

AFLP and SSR fingerprinting to evaluate genetic diversity among bread wheat cultivars in Tunisia

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Summary

Bread wheat (*Triticum aestivum* L.) is an economically and nutritionally important cereal crop in the Mediterranean region. Characterization of wheat germplasm by means of DNA fingerprinting techniques provides a tool to assess genetic diversity and to identify varieties. In this study, six Tunisian bread wheat cultivars were characterized by AFLP and SSR markers. Five AFLP primer pairs showed clear different patterns and seems to be the most suitable for analysis of the bread wheat varieties. Three SSR primers were polymorphic with more than two alleles. The pairwise genetic similarities (GS) based on these molecular markers were calculated and used to construct a dendrogram that allowed the discrimination of the six cultivars. The GS among the six varieties ranged from 0.79 to 0.36. The six varieties used in this work clustered into four groups using either AFLP or SSR markers. A high GS was found between Tebecca and Vaga Varieties which have a similar pedigree.

Keywords: Amplified fragment length polymorphism, microsatellites SSR, genetic markers, wheat (*Triticum aestivum*), genetic similarity.

Abbreviations: AFLP, amplified fragment length polymorphism; CTAB, hexadecyltrimethylammonium bromide; SSR, simple sequence repeat; TBE, Tris-borate-EDTA; TE, Tris-EDTA; CIMMYT, International Maize and Wheat Improvement Center.

Introduction

Progenitors of *Triticum* sp. (*aestivum* and *durum*), cultivated wheat, are native to the Mediterranean region and are a basis of human nutrition in many countries such as Tunisia. Successful strategies in plant breeding to produce higher yield, increased resistance to biotic and abiotic stresses, better quality and other beneficial properties are based on the available diversity of genetic resources. Established process of plant breeding, such as crossing and selection have been broadened by biotechnological methods over the past two decades. The potential of the molecular markers, such as AFLP (Amplified Fragment Length Polymorphism, Vos et al., 1995) and SSR (also called microsatellites, Röder et al., 1995), should assist the screening and the selection of genetic resources and increase the efficiency of breeding programs. Identification and registration of bread wheat cultivars is mainly based on morphological and physiological characteristics. Analyses of molecular markers offer an

attractive way to evaluate potential genetic variation, providing a basis for genetic improvement. Environmental adaptation involves many complex traits (Allard, 1996). The use of germplasm developed within the same region targeted for cultivar improvement reduces the risk of losing essential adaptive characteristics through recombination.

Restriction fragment length polymorphism (RFLP, Botstein *et al.*, 1980) was one of the first DNA marker techniques used to characterize wheat cultivars (Vaccino *et al.*, 1993) and to assess genetic diversity (Kim and Ward, 1997; Paull *et al.*, 1998). However, the relatively low level of polymorphism observed among elite wheat cultivars (Bryan *et al.*, 1999), the complexity and cost of the technique, limit the use of RFLP for routine cultivar identification.

The AFLP technique was used successfully to evaluate genetic diversity and genetic relationship in wheat (Heun *et al.*, 1997; Barrett and Kidwell, 1998; Domini *et al.*, 2000). In wheat, SSR markers provide also a great level of polymorphism (Röder *et al.*, 1995; Plaschke *et al.*, 1995). The SSR technique gained rapid acceptability because of its codominant nature, reproducibility, and high information content (De Loose and Gheysen, 1995).

In this study, we present the characterization of six bread wheat cultivars from Tunisia using AFLP and SSR markers. The objectives of this work were to (i) use AFLP and SSR markers to assess levels of genetic diversity among a representative sample of bread wheat cultivars adapted to low rainfall area, and (ii) to construct a common dendrogram depicting the relationships between all the main bread wheat varieties used in Tunisia.

Materials and Methods

Plant materials

The plant material consisted of six varieties of bread wheat, received from INRAT (Institut National de Recherche Agronomique de Tunis) and had the following phenotypic characteristics:

FlorencexAurore: This variety, introduced in 1922, is early, moderately resistant to lodging and drought, resistant to black rust and tolerant to powdery mildew and Septoria.

Ariana 66: This variety, introduced in 1963, is mid-early, moderately sensitive to powdery mildew, to Septoria and to brown and black rust. It is resistant to yellow rust and moderately to lodging. It has a high yield in optimal conditions.

Salambo 80: This variety, introduced in 1970, is early, moderately resistant to Septoria, to brown and black rust, resistant to yellow rust and to lodging. It utilizes excessive nitrogen fertilizers and has a broad spectrum of adaptation and good production in areas with intermediate rainfall conditions.

Byrsa 87: This variety, introduced in 1982, is mid-early, resistant to lodging, to Septoria and to yellow rust. This variety is highly productive and utilizes the excessive nitrogen fertilizers. It is convenient in intensive culture or with topping-up irrigation.

Tebecca 96: This variety, introduced in 1990, is resistant to Septoria, brown rust, black rust and powdery mildew. It is well adapted to low rainfall areas.

Vaga: This variety, introduced in 1985, has a good level of tolerance to Septoria, to brown and yellow rusts and to powdery mildew. It has a large spectrum of adaptation and good production in favourable conditions.

These varieties were selected based on their behavior under drought and salt stress conditions and used throughout this study. Plants were grown in a greenhouse (16/8 h daily light period,

25 °C temperature (night/day) and 60 to 70 % of relative humidity). Seeds of each line were germinated in compost soil (leaf-mould/stable-litter/sand, 1/1/1) in a greenhouse.

DNA extraction

Leaves of three-week-old wheat seedlings were collected in the greenhouse, frozen in liquid nitrogen then powdered with a mortar and pestle. DNA was extracted according to the CTAB protocol published by Saghai-Marooft *et al.* (1984) with minor modifications. The RNA was removed by the addition of 40 µL of 10 mg/ml RNase A and was incubated at 37°C during 30 min. 0.25 ml of 10 mg/ml proteinase K was then added and the solution incubated at 50°C for 20 min. The DNA was then phenol extracted and centrifuged at 13,000 rpm for 10 min. The DNA was precipitated with 2 volumes of ethanol and 1/10 volume of 3 M sodium acetate for 3 h at -20°C. After washing with 70% ethanol, the DNA was centrifuged and finally resuspended in 50 µL double distilled water.

AFLP reactions

Wheat DNA (0.5 µg) was digested with *MseI* (frequent cutter) and *EcoRI* (rare cutter) restriction enzymes. Restricted DNA and control DNA preparations were analysed on 1% agarose gel using 0.5x TBE buffer and stained with ethidium bromide. The digested DNA was ligated to *EcoRI* (5 pmol) and *MseI* (50 pmol) adapters with 1 U of T4 DNA ligase (Amersham). Two PCR amplification steps were performed for AFLP. In the preamplification, primers were used with one selective nucleotide [*EcoRI*(+1) and *MseI*(+1)]. PCR conditions for this first step are as follow: a 30 s DNA denaturation step at 94°C, a 30 s annealing step at 60°C, and 1 min extension step at 72°C. The reaction was performed for 30 cycles. The second PCR amplification step, was done with 1/100 of the first PCR-amplified products, using primers with three selective nucleotides [*EcoRI*(+3) and *MseI*(+3)]. PCR conditions for this second step are as follow: a 30 s DNA denaturation step at 94°C, a 30 s annealing step at 65°C, and 1 min extension step at 72°C. The annealing temperature was subsequently reduced in each cycle by 1°C for the next 12 cycles, and was continued at 56°C for the remaining 23 cycles. The AFLP products were separated on 6% (w/v) polyacrylamide gels. The gel was pre-warmed at 50°C, and 3.5 µL samples were loaded and the gel was run at 60 W (1400-1500 V) in 0.5x TBE. After electrophoresis the gel was stained with silver nitrate. For AFLP reactions six *EcoRI* primers (E36, E37, E39, E40, E41 and E42) were used in combination with three *MseI* primers (M40, M42 and M43), all of them are with three selective bases.

AFLP analysis

Banding patterns of the six bread wheat varieties were analysed by scoring the relative migration of all major bands. We consider that every band corresponds to a single dominant marker locus (presence/absence polymorphism).

Microsatellites reactions

PCR reactions were performed in a volume of 25 µL in a Perkin-Elmer 9600 thermocycler. The reaction mixture contained 0.2 mM of each deoxynucleotide, 1.5 mM MgCl₂, 1 U *Taq*-polymerase, 200 nM of each primer and 100 ng template DNA. Typically, 35 cycles were

performed with 1 min 96°C, 1 min 60°C (in some cases 50°C) and 2 min 72°C and a final elongation step of 10 min at 72°C. PCR products were separated in 8% denaturing polyacrylamide gels. The sequence of the primers used to generate microsatellite markers are given in Table 1.

Table 1: Microsatellite primer sequences, annealing temperature and repeat types (Röder et al., 1995)

Markers	Primers	Annealing Temperature (°C)	Repeat type
WMS 2	5'CTGCAAGCCTGTGATCAACT3' 5'CATTCTCAATGATCGAACA3'	50	(CA) ₁₈
WMS 3	5'GCAGCGGCACTGGTACATTT3' 5'AATATCGCATCACTATCCCA3'	50	(CA) ₁₈
WMS 43	5'CACCGACGGTTTCCCTAGAGT3' 5'GGTGAGTGCAAATGTCATGTG3'	60	(CA) ₂₂
WMS 44	5'GTTGAGCTTTTCAGTTCGGC3' 5'ACTGGCATCCACTGAGCTG3'	60	(GA) ₂₈
WMS 46	5'GCACGTGAATGGATTGGAC3' 5'TGACCCAATAGTGGTGGTCA3'	60	(GA) ₂ GC(GA) ₃₃

Results and Discussion

Data evaluation of AFLP markers

All 18 primer combinations produced high number of bands, ranging between 50 and 100. The level of polymorphism varied from one primer combination to another. Five primer pairs showed clear different patterns and seem to be the most suitable for AFLP analysis of our bread wheat varieties. These were: E36/M42, E37/M42, E39/M42, E40/M42 and E40/M43. They produced a total of 39 polymorphic clear and reproducible bands which were therefore scored as 1/0 polymorphisms in the six varieties.

Data evaluation of SSR markers

Five primer-pairs were used to generate SSR fingerprints of the six Tunisian bread wheat varieties. Two of them showed no polymorphism and the three others were polymorphic with at least two alleles. The polymorphism information content (PIC) was calculated for each primer-pair (marker) as follows:

$$PIC = 1 - \sum_{i=1} P_{ij}^2$$
, where P_{ij} is the frequency of the allele j for the i^{th} marker.

This gives an average value of 36.6%. PIC value could be interpreted as the probability that two alleles at a given locus are different between two varieties taken at random. This shows that we have a relatively high level of polymorphism (as expected) in our six cultivars. Röder et al. (1995) obtained an average PIC of 54% among 18 bread wheat accessions.

Genetic distances and hierarchical classification

We calculated the pairwise genetic similarities for AFLP and SSR markers separately using the simple match coefficient. The genetic similarity coefficient (GS) between two varieties is calculated as the ratio of the number of bands which have similar status in both varieties (present or absent in both) over the total number of bands. Thus, GS varies from 0 (no similarity) to 1 (identity). Based on the matrix of GS values, we used the UPGMA clustering method to obtain the dendrogram, depicting genetic relatedness of the cultivars (NTSYS software was used in all analysis).

Based on the AFLP markers, the two nearest lines were Ariana and Byrsa (GS=0.79), while the two distant lines were FlorencexAurore and Salambo (GS=0.36) with an average value of 0.60. For the SSR markers, GS values varied from 1 (between FlorencexAurore and Salambo) to 0.20 (between Tebecca and three of the five remaining varieties) with an average value of 0.51 showing that less diversity is revealed by SSR markers.

The dendrogram produced for AFLP markers allows to set up four groups (Figure 1a):

- ❖ The first group consists of Tebecca and Vaga (GS=0.77), which are two recently introduced cultivars with similar agro-morphological characteristics. They both derive from crosses done by CIMMYT involving a common line as a parent (Buc "S"). The kinship coefficient between these cultivars, calculated based on their pedigree, is 0.125, showing that these two varieties share one eight of their genome, which partly explains the high genetic similarity obtained by AFLP markers.
- ❖ The second group is made up of Ariana and Byrsa, which do not have a pedigree relation but share many common characteristics, such as their mid-earliness and high yielding potential in favourable conditions.
- ❖ FlorencexAurore has nearly equal GS values (around 0.60) with all the four aforementioned varieties while being less similar to Salambo (GS=0.31). This old tall variety has very little in common (if any) with these relatively recently introduced ones and the explanation of the high genetic similarity observed remains unclear.
- ❖ Salambo has high similarity with Tebecca (GS=0.70) and Vaga (GS=0.56) while is quite different than the other three cultivars (average GS=0.35).

With SSR markers quite different picture of genetic diversity was obtained (Figure 1b). Four groups could be defined again but the only one which is consistent with the AFLP clustering is composed of Tebecca and Vaga. In fact we obtained GS=0.80, a value which is very close to the result of AFLP markers. The correlation coefficient between GS values calculated on AFLP and SSR data was $r=-0.19$ (non significant at 5% level). We should emphasize that the poor correspondence of the SSR distances (correlation coefficient and dendrogram) with the AFLP ones is clearly linked to the fact that few SSR markers were used in this study (only three were polymorphic).

We finally analysed together AFLP and SSR data. The dendrogram produced in Figure 3c has quite good consistency with that of AFLP (Figure 3a) and shows, as expected, that Tebecca and Vaga cluster together (GS=0.69) and at less degree Ariana and Byrsa (GS=0.66)

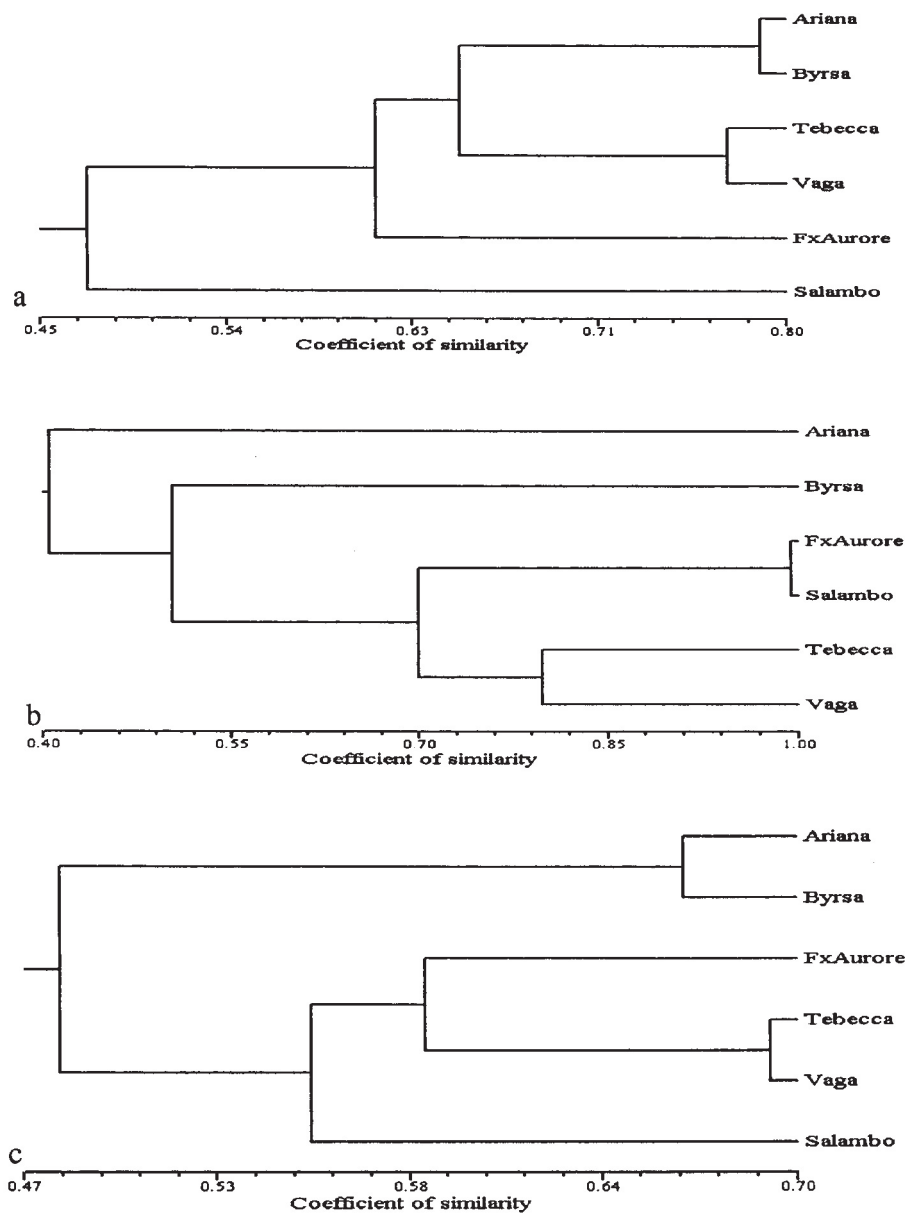


Figure 1: UPGMA dendrograms depicting patterns of genetic diversity estimated by (a) 18 AFLP primer combinations, (b) five SSR primers and (c) combined AFLP and SSR markers among six bread wheat cultivars from Tunisia.

Conclusions

Our results using both AFLP and SSR techniques allowed to discriminate wheat varieties and to reveal genetic similarities among the six major cultivars of bread wheat used in Tunisia. A high genetic similarity is found between Vaga and Tebecca which is expected based on the pedigree information. However the high similarity between Arian and Byrsa revealed by AFLP markers is very interesting since these varieties do not share any common history or parent. Ariana is a local cultivar and was introduced from France in 1963 and derives from a cross between two French varieties whereas Byrsa has been used since 1987 and was introduced in 1982 from a cross done at CIMMYT having no relation with that of Ariana. Moreover the two cultivars do not show similar phenotypic characteristics.

- In the present study, AFLP and SSR markers could be identified for the differentiation and the selection of bread wheat varieties based on the polymorphism rates.
- Primer combinations should be used for genetic diversity assessment for the wheat varieties.
- The AFLP and SSR molecular markers allowed us to construct a common dendrogram depicting the relationships between all the main bread wheat varieties used in Tunisia.
- The AFLP technique is a very effective tool to reveal polymorphisms and could be used as criteria for practical breeding.
- Microsatellites may be a useful alternative or complementary type of molecular marker. They can be used as cultivar specific markers for varietal identification in wheat.

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