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MOLECULAR DIVERSITY IN TUNISIAN DURUM WHEAT ACCESSIONS BASED ON MICROSATELLITE MARKERS ANALYSIS

Meriam Nefzaoui[1], Sripada Mahabala Udupa[2], Mohamed Salah Gharbi[3], Mariem Bouhadida[3] and Driss Iraqi[2]

[1]Institut Supérieur des Sciences Biologiques Appliquées de Tunis, Université El Manar, Tunis, Tunisia
E-mail : meriam.nef@gmail.com

[2] INRA Morocco/ICARDA Biotechnology Laboratory, Rabat, Morocco

[3] Institut National de la Recherche Agronomique de Tunis, Rue Hedi Karray, 2036 Ariana, Tunisia

ABSTRACT

In Tunisia, durum wheat (*Triticum durum* Desf.) varieties and landraces adapted to different ecological regions are available for cultivation by farmers. However, many of these varieties and landraces exhibit highly similar morphologies, which make their identification and characterization difficult. In this study, we identified microsatellites from the A and B genomes of bread wheat (*Triticum aestivum* L.) that are useful for the identification, characterization and genetic relationship estimation of the main durum wheat varieties and landraces of Tunisia. These markers amplified a total of 24 alleles, with a number of alleles per locus varying from 2 (for *Xpsp 2999, Xgwm 193* and *Xgwm 130*) to 3 (for *Xgwm 136, Xgwm 389, Xgwm 610, Xgwm 493, Xgwm 273* and *Xgwm 89*) and an average of 2.667. The Polymorphism Information Content (PIC) varied between 0.1103 for Xgwm193 to 0.556 for Xgwm493, with an average value of 0.363. Average genetic diversity among the accessions was 0.422. These results indicate that microsatellite primers from bread wheat can be effectively applied in durum wheat to assess genetic diversity and to differentiate between accessions. All of the varieties were identified with the 9 microsatellite markers used in this study. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram based on Nei's genetic distance placed the accessions into 3 different clusters.

Key words: *Triticum durum* Desf., molecular markers, simple sequence repeats (SSR), genetic diversity, genetic distance.

INTRODUCTION

Durum wheat (*Triticum durum* Desf.) is a member of the *Gramineae* family, and of the *Triticeae* tribe and belongs to the genus *Triticum*. Durum wheat is an allotetraploid (two genomes: AABB) and with a total of 28 chromosomes (2n=4x=28). Based on cytological and molecular analysis, *Triticum durum* Desf. is believed to have originated from the natural hybridization of *Triticum monococcum* L. subsp. *boeoticum* (Boiss.) (Synonym: *Triticum urartu*: AA) with an unknown diploid wheat species containing the B genome (Feldman, 1976). Durum wheat is considered as the second most important *Triticum* species, next to common wheat (*Triticum aestivum* L.).

Wheat is one of the most economically important cultivated crops in the world. World production of durum wheat is about 27 million tons from some 17 million ha (Nefzaoui et al., 2012). In Tunisia, it covers an average area of 600 000 ha, with an average total production of 1.0-1.2 million tons (Nefzaoui et al., 2012).

The high protein content and specific gluten properties make durum good for special uses. The principal use of durum wheat grain is the production of semolina for use in pasta products. In North Africa, and particularly in Tunisia, durum is preferred for the production of couscous and "burgul", in addition to macaroni. Traditional breads are also made out of durum flour.

In Tunisia, durum wheat varieties and landraces adapted to different ecological regions are being cultivated by farmers. Many of these varieties and landraces exhibit highly similar morphologies, which makes their

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identification and characterization difficult. Most of the varieties grown in marginal areas are landraces and they carry useful variability for genetic improvement. A good characterization of existing genetic resources helps to an effective choice of parental materials for hybridization and suitable germplasm development.

Genetic diversity of wheat genotypes has been largely evaluated using morphological and protein variation. In most countries, characterization and identification of commercial wheat varieties are generally based on morphological and phenological observations according to UPOV (Unité pour la Protection des Obtentions Végétales) and IPGRI (International Plant Genetic Resources Institute) descriptors.

Microsatellite markers (SSRs) have gradually evolved into appropriate markers for molecular identification, characterization and genetic diversity studies in crop plants including wheat.

Consisting of tandem repeats of mono-, bi-, tri- or tetra-nucleotides in the eukaryotic genome, these molecular markers present a very high polymorphism based on the number of the nucleotide motif repeats (Morgante and Olivieri, 1993). In addition, they have a co-dominant inheritance and are reproducible and easily detected by PCR (polymerase chain reaction) tools.

This technique has been used in crop species including rice, wheat, maize, barley, rapeseed, soybean, potato and other crops, for characterization, identification and documentation (Rahman et al., 2006, 2007).

The present study employed 9 microsatellite markers distributed across the A and B genomes of bread wheat (*Triticum aestivum* L.) to characterize 16 durum wheat varieties and landraces and determine the usefulness of these markers for cultivar identification, characterization and genetic diversity analysis.

MATERIAL AND METHODS

Plant materials

Sixteen accessions of durum wheat consisting in both varieties and advanced lines developed at the Durum Wheat Breeding Program of the National Institute for Agronomic Research of Tunisia (INRAT) were studied. These included three old varieties or landraces (Chili, Mahmoudi and Agili 2), three varieties (INRAT 69, Maghrebi and Ben Bachir) developed during the 1960s and 1970s through hybridization of local varieties with semi-dwarf types, eight current semi-dwarf high yielding varieties (Karim, Yavaros, Razzak, Khiar, Nasr, Oum Rabiaa, Maali and Salim), and two new breeding lines (INRAT100 and INRAT102), recently developed at INRAT, but not yet included in the Tunisia national catalogue of plant varieties.

DNA extraction and amplification

Fresh young leaves were ground to powder with liquid nitrogen using a mortar and pestle. Genomic DNA was isolated from leaf samples using the CTAB (CethylTrimethyl Ammonium Bromide) extraction method described by Saghai-Maroof et al. (1984) and modified by Udupa et al. (1998). DNA quality was examined by electrophoresis in 1% agarose and estimated by visual comparison of DNA bands on gel with known concentrations of phage lambda DNA.

Nine bread wheat microsatellite markers belonging to chromosomes 1A, 1B, 3B, 4A, 6B, and 7A (Table 1) were selected for genotyping. DNA amplification reaction were carried out in a final volume of 10 µl containing 1 µl of template DNA (20ng/µl of DNA) and 9 µl of the PCR master mix composed of 1 µl of 10 X PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$), 1 µl of 0.2 mM dNTPs, 1 µl of 10 pmole/µl of forward and reverse primers and 0.2 µl of *Taq* DNA Polymerase (Roche).

The PCR amplification was carried out in a master cycler gradient (Eppendorf) using the following temperature cycles: 1 cycle of 5 min at 94°C (pre-denaturation) followed by 35 cycles of: (1) denaturation of the double-stranded DNA during 30 s at 94°C; (2) annealing of primers to DNA during 30 s at 59°C and (3) elongation step during 45 s at 72°C. The last cycle was followed by a final incubation for 5 min at 72°C and the PCR products were stored at 4°C before analysis.

The DNA amplification products were loaded on 8% non-denaturing polyacrylamide gels in 1 x TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA). Gels were run at 180 V, stained by ethidium bromide and then visualized under UV. Fragment sizes were estimated with the 100 bp ladder (Invitrogen) DNA sizing markers.

Data analysis

Based on the gel analysis, alleles were detected. The genetic diversity index (*H*) was calculated for all the loci studied according to the formula of Nei (1987):

$$H = n(1-\Sigma p_i^2)/(n-1)$$

where *"n"* is the number of analysed genotypes and "p_i" is the frequency of i^{th} allele.

The polymorphism information content (*PIC*) for each marker was also determined, using the following equation of Botstein et al. (1980):

$$\widehat{PIC}_l = 1 - \sum_{u=1}^{k} \tilde{p}_{lu}{}^2 - \sum_{u=1}^{k-1} \sum_{v=u+1}^{k} 2\tilde{p}_{lu}^2 \tilde{p}_{lv}^2$$

where p_i is the frequency of the i^{th} allele in the set of 16 genotypes. Those parameters served to evaluate and summarize the information derived from the microsatellites markers (Table 1).

Table 1. Description of 9 wheat microsatellite markers, their chromosomal location and left and right primers

Microsatellite marker name	Chromosome location	Left primer (5'→ 3')	Right primer (5'→ 3')
Xgwm130	7A	AGCTCTGCTTCACGAGGAAG	CTCCTCTTTATATCGCGTCCC
Xgwm136	1A	GACAGCACCTTGCCCTTTG	CATCGGCAACATGCTCATC
Xgwm193	6B	CTTTGTGCACCTCTCTCTCC	AATTGTGTTGATGATTTGGGG
Xgwm273	7A	ATTGGACGGACAGATGCTTT	AGCAGTGAGGAAGGGGATC
Xgwm389	3B	ATCATGTCGATCTCCTTGACG	TGCCATGCACATTAGCAGAT
Xgwm493	1B	TTCCCATAACTAAAACCGCG	GGAACATCATTTCTGGACTTTG
Xgwm610	4A	CTGCCTTCTCCATGGTTTGT	AATGGCCAAAGGTTATGAAGG
XPSP2999	1A	TCCCGCCATGAGTCAATC	TTGGGAGACACATTGGCC
Xwmc89	4A	ATGTCCACGTGCTAGGGAGGTA	TTGCCTCCCAAGACGAAATAAC

The binary matrix was obtained from the reading of the electrophoretic patterns corresponding to all the microsatellites analysed. Amplified fragments for each locus were scored as present allele (1) or absent allele (0). The binary matrix was used to calculate the genetic distance between each pair of accessions using the formula of Jin and Chakraborty (1993):

$$D_{SAB} = 1 - [2 P_{SAB} / P_{SAX} + P_{SAY}]$$

where: D_{SAB} is the average proportion of alleles shared between populations *X* and *Y*; P_{SAB}, P_{SAX} and P_{SAY} are calculated by all possible combinations of accessions.

A dendrogram was constructed using data of the genetic distance matrix and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering (Sokal and Michener, 1958). All the analyses were performed using the software Power Marker version 3.0 (Liu and Muse, 2005).

RESULTS AND DISCUSSION

Microsatellite diversity

The 16 durum wheat accessions were analysed using 9 microsatellite markers. The microsatellite markers studied amplified each one single-locus. A total of 24 alleles were amplified with a range of 2 to 3 alleles per locus, with a mean value of 2.6 alleles per locus (Table 2). All the microsatellites employed in this study revealed polymorphism and enabled unambiguous discrimination among the durum wheat varieties. Six of the nine SSR markers belonged to A genome and 3 to B genome (Table 1).

The genetic diversity index (*H*) for microsatellites from A genome ranged from 0.22 (*Xpsp 2999*) to 0.60 (*Xgwm 610*) with a mean value of 0.44. For microsatellites from B genome, the genetic diversity index (*H*) varied from 0.12 (*Xgwm 193*) to 0.63 (*Xgwm 493)*, with a mean value of 0.38. These results show that the genetic diversity among the 16 accessions studied is slightly higher in *A* genome than in B genome.

Table 2. Number of alleles, major allele frequency, genetic diversity index and polymorphism information content (PIC) for the microsatellite markers used in the study

Wheat microsatellite marker name	Chromosome location	N°. of alleles	Major allele frequency	H*	PIC**
Xgwm 136	1A	3	0.56	0.54	0.45
Xgwm 389	3B	3	0.75	0.40	0.35
Xgwm 610	4A	3	0.50	0.60	0.51
Xpsp 2999	1A	2	0.87	0.22	0.19
Xgwm 193	6B	2	0.94	0.12	0.11
Xgwm 493	1B	3	0.44	0.63	0.55
Xgwm 273	7A	3	0.81	0.32	0.29
Xgwm 89	4A	3	0.69	0.47	0.43
Xgwm 130	7A	2	0.53	0.50	0.37
Total	-	24	-	3.79	3.37
Mean$^{(A)***}$	-	2.6	-	0.44	0.37
Mean$^{(B)***}$	-	2.6	-	0.38	0.34
Mean $^{(C)***}$	-	2.6	0.68	0.42	0.36

*H: genetic diversity index; ** PIC: polymorphism information content;
***mean (A): mean for A genome; Mean (B): mean for B genome; Mean (C): mean for C genome.

The most informative locus of this study was *Xgwm 493* located in the B genome, with a PIC value of 0.55, while the less informative one was *Xgwm 193* also in the B genome with a PIC value of 0.11. Roussel et al. (2004) also found that the highest PIC value occurred in the B genome using SSR markers for molecular diversity in French bread wheat accessions. The highest PIC value and the highest genetic diversity index (*H*) were observed for *Xgwm 493* from the B genome. Our results indicated that the two parameters, *PIC* and *H*, were very useful criteria for the identification of microsatellite markers that enable unambiguous discrimination among related durum wheat accessions. These results showed that the number of loci necessary to characterize a durum wheat collection can be effectively reduced by using the most informative ones. The highest values of the major allele frequency in each locus were observed for *Xgwm 193* (0.*94) and Xpsp 2999 (0.87),* while the lowest values were observed for *Xgwm* 493 (0.44) and *Xgwm 610* (0.50) (Table 2). The less informative markers with the lowest genetic diversity index showed the highest frequencies of major alleles, and the most informative ones with the highest index of genetic diversity showed the lowest frequencies.

According to Botstein et al. (1980), a value of *PIC* greater than 0.5 corresponds to a very informative marker; PIC values between 0.5 and 0.25 correspond to a reasonably informative marker, and a PIC value less than or equal to 0.25 reflects a poorly informative marker. Therefore, and according to Table 2, the markers used in this study, with the exception of two markers (*Xgwm 193* and *Xpsp 2999*), were either highly informative markers or informative.

Genetic relationships among cultivars

Genetic distances (Table 3) and the dendrogram (Figure 1) were developed using the UPGMA methodology.

The dendrogram showed a clear separation between all accessions, which is a proof that the microsatellites used are sufficient to identify, discriminate and group the Tunisian durum wheat varieties and landraces. Similar results have been reported by Plaschke et al. (1995) who showed that a small number of markers are able to discriminate closely related wheat accessions.

The dendrogram enabled us to classify the studied genotypes into two large groups, with the exclusion of Maghrebi which is positioned outside of these groups. The first group was composed of only three old varieties, i.e. Agili 2, Chili and INRAT69. The second group comprised the remaining twelve entries. This latter group itself was subdivided into two subgroups; the first one formed of only two varieties Ben Bachir and Oum Rabiaa, while the second subgroup comprised all semi-dwarf varieties (Razzak, Karim, Salim Yavaros, Maali, INRAT100, INRAT102, Khiar, and Nasr) plus the old variety Mahmoudi.

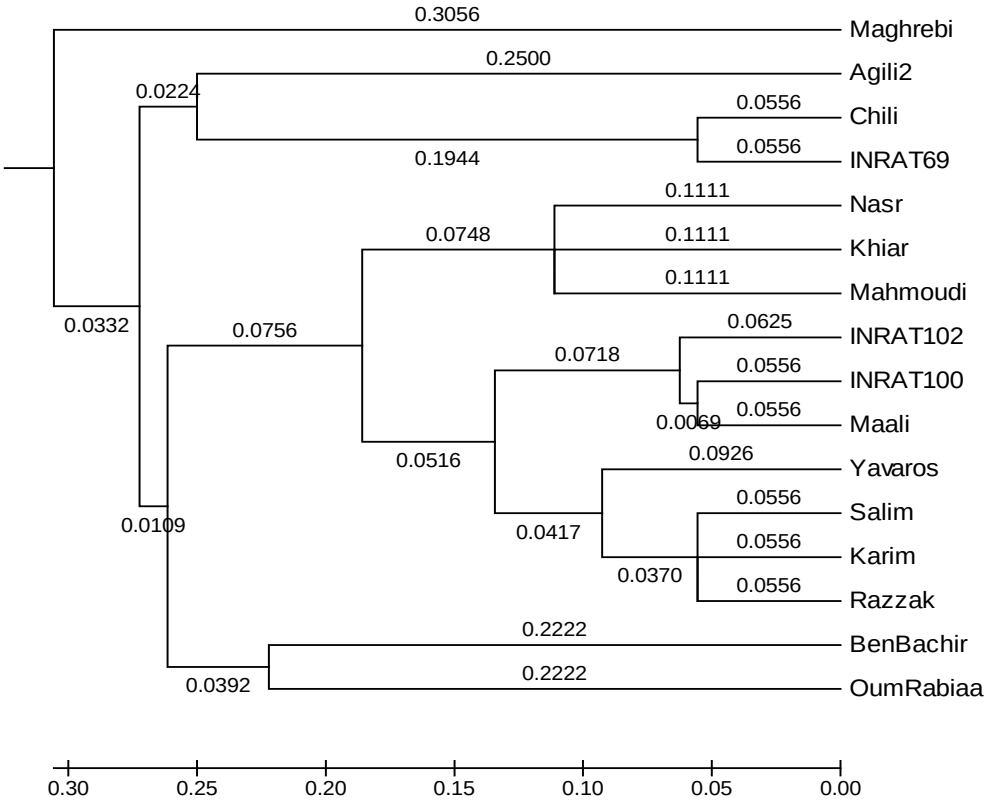

Figure 1. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram showing relationships among 16 durum wheat genotypes

The genetic distance matrix (Table 3) showed some genetic variability among the group of entries included in the study. In fact, the genetic distance index ranged between 0.111 and 0.778, indicating a good diversity among the investigated varieties. The smallest genetic distance (0.111) was observed between the variety Chili and variety INRAT 69. Chili is an old variety that has been grown in Tunisia since the 1930s. The second variety

is a selection derived from a cross involving Mahmoudi 981, a variety phenotypically very similar to Chili. The same value of genetic distance was found between the new variety Maali and the breeding line INRAT100 that share at least three parents in their pedigree.

Also, varieties Maali and Karim are closely related genetically with a similar genetic distance of 0.111. This is likely explained by the fact that the cultivar Yavaros, a sister line of Karim is also a parent of variety Maali. A similar genetic distance separates variety Razzak from its male parent Karim crossed with the female line Dmx69-331. A similarly low genetic distance (0.111) was also found between the varieties Salim and Razzak. This is explained by the fact that the variety Razzak is one of the progenitors of the cross that generated Salim.

A low genetic distance (0.125) was found between lines INRAT 100 and INRAT 102. These two lines share the same female parent "SOMO" S" /" S" STN / 3/TEZ" S" / YAV79 //" S" HUI / 4/CHEN/ALTAR84", which explains the low genetic distance that separates them. A similar genetic distance was found between the breeding line INRAT 102 and variety Maali, which was quite expected, given that Maali is the male parent of INRAT 102.

The largest genetic distance (0.778) was observed between Yavaros and Agili 2; the first being a variety that is semi-dwarf, early, and highly productive, in contrast to the second (Agili 2) which is an old, late and low-yielding variety. A similar genetic distance (0.778) is observed between the old cultivar Maghrebi and each of the four varieties Mahmoudi, Agili2, Oum Rabiaa, and Khiar, suggesting that they belong to gene pools different from Maghrebi's.

The present study, covering old varieties, very recent varieties such as Maali and Salim, two new breeding lines not yet listed in the national Tunisian plant variety catalogue, as well as intermediate durum wheat varieties, is a very recent assessment of the genetic diversity in durum wheat for old cultivars, modern cultivars and future varieties that may cover Tunisian lands for years to come.

Table 3: Matrix of genetic distances among 16 Tunisian durum wheat accessions

	Agili 2	Ben Bachir	Chili	INRAT 100	INRAT 102	INRAT 69	Karim	Khiar	Maali	Maghrebi	Mahmoudi	Nasr	Oum Rabiaa	Razzak	Salim	Yavaros
Agili 2	0,000															
Ben Bachir	0,556	0,000														
Chili	0,556	0,444	0,000													
INRAT 100	0,444	0,444	0,444	0,000												
INRAT 102	0,625	0,375	0,375	0,125	0,000											
INRAT 69	0,444	0,556	0,111	0,556	0,500	0,000										
Karim	0,556	0,444	0,556	0,222	0,250	0,667	0,000									
Khiar	0,444	0,444	0,333	0,222	0,375	0,444	0,222	0,000								
Maali	0,556	0,556	0,556	0,111	0,125	0,667	0,111	0,333	0,000							
Maghrebi	0,778	0,556	0,444	0,667	0,500	0,556	0,556	0,778	0,556	0,000						
Mahmoudi	0,444	0,667	0,333	0,333	0,500	0,444	0,444	0,222	0,444	0,778	0,000					
Nasr	0,444	0,667	0,333	0,333	0,375	0,444	0,222	0,222	0,222	0,556	0,222	0,000				
Oum Rabiaa	0,667	0,444	0,667	0,667	0,750	0,778	0,444	0,444	0,556	0,778	0,556	0,444	0,000			
Razzak	0,667	0,444	0,556	0,333	0,250	0,667	0,111	0,333	0,222	0,556	0,556	0,333	0,556	0,000		
Salim	0,667	0,333	0,444	0,333	0,125	0,556	0,111	0,333	0,222	0,444	0,556	0,333	0,556	0,111	0,000	
Yavaros	0,778	0,556	0,667	0,444	0,375	0,778	0,222	0,444	0,333	0,667	0,444	0,444	0,556	0,111	0,222	0,00

CONCLUSIONS

Microsatellite markers are informative descriptors of the genetic variability of Tunisian cultivated varieties of durum wheat studied for the purpose of cultivar identification. The high genetic variability of durum wheat will be exploited in the breeding programs. This study on the use of microsatellite markers has further confirmed these markers as a powerful tool for identification and characterization of intra-specific variations among durum wheat varieties and landraces. Molecular characterization data would be of enormous assistance for the establishment and defense of intellectual property rights (IPR). The information on genetic distance will also enable selection of maximized diversity in parents and will assist in broadening the germplasm base of future durum wheat breeding programs.

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