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Evaluation of classification methods in durum wheat

S.T.Kotzamanidis[1], A.Mavromatis[2], A.Korkovelos[2], V.Greveniotis[3], D.Hasioti[2], C.G.Ipsilandis[4]*

[1]NAGREF, Cereal Institute, Thermi 57001, Thessaloniki, (GREECE)

[2]Department of Plant Production, University of Thessaly, N. Ionia, Volos 38446, (GREECE)

[3]Dpt. of Agriculture Development, Democritus University of Thrace, Orestiada 68200, (GREECE)

[4]Dpt. of Agriculture, Prefecture of Thessaloniki, A. Papanastasiou 63, 54453 Thessaloniki, (GREECE)

E-mail: ipsigene@gmail.com

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ABSTRACT

In this study, evaluation of classification methods in durum wheat involved cluster analyses on data from RAPD and SSR techniques, morphological characteristics according to CPVO and various agronomic characteristics, including yield. Molecular methods exhibited the greatest r-coefficient (r= 0.499). The SSR method showed a satisfying correlation to agronomic data (r= 0.439). Field methods exhibited a lower correlation (r= 0.395). CPVO method showed no correlation to molecular methods. Electrophoregrams of the gliadin proteins showed that the first step for selecting promising varieties is the presence (in both parents of a cross) of band 42. All the methods had errors in estimating genetic distances between all the possible pairs of the varieties used, since in many cases, closely related varieties showed great genetic distances and the opposite, not related varieties showed low genetic distances. For this, it is obvious that none of these methods alone could predict the promising crosses. If the relation of the varieties is known from pedigree records, then data from the methods used may become satisfactory reliable. In combination to quality data from electrophoregrams of the gliadin proteins, these data are very useful for parent selection. SSR and agronomic methods were more capable for discovering of promising crosses. SSR method seems to be more effective than agronomic method, since easily and rapidly a breeder may have data that could indicate the most promising parents, while in our study, CPVO and RAPD data proved less effective.

KEYWORDS

RAPD;
SSR;
Morphological;
Agronomic;
Classification.

INTRODUCTION

In the last decade the narrowing of genetic base exploited for crop improvement and the need for introgression of further variation become a matter of great consideration[1]. According to Gepts[2], genetic diversity can be evaluated with morphological traits, seed proteins, isozymes and DNA markers. Estimation of genetic diversity between durum wheat cultivars is based on different types of data, usually involving morphological traits and genetic analyses based on various genetic markers such as RAPD markers, even though

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there was not found any significant correlation between molecular methods and data from morphological traits[3]. In general, molecular data and data from morphological traits have low or no correlation at all, depending on the number and choice of morphological characters[3]. Morphological characters limited in number, are not reliable and their relationship to other data may be influenced by environmental conditions[4]. Genetic markers proved to be more effective than morphological traits for classification purposes[5,6]. Landraces of durum wheat can contribute in genetic diversity[7]. For this reason agronomically useful traits must be evaluated for effective contribution in breeding programs[8], although lack of genetic diversity was found in Greek durum wheat landraces using RAPD data[6].

Simple Sequence Repeats (SSR) or microsatellites are small DNA segments that are abundant, dispersed throughout the genome and show higher level of polymorphisms compared to other genetic markers. Due to their advantages compared with other types of molecular markers, microsatellites have recently become important genetic markers in cereals, including wheat[9]. Theoretically, the SSR assays are more robust than RAPDs and more transferable than AFLPs (dominant marker) where the polymorphisms are often diffícu1t to transfer to more sequence specific PCR applications. Microsatellite markers were used to detect the genetic diversity of wheat[10-13]. Classification of genotypes based on morphological data and RAPD markers found to be satisfactory with similar results and correlation between morphological and genetic data of 0.63[14]. According to Mitrick *et al.*[5], morphological data were less effective for classification than RAPD markers. In general, correlations between different types of data (RAPD or RFLP and morphological traits) were low and sometimes non-significant in case of yield[15,4,16,6].

The purpose of this study was to compare classification methods based on morphological data, data from agronomic characteristics and from genetic markers such as RAPDs and SSRs, for evaluation of durum wheat germplasm.

MATERIALS AND METHODS

Evaluation of classification methods in durum wheat involved cluster analyses on data from RAPD and SSR techniques, morphological characteristics according to CPVO and various agronomic and quality characteristics involving yield.

Molecular analysis

(a) DNA extraction and PCR amplification

Total genomic DNA (0.2g) was extracted from young leaves as a bulk of ten individual wheat seedlings using a modified CTAB method[17]. The varieties used were: Anna, Athos, Aias, Kallithea, Mexicali81, Papadakis, Pontos, Selas, Sifnos, Skiros, Sapfo, Santa, Sarti, Samos, Syros, Skiti.

(b) RAPD primers and data analysis

For molecular analysis of genotypes were used in total 21 decamer sequences provided by Operon Technology, USA. The primers used in this study are listed in TABLE 1. The PCR amplification reactions were performed using 30 ng template DNA at a 25 µl volume reaction containing: 0.4 m*M* RAPDs primers, 0.2 m*M* dNTPs, 1.5 m*M* $MgCl_2$, 1 x PCR buffer and 1 U *Taq* DNA polymerase. In case of RAPD, the primary cycle lasted 6 min under 94 °C. Denaturation lasted 1 min at 94 °C. Annealing lasted 1 min under 38 °C. Extension lasted 1.5 min under 72 °C. Hybridization lasted 7 min under 72 °C.

TABLE 1 : The nucleotide sequences of the 21 RAPD primers

RAPD primer	Sequence (5´ to 3´)	RAPD primer	Sequence (5´ to 3´)
OPC-03	5'-GGGGGTCTTT-3'	OPA-07	5'-GAAACGGGTG-3'
OPC-06	5'-GAACGGACTC-3'	OPA-08	5'-GTGACGTAGG-3'
OPC-07	5'-GTCCCGACGA-3'	OPA-17	5'-GACCGCTTGT-3'
OPC-08	5'-TGGACCGGTG-3'	OPB-08	5'-GTCCACACGG-3'
OPC-09	5'-CTCACCGTCC-3'	OPB-10	5'-CTGCTGGGAC-3'
OPC-11	5'-AAAGCTGCGG-3'	OPN-04	5'-GACCGACCCA-3'
OPC-14	5'-TGCGTGCTTG-3'	OPO-04	5'-AAGTCCGCTC-3'
OPC-15	5'-GACGGATCAG-3'	OPO-06	5'-CCACGGGAAG-3'
OPC-16	5'-CACACTCCAG-3'	OPO-12	5'-CAGTGCTGTG-3'
OPC-17	5'-TTCCCCCCAG-3'	OPO-15	5'-TGGCGTCCTT-3'
OPE-02	5´-GGTGCGGGAA-3´		

The analyses of data were performed in NTSYS and Statistica software packages, after the coding of molecular data. The presence or the absence of a particular DNA fragment was scored with (1) and (0) respectively. The calculation of genetic similarity was performed based on the coefficients of Jaccard[18] and

Dice[19]. The matrices that obtained were used for the construction of dendrograms with UPGMA methods.

(c) SSRs and data analysis

In this study, 13 random microsatellites (SSRs) were used (TABLE 2). DNA amplifications were carried out in 30μL reaction mixtures, each, 10 mM Tris-HCl, pH 9.0, 50 mM KCl 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 17 mM of each primer containing 100 ng template DNA, and 1 Unit Taq DNA polymerase (Minotec) using the following PCR profile in a Eppendorf DNA thermolcycler. In case of SSR, the primary cycle lasted 6 min under 94 °C. Denaturation lasted 50 sec at 94 °C. Annealing lasted 50 sec under 55 °C. Extension lasted 50 sec under 72 °C. Hybridization lasted 8 min under 72 °C.

TABLE 2 : Sequences of SSR primers

SSR primer	Sequence (left)	Sequence (right)
Xgwm33-1A	GGA GTC ACA CTT GTT TGT GCA	CAC TGC ACA CCT AAC TAC CTG
Xgwm136-1A	GAC AGC ACC TTG CCC TTT G	CAT CGG CAA CAT GCT CAT C
Xgwm193-6B	CTT TGT GC ACCT CTC TCT CC	AAT TGT GTT GAT GAT TTG GGG
Xgwm361-6B	GTA ACT TGT TGC CAA AGG GG	ACA AAG TGG CAA AAG GAG ACA
Xgwm644-7B	GTG GGT CAA GGC CAA GG	AGG AGT AGC GTG AGG GGC
Wms 297-7B	ATC GTC ACG TAT TTT GCA ATG	TGC GTA AGT CTA GCA TTT TCT
Wmc 256-6A	CCA AAT CTT CGA ACA AGA ACCC	ACC GAT CGA TGG TGT ATA CTGA
Wms 135	TGT CAA CAT CGT TTT GAA AAGG	ACA CTG TCA ACC TGG CAA TG
Wmc 233-5D	GAC GTC AAG AAT CTT CGT CGGA	ATC TGC TGA GCA GAT CGT GGTT
Wms 375-4B	ATT GGC GAC TCT AGC ATA TACG	GGG ATG TCT GTT CCA TCT TAGC
Wmc 25-2A	TCT GGC CAG GAT CAA TAT TACT	TAA GAT ACA TAG ATC CAA CACC
Wms 52-3D	CTA TGA GGC GGA GGT TGA AG	TGC GGT GCT CTT CCA TTT
Wms 234	GAG TCC TGA TGT GAA GCT GTTG	CTC ATT GGG GTG TGT ACG TG

The amplification products separated by gel electrophoresis on 6% denaturing polyacrylamide sequencing gels (0.4 mm thick, 50 cm long, OWL products), containing 7M urea and run with 0.5X TBE buffer for a constant power of 85 W for 3h. PCR products were mixed with equal volume of loading buffer and incubated at 95 ºC for 5 min and cooled on ice before loading and run as single strand DNA. The gel was fixed, stained and dried with a DNA silver staining method (Promega, Madison, WI, USA) and allele length determination was made by comparing the amplification fragments with two molecular markers 20 bp and 100 bp available from Sigma.

Number of alleles (N) per locus and their frequency were estimated. Microsatellite effectiveness for differentiating among species, which will be referred to herein as microsatellite's screening ability (MSA) was based on the following parameters: Diversity Indices (DI)[20], Probability of Identity (I)[21] and allelic polymorphic information content (PIC)[22].

For genetic diversity and phylogenetic relationship determination, all gels were classified in a binary format matrix where the presence of a band scored unit (1) and the absence zero (0). Based on the transformed data, the similarity coefficients[23] between species were estimated using the SIMQUAL program. Furthermore, a cluster analysis using the unweighted pair group method with arithmetic mean neighbor joining (NJ) procedures was performed. Then the correlation coefficient between similarity and cophenetic matrices for each similarity matrix and clustering procedure was estimated as well using Mantel's test. The previously mentioned data analyses were done using NT-SYS software, version 2.02I[24]. Combined SSR-RAPD analysis and clustering followed, based on Jaccard Coefficient and unweighted pair group method with arithmetic mean neighbor joining (NJ).

Protein electrophoresis for gliadin analysis

Gliadin extraction was performed as described by Autran[25]. Polyacrylamide gel electrophoresis (PAGE) was carried out according to the procedure of Bushuk and Zillman[26], except for the use of 0.05 M HCOOH as buffer system adjusted to pH 3.1

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with 0.01 M NaOH[27]. After staining, electrophoregrams were examined for bands 42 (weak gluten) and 45 (strong gluten).

Morphological characteristics according to CPVO

Measurements and evaluation of cultivars involved three years in test fields of the farm of NAGREF in Thessaloniki and two years in test fields of the Variety Research Institute of Cultivated Plants. Morphological characteristics according to CPVO tables were measured for all these years. In total, 26 characteristics were measured[28].

For this purpose 1000 rows were sown, 1m long and 0.25m apart. Each year, observations were taken only between plants with uniform expression of the desired characteristics. 20 plants were used for each characteristic within 200 uniform rows. During the growing season, the selected plants were under surveillance for all morphological characteristics. The varieties used were: Anna, Athos, Aias, Kallithea, Mexicali81, Papadakis, Pontos, Selas, Sifnos, Skiros, Sapfo, Santa, Sarti, Samos, Syros, Skiti. Their pedigree is presented in TABLE 3.

Calculation of genetic distances was based on the Euclidean model of distances with unweighted pair-group average and cluster analyses were performed on SPSS and Statistica software packages.

Agronomic characteristics

In two different sites (the farm of NAGREF in Thessaloniki and the experimental station of Agios Mamas) there were conducted experiments to measure various agronomic and quality characteristics for two growing seasons (2003-04 and 2004-05). Randomized Complete Block designs with four replications were used and each plot consisted of 7 rows, 4m long and 0.25m apart. Within each plot two inner rows were used for measurements (outside rows served as borders). Measurements were conducted in specified parts of each row, 50cm long for both sides of the row and for all replications. Agronomic characteristics measured were: number of tillers per plant, total number of reproductive tillers per plant, number of kernels on the spike, kernel weight per spike. Especially for the two last measurements, 5 spikes were used separately within the specified areas of the two rows in the plots and means were calculated. The rest of measurements involved silking, plant height, 1000-kernel weight, yield, total protein, vitreousity and black points. The varieties used were: Anna, Athos, Aias, Kallithea, Mexicali81, Papadakis, Pontos, Selas, Sifnos, Skiros, Mavragani Iraklio, Myrina, Kornos, Limnos, Simeto, Simi (TABLE 3).

TABLE 3 : Pedigree of durum wheat varieties

Variety	Pedigree
Papadakis	Athos/Mexicali81//Mexicali81(BC)
Aias	Selection from Yavaros
Pontos	Selection from Mexicali81
Anna	Mexicali81/Santa
Mexicali81	Selection from Mexicali75 (61.130/Leeds//Jori3/GDOVZ469)
Athos	Selection from Appulo
Sifnos	Limnos/Mexicali81
Selas	Selection from Stork "S" (CIMMYT)
Kallithea	Selection from Capeiti 8
Skiros	Selection from WAHA "S"-PL "S"-RUFF / GTA "S"-ROL (ICARDA)
Sapfo	Selection from Crane
Santa	Mutation after irradiation of Methoni
Sarti	Mutation after irradiation of Methoni
Samos	Selection from CR "S" (21563/61.130/Leeds) (ICARDA)
Siros	Selection from PLC "S"-Ruff / GTA "S"-ROL (CIMMYT)
Skiti	Selection from CR "S"/T.DIC "S" VERNUM-GLL "S" (ICARDA)
Mavragani Heraclio	Iraklio, Greek landrace from Crete
Mirina	Greek landrace selection
Kornos	Greek landrace selection
Limnos	Greek landrace selection
Symi	Greek landrace selection
Simeto	Capeiti 8 / Valnova

ANOVA, clustering and calculation of genetic distances were based on Snedecor and Cochran[29] and SPSS manual[30], and were performed on SPSS and Statistica software packages. Genetic distances were calculated according the Euclidean model of distances with unweighted pair-group average[31]. Data were standardized to become comparable. Additionally, correlations were calculated from common varieties' data[29,30] between the tables of genetic distances found by the above-mentioned methods.

RESULTS AND DISCUSSION

Evaluation of classification methods in durum wheat involved cluster analyses on data from RAPD and SSR techniques, morphological characteristics according to CPVO and agronomic characteristics (Figures 1, 2, 3, 4 and 5). The precision of molecular data analyses was relative high (The RAPD matrix correlation: r = 0.88827, approximate Mantel t-test: t = 4.3670, SSR Matrix correlation: r = 0.84500, approximate Mantel t-test: t = 4.6065, combined RAPD/SSR analysis matrix correlation: r = 0.91586, approximate Mantel t-test: t = 4.1877, pr. random Z<obs. Z: p = 1.0).

In RAPD and SSR methods, Mexicali 81 exhibited the greatest genetic distances in most cases, but exhibited erroneously great genetic distances (Figures 4, 5, 6) to related varieties (according to pedigree in TABLE 3) such as Papadakis, Pontos, Anna in RAPD and SSR respectively and Selas in RAPD. Additionally, varieties pair Pontos and Aias in RAPD and SSR and varieties pair Selas and Aias in SSR showed erroneously the lowest genetic distances, although not related to each other. In bread wheat (*Triticum aestivum* L.), genetic distances based on RAPD markers found to have no correlation with hybrid performance and heterosis leading to the conclusion that it is impossible to predict performance from RAPD data[16]. Maccaferri *et al*.[32] stated that, if the results did not agree with the registered parentages, SSR markers could provide information to identify the most probable parents. For CPVO data, Mexicali 81 exhibited the greatest euclidean distances in most pairs, but exhibited erroneously great genetic distances to related varieties Papadakis, Pontos, Anna (Figure 1). Additionally, pairs of varieties such as Pontos and Aias showed erroneously the lowest genetic distance although not related to each other. For agronomic and quality data Mexicali 81 exhibited the greatest euclidean distances in most pairs but ex-

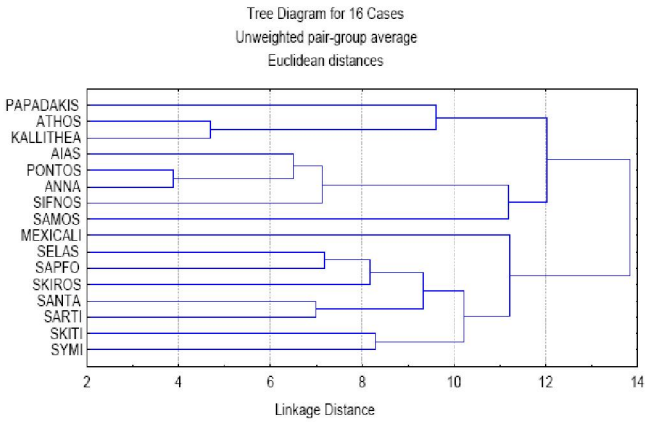

Figure 1 : Dendrogram of durum wheat cultivars based on CPVO euclidean distances

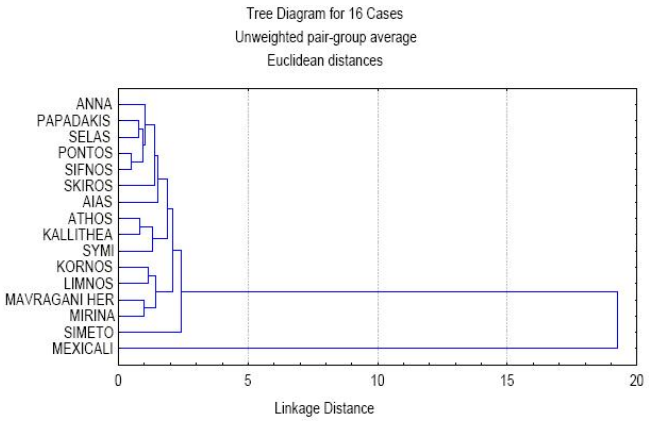

Figure 2 : Dendrogram of durum wheat cultivars based on agronomic euclidean distances

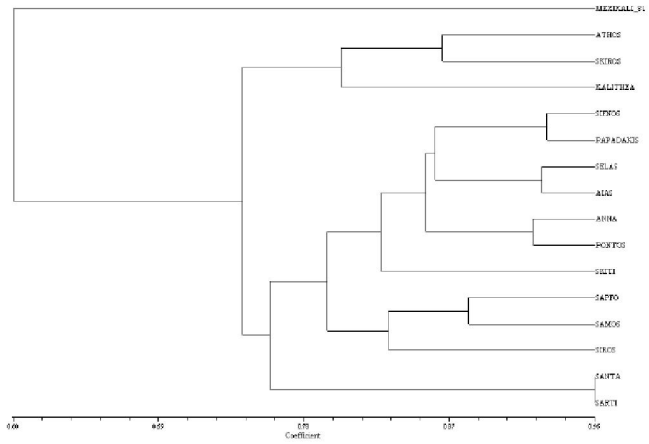

Figure 3 : Dendrogram of durum wheat cultivars based on SSR molecular markers

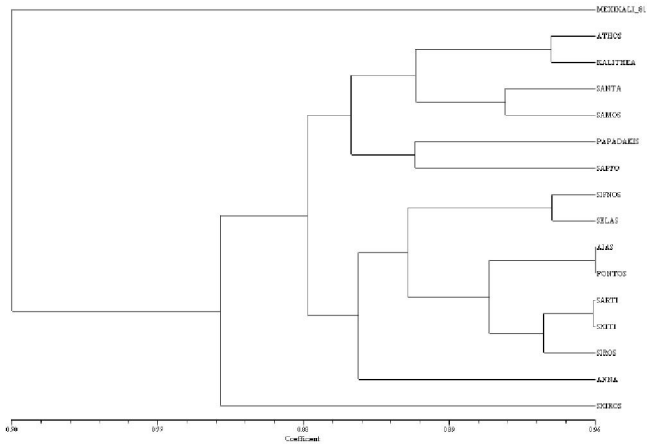

Figure 4 : Dendrogram of durum wheat cultivars based on RAPD molecular markers

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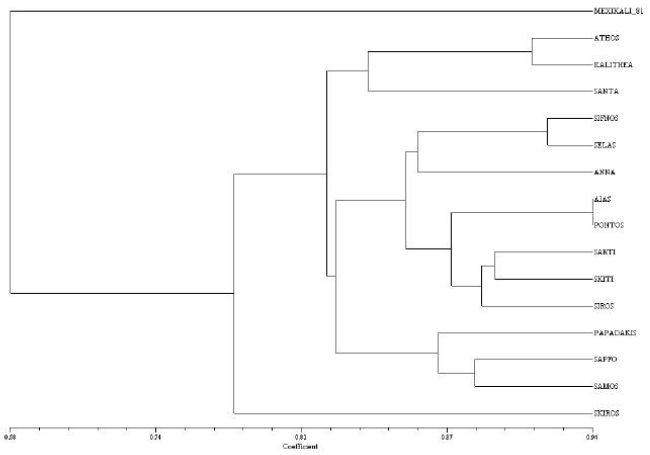

Figure 5 : Dendrogram of durum wheat cultivars based on combined RAPD and SSR molecular analysis

hibited erroneously great genetic distances to related varieties Papadakis, Pontos, Anna, Sifnos and Selas. Additionally, pairs of varieties Pontos and Aias, Selas and Aias showed erroneously the lowest genetic distances (Figure 2). Smith and Smith (1989)[33], using RFLPs and pedigree data concluded that morphological characters might be unreliable. Correlations of data, between all methods used are presented in TABLE 4. Molecular methods exhibited the greatest r-coefficient (r= 0.499). The SSR method showed a satisfying correlation[15] to agronomic data (r= 0.439). Field methods exhibited a lower correlation (r= 0.395). CPVO method showed no correlation to molecular methods.

TABLE 4 : Correlations (r coefficients) for all methods used

	CPVO	agronomic	SSR
agronomic	0.395		
SSR	0.281 (ns)	0.439	
RAPD	0.142 (ns)	0.361	0.499
Combined SSR/RAPD	0.242 (ns)	0.342	

Electrophoregrams of the gliadin proteins (of all the cultivars examined) showed that varieties Aias, Simeto and Pontos had band 42 instead of band 45, while all the others possessed band 45 but not band 42. Gliadins may be used for evaluating quality of durum wheat[34,35]. If, additionally to yield, quality of gluten is the target, then the presence of band 45 in gliadin electrophoregrams may reveal the best varieties. Thus, gliadin electrophoresis may be the first step for selecting promising varieties by rejecting low quality materials when both parents of a cross develop band 42.

Concluding, all the methods had errors in estimating genetic distances between all the possible pairs of the varieties used, since in many cases, closely related varieties showed great genetic distances and the opposite, not related varieties showed low genetic distances. For this, it is obvious that none of these methods alone could predict the promising crosses. If the relation of the varieties is known from pedigree records, then data from the methods used may become satisfactory reliable. In combination to quality data from electrophoregrams of the gliadin proteins, these data are very useful for parent selection. With this in mind SSR and agronomic methods were more capable for discovering of promising crosses. SSR method seems to be more effective than agronomic method, since easily and rapidly a breeder may have data that could indicate the most promising parents, while in our study, CPVO and RAPD data proved less effective.

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