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Genetic diversity in barley genetic diversity in local Tunisian barley based on RAPD and SSR analysis

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Abstract

The genetic variation and relationships among 12 local barleys and the varieties Martin and Manel were evaluated using random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR). A high level of polymorphism was found with both RAPD and SSR markers and the mean polymorphism information content (PIC) values were 0.477 and 0.533 for RAPD and SSR markers, respectively. In RAPD analyses, 69 out of 93 bands (74%) were polymorphic. The number of alleles ranged from 4 to 10 per primer, with an average of 6.2 per primer. The RAPD-based genetic dissimilarity (RAPD-GD) ranged from 0.114 to 0.933, with the mean of 0.523. In SSR analyses, a total of 43 alleles were detected, among which 39 alleles (90.7%) were polymorphic. The number of alleles per primer ranged from 2 to 4 with an average of 2.87 alleles per SSR primer. The SSR-derived genetic dissimilarity (SSR-GD) ranged from 0.423 to 0.910, with the mean of 0.665. SSR was better than RAPD to detect genetic diversity among the barley accessions. A poor correlation (r = 0.193) was found between both sets of genetic similarity data, suggesting that both sets of markers revealed unrelated estimates of genetic relationships.

Key words: *Barley, RAPD, SSR, Genetic diversity*

Introduction

Barley, *Hordeum vulgare* L., is one of the principal cereal crops in the world and is cultivated in all temperate areas (von Bothmer et al. 1995). Wild barleys, *H. vulgare* ssp. *spontaneum* and *H. vulgare* ssp *agriocrithon*, are the primary gene pool of cultivated barley (*H. vulgare* ssp. *vulgare*). The total number of barley accessions in the Genbanks, including redundant materials, is estimated to be about 280,000 (Plucknett et al. 1987). Barley is a major cereal crop in Tunisia and is of great importance as forage species. It had been the subject of intensive genome mapping and quantitative trait dissection efforts. Barley is raking fourth in the world after rice, wheat and maize (Forster et al., 2000). Local barley is of six rows ear and presenting a genome of 2n = 2x = 14. In Tunisia, since die begening of the cereal improvement program, we have registered only 15 varieties which make a narrow genetic diversity. In the opposite, we have more than 30 varieties of *durum* and bread wheat officially recorded (El Faleh, 1998).

Genetic barley erosion could be avoided through the establishment of a local genotype ressources collection and conservation. Consequently, genetic diversity, identification and maintain of our local ressources should be achieved to be used in breeding programs.

Molecular markers have been proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. It has been shown that different markers might reveal different classes of variation (Powell et al. 1996; Russell et al. 1997). It is correlated with the genome fraction surveyed by each

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kind of marker, their distribution throughout the genome and the extent of the DNA target which is analyzed by each specific assay (Dávila et al. 1999b). The advent of the polymerase chain reaction (PCR) favored the development of different molecular techniques such as random amplified of polymorphic DNA (RAPD), simple sequence repeats (SSR or microsatellite), sequence tagged sites (STS), random amplified microsatellite polymorphism (RAMP) and inter-simple sequence repeat polymorphic DNA (ISSR), and so on (Saiki et al. 1988; Welsh and McCleland 1990; Williams et al. 1990; Akkaya et al. 1992; Tragoonrung et al. 1992; Zietkiewicz et al. 1994; Wu et al. 1994; Nagaoka and Ogihara 1997). These molecular markers had been used in barley for detecting genetic diversity, genotype identification, genetic mapping (Sánchez de la Hoz et al. 1996; Matus and Hayes 2002; Dávila et al. 1999a, 1999b; Dávila et al. 1998; Tragoonrung et al. 1992; Tanyolac 2003; Fernández et al. 2002; Struss and Plieske 1998). Of these techniques, RAPD has several advantages, such as simplicity of use, low cost, and the use of small amount of plant material, etc. RAPDs were proved to be useful as genetic markers in the case of self-pollinating species with a relatively low level of intraspecific polymorphism, such as hexaploid wheat (Devos and Gale 1992; Joshi and Nguyen 1993) and cultivated barley (Barua et al. 1993; Chalmers et al. 1993; Tinker et al. 1993). ISSR markers, which involve PCR amplifications of DNA using a primer composed of a microsatellite sequence anchored at 3' or 5' end by 2-4 arbitrary, could be used to assess genetic diversity (Qian et al. 2001).

No big information is available in genetic variability among Tunisian barley germoplasm at both molecular and morphological level. In fact, Abdellaoui et al (2007) showed a large diversity of local barley accessions using RAPD markers. Besides, Hamza et al (2004), working on 31 barley accessions, used 15 ISSR markers and shown 0.53 PIC value. Also Belgouthi (2007), working on 12 local barley genotypes using 10 SSR markers, showed a PIC value of 0.5.

The objectives of this study are to (1) reveal the SSR-based genetic diversity in a barley germplasm from Tunisia, (2) compare RAPD and SSR diversity in the studied materials, and (3) assess the genetic diversity within the selected accessions of the barley landraces as compared to that in its wild relatives by using RAPD and SSR molecular markers.

2. Materials and methods

2.1. Plant material

Twelve local winter barley accessions *(Hordeum vulgare,L.)* were collected from different Tunisian regions and named according to their origin. In addition, cultivated varieties `Martin'and 'Manel' were used as a control. These accessions were obtained after prospection carried out in different Tunisian bioclimatic regions (Table 1). Seeds of each accession were sown in pots in three replications. The experiment was carried out at field capacity in the National Agronomic Research Institute of Tunisia (INRAT).

Table 1. Accessions' origin, bioclimatic stage and rainfall (Monthly Bulletin of the National Meteorological Institute from 1975 to 2004)

Accessions	origin	Bioclimatic stage	Rainfall (mm)
Tozeur 1	Tozeur	Sahara	150
Tozeur 2	Tozeur	Sahara	150
Kébill 1	Kébilli	Sahara	150
Kébilli 2	Kébilli	Sahara	150
Kébilli 3	Kébilli	Sahara	150
Kasserine	Kasserine	Arid sup	300
Sidi Bouzid	Sidi Bouzid	Arid sup	300
Jendouba 1	Jendouba	Humid inf	800
Jendouba 2	Jendouba	Humid inf	800
Manel	Jendouba	Humid inf	800
Kalaâ	Kalaât El Andalous	Sub-humid	600
Kélibia 1	Kélibia	Sub-humid	600
Kélibia 2	Kélibia	Sub-humid	600
Martin	Introduced from Algeria (1931)		

2.1. DNA Extraction

The DNA was extracted and purified from 100 mg of fresh leaves, using a CTAB (Cetyl himethyl ammonium Bromide) method (Webb and Knapp, 1990). DNA was then quantified at 260 nm using a spectrophotometer (standard CECIL CE2501 sertes 2000/3000).

3.1. PCR amplification and electrophoresis

A total of fifteen 10-mer oligonucleotides with arbitrary sequence from Operon (kits A, B, D,F, H, and J) were used in RAPD analysis (Table 2) and 15 primer pairs were used in SSR analysis (Table 3). The PCR reaction mixture consisted of 20-50ng genomic DNA, 1×PCR buffer, 2.0 mmol/L $MgCl_2$, 100 µmol/L of each dNTP, 0.1 µmol/L primer and 1U *Taq* polymerase in a 25µL volume. The amplification protocol was 94 ºC for 4 min to pre-denature, followed by 45 cycles of 94 ºC for 1 min, 36 ºC (for RAPD analysis) or melting temperatures (for SSR analysis) for 1 min and 72 ºC for 1 min, with a final extension at 72 ºC for 10 min. Amplification products were fractionated on 1.5% agarose gel, electrophoresis was carried out at a constant voltage of 100 v for 2 hrs (for RAPD analysis) or 6% denatured polyacrylamide gels and the electrophoresis was conducted at 1200 v and 45 C° (for SSR analysis). The DNA bands were visualized according to the slightly modified methode of Saker et al. (2005) in 1X TBE buffer containing 0.01% of ethiduim bromide for (RAPD) or by silver staining of gels according to Cho et al. (1996).

RAPD and ISSR data were scored for presence (1), absence (0) or as a missing observation (9), and each band was regarded as a locus. Two matrices, one for each marker, were generated. The genetic dissimilarities (GD) were calculated according to Nei and Li (1979). Based on the dissimilarity matrix, a dendrogram showing the genetic relationships between genotypes was constructed using the unweighted pairgroup method with arithmetic average (UPGMA) (Sneath and Sokal, 1973) though the software NTSYS-pc version1.80 (Rohlf, 1993). Polymorphic information content (PIC) values were calculated for each RAPD and SSR primer according to the formula: $PIC = 1-\Sigma(P_{ij})^2$, where P_{ij} is the frequency of the i^{th} pattern revealed by the j^{th} primer summed across all patterns revealed by the primers (Botstein et al. 1980). The similarity between matrices based on different marker system (RAPD and SSR) was calculated using the standardized Mantel coefficient (Mantel 1967). The significance level for the correlation coefficient was calculated following Sokal and Rohlf (1995).

3. Results

In RAPD analysis, a total of 93 bands was detected, among which 69 bands (74%) were polymorphic with the mean of 4.6 per primer (Table 2). For each primer, the number of bands ranged from 4 to 10, with an average of 6.2. The average polymorphic information content (PIC) was 0.447, ranging from 0.111 to 0.854. The lowest and the highest PIC values were recorded for primers OPH13, OPF03 and OPD20, respectively.

Table 2. RAPD primers' sequence, number of total band, number of polymorphic bands and polymorphic information content value generated by each primer

primers	Primers sequences 5'---- 3'	Number of total bands	Number of Polymorphic bands	Polymorphic information content value
OPD02	GGACCCAACC	8	6	0.623
OPD10	GGTCTACACC	6	5	0.525
OPD18	GAGAGCCAAC	4	2	0.111
OPD20	ACCCGGTCAC	9	7	0.821
OPG12	CAGCTCACGA	8	6	0.756
OPG14	GGATGAGACC	5	2	0.312
OPG10	AGGGCCGTCT	8	3	0.501
OPJ10	AAGCCCGAGG	4	2	0.133
OPF03	CCTGATCACC	10	8	0.814
OPH13	GACGCCACAC	9	7	0.854
OPE03	CCAGATGCAC	4	3	0.214
OPE07	AGATGCAGCC	5	3	0.112
OPE12	TTATCGCCCC	8	5	0.554
OPB05	TGCGCCCTTC	7	6	0.514
OPB18	CCACAGCAGT	6	4	0.321
Total		93	69	7.165
Average		6.2	4.6	0.447

In SSR analysis, a total of 43 bands were observed, with 2.87 bands per primer (Table 3). Thirty nine out of 43 bands (84.7%) were polymorphic, among which 2 to 4 polymorphic bands were detected by each primer. The average PIC was 0.533, and the lowest and highest PIC values were 0.133 (SSR12) and 0.974 (SSR4), respectively. Three SSR primers (i.e. SSR1, SSR2, and SSR5) had the higher PIC values.

Table 3. SSR code number and characteristics of the 15 selected SSR primers, number of loci and polymorphic loci and polymorphic information content value generated by each primer.

Primer code	Primer sequence	Number of loci	Polymorphic loci	Polymorphic information content value
GB391	F: AgCTCCTTTCCTCCCTTCC R: CCAACATCTCCTCCTCCTgA	3	3	0.867
VITR1	F: CCACTTgCCAAACACTAgACCC R: TTCATgCAgATCgggCCAC	3	2	0.949
MAG149	F: CAAgCCAACAgggTAgTC R: ATTCggTTTCTAgAggAAgAA	2	2	0.49
GB371	F: CACCAAgTTCACCTCgTCCT R: TTATTCAggCAGCACCATTg	3	3	0.439
BMAC624	F: AAAAgCATTCAACTTCATAAgA R: CAACgCCATCACgTAATA	2	2	0.934
MAG210	F: ACCTACAgTTCAATAgCTAgTACC R: gCACAAAACgATTACATCATA	4	4	0.617
MAG13	F: AAggggAATCAAAATgggAg R: TCgAATAggTCTCCgAAgAAA	3	2	0.485
GB318	F: CggCTCAAggTCTCTTCTTC R: TATCTCAgATgCCCCTTTCC	4	4	0.582
GB357	F: gCTCCAgggCTCCTCTTC R: AgCTCTCTCTgCACgTCCTT	2	2	0.459
V13GEIII	F: AggAACCCTACgCCTTACgAg R: AggACCgAgAgTggTggTgg	2	2	0.459
VB32D	F: ggTAgCAgACCgATggATgT R: ACTCTgACACgCACgAACAC	4	4	0.332
GB402	F: CAAgCAAgCAAgCAgAgAgA R: AACTTgTggCTCTgCgACTC	3	2	0.974
VGLUEND	F: TTCgCCTCCATCCCACAAAg R: gCAgAACgAAAgCgACATgC	2	2	0.337
GB384	F: CTgCTgTTgCTgTTgTCgTT R: ACTCggggTCCTTgAgTATg	3	2	0.133
MS1	F: CTgACCCTTTgCTTAACATgC R: TCAgCgTgACAAACAATAAAgg	3	3	0.163
Total		43	39	7.99
Average		2.87	2.6	0.533

All the 77 bands, generated from 15 RAPD primers, were subjected to calculate the genetic dissimilarity index (RAPD-GD) among the 14 accessions. The RAPD-GD value ranged from 0.114 to 0.933, with the mean of 0.523. The highest genetic similarity was found between Tozeur 2 (from South) and Kébilli 3 (from South), while the lowest genetic similarity was observed between Manel (from North) and Tozeur 1 (from South).

The dendrogram of genetic distances (Fig. 1) was constructed based on UPGMA Method using midpoint joining procedure of Nei and Li, (1979) dissimilarity matrix. According to genetic distances dendrogram obtained by RAPD markers and referring to a similarity rate of 65, we distinguished five groups. The first group is composed of two sub-groups ('Tozeur 2', `Kébelli 3', 'Kalaâ', `Martin' and 'Kélibia 2') and ('Jendouba 1', 'Jendouba 2' and `Sidi Bouzid'). The similarity percentage between accessions of the first sub-group varies between 77.42 and 90.77; but that of the second sub-group was ranged between 68 and 76. The accessions 'Kébelli l', 'Kébelli 2' and 'Kassrine' formed the second group. Their similarities vary from 73.17 to 82.93. Finally, the accessions 'Tozeur 1', 'Kélibia l' and 'Manel' formed groups III, IV and V, respectively. The Manel's distance in comparison with 'Tozeur l' and 'Kélibia l' is of 93.33 and 75 respectively. Despite being collected from the came region, the accession 'Tozeur l' is distant from 'Tozeur 2'; 'Kébilli 3 ' is distant from `Kébilli l' and 'Kébilli 2' since they are classified in different groups. The same case is observed for the accessions 'Kélibia l' and 'Kélibia 2'. It's also to be remarked that the group consisted of Manel is very far away from the others.

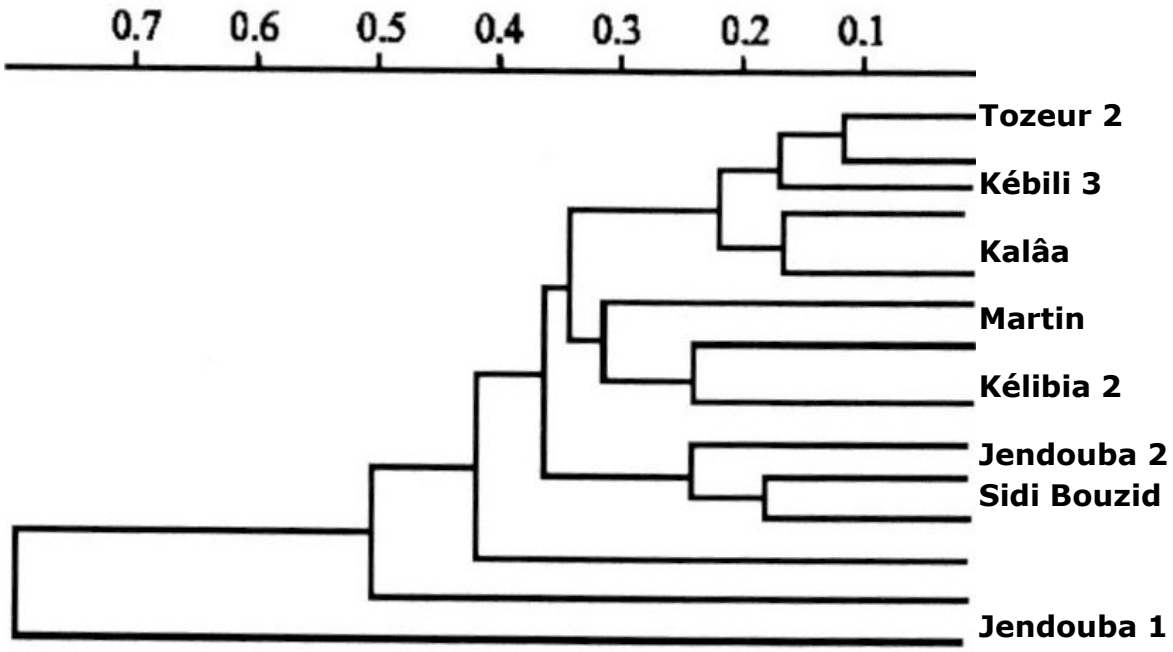

Figure 1. Phylogenetic dissimilarity distance generated by RAPD markers using UPGMA procedure according to Nei and Li (1979).

Fig. 2 was the dendrogram generated from SSR data.The obtained profiles were analyzed showing a genetic dissimilarity matrix between local barley accessions. A dendogram of dissimilarity was built. Phylogenetic constructions were based on UPGMA method. The obtained dendrogram showed 4 groups. The first group gathered the accessions 'Sidi Bouzid', 'Jendouba l', 'Jendouba 2' and Manel. The GD between Sidi Bouzid and Jendouba 1 is 17.65, which means that these accessions may have a common parent as they share the same morphological traits (growth habit, ear attitude, ear density and sterile spikelet attitude). Although they were collected from different origins, accessions of Jendouba presented a GD< 30 related to Manel. In fact they showed some common morphological traits especially ear attitude, car density and sterile spikelet attitude. They differed only in growth habit, which is usually affected by environmental conditions, as they were collected from different climatic stage. This relative small GD could be explained by exchange of seeds between farmers.

The second group consisted of Kélibia 1, Kélibia 2, Kalaâ and Martin. The first two accessions belonging to the same origin showed a GD<20 which means that farmers sowed mixture of seeds. However, Kélibia 1 and Kalaâ were genetically different only by 16.29, which means that they are probably relatives. Also, Martin, an introduced variety from Algeria; presented a GD<30 with the other accessions of the same group. This could be explained by the fact that these accessions derived from Martin variety as they herited some morphological traits such as ear attitude and sterile spikelet attitude.

The third group formed by Kébilli 3 and the last one associated Kébilli 1, Kébilli 2, Tozeur 1, Tozeur 2 and Kasserine. This group gathered accessions originating from the south of Tunisia except Kasserine that belong to the Center. The smallest GD (15) was noticed between Tozeur 1 and Kébilli 1. These two accessions were similar in the morphological traits. The other accessions presented GD situated between 20 and 30 and shared almost of the morphological traits. It is to be noticed that GD between east northern accessions (Kélibia 1, Kélibia 2 and Kalaâ) and southern ones is very important, it's about 72.89. The Martin variety presented also a GD>60 related to these accessions, which means that farmers don't saw Martin in the South of Tunisia since it's characterized by drought sensitivity. However, this variety is well spread in North of Tunisia characterized by a subhumid to humid inferior climate.

The relationship between the distances based on RAPD and the SSR markers matrices was calculated using the Mantel test (Mantel 1967). In fact we found a low matrix correlation coefficient (r = 0.176) Indicating that both sets of markers revealed the unrelated estimates of genetic relationships.

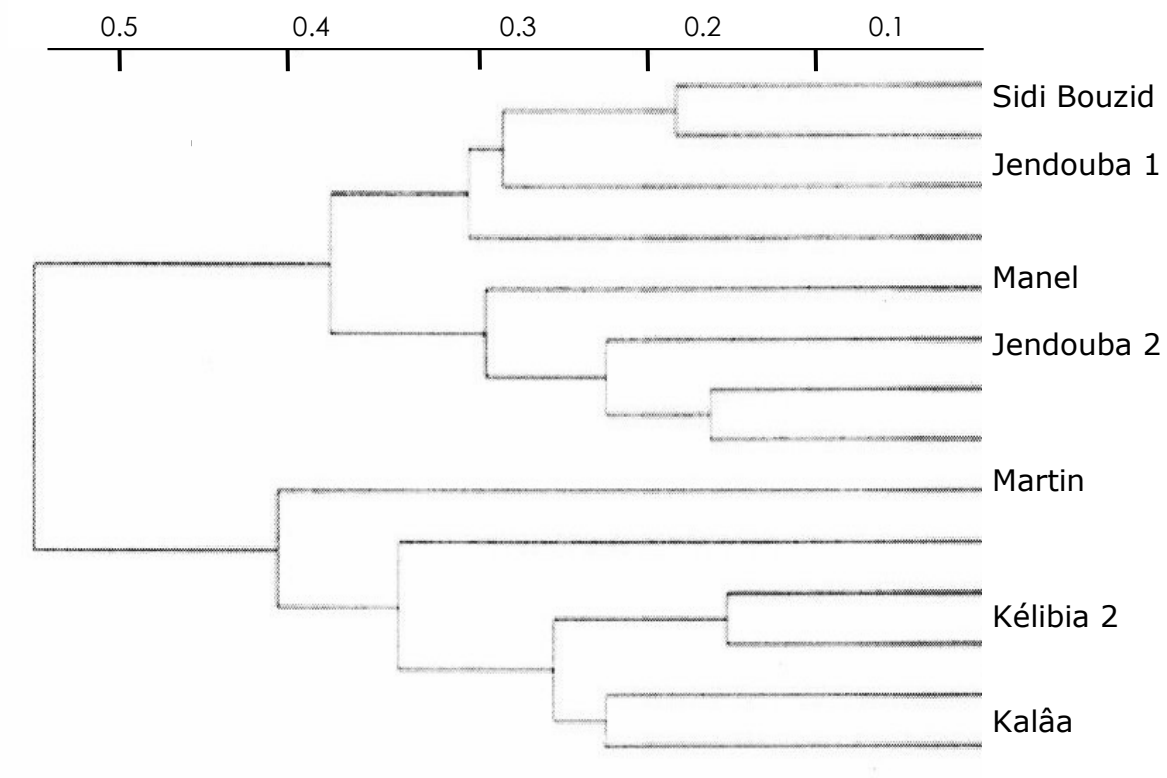

Figure 2. Phylogenetic dissimilarity distance generated by SSR markers using UPGMA procedure according to Nei and Li (1979).

4. Conclusions

In this study, we used a minimum of ten individuals for representing the each barley accession. The results indicated that the percentage of SSR polymorphic bands (83.9%) was higher than that of RAPD (74%). The mean number of amplification RAPD bands (4.2) was more than that of SSR (2.87). However, the mean polymorphism information content (PIC) (0.447) in SSR analysis was higher than that in RAPD analysis (0.533). The results in this study suggested that the SSR markers were superior to RAPD markers in the capacity of revealing more informative bands in a single amplification. The similar results were observed by (Sosinski et al. 2000; Cheng et al.2004)

Due to its worldwide distribution, the valuation of the genetic diversity among barley germplasm from different countries has been performed (Tanyolac 2003; Liu et al. 2002; Fernández et al. 2002; Matus et al. 2002; Dávila et al. 1999a, 1999b; Dávila et al. 1998; Konishi 2001; Bustos et al. 1998; Bjornstad et al. 1997; Chen et al. 2000b; Feng et al. 2003). Bernard et al. (1997) analyzed the genetic diversity in 88 genotypes from 20 populations of wild barley from Israel, Turkey and Iran by RAPD markers. When the total genetic diversity was estimated, 75% of the variation detected was partitioned within the 88 genotypes and 25% among the populations. When variation between countries was assessed, no substantial differences were found, because most of the variation detected (97%) was partitioned within the 20 populations and the remainder among the countries. Russell et al. (1997) found that the average genetic diversity based on RAPD analyses of eighteen accessions from Netherlands, France, Great Britain, Germany and Italy was 0.521. Bahattin (2003) assayed 15 wild barley populations from west Turkey by using RAPD and ISSR markers. The results revealed that the average genetic similarity was 0.27 and the genetic variation was higher than that found by Nevo et al. (1979) and Nevo et al. (1986) both using isozyme markers. In the present study, the average genetic dissimilarities of barley accessions from Tunisia based on RAPD and SSR markers were 0.523 and 0.674, respectively, Baird et al. (1996) also found similar results. The genetic variation found in this study was equivalent with that found by Chen et al. (2000a) (0.81), Shi et al. (2004) (0.631) and Chen et al. (2000b) (0.746) both using RAPD markers to analyze the genetic variation of different barley populations from China. But the variation was relatively lower than that from other country (Russell et al. 1997; Bahattin 2003).

In this study, it was obvious that the dendrogram based on RAPD markers was not in accord with the dendrogram based on SSR markers. The dendrogram generated by the RAPD matrix agrees better with the groups of the genotypes than the dendrogram generated by the SSR results (Fig. 1). Most of the accessions were closely related. However, the dendrogram generated by the SSR matrix seemed to be effective to discriminate local barleys defined as

accessions or populations geographically based (Ben Hmida, 2000). It is also a valuable tool for assessing genetic diversity levels. In fact in our study, dendrogram obtained by SSR markers classified the studied barley accessions according to climatic stage and some morphological traits especially ear attitude, ear density and sterile spikelet attitude, which could be genetically herited independently from the environmental conditions. (Yang et al.2001) found the dendrogram generated by the SSR matrix agrees better with the genealogy and the known pedigree of the barley cultivars than the dendrogram generated by the RAPD results. Wu et al. (2004) found that the data based on RAPD were more correlated with the geographic distribution of the genus *Houttuynia Thunb*, while the data based on SSRs were closely related with their number of chromosomes. Although both RAPD and SSR methods compare the genetic diversity of different barley groups based on DNA marker information, the results were different. This could be a result of the selection of accessions and the number of cultivars included within each variety group. In addition, the different molecular techniques might also affect the result (Tian et al.2008). It could be partially explained by the importance of the number of loci and their coverage of the overall genome and obtained reliable estimates of genetic relationship among the studied materials (Fernández et al. 2002). Similar results had been observed by Loarce et al. (1996). On the other hand, the relationship observed using molecular markers may provide information on the history and biology of cultivars, but it does not necessarily reflect what may be observed with respect to agronomic traits (Métais et al. 2000). The selection process leads to an accumulation of best alleles for the traits under selection. RAPDs and SSRs are dispersed throughout the genome and their association with agronomic traits is influenced by the breeder only in the region under selection pressure. The other loci are subjected to random genetic drift (Fernández et al. 2002). Another explanation could be the putatively similar bands originating for RAPDs in different bulked samples were not necessarily homologous although they shared the same size in base pairs (Karp et al. 1997). This situation might lead to wrong results when calculating genetic relationships (Fernández et al. 2002).

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