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Genetic Diversity of Tunisian Barley Accessions Based on Microsatellite Markers

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Abstract: Genetic diversity can be measured by several criteria, including phenotype, pedigree, allelic diversity at marker loci and allelic diversity at loci controlling phenotypes of interest. Abundance, high level of polymorphism and ease of genotyping make simple sequence repeats (SSRs) an excellent molecular marker system for genetics diversity analyses. In this study, we used a three of mapped SSRs to examine the genetic diversity of Tunisian barley accessions and to establish phylogenetic relationships among them. These primers produce a total of 9 bands witch 7 loci polymorphic, the percentage of polymorphism ranged from 14.28 to 42.85%. The clustering grouped the studied accessions into 3 clusters with no correlation to geographical origins. The CFA permit to group the various populations by projected them in a plan formed by two axes (F_1, F_2), present 3 groups which are similar with those obtained by hierarchical classification. Present results demonstrate that this SSR marker was highly informative and was useful in generating a meaningful classification of barley germplasm.

Key words: *Hordeum vulgare* L., genetic diversity, microsatellite marker

INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the most important crop species in the word and has been subject to considerable genetic study. It is a diploid (2n = 2x = 14), largely self-fertilizing species (Szucs *et al*., 2000). Barley is cultivated on about 450,000 hectares in Tunisia. During centuries, early domestication and local knowledge have generated diverse local barley used mainly for feed and lowly for food.

In semi-arid regions, barley is mostly cultivated by sheep owners and gazed one or two time as early winter crop when forage and pasture are not available the conservation and use of plant genetic resources are essential to the continued maintenance and improvement of agricultural production, sustainable.

Advances in DNA technology have greatly increased the number and type of molecular markers available for plant genetic diversity studies. It has been shown that different markers might uncover different classes of variation (Powell *et al*., 1996). This is correlated with the genome fraction surveyed by each kind of marker (coding vs. non coding; single sequences vs. repeated sequences), their distribution throughout the genome and the extent of the DNA target which is analysed by each specific assay.

For detection of genetic variation in barleys, an array of molecular markers is available (Varshney *et al*., 2007). In fact, among different classes of molecular markers available, the simple sequence repeat (SSR) or microsatellite (derived from genomic DNA) and AFLP markers have been used separately as well as in combination in many studies (Maestri *et al*., 2002; Matus and Hayes, 2002; Turpeinen *et al*., 2003; Nevo *et al*., 2005). In recent years, with increasing efforts to develop EST (expressed sequence tag) resources for crop plants including barley, a new class of locus-specific DNA markers called functional molecular markers have been developed (Anderson and Lübberstedt, 2003).

These include EST derived SSR (EST-SSR) and SNP (EST-SNP) markers which are easy to develop utilizing the EST resources and mirrors the functional genomic component (Varshney *et al*., 2005; Varshney *et al*., 2006). These markers, at present, are gaining momentum for estimating the barley genetic diversity in genebank collections and natural as well as breeding populations of barley (Russell *et al*., 2004; Khlestkina *et al*., 2006).

At present, Simple Sequence Repeats (SSR), also called microsatellites, have proven to be a highly informative marker system in eukaryotes including plants (Roder *et al*., 1995).

However, the time and cost necessary to identify sequences containing SSRs and to design flanking primers prevents the broad use of microsatellites in many plant groups.

Alternatively, it has been proposed that the polymorphism associated with SSRs could be revealed by using primers complementary to SSR itself (Charters *et al*., 1996; Nagaoka and Ogihara, 1997; Kojima *et al*., 1998).

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MATERIALS AND METHODS

Plant material: Barley genotype used in this study is variety Ardhaoui from 6 row selected from different regions in south Tunisia. The accessions numbers, country of origin are shown in Table 1.

This study was carried out in experiment field of Institut des Régions Arides of Medenine.

Seeds of different genotypes were sowed in January and the collect of leaves was carried to May to June 2005.

DNA extraction: Genomic DNA was extracted according to the previously described protocol of modified chloroform- isoamylic alcohol extraction (Aras *et al*., 2003). The plant tissues were homogenized in 1 mL of prewarmed (to 65°C) buffer (2% SDS; 500 mM NaCl; 20 mM EDTA; 1% MBSK; 100 mM boric acid), the homogenate was incubated for 30 min at 65°C. The DNA was extracted with the same volume of a chloroform/isoamilic alcohol mixture (24:1), the suspension was centrifuged and the water phase separated. The DNA was precipitated with equal volume of isopropanol and washed in 70% ethanol, finally the DNA was dissolved in 200 μL deionised water.

DNA concentration was determined by both spectrophotometry at 260 nm and 1% agarose gel electrophoresis.

SSR analysis: SSR designation, SSR motif and annealing temperatures are shown in Table 2. Optimization of each reaction was carried out by trying a range of annealing temperatures. PCR reactions were performed in a total volume of 20 μL containing 20 ng of DNA 0.8 μL of each primer (50 ng μL^{-1}), 0.4 μL of dNTPs (10 mM), 0.2 μL Taq DNA polymerase (5 U μL^{-1}), 0.2 μL of $MgCl_2$ (25 mM) and 2 μL of PCR buffer (10 mM). DNA amplification was carried out using a thermocycler (geninus) programmed with 5 min at 94°C for initial denaturation flowed by 30 cycles of 35 sec at 94°C, 1 min at 54°C, 1 min at 72°C and a final 7 min extension at 72°C. After amplification the DNA fragments were separated by electrophoresis for about 2 h under constant voltage (60) in 3% agarose gel submersed in 1x TBE buffer. The gels were stained with ethidium bromide solution and observed under ultraviolet light each gel was photo documented using the image capturing system bio print.

Statistical analysis: Data were scored as presence or absence of bands. Statistical analysis was performed using the gel pro- analyzer. Based on the matrix of genetic similarity (Russell and Rao), cluster analyses were performed using unweirghted pair/group method with SPSS version 12 software. This grouping was performed by CFA with Stat Box version 3.4 Software.

Table 1: Barley cultivars studied with their origin

No.	Designation	Lieu of collection
1	El Morra	Tataouine
2	Tataouine Ejdida	Tataouine
3	Oued El Khil 2	Ben Keddache
4	Gasbett Gomri	Gomrassen
5	Lamaat	Tataouine
6	El Ferch 1	Tataouine
7	Kasar Ouled Boubaker	Tataouine
8	Swittir	Medenine
9	Bir Ezwai 3	Medenine
10	Bir 30	Tataouine
11	Dkilet Toujene	Gabes
12	Belkhir 3	Gafsa
13	Gomrassen 2	Tataouine
14	Manzel Mgor 3	Ben Khddache
15	El Ferch 2	Tataouine
16	Ben Gzayel	Medenine
17	Mazreet Ben Slama	Gabes
18	Swittir 1	Medenine

Table 2: Repeat motif, annealing temperature and chromosomal locations for the SSR primers employed

Primers	L	R	T_m	Localisation
1	AGAGCAACTACCAGT	GTCGAAGGAGAA GCGGCCCTGGAT	52	Chromosome 1
2	CCGGTCGGTGCAGAA GAG	AAATGCAAGCTA AATGGGCGATAT	50	Chromosome 6
3	CGATCAAGGACA TTT GGGTAAT	AACTCTTCGGGT TCAACCAATA	46	Chromosome 6

RESULTS AND DISCUSSION

The three primers were screen for their ability to generate SSR polymorphic DNA bands using the accessions total DNA (Fig. 1-3). The number of total bands and their polymorphism were present in Table 3.

The three primers used produced 7 polymorphic bands, percentage of bands polymorphs ranged from 14.28 to 42.85%.

Cluster analysis (Fig. 4) divided the 18 cultivars into 3 groups:

- Group 1 formed by 6 cultivars characterizing by the presence of 3 locus at 80, 100 and 125 pb and absence of majority of locus (75, 85, 90 and 150 pb)
- Group 2 regroups about 9 cultivars characterizing by the presence of 2 locus at 75 and 150 pb and absence of majority of locus (80, 85, 90,100 and 125 pb)
- Group 3 contained cultivars Swittir 1 and Mazreet ben slama characterizing by the presence of bands who molecular mass are 85 and 90 pb and absence of author locus

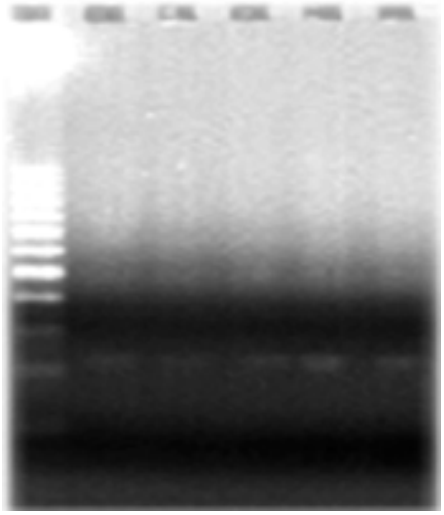

Fig. 1: Electrophoresis pattern obtained by SSR primer sequence (L: 5'-AGAGCAACTACCAGT-3') (1-5 = cultivars, L: 330 pb DNA ladder)

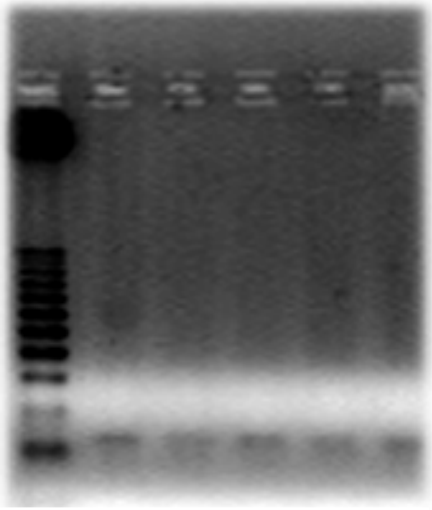

Fig. 2: Electrophoresis pattern obtained by SSR primer sequence (L: 5'-CCGGTCGGTGCAGAAGAG-3') (1-5 = cultivars, L is 330 pb DNA ladder)

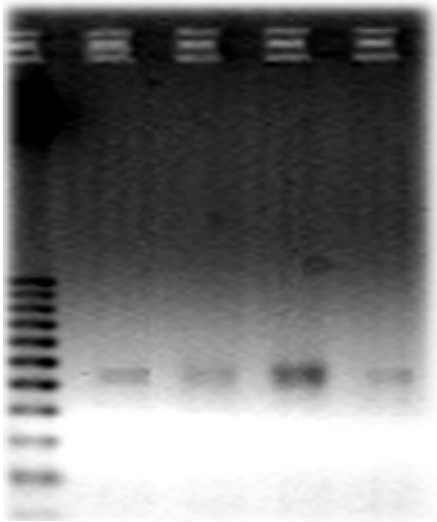

Fig. 3: Electrophoresis pattern obtained by SSR primer sequence (L: 5'-CGATCAAGGACA TTTGGGTAAT-3') (1-5 = cultivars, L is 330 pb DNA ladder)

Table 3: Number of bands and percentage of polymorphism for different cultivars

Cultivars	No. of bands	Percentage of polymorphism
1	2	28.57
2	3	42.85
3	3	42.85
4	3	42.85
5	2	28.57
6	2	28.57
7	3	42.85
8	1	14.28
9	2	28.57
10	2	28.57
11	3	42.85
12	2	28.57
13	2	28.57
14	1	14.28
15	2	28.57
16	2	28.57
17	2	28.57
18	1	14.28

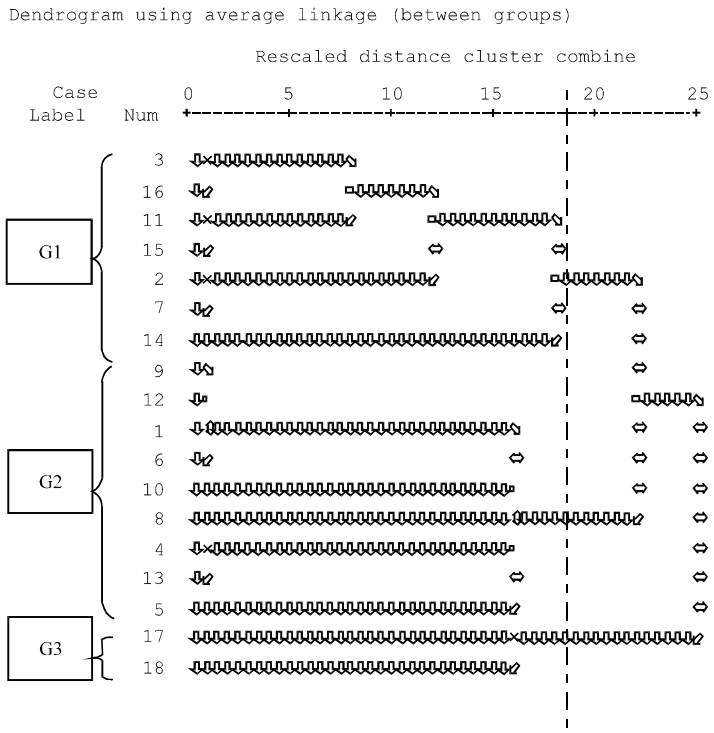

Fig. 4: Dendrogram of 18 barley cultivars based in Russell and Rao similarity index

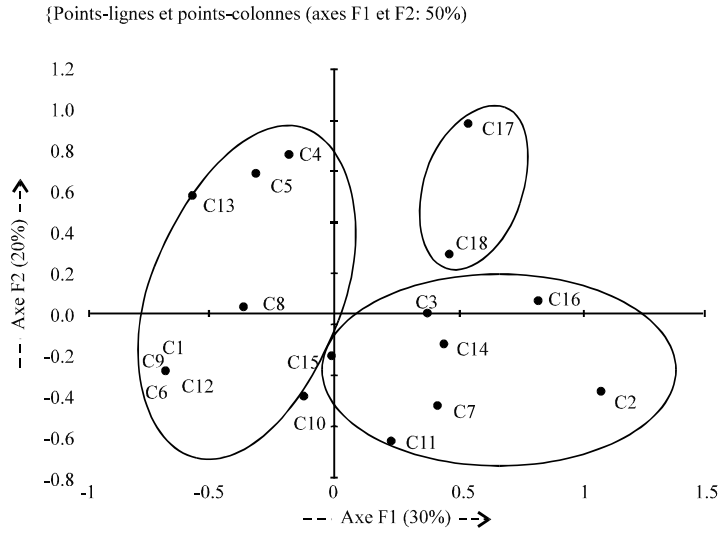

Fig. 5: Provision of the various populations studied according to F_1 axes' and F_2 of the factorial analysis of correspondence by combining the three primers

The CFA permit to group the various populations by projected them in a plan formed by two axes (F_1, F_2), it present 3 groups which are similar with those obtained by hierarchical classification (Fig. 5).

The matrix of the distances from Nei, were considered on the basis of allelic frequency, making it possible to consider the genetic divergence between populations of the same species (Fig. 4). The matrix of the distances estimated at the 18 cultivars study varies between 0 to 1. The lowest value is recorded between the cultivars El Morra and El Ferch 1 from Tataouine, Bir Ezwai 3 from Medenine and Belkhir 3 from Gafsa which presents the maximum of similarity. On the other hand the cultivars Manzel Mgor 3 from Ben Khddache and Swittir 1 from

Medenine are most divergent compared to the others (maximum distance equal to 1). The average of the distances genetics is 0.7622. These results suggest the existence of great variability on the level of the AND of the cultivars study.

Due to its worldwide distribution, the evaluation of the genetic diversity among barley germplasm from different countries has been performed by many authors (Liu *et al*., 2002).

The utilization of microsatellites markers in order to study diversity of different cultivars of barley, revel the presence of 7 loci.

This study provides evidence that the SSR procedure is an informative and suitable approach to the examination of the molecular polymorphism and the phylogenic relationships in the cultivated barley.

One of the advantages of a highly variable marker system like SSRs which detect codominant single locus variation is the ability to directly detect the level of heterozygosity in populations. Several studies were made SSR to characterize some cultivars of barley (Salvo *et al*., 2004; Canci *et al*., 2003), their application in both linkage and diversity studies will provide a common reference that will facilitate the rapid integration of mapping data from different populations with that from ecological and biodiversity studies in barley.

This SSR technique have been also used with success for the characterization of genetic polymorphism for authors species for *Medicago sativa* (Julier *et al*., 2005) and tomato (Areshchenkova and Ganal, 1999).

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