Molecular markers for salt tolerant wild barley *Hordeum spontaneum*

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Abstract: The present study aims at detecting molecular markers, based on RAPD, ISSR and AFLP, for the salt-tolerant wild barley *Hordeum spontaneum* as a little is known about its genetic structure and function. Barley is one of the most important cereal crops all over the world. Therefore, the identification of molecular markers for the salt-tolerant wild species is crucial for the future development of tolerant domesticated varieties of *H. vulgare*. For comparison, the study involved seven domesticated barley cultivars. Across the three types of molecular markers, a total of 26 species-specific distinguished *H. spontaneum* from *H. vulgare*. RAPD markers revealed the highest expected heterozygosity H_e, E and marker index (MI) values, while ISSR indicated the lowest values. These results indicate the reliability of ISSR as a molecular marker in distinguishing wild and cultivated barley species. Some of these markers can be linked to salt stress tolerance genes in *H. spontaneum* that can be transferred to domesticated barley (*H. vulgare*) through marker-assisted selection (MAS).
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1. Introduction

Barley is one of the most important cereal crops all over the world, which ranks fourth in terms of productivity and area of cultivation (Schulte *et al*., 2009). In comparison to other cereals, barley species typically grow in low input and climatically marginal areas (Ceccarelli, 1996, Kausar *et al*., 2012) because of high water use and transpiration efficiencies (Lopez-Castaneda & Richards, 1994). Cultivated barley is a member of the genus *Hordeum* namely *H. vulgare*, which is descended from wild barley (*Hordeum spontaneum*). *H. vulgare* is well-studied in terms of genetics, genomics, and breeding, however, a little is known about the genetic makeup and genome function of its wild descendant (*Hordeum spontaneum*). Identifying molecular markers for this salt-tolerant wild species is essential in breeding programs for the future development of salt stress tolerant crop cultivars. The genome of barley genus (*Hordeum*) has been estimated to weight about 5.5 picograms (pg) of DNA in the haploid (n = 7) nucleus (Bennett & Smith, 1976) in which CG comprises about 41% (Chakrabarti & Subrahmanyam, 1985, Bahieldin *et al*., 2006). Barley genome consists of a complex mixture of unique (20-30%) and repeated nucleotide sequences. The latter is subdivided into several classes, where about 6% exists as inverted repeats of 300-3000 bp, while the rest ranged in size from 400-700 bp. About 20% of repeated sequences arranged in random, while they are mostly interspersed among themselves and/or among unique sequences (Bahieldin *et al*., 2006).

Progress in plant breeding and cultivar identification mostly relies on morphological characteristics that require extensive observations of individuals (Wrigley *et al*., 1987). Factors, like the environment, multigenic and quantitative inheritance or partial and complete dominance virtually confound gene expression. Although protein and isozyme markers were used in many crops, major limitations are the lack of polymorphism among closely-related genotypes and the variation of protein content and type among different tissues and developmental stages under different environmental conditions (Beckmann & Soller, 1983). DNA-based genetic markers are recently integrated into several plant systems and expected to play a very important role in the future of plant

breeding (marker assisted selection or MAS) and molecular genetics analysis.

Polymerase chain reaction (PCR) was initiated as a genetic assay based on selective DNA amplification (Saiki *et al*., 1988, Innis *et al*., 1990). Among the different types of PCR-based molecular markers, random amplified polymorphic DNAs (RAPDs) are useful for the assessment of genetic diversity among rare species (Williams *et al*., 1990) because of their simplicity, speed and relatively low cost as compared to other molecular markers. RAPDs are used extensively in analyzing genetic diversity (Artyukova *et al*., 2004, Sureja *et al*., 2006, Guerra *et al*., 2010). Also, inter simple sequence repeats (ISSRs) were developed as an anonymous approach accessing variation in the numerous microsatellite regions dispersed throughout the genome (Zietkiewicz *et al*., 1994). ISSRs are based on the amplification of DNA regions between inversely oriented SSRs or microsatellites (Bussell *et al*., 2005). The ISSR markers are simple and reproducible. They require small amounts of DNA and do not require information on DNA sequence. ISSR primers are designed from SSR motifs and can be undertaken for any plant species containing a sufficient number and distribution of SSR motifs in the genome (Buhulikar *et al*., 2004). Therefore, ISSRs are widely used in many respects such as the study of genetic diversity in barley (Brantestem *et al*., 2004) and cultivar identification in tobacco (Denduangboripant *et al*., 2010). Microsatellites are very short stretches of DNA that are "hypervariable", expressed as different variants within populations and among different species. They are characterized by mono-, di- or trinucleotide repeats that have 4-10 repeat unit side-by-side (Morgante and Olivieri, 1993). Amplified fragment length polymorphism (AFLP) utilizes fragments of DNA amplified using primers from restriction digested genomic DNA (Vos *et al*., 1995). AFLP provides the highest levels of resolution to allow delineation of complex genetic structures, to differentiate individuals in a population in gene flow experiments, and also to register plant varieties (Powell *et al*., 1996, Law *et al*., 1998, Barker *et al*., 1999, Aparajita & Rout, 2010, Misra *et al*., 2010).

The present study aims at the evaluating the usefulness of molecular markers, i.e., RAPD, ISSR and AFLP, in characterizing the salt-tolerant wild barley (*H. spontaneum*) as compared to the cultivated barley (*H. vulgare*) and in detecting possible species-specific markers to be utilized in the future breeding for salt tolerance in barley.

2. Materials and Methods

Plant material

The study involved the wild barley of *H. spontaneum* as well as seven domesticated cultivars (Table 1) of *H. vulgare* of Egyptian origin to be compared on the molecular levels. Genotype- or species-specific molecular markers for the salt-tolerant genotype were also detected. Names and some pedigrees of the domesticated cultivars are shown in Table 1. Relatedness of the cultivars with no available pedigrees will be detected based on the molecular analyses. Seeds of the wild species were collected from wild habitat at Rafah region near the north coast of Sinai, Egypt. Seeds of each genotype were collected from plants in three locations (populations). Ten plants of different genotypes were selected in each location based on morphological homogeneity.

Table 1. Names and pedigrees of the tested genotypes.

Serial no.	Genotype name	Pedigree
1	*H. spontaneum*	Wild barley
2	Giza 123	Giza 117/FAO 86
3	Giza 124	Giza 117/Bahteem 52// Giza 118/FAO 86
4	Giza 125	Sister line to Giza 124
5	Giza 129	N/A
6	Giza 130	N/A
7	Giza 131	N/A
8	Giza 2000	Giza 117/Bahteem 52// Giza 118/FAO 86*Giza 121

Genomic DNA extraction and purification

Extraction of total DNA was performed using the modified procedure of Gawel and Jarret (1991). The minimum number of plants to be bulked for each genotype to saturate polymorphisms within each cultivar was determined (data shown upon request). To remove RNA contamination, RNase A (10 mg/ml, Sigma, USA) was added to the DNA solution and incubated at 37^{o}C for 30 min. Estimation of the DNA concentration in different samples was done by measuring optical density at 260 nm according to the following equation:

Concentration (ug/ml) = OD_{260} X 50x dilution factor

Random amplified polymorphic DNA (RAPD)

A set of 20 random 10*mer* primers (Operon Technology, USA) from groups A, B, C and O was used in detecting polymorphism among different genotypes but three only were successful in generating reproducible and reliable amplicons. Therefore, triple primer RAPD was used to recover polymorphic and reliable amplicons. The amplification reaction was carried out in 25 µl reaction volume containing 1x PCR buffer, 4 mM $MgCl_2$, 0.2 mM dNTPs, 21 pmole primer(s), 2 units Taq DNA polymerase and 25 ng template DNA. Names of primers for single primer RAPD are OP-A05, OP-C16 and OP-O07. Combinations of primers for triple primer RAPD are OP-A02/OP-C08/OP-C10, OP-A03/OP-B10/OP-C10, OP-A18/OP-B03/OP-C06, OP-A09/OP-B04/OP-O09,

OP-A17/OP-B09/OP-C07, OP-A08/OP-C14/OP-O06, OP-A16/OP-B07/OP-C16 and OP-A15/OP-C02/OP-O07.

PCR amplification was performed in a Perkin Elmer 2400 thermocycler (Germany), programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 37°C for 2 min, and an extension step at 72°C for 2 min, followed by extension cycle for 7 min at 72°C in the final cycle.

Inter simple sequence repeat (ISSR)

Thirty primers for ISSR were used in the study but only 14 were successful in generating reproducible and reliable amplicons for different genotypes. Names and sequences of the selected primers are shown in Table 2. PCR analysis was performed in 25 µl reaction and amplification was programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 40°C for 2 min, and an extension step at 72°C for 2 min, followed by extension cycle for 7 min at 72°C in the final cycle.

Table 2. List of ISSR primers and their nucleotide sequences.

No.	Name	Sequence	No.	Name	Sequence
1	814	$(CT)_8TG$	8	HB8	$(GA)_6GG$
2	844A	$(CT)_8AC$	9	HB9	$(GT)_6GG$
3	844B	$(CT)_8GC$	10	HB10	$(GA)_6CC$
4	17898A	$(CA)_6AC$	11	HB11	$(GT)_6CC$
5	17898B	$(CA)_6GT$	12	HB12	$(CAC)_3GC$
6	17899A	$(CA)_6AG$	13	HB13	$(GAG)_3GC$
7	17899B	$(CA)_6GG$	14	HB14	$(CTC)_3GC$

Amplified fragment length polymorphism (AFLP)

AFLP analysis was performed using the AFLP Analysis System I (Invitrogen, cat. no. 10544-013) according to the manufacturer's protocol. Genomic DNA samples were digested with *Eco*RI and *Mse*I restriction enzymes in which *Eco*RI and *Mse*I adapters were ligated to the digested DNA fragments. Pre-amplification was carried out using *Eco*RI primer plus one extension base at the 3' position (A) and *Mse*I primer plus one extension base at the 3' position (C) to amplify fragments that contain complementary sequences. Five combinations of *Eco*RI primers plus three extension bases and *Mse*I primers plus three extension bases were used to selectively amplify the DNA fragments matching the primer-extension sequence. Only, two combinations succeeded to recover good quality polymorphic patterns. These two combinations are M-CCA/E-ACT and M-CAC/E-ACA.

Detection of PCR products

The products of both RAPD and ISSR were detected using electrophoresis on agarose gel (1.2% in 1x TBE buffer), stained with ethidium bromide (0.3 ug/ml), then visually examined with UV transilluminator and photographed using a CCD camera (UVP, UK). AFLP products were detected by capillary electrophoresis and virtual gels were prepared and analyzed. Fragments were separated and sized on an ABI 3500 DNA sequencer (Applied Biosystems, Foster city, California). Using the program Genemapper 4.1 (Applied Biosystems), a genetic fingerprint was produced for each individual sample by scoring for the presence or absence of a standardized set of markers between 50 and 600 base pairs in size (Rogers, 2008).

Data analysis

The bands recovered by different techniques were considered reproducible and scorable only after observing and comparing them in three separate amplifications for each primer. Clear, unambiguous and reproducible bands recovered through different techniques were considered for scoring. Each band was considered a single locus. Data were scored as (1) for the presence and (0) for the absence of a given DNA band. Band size was estimated by comparing with 100-bp ladder (Bioron, Germany) using Gel Works 1D advanced gel documentation system (UVP, UK). The binary data matrices were entered into the TFPGA (Ver. 1.3) and analyzed using qualitative routine to generate similarity coefficient. Dissimilarity coefficients were used to construct a dendrogram using un-weighted pair group method with arithmetic average (UPGMA) and sequential hierarchical and nested clustering (SHAN) routine.

Matrix comparison

Similarity matrix produced by RAPD, ISSR and AFLP were compared based on the genetic distance of the TFPGA, the normalized Mantel statistic (Mantel, 1967). The PIC (polymorphism information content) was calculated by applying the following formula given by Powell *et al*. (1996) and Smith *et al*. (1997):

$$PIC = 1 - \sum_{i=1}^{n} fi^2$$

Where, fi is the frequency of the i^{th} amplicon. The number of amplicons refers to the number of scored bands. The frequency of an amplicon was obtained by

dividing the number of cultivars, where it was found, by the total number of cultivars. The PIC value provides an estimate of the discriminating power of a marker. Marker index (MI) was calculated for each primer as the product of PIC and the number of polymorphic bands.

Analysis of molecular variance (AMOVA)

Analysis of molecular variance (AMOVA) is a method of estimating population differentiation directly from molecular data and testing hypotheses about such a differentiation. A variety of molecular marker data (for example, RAPD or AFLP), direct sequence data, or phylogenetic trees may be analyzed using this method (Excoffier *et al*., 1992). AMOVA was performed using GENALEX 6 (genetic analysis in excel, Peakall & Smouse, 2006) in RAPD, ISSR and AFLP to partition the total molecular variance between and within populations.

3. Results and Discussion

In this work, RAPD, ISSR and AFLP molecular techniques were utilized to analyze germplasms of wild (*H. spontaneum*) and the seven cultivated (*H. vulgare*) barley plants (Table 1, Figures 1 & 2). Generally, the optimal number of primers required to discriminate among genomic DNAs of different plant genotypes depends on the reproducibility of data and level of polymorphism obtained by the type of molecular analysis (e.g., RAPD, ISSR, AFLP, RFLP, etc.). Arguments about the value of genetic distance required to classify correlated plants accessions as distinct cultivars have been raised by several authors (Cabrita *et al*., 2001, Papadopoulou *et al*., 2002). In the present study, primers (11 single and triple for RAPD, 14 for ISSR and two combinations for AFLP) with informative patterns were selected. Selection of primers was based on the number of amplicons recovered through PCR and the stability (reproducibility) of the patterns. These primers were used in the characterization of eight genotypes belonging to the two plants species as well as the level of polymorphism. Less than 10% intra-plants polymorphism (within) was found across the three types of analyses for the plants of the same genotype (data provided upon request). As being dominant markers, pooling (bulk DNA) strategy in ISSR, RAPD and AFLP analyses is ideal to saturate such an intra-plants polymorphism with no effects on the accuracy of the obtained results. Mengoni *et al*. (2000) indicated that this level of intra-plant polymorphism, following the procedure of AMOVA (Excoffier *et al*., 1992), is statistically insignificant and acceptable.

Identification of species- and cultivar-specific molecular markers

A high level of polymorphism was generated utilizing the 11 RAPD with single and triple primers. A total of 531 amplicons, across genotypes and primers, were separated on agarose gel electrophoresis across genotypes. Of these, 222 bands were polymorphic (42%) and the rest were monomorphic (58%). The highest number of amplicons was generated from G2000 (51 amplicons), while *H. spontaneum* generated the lowest (37 amplicons). The highest number of genotype-specific markers (9, see Table 3), due to the presence of a unique band for a given plant species (positive marker), was scored for *H. spontaneum* (species-specific markers), while the lowest number was scored for G124 (4) followed by G125 (6) (cultivar-specific markers).

ISSR is a relatively more recent class of molecular markers, which is based on inter tandem repeats of short DNA sequences. Such repeats were proven to be highly polymorphic even among closely-related genotypes due to the lack of functional constraints in these non-functioning DNA regions that was thought to result in the evolutionary changes in their DNA structures. Accordingly, a high level of polymorphism was generated utilizing the 14 ISSR primers. A total of 736 amplicons were obtained in which 418 of them were polymorphic (57%) and the rest were monomorphic (43%). The highest number of amplicons was generated from G131 (109 amplicons), while *H. spontaneum* plant gene rated the lowest (75 amplicons). The highest number of genotype-specific markers (Table 3) was scored for *H. spontaneum* (13), while the lowest number was scored for G129 (6).

Two combinations were used in the AFLP analysis and revealed 789 amplicons, as little as 355 of them were polymorphic (45%) among the different genotypes. The highest number of amplicons was generated from G123 (112 amplicons), while G2000 generated the lowest (98 amplicons). The highest number of genotype-specific markers (7) was scored for G123, while the lowest number of genotype-specific markers (4) was scored for *H. spontaneum*. In conclusion, the 14 ISSR primers used in the present study allowed for the highest rate of distinction, as compared to AFLPs and RAPDs, between the two barley species, on one hand, and among the different cultivars, on the other hand. A number of 26 species-specific markers were detected that can be used in distinguishing *H. spontaneum* from *H. vulgare* (Table 3).

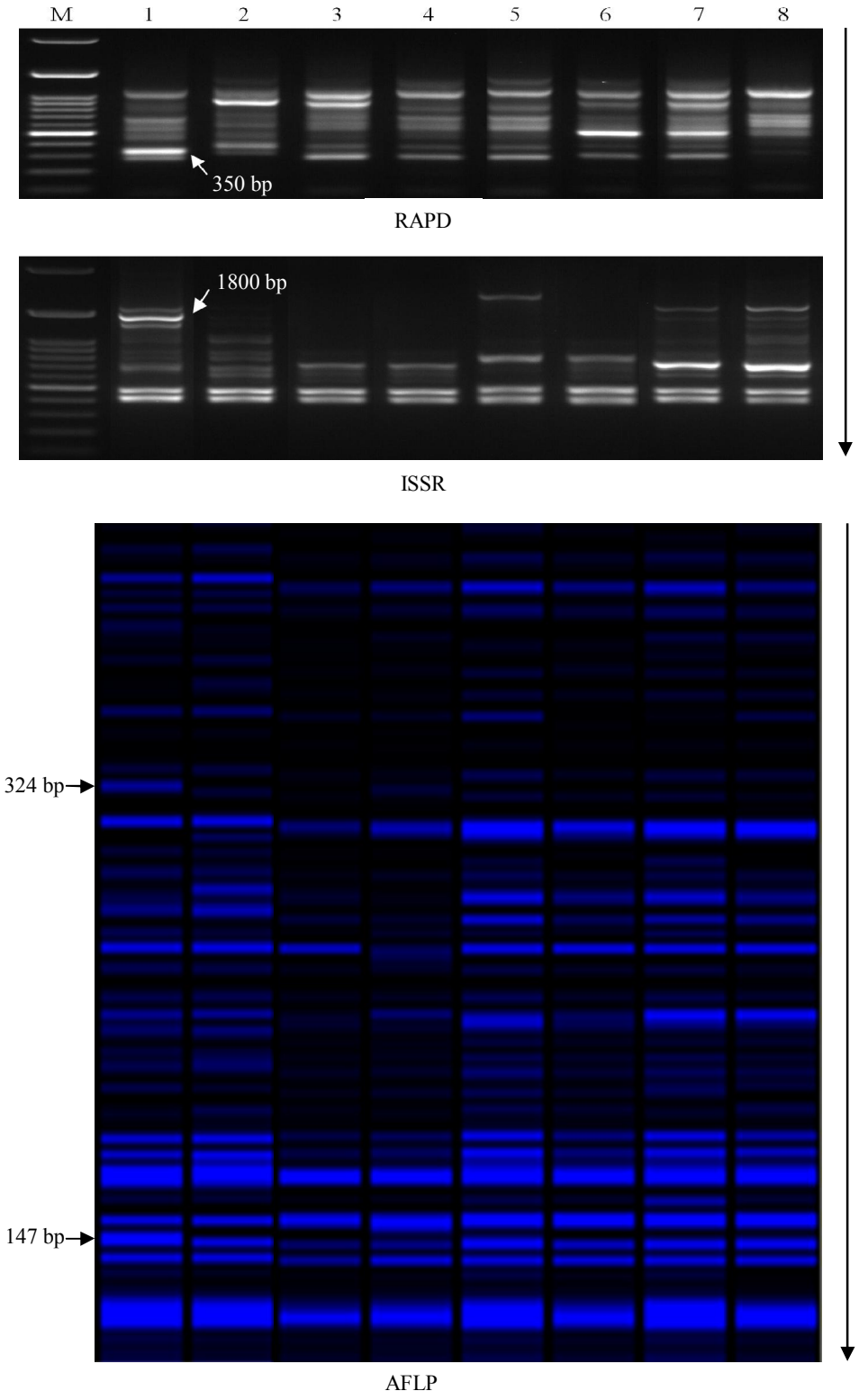

Figure 1: Models of different marker profiles (RAPD with triple primers OP-A02/OP-C08/OP-C10 (350 bp), ISSR with primer 844B (1800 bp) and AFLP with primer combination M-CCA/E-ACT (147 and 324 bp) of the eight genotypes (1-8, see Table 1). M refers to DNA standard (100-bp ladder, Bioron). Arrows indicate direction of migrated PCR products.

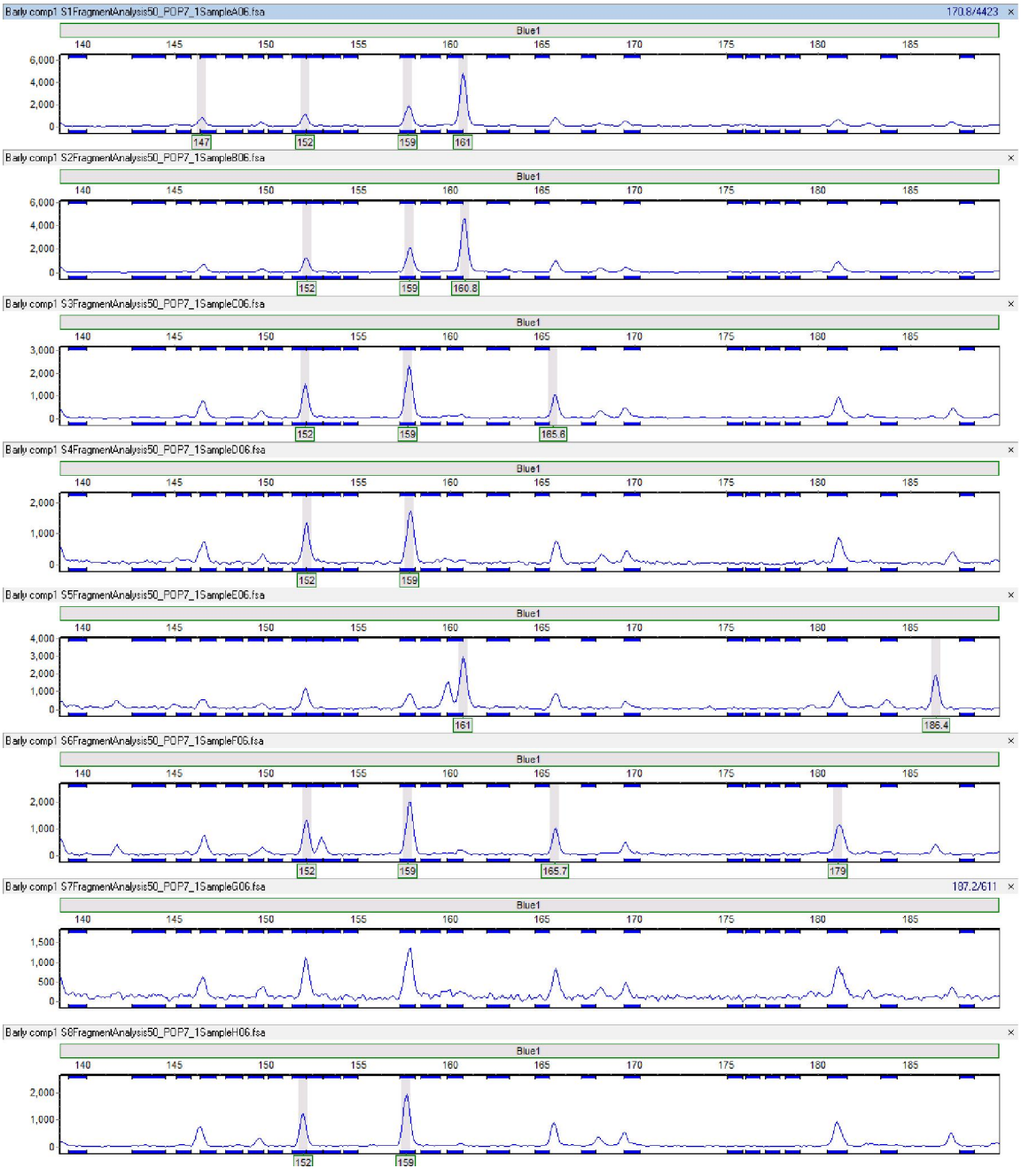

Figure 2: A section of the electrophoregram (140-185 bp) of the AFLP capillary run with primer combination M-CCA/E-ACT showing a specific marker with MW of 147 bp for *H. spontaneum* (1-8, see Table 1). Arrow indicates direction of migrated PCR products.

Genetic relationships and cluster analysis

The genetic similarities between the two species and among the seven cultivars of *H. vulgare* species, based on Nei's method (Nei's, 1978), within and across markers are shown in Table 4 and Figure 3. The results of similarity indices and dendrograms within RAPD, ISSR, AFLP and across markers indicated mean distances between the two species of 82.9, 75.0, 80.3 and 79.7, respectively. It was obvious that ISSR marker was the type of markers resulted in the higher number of positive species-specific markers (13 for *H. spontaneum* and 12 for *H. vulgare* cv. G123) when compared to the other two types of markers. However, the results of the other two marker types as well as across types of markers were able to separate the two species in two clusters. The results of the domesticated

cultivars indicated that G124/G125 were the most closely related genotypes, followed by G129/G130 and G129/G131 within and across types of markers. Although information on the ancestors for G131 is not available, it was shown that this cultivar is closely related to G2000. These results are unexplained as G2000 is known to be closely related to G124, G125, then G123. The three cultivars G129, G130 and G131 seem to be closely related. We can conclude that use of more RAPD primers and AFLP combinations might result in more polymorphic and species-specific markers.

The partition of variation within species was studied with the analysis of the Dice's distance matrix by the analysis of molecular variance (AMOVA) approach. A hierarchical analysis of genetic diversity using a two-way nested AMOVA was performed. Results from AMOVA within and among population are shown at Table 5. Data indicated that 94% of the genetic variation is attributed to differences among populations, while 6% of the genetic variation is attributed to differences within populations. The values of MS indicated the high level of polymorphism among genotypes and the low level of experimental error. This reflects the homogeneity in leaf samples collected for the study as a perfect representative of the target genotypes.

The average of heterozygosity (H_e), the effective multiplex ratio (E), and the marker index (MI) were computed for each assay based on experimental data (Table 6). RAPD revealed the highest H_e (0.48) as compared to AFLP (0.38), then ISSR (0.36). The obtained results in the present investigation agreed with these of Powell *et al*. (1996). Muzher (2005) found that H_e of RAPD was more than AFLP and ISSR. With regard to the E value, ISSR indicated the lowest value (15), while AFLP (28) and RAPD (45) indicated higher values. Concerning MI, ISSR revealed the lowest value (4.33) compared with AFLP (9.21), then RAPD (16.35). In general, the results of ISSR unexpectedly can be considered more reliable than AFLP and RAPD. Reliability of AFLP can be improved if more combinations were used in characterizing species or cultivars on the molecular levels. It could be concluded that markers differ in their ability to differentiate individuals, the mechanism of detecting polymorphism, genome coverage, and the ease of application. They can be complementary to each other depending on technical availability. Some of these markers can be linked to salt stress tolerance genes in *H. spontaneum* that can be transferred to domesticated barley (*H. vulgare*) through marker-assisted selection or MAS (Ribaut & Hoisington, 1998, Zhong *et al*., 2006, Miedaner & Korzun, 2012). There are some efforts towards breeding salinity tolerance in plant via MAS (Thomson *et al*., 2010, Singh *et al*., 2011, Ashraf *et al*., 2012) that can be duplicated for the development of salt-tolerant domesticated cultivars of barley.

Table 3. List of specific markers for *H. spontaneum* for different marker types. The table indicates the type and number of markers along with their molecular weights (MW) in bp.

Marker type	Marker name	No.	MW (bp)
RAPD	A02/C08/C10	1	350
	A03/B10/C10	1	560
	A18/B03/C06	-	-
	A09/B04/O09	2	420, 890
	A17/B09/C07	1	730
	A08/C14/O06	1	520
	A16/B07/C16	1	1130
	A15/C02/O07	2	1400, 1240
	Total	**9**	
ISSR	814	-	-
	844A	1	810
	844B	1	1800
	17898A	1	550
	17898B	1	1030
	17899A	-	-
	17899B	1	1270
	HB8	1	860
	HB9	1	1330
	HB10	1	1110
	HB11	-	-
	HB12	2	370, 1200
	HB13	1	460
	HB14	2	650, 1440
	Total	**13**	
AFLP	M-CCA/E-ACT	2	147, 324
	M-CAC/E-ACA	2	190, 330
	Total	**4**	

Table 4. Similarity matrixes based on molecular data for the eight barley genotypes.

a. RAPD

Genotype	*H. spon*	G123	G124	G125	G129	G130	G131	G2000
H. spon	1.00							
G123	0.80	1.00						
G124	0.86	0.92	1.00					
G125	0.83	0.91	0.90	1.00				
G129	0.80	0.84	0.85	0.80	1.00			
G130	0.85	0.85	0.87	0.84	0.89	1.00		
G131	0.85	0.88	0.89	0.87	0.93	0.90	1.00	
G2000	0.81	0.87	0.88	0.90	0.83	0.84	0.85	1.00

b. ISSR

Genotype	*H. spon*	G123	G124	G125	G129	G130	G131	G2000
H. spon	1.00							
G123	0.74	1.00						
G124	0.72	0.87	1.00					
G125	0.71	0.86	0.90	1.00				
G129	0.82	0.83	0.84	0.82	1.00			
G130	0.82	0.84	0.86	0.84	0.89	1.00		
G131	0.74	0.87	0.88	0.87	0.87	0.90	1.00	
G2000	0.70	0.86	0.87	0.88	0.81	0.80	0.91	1.00

c. AFLP

Genotype	*H. spon*	G123	G124	G125	G129	G130	G131	G2000
H. spon	1.00							
G123	0.83	1.00						
G124	0.77	0.88	1.00					
G125	0.79	0.89	0.91	1.00				
G129	0.80	0.84	0.82	0.84	1.00			
G130	0.81	0.80	0.85	0.83	0.81	1.00		
G131	0.81	0.86	0.84	0.86	0.87	0.78	1.00	
G2000	0.81	0.89	0.89	0.88	0.80	0.86	0.86	1.00

Overall

Genotype	*H. spon*	G123	G124	G125	G129	G130	G131	G2000
H. spon	1.00							
G123	0.79	1.00						
G124	0.76	0.87	1.00					
G125	0.77	0.89	0.91	1.00				
G129	0.82	0.81	0.80	0.79	1.00			
G130	0.83	0.84	0.82	0.82	0.88	1.00		
G131	0.81	0.81	0.89	0.84	0.85	0.84	1.00	
G2000	0.80	0.83	0.88	0.85	0.81	0.81	0.88	1.00

Table 5: Analysis of molecular variance (AMOVA) of the different barley genotypes.

Source	*d.f.**	*S.S.***	*M.S.****	*%*
Among Pops	7	5.238	0.748	96
Within Pops	664	122.738	0.185	4
Total	671	127.976	0.198	

**d.f.* = Degrees of freedom, ***S.S.* = Sum of squares, ****M.S.* = Mean square

Table 6: Polymorphism information content (PIC), expected heterozygosity for polymorphic products (He), effective multiplex ratio (E) and the marker index (MI) of each marker type used across genotypes.

Marker type	PIC	He	E	MI
ISSR	0.30	0.36	15	4.33
RAPD	0.36	0.48	45	16.35
AFLP	0.31	0.38	28	9.21

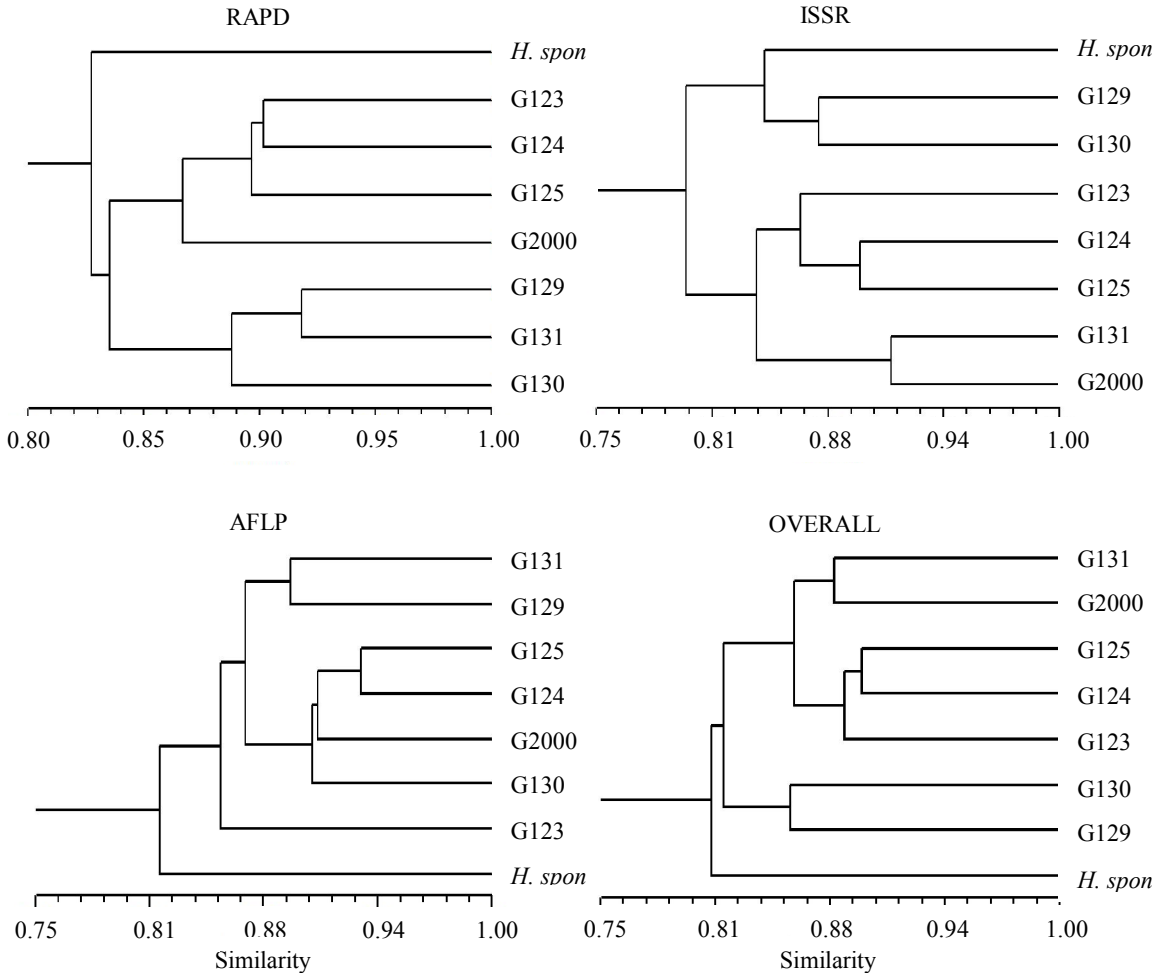

Figure 3: Dendrogram based on algorithm of unweighted pair group method with arithmetic averages between species and among cultivars.

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