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Genetic diversity analysis of different barley (*Hordeum vulgare* L.) genotypes from arid and humid regions using ISSR and RAPD markers

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Abstract - The genetic variability analysis of eight barley genotypes, of various origin and pedigree, were achieved using 9 ISSR (Inter-simple sequence repeat) and 9 RAPD (Random amplified polymorphic DNA) primers. ISSR and RAPD markers revealed 72.2% and 61% polymorphic bands, respectively. The relative efficiencies of these marker systems were compared by means of different parameters such as effective multiplex ratio (EMR), marker index (MI) and polymorphic information content (PIC). All the examined parameters found to be higher in ISSR system.
A significant correlation was observed between the Jaccard's dissimilarity matrices based on ISSR and RAPD markers, as revealed by Mantel test using the Pearson correlation coefficient ($r = 0.67; P < 0.05$). Cluster analysis on ISSR and RAPD data clearly discriminated the genotypes in terms of their genetic background and geographical origin and both of clustering provides consistent results of their qualifications.

Keywords: *Hordeum vulgare* L.; Genetic variability analysis; Molecular markers, Discriminating power.

1. Introduction

Barley (*Hordeum vulgare* L.) is one of the earliest cultivated crops. It remains one of the major cereal crops grown in the world. Barley is a versatile crop due to its broad range of adaptability. It is cultivated in highly diverse regions of the world. It has a short growing season which makes it more drought and salinity tolerant compared to other cereals (Newton et al. 2011).
The genus *Hordeum* is considered as a pre-eminent plant for experimental genetics because of the simpler genomic constitution. Due to true diploid nature along with the similarity of its genome to that of other grain cereals, barley is considered a good genetic model for *Triticeae* and more genetically complex cereal crops like hexaploid bread wheat (Kleinhofs and Han 2002).
The genetic variability among barley genotypes makes them a valuable resource as potential donors of genes for the development and maintenance of modern crop varieties. Thus, the knowledge and understanding of this genetic diversity serve as a basis for making decisions related to the conservation and the use of the germplasm collection in genetic improvement. DNA-based molecular markers are tools that might help plant breeders to directly evaluate genetic variation among relatives without effect of environmental factors. In addition, DNA techniques allow the assessment of a theoretically unlimited number of polymorphic marker loci (Jones et al. 1997).
Among these molecular markers, RAPD (Random amplified polymorphic DNA), which depends on random amplification of DNA with short primers, offers a cheap and simple DNA-based marker that is quick and easy to assay. This procedure requires only small amounts of DNA and doesn't need primer construction, since random primers are already commercially available. In general, RAPD can provide valuable data about genetic variations within and among populations of a species, given that data are treated correctly (Collignon et al. 2002). The main disadvantage of RAPD techniques is their low reproducibility.
Moreover, ISSR (Inter-simple sequence repeat) markers are very useful for estimating the genetic diversity in plants. ISSR analysis involves the polymerase chain reaction (PCR) amplification of regions between adjacent, inversely oriented microsatellites, using a single simple sequence repeat (SSR) motifs

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(di-, tri-,tetra-, or penta nucleotides) containing primers anchored at the 3' or 5' end by two to four arbitrary, often degenerate nucleotides (Zietkiewicz et al. 1994).
Both markers, ISSR and RAPD, are widely used for genetic diversity studies in several species such as wheat (Liu et al. 1999; Sofalian et al. 2009), barley (Hou et al. 2005; Tanyolac 2003), cotton (Rana et al. 2007) and triticale (Sozen 2010).
Therefore, in the present research we used ISSR and RAPD markers to investigate the genetic diversity among 8 barley genotypes from North Africa (Tunisia), Middle East (Jordan) and from Europe (United Kingdom).The objectives of this study were: to distinguish these genotypes, to estimate the genetic diversity and relationship among these barley resources, and to compare the usefulness and the effectiveness of ISSR and RAPD markers for the assessment of genetic diversity of barley genotypes.

2. Materials and Methods

2.1. Plant materials

In the present study, 8 barley cultivars of diverse geographic origins were used. Names, pedigree, and some characteristics of these tested barley varieties are presented in Table 1. Seeds of Jordanian and United Kingdom varieties were obtained from John Innes Center (Norwich, UK).

Table1. Names, pedigree and some characteristics of barley varieties used in the study

Code	Name	Abbreviation	Row type	Description	Pedigree/ Source
1	Ardhaoui	Ar	6	Local Tunisian barley landrace adapted to the driest part of the country.	Local Tunisian variety
2	Martin	Mr	6	An old genotype derived from a local germplasm collected from Algeria and was cultivated in Tunisia (semi-arid region) since 1931.	Unknown Algerian population
3	Mutah	Mu	2	A new released Jordanian cultivar (2004) well adapted to dry conditions.	Roho-A.Abiad-6250
4	Rum	Rm	6	An old released Jordanian cultivar (1986), originated from CIMMYT (Mexico) well adapted to dry conditions.	Harbin-Arivat x Attiki CYB 19-1A-0A-0A-0A
5	Maythorpe	My	2	A United kingdom variety (from humid regions).	Maja x Irish Goldthorpe
6	Golden promise	Gp	2	A direct mutant of Maythorpe (by gamma- ray irradiation in 1956) (Walia et al. 2007)	Maythorpe
7	Oxbridge	Ox	2	A United kingdom malting variety that has a good resistance to mildew and *Rhynchosporium.*	Tavern x Chime
8	NFC-Tipple	Tp	2	A United kingdom malting variety that has a good resistance to mildew and brown rust.	(NFC 497-12 x Cork) x Vortex

2.2. DNA extraction

Total genomic DNA was extracted from 6-day-old seedlings (about 1g fresh weight) following a protocol, as described by Edwards et al. (1991).The DNA quality was checked by staining DNA with ethidium bromide (0.1µg/ml) after electrophoresis in 1% agarose gel at 100V for 1hour in 0.5xTBE buffer.

2.3. RAPD analysis

RAPD analysis was carried out with 15 decamer random primers from “Operon molecular for life”. Only nine primers that gave clear and polymorphic amplification patterns were used for genetic diversity analysis. These primers, their names, sequences, and annealing temperatures are listed in Table2 (Guasmi et al. 2012).
PCR (Polymerization Chain Reaction) reactions were performed in a 20µL volume containing 50ng genomic DNA, 2mM $MgCl_2$, 200µM each of dATP, dCTP, dGTP and dTTP, 0.8µM of each primer (Forward and Reverse), 1U *Taq* DNA polymerase (Thermo Fisher) and 4µl of 10X Taq buffer (with $(NH_4)_2SO_4$). PCRs were performed in a thermocycler ‘Cleaver scientific Ltd’. The PCR conditions included initial denaturation at 94°C for 3 min, followed by 45 cycles: denaturation at 94°C for 30sec, annealing at Tm (°C) of each primer (45sec) and extension at 72°C for 2 min, with a final extension at

72°C for 7 min. The amplified products were subjected to electrophoresis at 80 volt on 2% agarose gel. Bands in gels stained with ethidium bromide were visualized under ultraviolet light using UVP gel documentation system. In order to evaluate the reproducibility of the DNA profile, DNA isolation and PCR reactions were carried out 3 times, and only well-defined and reproducible bands were scored (Pérez de la Torre et al. 2012).

Table2. RAPD markers, sequence of primer, Total number (TB) and number of polymorphic bands (PB), percentage of polymorphism (PP), polymorphism information content (PIC), and marker index (MI).

RAPD Primer	Sequence	Tm(°C)	TB	PB	PP	PIC	MI
AX16	GTCTGTGGGG	34	5	1	20	0.12	0.02
AF14	GGTGCGCACT	34	10	8	80	0.36	2.3
W07	CTGGACGTCA	32	3	2	66.6	0.33	0.44
By 15	CTCACCGTCC	34	12	6	50	0.28	0.84
LG 13	GTTGCCAGCC	36	11	5	45.4	0.26	0.59
OPD 02	GGACCCAACC	36	8	5	62.5	0.3	0.94
OPA04	AATCGGGCTG	39.5	5	4	80	0.31	0.99
UBC 534	CACCCCCTGC	47.7	9	7	77.7	0.36	1.96
UBC 402	CCCGCCGTTG	47	6	4	66.6	0.28	0.75
Average of value			7.67	4.67	61	0.29	0.98

2.4. ISSR analysis

The reaction mixture contained the following in a total volume of 20µl: 100ng of template DNA, 1.2µM of each primer, 0,2 mM of each desoxyribonucleotide (dNTP), 1U *Taq* DNApolymerase (Thermo Fisher), 2.5 µl of 10X Taq buffer (with $(NH_4)_2SO_4$) and 2mM of MgCl2. DNA amplification was carried out using a Thermolcycler (gen-Amp PCR system 9700) and reactions were submitted to the following PCR program; 94ºC for 3 min to pre-denature followed by 35 cycles, each with denaturation at 94ºC for 1min, primer annealing at Tm(°C) for 1min and extension at 72ºC for 2min followed by a final extension at 72ºC for 5min (Guasmi et al. 2012).
ISSRs primers, their names, sequences, and annealing temperatures used in PCR reactions are listed in Table3.
The PCR products were loaded on 2% agarose gel and stained with ethidium bromide. The run was performed at a constant voltage of 80 volt and amplified products were detected under ultraviolet light by UVP Gel documentation system. The bands were considered reproducible and scorable only after observing and comparing them in three separate amplifications for each primer and each cultivar.

Table3. ISSR markers, sequence of primer, Total number (TB) and number of polymorphic bands (PB), percentage of polymorphism (PP), polymorphism information content (PIC) and marker index (MI).

ISSR Primer	Sequence	Tm (°C)	TB	PB	PP	PIC	MI
A1	AGAGAGAGAGAGAGAGC	53	3	2	66.6	0.38	0.51
Am1	AGAGAGAGAGAGAGAGT	53	7	5	71.4	0.42	1.5
Am2	TCTCTCTCTCTCTCTCC	53	9	7	77.7	0.36	1.96
UBC888	BDBCACACACACACACA	55	3	2	66.6	0.45	0.6
UBC889	DBDACACACACACACAC	55	7	5	71.4	0.35	1.25
UBC890	VHVGTGTGTGTGTGTGT	56	4	3	75	0.32	0.72
Am3	GAGAGAGAGAGAGAGAT	45	6	5	83.3	0.4	1.67
Am9	ACACACACACACACACYA	53	4	3	75	0.39	0.88
Am5	CACACACACACACACAG	53	8	5	62.5	0.36	1.13
Average of value			5.67	4.11	72.2	0.38	1.13

2.5. Statistical analysis

Amplified fragments using ISSR and RAPD markers were classified as present (represented by 1) or absent (represented by 0) in each cultivar.

For both marker systems, only fragments between 100bp and about 1000bp were scored. The bands were considered reproducible and scorable only after observing and comparing them in three separate amplifications for each primer and each cultivar. Clear and intense bands were scored while faint bands against background smear were not considered for further analysis.

A dissimilarity matrix was generated with DARwin software (version 6.0.12) using the Jaccard coefficient (Perrier et al. 2003; Perrier and Jacquemoud 2006). A dendrogram was then constructed on the basis of unweighted neighbor-joining method.

The robustness of the tree was estimated by the Bootstrap method with 2000 replicates. A bootstrap value of 70% or greater was considered as strong support.

Correlation between the distance matrices obtained from RAPD and ISSR data was calculated by Mantel test at p= 5% with 5000 permutations (Mantel 1967) using XLSTAT 2014 software.

Discriminating power of each DNA marker was assessed by means of three parameters; polymorphic information content (PIC), effective multiplex ratio (EMR), and marker index (MI).

The polymorphism information content (PIC) value for each locus was calculated according to (Roldán-Ruiz et al. 2000); $PIC_i = 2f_i (1 - f_i)$ where PIC_i is the PIC of the locus i, f_i is the frequency of the amplified fragments (band present), and $(1 - f_i)$ is the frequency of non-amplified fragments (band absent). The frequency was calculated as the ratio between the number of amplified bands at each locus and the total number of accessions. The PIC of each primer was calculated using the average PIC value from all loci of each primer.

The Marker Index was calculated as proposed by Varshney et al. (2007); MI = EMR x PIC; where the EMR (Effective Multiplex Ratio) is the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay. The Diversity Index (DI) is the average PIC values obtained for all the markers (Powell et al. 1996).

3. Results and discussion

3.1. RAPD and ISSR markers informativeness

In the present investigation, we employed RAPD and ISSR markers to differentiate the eight tested barley genotypes and to determine the molecular relationships among these cultivars.

Examples of representative gel images showing the amplification profiles of the eight genotypes by using OPD-02 (RAPD) and Am5 (ISSR) primers are represented in Figure 1.

A total of 15 RAPD primers were tested, 9 of which revealed reproducible polymorphic patterns and were used for further analysis. Amplification products using RAPD primers yielded a total of 69 scorable bands, of which 42 bands (61%) were polymorphic with an average of 4.67 polymorphic bands per primer (Table2 and 6).The highest number of the polymorphic bands (PB) (8) was obtained with AF14 primer, while the lowest number (1) was generated by AX16 primer.

The analysis of the pre-screening data using 3cultivars and12 ISSR primers showed that 3 primers failed to generate any amplification product. The 9 ISSR primers evaluated in eight barley cultivars were found to give clear and polymorphic patterns. These primers revealed high levels of diversity, detecting a total of 51 loci, 37 of them polymorphic (72.2%), averaging 4.11 polymorphic loci per primer (Table3).The number of the polymorphic bands (PB) generated by each used ISSR primers ranged from 2 (A1) to 7 (Am2) (Table3).

The mean number of amplification RAPD bands (7.67) was more than that of ISSR (5.67). And, the average number of polymorphic bands (4.67) detected by 9 RAPD primers was higher than that of the 9 ISSR primers (4.11) (Table2 and 3).

However, the percentage of ISSR polymorphic bands (72.2%) was higher than that of RAPD (61%) (Table 6).Similar results were reported by Tonk et al. (2014) who recommended ISSR in respect to higher polymorphism in triticale genotypes. However, Guasmi et al. (2012) found that RAPD markers were superior to ISSR markers in the capacity of revealing more informative bands in a single amplification in *Hordeum vulgare*.

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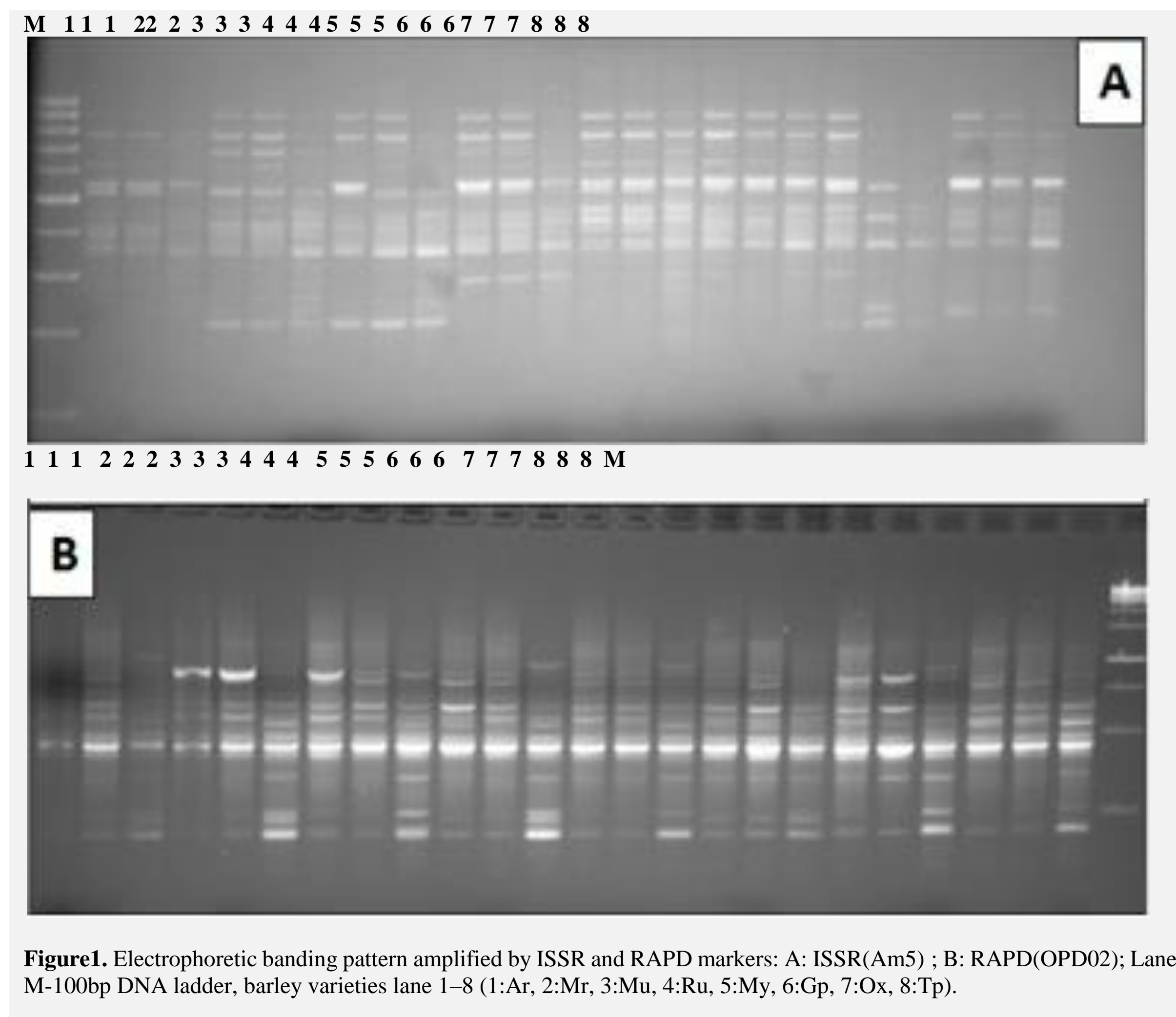

Figure1. Electrophoretic banding pattern amplified by ISSR and RAPD markers: A: ISSR(Am5) ; B: RAPD(OPD02); Lane M-100bp DNA ladder, barley varieties lane 1–8 (1:Ar, 2:Mr, 3:Mu, 4:Ru, 5:My, 6:Gp, 7:Ox, 8:Tp).

3.2. RAPD and ISSR markers performance

The information on the genetic profile of each cultivar obtained using the nine ISSR and the nine RAPD primers were used to assess the marker performance through evaluation of three parameters: PIC, EMR and marker index (MI).

To determine PIC values of each ISSR and RAPD primer, we analyzed the mean of PIC values for all loci of each ISSR or RAPD primer.

PIC values of the ISSR primers ranged from 0.32 for UBC890 to 0.45 for UBC888. The average PIC value was identified 0.38 (Table3). The effective multiplex ratios (EMR) and marker index (MI) of ISSR analysis was detected 2.98 and 1.13, respectively (Table 6).

The polymorphic information content (PIC) value of RAPD primers varied from 0.12 for AX16 to 0.36 for AF14 with an average of 0.29 (Table 2). For RAPD analysis in barley genotypes, effective multiplex ratios (EMR) were estimated at 2.84, while marker index (MI) was identified 0.82 (Table 6).

The highest possible PIC value in the case of dominant marker is (0.5) (Roldán-Ruiz et al. 2000).In this study, the highest PIC values (>0.35) was obtained for 7 out of 9 ISSR loci and for 2 out of 9 RAPD loci. In comparison of average PIC values of ISSR and RAPD, it was revealed that ISSR markers exhibited higher level of polymorphism.

Our result corroborates those of Fernández et al. (2002), Hou et al. (2005) and Tonk et al. (2014) who found higher average PIC value in ISSR compared to RAPD.

It was revealed that the ISSR makers had higher effective multiplex ratio (EMR) than RAPD. Furthermore, the marker index value (MI) which is an important comparison scale, found to be higher in ISSR system (1.13) in comparison to RAPD (0.82) in barley genotypes (Table6).

MI values varied from 0.51 to 1.96 for ISSR markers and from 0.02 to 2.3 for RAPD markers and had been used to evaluate utility and efficiency of these markers. Similar results were found by Myśków et al. (2011)who revealed that MI value of ISSR was higher (1.42) than that of the RAPD (1.16) in rye inbred lines. However, Khaled et al. (2015) stated higher marker index value in RAPD in comparison with ISSR in bread wheat.

3.3. Phylogenetic relationships and cluster analysis

Jaccard's pair-wise dissimilarity coefficient values were calculated for both ISSR and RAPD data. Genetic dissimilarity (GD) obtained from ISSR data across the 8 barley genotypes varied from 0.15 (Gp vs. My) to 0.73 (Ar vs. Ox) (Table 4).

Table 4. Jaccard's dissimilarity matrix based on ISSR data for the eight barley genotypes(1: Ar, 2: Mr, 3: Mu, 4:Ru, 5:My, 6:Gp, 7:Ox, 8:Tp)

	1	2	3	4	5	6	7	8
1	0							
2	0.37	0						
3	0.38	0.51	0					
4	0.51	0.54	0.45	0				
5	0.69	0.6	0.5	0.71	0			
6	0.72	0.58	0.58	0.66	0.15	0		
7	0.73	0.64	0.5	0.67	0.34	0.39	0	
8	0.69	0.64	0.54	0.67	0.34	0.39	0.19	0

In the case of RAPD, GD ranged from 0.19 (Gp vs. My) to 0.72 (Mr vs. Ox) (Table 5). In both ISSR and RAPD data, Golden Promise presented the lowest genetic distance face to the genotype Maythorpe (Table 4 and 5).

Table 5. Dissimilarity matrix based on RAPD data for the eight barley genotypes(1: Ar, 2: Mr, 3: Mu, 4:Ru, 5:My, 6:Gp, 7:Ox, 8:Tp)

	1	2	3	4	5	6	7	8
1	0							
2	0.54	0						
3	0.71	0.55	0					
4	0.57	0.51	0.24	0				
5	0.63	0.62	0.64	0.64	0			
6	0.58	0.61	0.54	0.55	0.19	0		
7	0.65	0.72	0.7	0.7	0.38	0.39	0	
8	0.57	0.55	0.57	0.54	0.31	0.2	0.37	0

The level of polymorphism between the two genotypes was explored by Walia et al. (2007) . The results confirm that Golden Promise and Maythorpe are genetically very closely related.

The average GD values of barley genotypes were found 0.52 for both ISSR and RAPD markers. These results further showed a moderate level of genetic similarity across the tested genotypes. 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.

The dendrograms obtained by the unweighted neighbor-joining method using genetic dissimilarities based on ISSR and RAPD data are shown in Figure 2 and Figure 3, respectively.

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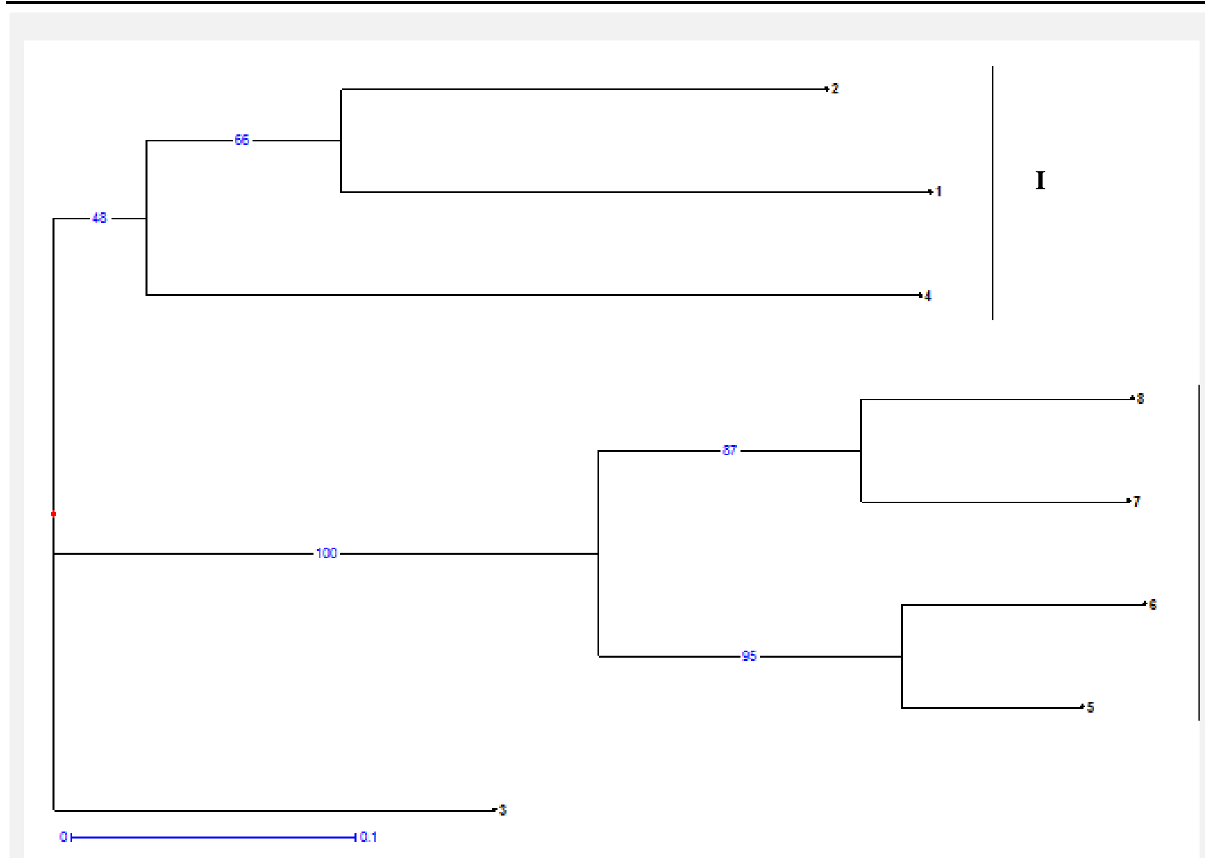

Figure2. Dendrogram generated by unweighted neighbor-joining method using genetic distance matrix based on ISSR data showing relationship among 8 barley genotypes (1:Ar, 2:Mr, 3:Mu, 4:Ru, 5:My, 6:Gp, 7:Ox, 8:Tp).

The bootstrap values are reported on the 2 dendrograms (Figure 2 and 3) and only three and two nodes among five had bootstrap values higher than 70% in ISSR and RAPD dendrograms respectively. The genotypes clustered similarly with little differences in both dendrograms. In RAPD dendrogram, the genotypes grouped in two main clusters (Figure 3). The first cluster consisted of the Tunisian and the Jordanian cultivars while the other involved the varieties originated from the UK. These results suggest that Tunisian and Jordanian cultivars are related. The first cluster showed an average genetic diversity of approximately 51%. The second cluster consisting of cultivars from the UK recorded a genetic similarity of 69% between them, and an average diversity of approximately 61 % regarding the first cluster. In ISSR dendrogram (Figure 2), the major difference is that the varieties originated from Tunisia and from Jordan grouped in same cluster except the variety Mutah. Similarly, the lines and varieties from the United Kingdom were grouped in the same cluster in ISSR dendrogram. The Mutah variety was separately placed in the dendrogram and was closer to the cluster containing the UK varieties, which can be explained by the fact that this genotype (Mu) from Jordan, is two-rowed barley as well as the UK varieties.

In the first cluster of ISSR dendrogram, the Tunisian cultivars (Ar and Mr) were grouped in the same sub-cluster. Martin variety is an old genotype derived from a local germplasm collected from Algeria and was cultivated in Tunisia since 1931. This variety and the local germplasm Ardhaoui showed a similarity coefficient of 63%. Being a North African germplasm it should have passed through the same evolutionary pressures and effects as our local germplasm since it has been cultivated in Algeria for long time and then it was introduced and improved in Tunisia. This could explain their genetic resemblance (Chaabane et al. 2009).

The clustering patterns based on the two different marker systems were primarily related to the geographical distribution of the genotypes. These results support the fact that region specific variations exist, which can be explained by a long-term adaptation process to soil and climate, multiple generations of selection and overexploitation (Velasco-Ramírez et al. 2014).

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The six-rows/two-rows cultivars used in this study were exactly discriminated in ISSR dendrograms. However, in RAPD dendrogram, the genotypes were separated according to their origins. Fernández et al. (2002)found the dendrogram generated by the ISSR matrix agrees better with the genealogy and the known pedigree of the barley cultivars than the dendrogram generated by the RAPD results.

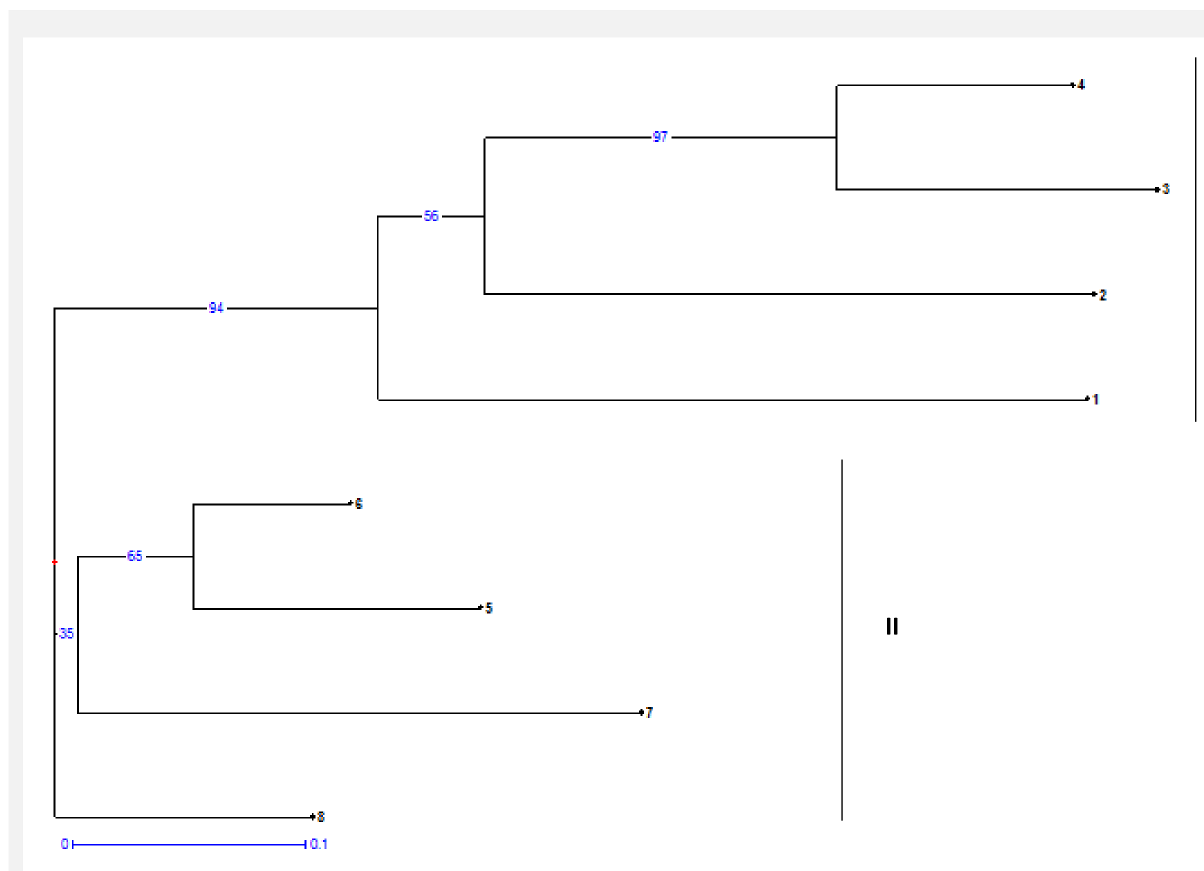

Figure3. Dendrogram generated by unweighted neighbor-joining using genetic distance matrixbased on RAPD datashowing relationship among 8 barley varieties (1:Ar, 2:Mr, 3:Mu, 4:Ru, 5:My, 6:Gp, 7:Ox, 8:Tp).

3.4. Association of ISSR and RAPD data

In order to determine the correlation among dissimilarity matrices of the marker systems (ISSR and RAPD), a Mantel test was carried out to measure the degree of relationship between them. Results showed that this correlation was significant ($r = 0.67$; $P < 0.05$).Tonk et al. (2014)reported a highly significant correlation ($r = 0.86$) between the Jaccard's similarity matrices of RAPD and ISSR in Triticale genotypes. But, Hou et al. (2005) reported a poor correlation ($r = 0.113$) between RAPD and ISSR markers in barley accessions from west China.

Table 6. Comparison of the discriminating potential of ISSR and RAPD marker system.

	ISSR	RAPD
Number of assay (primers)	9	9
Total number of markers	51	69
Number of polymorphic markers	37	42
Percentage of polymorphism	72.2	61
PIC-range of values	0.32-0.45	0.12-0.36
DI (Diversity Index)	0.38	0.29
EMR (Effective Multiplex Ratio)	2.98	2.84
MI (Marker Index= DI x EMR)	1.13	0.82

4. Conclusion

The present investigation was aimed at analyzing the genetic diversity present among the eight barley genotypes (*Hordeum vulgare* L.) using ISSR and RAPD markers.

In conclusion, the ISSR technique seems to be convenient tool to access genetic variability among barley genotypes compared to RAPD technique. However, the results of both techniques are slightly different in comparison of efficiency of markers (Table 6).

The elucidation of the relationships among the eight barley genotypes is an important resource for the breeding and management of barley germplasm.

Our long-term objective is to determine the usefulness of molecular marker diversity as a tool for gene discovery. However, more genetic studies must be conducted, employing larger samplings of germplasm collections.

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