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Assessment of genetic diversity of wheat (*Triticum aestivum* L.) using agro-morphological characters and microsatellite markers

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Abstract

Genetic diversity of ten bread wheat (*Triticum aestivum* L.) varieties was evaluated at the DNA level using 16 wheat microsatellites molecular markers (Simple Sequence Repeat-SSR) and 6 agro-morphological characters. SSR bands were scored across all genotypes, for presence (1) or absence (0) and transformed into 0/1 binary matrix. A pair-wise similarity matrix was generated with the software NTSYS. The Polymorphism Information Content (PIC) ranged from 0.13 to 0.70 respectively for the primer *WMC 24* and *WMC 50* with an average of 0.48 and 0.49 per primer pair. The similarity coefficient between cultivars ranged from 0.33 and 0.90 with an average of 0.63. Most of the genotypes showed a high degree of genetic similarity. The highest genetic distance value of 0.90 has been scored between Milan/S87230/babax and Angi-4. The lowest genetic distance value of 0.33 has been scored between Hammam1 and Attila2Pastor. Genetic similarity values between genotypes, calculated by the molecular derived data, were used to produce a dendrogram. The genotypes were clustered in four clear groups according to their origin, pedigree and in some cases to phenotypic characters similarities. The morpho-agronomical variability was analyzed using the Principal Component Analysis (PCA).The results demonstrate the utility of microsatellite markers for detecting polymorphism to estimate genetic diversity.

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Introduction

Wheat (*Triticum aestivum* L.) is one of the most important staple crops world-wide, with a total production of over 600 million tons annually. Since the 'green revolution' in 1960s, wheat grain yield has increased by 40% because of the adoption of semi-dwarf varieties that contain the reduced height genes Rht1 and Rht2 (Peng *et al.*, 1999). This substantial improvement in productivity has been accompanied by improvement in quality. However wheat consumption is quickly increasing especially in developing countries. It is estimated that the global demand for wheat production will increase by a further 40% before 2020 (Rajaram, 2005). One of the strategies for achieving this productivity goal is the implementation of molecular tools in plant breeding.

The common wheat is a domestically cultivated grass and a polyploidy in all over the world. Bread wheat (*Triticum aestivum* L.) is a staple food and the major source of calories and protein for a large world population, and is among the most important grain crops in Algeria. Wheat contains approximately one half of the calories in human's food and can also fulfill the huge part of their nutritional necessities. The genetic variability in bread wheat is important for a better improvement of this crop and for the increase of cereal yield in the context of sustainable agriculture to face human needs in the next decades. *Triticum aestivum* L. (common wheat) belongs to the *Poaceae* family. This family is one of the most diverse and significant among the families of kingdom *Plantea*. The substantial increase in world's population demands a consistent increase in the production of wheat. The wheat research is very difficult, time consuming, extensive and is used to maximize the production of wheat grains. It can also be used to improve and get better yield of grain. However, there is still considerable space for the advancement and improvement in genetics of wheat to conquer the daily problems of increasing requirements of world population. Genetic manipulation and genetic diversity is a good path for increasing yield of wheat. Therefore, it is required to study and estimate different mode of inheritance and genetic variation in different parameters of plants to start the productive wheat breeding programs (Babay *et al.*, 2015).

Knowledge of genetic diversity in a crop species is fundamental to its improvement. Evaluation of genetic diversity levels among adapted, elite germplasm can provide predictive estimates of genetic variation among segregating progeny for pure-line cultivar development (Manjarrez-Sandoval *et al.*, 1997). Criteria for the estimation of genetic diversity can be different: pedigree records, morphological traits or molecular markers (Cox *et al.*, 1985).

The use of molecular markers for the evaluation of genetic diversity is receiving much attention. Many wheat scientists have studied genetic diversity in common wheat using different molecular markers (Chen *et al.*, 1994; Vollmann *et al.*, 2005). However, most of these marker systems show a low level of polymorphism in wheat, especially among cultivated lines and/or cultivars (Devos and Gale, 1992). Because SSRs are multiallelic, they have high potential for use in evolutionary studies (Schloetterer *et al.*, 1991; Chao *et al.*, 2007) and studies regarding genetic diversity and relationships. At present, microsatellites are one of the most promising molecular-marker categories that have the ability to identify or differentiate genotypes within a species. Their codominant inheritance, high level of polymorphism and easy handling make them extremely useful for many different applications (Devos *et al.*, 1995; Prasad *et al.*, 2000).

In the present study, we used the SSR markers and the agro-morphological traits to investigate the diversity among 10 genotypes within the ITGC germplasm collection in Constantine/ Algeria. The objectives of this study were to (*i*) use SSRs and agro-morphological characters to assess levels and patterns of genetic variability among a representative sample of local wheat genotypes, (*ii*) compare these genetic diversity estimates with other international wheat cultivars, (iii) and use wheat microsatellite markers for the characterization and assessment of the genetic diversity of ten wheat varieties.

Materials and methods

Plant material

This investigation was carried out at the experimental farm of ITGC (Technic Institute of High Culture) in Constantine/Algeria. Three local wheat varieties from ITGC, Constantine and seven introduce varieties were used to establish the experimental materials for this investigation. All wheat varieties, along with their pedigree and country of origin, are listed in Table 1.

The genotypes were sown and grown under rain fed condition in a randomized complete-block design with two replicates per genotype, at the experimental field of ITGC. Each variety was sown along 2 m-long rows at a density of 40 seeds per line with 5 cm between seeds. This plot was used to study morphology from the seedling stage to maturity.

Evaluation of agronomic Characters

Plants were selected at random for 6 morpho-agronomical characters measurements as follows: heading date HD (days), plant height PH (cm), number of ears per m^{-2} NEM, number of kernels per ear NKE, thousand kernels weight TKW (g) and grain yield GY (Q/Ha).

DNA Isolation

Total genomic DNA was extracted from frozen young leaves following a CTAB method modified and described by Ben Naceur (1998), and followed by an organic extraction in chloroform: isoamyl alcohol (24:1). DNA was purified by RNase (10 µg/ml) and its concentration was estimated using 0.8% agarose gel. DNA was dissolved and preserved in TE buffer (10 mMTris- HCl, 1 mM EDTA, pH 8.0).

DNA amplification

Amplification was performed using a thermocycler (Multigene optimax.), in a total volume of 25 µl containing 1.5mM MgCl2, 0.2 mM dNTPs, 0.25 µM of forward and reverse primers, 1U of *Taq* DNA polymerase (Go Taq, Promega; http://www.promega.com), 1x buffer and 50 ng/µl DNA. PCR consisted of one round of perdenaturation at 94°C for 3 min followed by 35 amplification cycles of: 2 min denaturation at 94°C, 1 min hybridization at Ta between 52-64°C (Table 2), 2 min of extension at 72°C. These cycles were followed by a final extension for 1 min at 72°C. PCR products were separated on a 2% agarose gel. To better discern some fine bands, we also used a 40% polyacrylamide gel prepared with 70 ml distilled water; 10 ml TBE (5x); 20 ml of 40% acrylamide (19:1; acrylamide: bisacrylamide); 800µl APS (ammonium persulfate (10x) and 80µl TEMED (tetramethyl ethylene diamine). DNA concentrations were estimated by comparison with 100-bp DNA ladder (Promega). The amplified product was visualized under UV light on a gel documentation system after staining the gel with 5 µl Ethidium bromide.

Microsatellite Markers Analysis

The reaction of DNA amplification by PCR was performed with 16 microsatellite primer pairs (*WMC 14, WMC15, WMC16, WMC17, WMC18, WMC19, WMC20, WMC21, WMC22, WMC23, WMC24, WMC25, WMC27, WMC48, WMC50 and WMC283*) reported in table 2.

Data collection

The agronomical variability was analyzed using the Principal Component Analysis (PCA). The Pearson correlation coefficients (α= 0.05, standard PCA without rotation of axes) were calculated by using the software XLSTAT 7.5.2 (Addinsoft, (www.xlstat.com/en/home).

Diversity Analysis

Genetic Similarity Estimation and Cluster Analysis

The SSR profiles were transformed into a binary matrix where the presence of the generated band at a precise level is scored as 1 and its absence is scored as 0. A data matrix was prepared for the analyses. A pair-wise similarity matrix was generated with the software NTSYS pc-2.02j (NTSYS-Numerical Taxonomy and Multivariate Analysis; ROHLF 1998). Estimates of genetic similarity (GS) among all genotypes were also calculated using Nei and Li (1979) coefficient of similarity between two individuals (i and j), according to the formula Nei and Li's coefficient = 2a/(b+c), where"a": is the number of shared bands in both samples i and j,"b": is the total number of bands of individual i and "c" the total number of bands of individual j.

The similarity matrix was used to construct a dendrogram by the unweighted pair group method arithmetic averages (UPGMA) procedure (Sokal and Michener, 1958). The goodness of fit of the clustering was tested using the MxCOMP program, which directly compares the original similarity matrix and the cophenetic value matrix, as suggested by Rholf (1998).

Marker Polymorphism

To measure the informativeness of the *SSR* markers, the polymorphism information content (PIC) for each *SSR* was calculated according to the formula:

$$\text{PIC} = 1 - \sum_{i=1}^{k} Pi^2$$

where k is the total number of alleles detected for a locus of a marker and *P* the *I* frequency of the *i* th allele in the set of 10 genotypes investigated.

Results and discussion

A total of 16 SSR primers were tested, although only 11 (*WMC 14, WMC 15, WMC 17, WMC 20, WMC 21, WMC 24, WMC 25, WMC 27, WMC 48, WMC 50, WMC 283*) produced polymorphic bands (Table 3).

Microsatellite Polymorphism

Eleven microsatellite markers for 11 loci were used to characterize and evaluate the genetic diversity of ten wheat genotypes (Table 3). A total of 34 alleles were detected. The number of alleles per locus ranged from two for *WMC 15, WMC 20*, *WMC 21, WMC 24*, *WMC 27*, *WMC 48* to 5 for *WMC 14* and *WMC 50 w*ith an average number of 3.2 alleles per locus.

Table 1. Variety name, country of origin and pedigree for the wheat varieties used in this study.

N°	Genotypes	Pedigrees	Origin
V1	Ain abid	ITGC/ Algeria	Algeria
V2	Arz	ITGC/ Algeria	Algeria
V3	Hidhab	ITGC/ Algeria	Algeria
V4	Hamam 1	ICW92-0455-1AP-1AP-2AP-3AP-0AP	ICARDA/Syria
V5	5119		SERASENE/France
V6	Milan/S87230/babax	CMSS97MO-3687T6040Y-03M-020Y-030M-015Y-38M	CIMMYT/Mexico
V7	Angi-4	ICW92-0326-12AP-1AP-2AP-3AP-0AP	ICARDA/Syria
V8	Cham 6	CM39992-8M-7Y-OM-0AP	CIMMYT/Mexico
V9	Attila	CM85-836-50Y-0M-OY-3M-0Y	CIMMYT/Mexico
V10	Attila/2 Pastor	CGSS97Y00042M-099T0PB-058Y-099M-099Y-099B	CIMMYT/Mexico

The maximum number of alleles was observed at *WMC 14* (Figure 1) and *WMC 50* and their size ranged from 219 to 239 bp. A similar pattern of allelic variation was also detected at other loci (Babay *et al.*, 2015). The landraces which are selected from local germplasm have a lower range of diversity; however, cultivars which are introduced would have a wide genetic diversity than both of wild genotypes or landraces. Furthermore, the detected genetic diversity for the ten bread wheat varieties is also lower than that reported by Plaschke *et al.* (1995) and Leisova *et al.* (2007) studying closely relatedEuropean wheat cultivars having an average of 12 alleles per locus.

The Polymorphism Information Content (PIC) ranged from 0.13 to 0.70 respectively for the primer *WMC 24* and *WMC 50* with an average of 0.48 and 0.49 per primer pair (Table 3).

These results are confirmed in two earlier studies on wheat where the PIC values ranged from 0.23 to 0.79 (Röder *et al.* 1995) and from 0.29 to 0.79 (Plaschke *et al.* 1995). In another recent study, the PIC mean value (0.30) for SSRs was lower than the mean value of 0.48 and 0.49 observed in the present study (Bohn *et al.* 1999). However, these results are much higher in wheat Tunisian genotypes (PIC) ranged from 0.33 to 0.94 respectively for the primer *WMC 25* and *WMC 50* (Babay *et al.*, 2015).

Assessment of relationships between cultivars

The similarity coefficient between cultivars ranged from 0.33 and 0.90 with an average of 0.63 using Nei and Li's method (Table 4). Most of genotypes showed a high degree of genetic similarity.

The lowest genetic distance value of 0.33 has been scored between Hammam1 and Attila/2Pastor. Milan/S87230/babax and Angi-4 were the most similar (0.90). The UPGMA among 10 genotypes of bread wheat was generated (Fig. 2).

Table 2. Description of tested SSR primers (http:www.wheat.pw.usda.gov/ggpages/SSR /WMC).

Primer	Sequences	Motifs	Chromosome location	Alleles size (pb)
WMC 14F	ACCCGTCACCGGTTTATGGATG	(CT) (CA)	7D	239
WMC 14R	TCCACTTCAAGATGGAGGGCAG			
WMC 15F	AGTCCGATTCGGACTCCTCAG	(CT) (CA)	4A	295
WMC 15R	GGACTAACCGAGGGTAGTTG			
WMC 16F	ACCGCCTGCATTCTCATCTAA	(CT)	4B	165
WMC 16R	GTGGCGCCATGGTAGAGATTG			
WMC 17F	ACCTGCAAGAAATTAGGAAC	(CA)	7A-7B	182
WMC 17R	CTAGTGTTTCAAATATGTCGA			
WMC 18F	CTGGGGCTTGGATCACGTCATT	(CA) (CT)	2D	237
WMC 18R	AGCCATGGACATGGTGTCCTTC			
WMC 19F	CTGACATGCGGCATTCACTTCC	(CA)	1A	153
WMC 19R	AGGCTTAGAACACACCGACACG			
WMC 20F	TTAAAAACACGCGGATCTTCTC	(CA)	1A	119
WMC 20R	GTACTCACATATTTCTCGGTCT			
WMC 21F	CGCTGCCGTGTAACTCAAAATC	$(GA)_{37}$		136
WMC 21R	AGTTAATTGGGCGCTCCAAGAA		-	
WMC 22F	ATCATTGGTTTCCTCTTCACTT	$(GT)_{24}$		169
WMC 22R	GTGGACTATTTAACATCTTCAT		-	
WMC 23F	ATTCGCTCATACGATAGGGTTG	$CT)_{22}(CT)_{18}$		314
WMC 23R	AGAGGCTGGTGTAGTTGGTTTG		-	
WMC 24F	GTGAGCAATTTTGATTATACTG	$(GT)_{28}$	1A	136-155
WMC 24R	TACCCTGATGCTGTAATATGTG			
WMC 25F	TCTGGCCAGGATCAATATTACT	$(GT)_{26}$	2B	166
WMC 25R	TAAGATACATAGATCCAACACC			
WMC 27F	AATAGAAACAGGTCACCATCCG	$(GT)_{25}$	2B-5B	352-398
WMC 27R	TAGAGCTGGAGTAGGGCCAAAG			
WMC 48F	GAGGGTTCTGAAATGTTTTGCC	$(GA)_{9}$	4B	139-190
WMC 48R	ACGTGCTAGGGAGGTATCTTGC			
WMC 50F	CTGCCGTCAGGCCAGGCTCACA	$(GT)_{10}(GT)_{16}$	3A	219-236
WMC 50R	CAACCAGCTAGCTGCCGCCGAA			
WMC 283F	CGTTGGCTGGGTTATATCATCT	$(CA)_{19}(CA)_{8}$	4A	
WMC 283R	GACCCGCGTGTAAGTGATAGGA			

The consensus tree showed that it divided the wheat genotypes into four main clusters, the first included wheat varieties AinAbid, Attila/2 Pastor and 5119. The second main cluster was divided into two sub-clusters. The first sub-cluster included wheat varieties Hidhab and Hamam 1.

The second one included cultivars Milan/S87230/babax, Angi-4 and Attila. The third group is formed by chem-6 and the last group is composed by a local genotype Arz.

The dendrogram presented in Figure 2 demonstrate the ability of microsatellites to detect large amount of genetic diversity in genotypes with expected narrow genetic pool.

The cophenetic coefficient was r = 0.68, indicating that there is good fit between dendrogram clusters and the similarity matrix.

In this study, a different approach was taken by analyzing a smaller number of wheat genotypes of diverse origin using a higher number of SSRs to provide better genome coverage. These findings clearly demonstrate the reliability, usefulness and efficiency of SSRs in analyzing genomic diversity (Salem *et al.*, 2008).

Genotypes with the most distinct data are likely to contain the greatest number of novel genes and are likely to carry unique and potentially agronomically useful genes.

Table 3. PIC values, alleles number and annealing temperature generated by polymorphic primers.

Primer	PIC	Ta (°C)	Nb. Alleles	Primer	PIC	Ta (°C)	Nb Alleles
WMC 14	0.66	58	5	*WMC 25*	0.61	52	3
WMC 15	0.37	55	2	*WMC 27*	0.26	55	2
WMC 17	0.33	54	5	*WMC 48*	0.48	64	2
WMC 20	0.49	54	2	*WMC 50*	0.70	60	5
WMC 21	0.13	55	2	*WMC 283*	0.56	60.4	3
WMC 24	0.42	52	2				

Agro - morphological Characters Analyses

The principal component analysis (PCA) was applied to the correlation matrix obtained from 6 agro-morphological characters measured on the 10 varieties of bread wheat. The PCA analysis revealed that a first component accounts for 39.6% of the total variation. It was determined mainly by heading time, plant height (PH) and grain yield (GY). The second component which contains only 26.7% of the information was highly correlated with number of kernels per ear (NKE) and thousand kernels weight (TKW).

Table 4. Similarity matrix for the seven bread wheat varieties based on their microsatellite markers.

	AinAbid	Arz	Hidhab	Hammam1	5119	Milan babax	Angi4	Cham6	Attila	Attila2Pastor
Ain Abid	1.000									
Arz	0.609	1.000								
Hidhab	0.560	0.455	1.000							
Hammam1	0.560	0.636	0.667	1.000						
5119	0.640	0.455	0.500	0.667	1.000					
MilanS87230babax	0.500	0.381	0.696	0.522	0.609	1.000				
Angi4	0.583	0.476	0.696	0.609	0.696	0.909	1.000			
Cham6	0.348	0.400	0.364	0.455	0.545	0.476	0.571	1.000		
Attila	0.385	0.435	0.480	0.640	0.560	0.667	0.750	0.696	1.000	
Attila2Pastor	0.720	0.545	0.500	0.333	0.667	0.609	0.696	0.545	0.480	1.000

The major correlated variability of genotypes is showed by the axes 1 and 2 which explained 66.37% of the total information and revealed 4 distinct groups (Fig. 3).

The PCA analysis showed that AinAbid, Attila/ 2 Pastor and 5119 have the lowest TKW and NKE. Low results recorded in these two parameters TKW and NKE have a great influence on the performance on cereals and finally creates a negative impact on ear fertility (Tambussi *et al.*, 2005).

Similar results were fond mainly in Mediterranean climate (Araus *et al.*, 2002). These genotypes composed the first group (G1).

The second group (G2) consists of 3 genotypes and includes two Algerian wheat cultivars (Hidhab, and Arz) the third genotype is Hammam1. These genotypes were high tillering and the later compared to other cultivars studied, but showed the lowest GY.

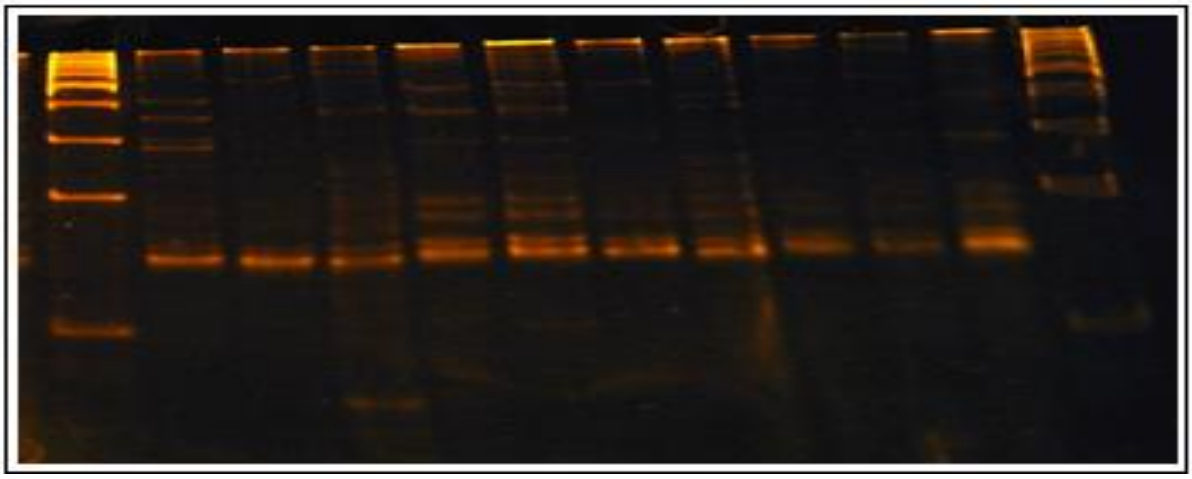

Fig. 1. Typical examples of SSR profile (*WMC 14*) obtained using genomic DNA template on polyacrylamide gel of ten genotypes of bread wheat.(M: marker 100-bp DNA ladder. V1: AinAbid; V2: Arz; V3: Hidhab; V4: Hamam1; V5: 5119; V6: Milan/S87230/babax; V7: Angi-4; V8: Cham6; V9: Attila; V10: Attila/2 Pastor).

This low yield could result from a longer phonological cycle and, therefore, a longer duration of vegetative disturbing the grain filling period (Ali *et al.* 2008; Yao *et al.*, 2014).

Conversely genotypes of the third group (G3) which include Attila, Angi-4 and Milan/S87230/babax were the earliest with short tillering but provide the most high grain yield.

So the shorter varieties are early maturing, these results seem to corroborate those of Gonzales *et al.* (2003) who observed that plant height is associated with their phenology. The height of the plant is very much influenced by the environment including the limiting factors of growth that are related to climate parameters such as rainfall and variability and temperature fluctuations that may impede the growth and development of plants (Khan *et al.,* 2010).

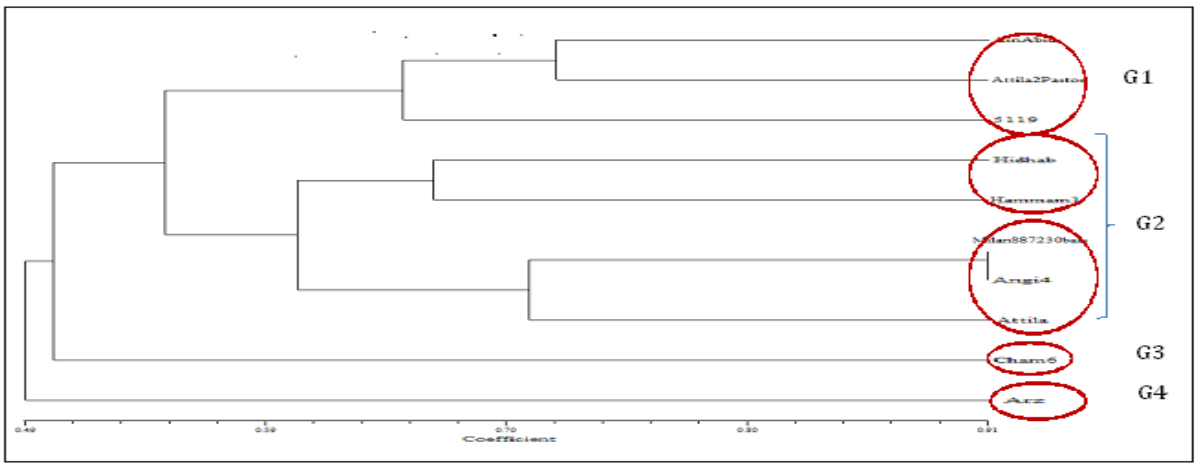

Fig. 2. Dendrogram based on data of 11 microsatellite primers from 10 bread wheat genotypes constructed by UPGMA.

The last group (G4) is formed by a single genotype Cham-6, this introduce variety records the highest values of TKW and NKE. High NKE increase the availability of assimilates for grain filling (Maydup *et al.*, 2010).

Genetic assessment has been greatly facilitated by molecular markers, which are good alternative methods mainly characterized by their rapidity, to detect polymorphism between different systematic levels.

Moreover, molecular markers are not influenced by environment factors like morphological traits (Hai *et al.*, 2015) and the genetic polymorphism observed using molecular markers may provide information on the history of cultivars, but it does not necessarily reflect what may be observed with respect to agronomic traits (Toklu *et al.*, 2015).

In this study, agro-morphological data analysis of the bread wheat genotypes was coupled with molecular analyses (SSR markers) to investigate the genetic relationships among 10 bread wheat genotypes.

The genotypes showed diverse agro-morphological traits and distinct SSR markers patterns. Both molecular and agro-morphological techniques are complementary to better characterize and study the diversity of wheat and all cereal species.

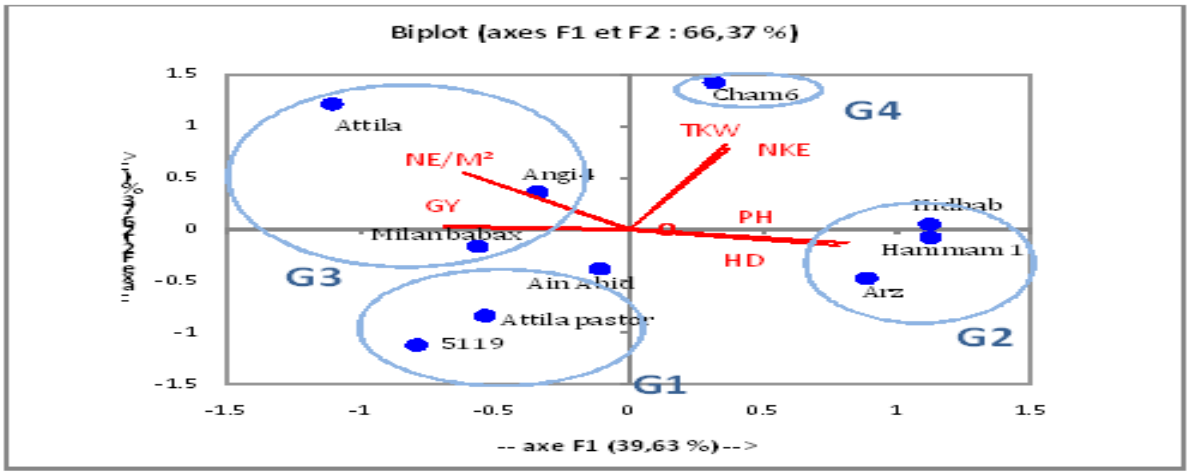

Fig. 3. Principal component analysis (PCA) of agro- morphological data in 10 genotypes of bread wheat. HD : heading date (days), PH : plant height (cm), NEM : nb. of ears per m^{-2}, NKE : nb. of kernels per ear, TKW: thousand kernels weight (g) and GY: grain yield (Q/Ha).

The genetic diversity obtained by SSR marker is relatively consistent and compatible with a pedigree and a geographical origin of genotype. SSR molecular-marker can be successfully employed in assaying the level of polymorphism and diversity.

The characterization of some bread wheat genotype using morphologic and SSR markers may provide useful information for long term improvement in yield, qualities, resistance (biotic and abiotic stresses) and for germplasm identification and conservation. This study shows that both morphological and molecular markers need to be studied concurrently to obtain better knowledge about similarities among varieties.

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