the adapted commercial cultivar Kronos alone, the second cluster included the new durum wheat genotypes as well as the commercial cultivar Benysowef'. These new durum wheat genotypes had one parent in common (Benysowef). Previously, SRAP markers have been used to detect the genetic diversity of some accessions of *Cucurbita maxima* from Spain (Ferriol et al., 2003). SRAP markers were employed to examine their potential for genetic diversity analyses in hard red winter wheat (Fufa et al., 2005). The potential of the sequence-related amplified polymorphism (SRAP) technique, which preferentially amplifies gene-rich regions, was evaluated to assess the genetic relationships among members of the *Saccharum* species (Suman et al., 2008). Recently, Wang et al. (2009) reported that SRAP is a new molecular marker which could provide high polymorphism and plentiful information. It is simple and has not the species-specific character. It had been widely

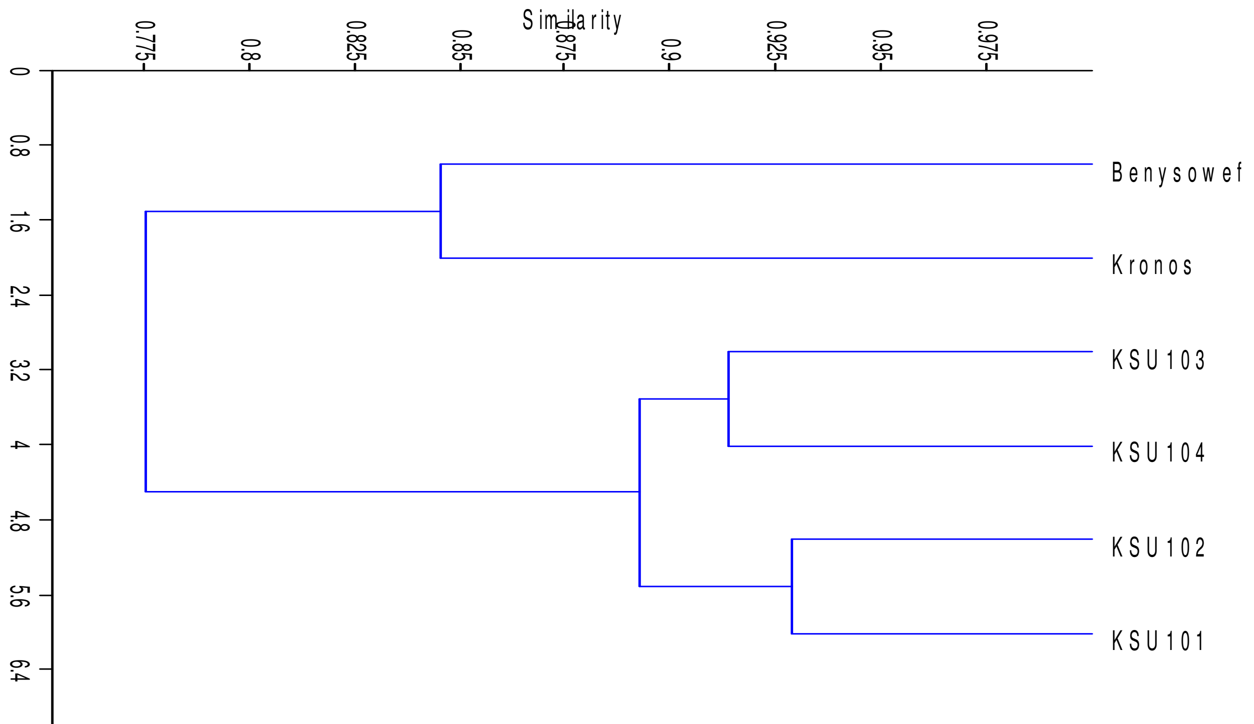

Figure 3. Dendrogram based on Jaccard similarity coefficient of 6 durum wheat genotypes, generated using SRAP markers. (the figure was not sited in the main work)

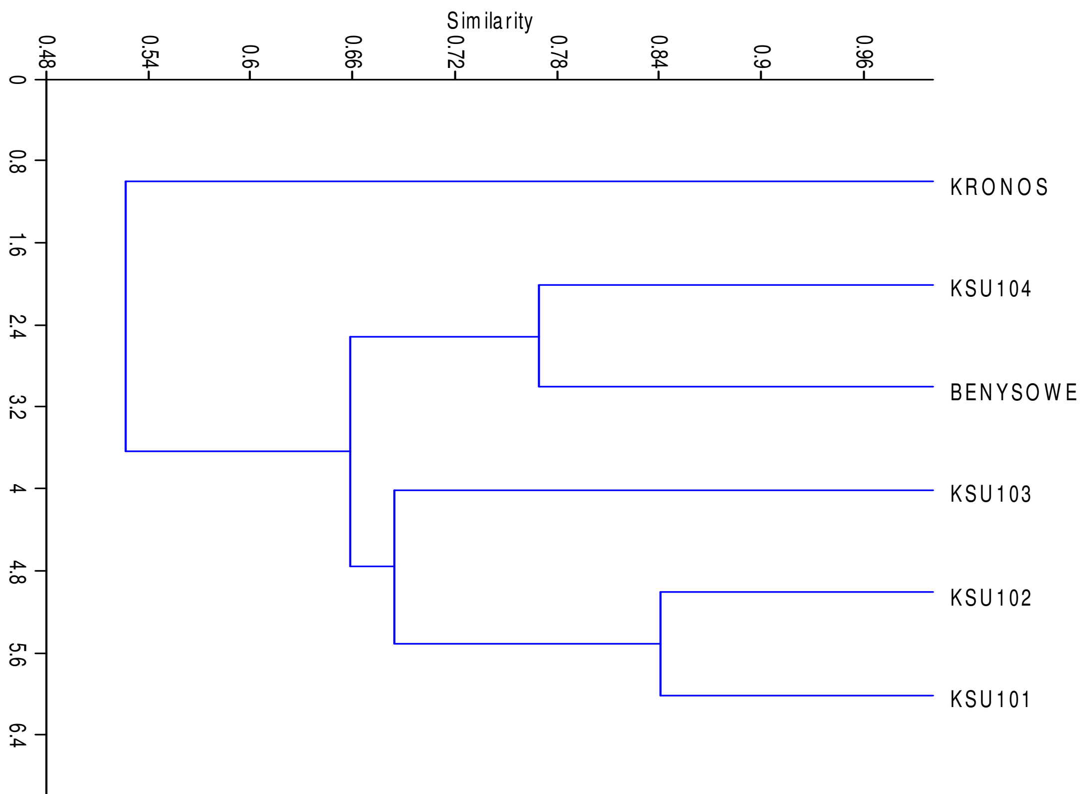

Figure 4. Dendrogram based on Jaccard similarity coefficient of 6 durum wheat genotypes, generated using TRAP markers. (The figure was not sited in the main work)

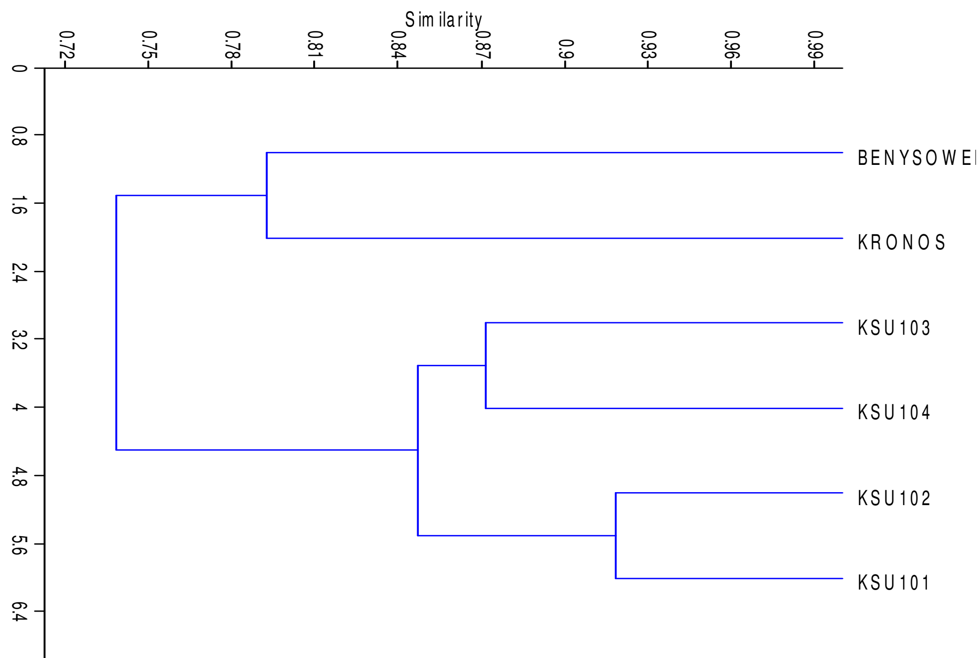

Figure 5. Dendrogram based on Jaccard similarity coefficient of 6 durum wheat genotypes, generated using SRAP and TRAP markers. (The figure was not sited in the main work)

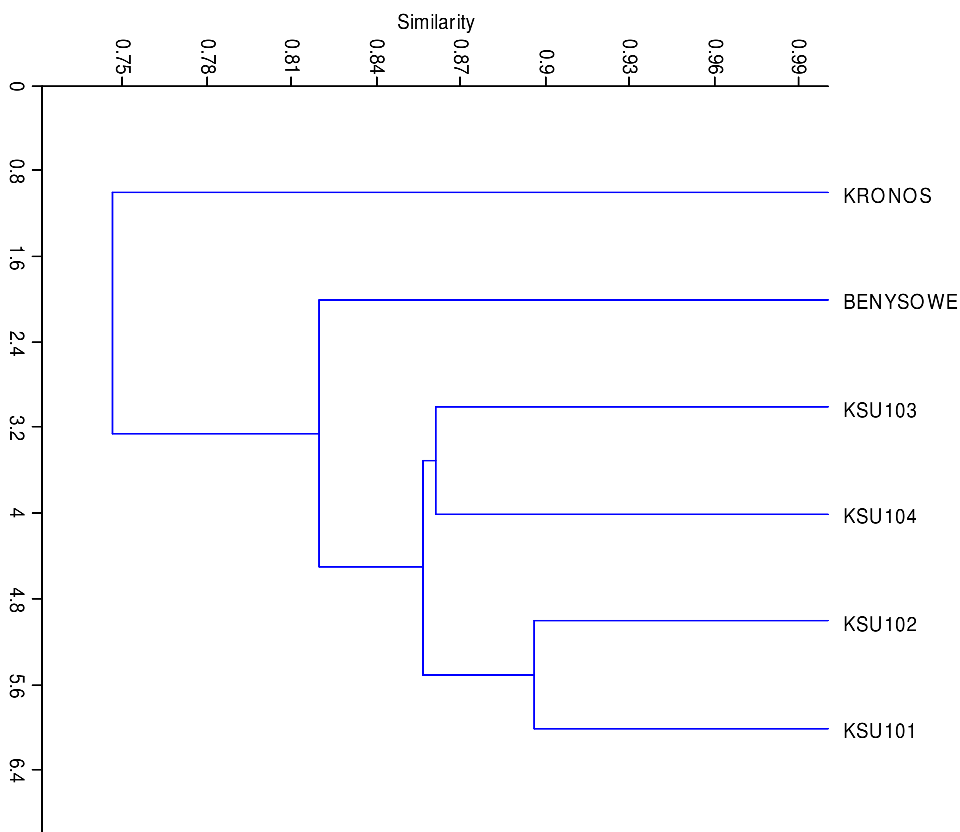

Figure 6. Dendrogram based on Jaccard similarity coefficient of 6 durum wheat genotypes, generated using combined molecular markers and morpho-agronomic traits. (The figure was not sited in the main work)

used for genetic diversity, comparing genome analysis and map construction. Previously, TRAP also was successfully used to estimate the genetic diversity in genetic stocks of tetraploid wheat (*T. turgidum* L., 2n =4, x =2 8, AABB genomes) (Xu et al., 2003).

In order to compare the extent of agreement among dendrograms derived from morphology and molecular markers, a distance matrix was constructed for each assay and compared, using the Mantel matrix correspondence test. Comparison of matrices of morpho-agronomic data and SRAP or TRAP or SRAP + TRAP matrix or SRAP +TRAP + morpho-agronomic matrix showed a significant correlation among dendrograms. The correlation coefficient between SRAP and TRAP matrix was highly significant. Additionally, both SRAP and TRAP matrices showed significantly positive correlation with TRAP + SRAP matrix. Moreover, both SRAP and TRAP matrices showed significantly positive correlation with TRAP + SRAP + morpho-agronomic matrix. Agrama and Tuinstra (2003) reported that, genetic diversity of sorghum measured using SSR and RAPD markers exhibited highly significant association with geographic origin and race classification. The correlation of pairwise distances between all pairs of genotypes for SSRs when compared with geographical and race was r = 0.51; the correlation for RAPDs with geographical and race data was r = 0.43. The correlation of pairwise distances among all pairs of sorghum genotypes for SSRs when compared with RAPDs was r = 0.79. Also, significant and positive correlation between distance matrices generated using morphological traits, end-use quality and molecular markers in wheat were reported (Fufa et al., 2005). Recently, Vieira et al. (2007) reported that the matrices obtained by morphological and molecular marker data analyses in wheat revealed a significant but moderate correlation (r = 0.47), indicating that such techniques sample distinct genome regions.

The moderate association between genetic distances estimated using molecular and phenotypic markers can be explained by a range of factors (Al-Doss et al., 2009; Barakat et al., 2010). Molecular analysis provides a wider genome sampling than the morphological analysis, since a study comparing both techniques rarely evaluates the same or even a similar, number of morphological and molecular markers. The association between estimates is also influenced by the fact that a large portion of the variation detected by molecular markers is non-adaptive and therefore, not subject to either natural or artificial selection. On the other hand, the phenotypic characters are subjected to both natural and artificial selection, aside from their high environmental dependence. Moreover, it is not always the case that two identical phenotypes are determined by the same genes, that is, distinct genes may lead to similar phenotypes. Thus, it is clear that such estimates are closer when there is an association between the loci controlling the targeted morphological traits (quantitative trait loci, or QTLs) and the evaluated bands and when a large number of morphological traits are evaluated.

In the present investigation, the characterized durum wheat genotypes were mainly classified according to morpho-agronomic traits under heat stress conditions, which were complex and multigenic characters. Such characters were environmentally affected and therefore, liable to subjective evaluation. In this sense, the molecular characterization was more efficient in the generation of an unbiased picture of diversity than an agronomic approach. However, the agronomic characterization was still important in wheat germplasm management and determination of molecular diversity should not be seen as replacing traditional characterization, but rather as a complement to it.

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