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Genetic Diversity in *Hordeum spontaneum* C. Koch of Northern Jordan (Ajloun Area) as Revealed by RAPD and AFLP Markers

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Abstract: The objectives of this study were to locate and collect wild barley accessions of the species (*Hordeum spontaneum* C. Koch) from certain regions in northern Jordan (Ajloun area) to characterize the molecular diversity of the collected barley accessions by analyzing the DNA amplification products using AFLP and RAPD molecular marker methodologies; evaluate the AFLP and RAPD methods to be used in barley as genetic markers and improve such techniques as suitable strategies for barley germplasm characterization. Fifty samples representing five populations of *Hordeum spontaneum* C. Koch from northern Jordan (Ajloun area) were included in this study and were subjected to RAPD and AFLP molecular markers analysis. Twenty-seven RAPD primers produced 288 amplification products of which 90 (31.3%) were polymorphic and six AFLP primers produced 300 amplification products of which 155 (51.7%) were polymorphic. Genetic diversity was larger among than within populations.

Key words: *Hordeum spontaneum*, AFLP, RAPD, genetic diversity

INTRODUCTION

The barley (*Hordeum* L.) is one of the most important crops in the world, ranking fifth in the world production that is used for animal feed, brewing malts and human consumption (Hayes *et al*., 2002).

Hordeum L. is a widely distributed genus of the tribe Triticeae of the Poaceae (Graminae) family. There are about 45 species and subspecies, most of which represent weedy annual or perennial grasses, found throughout the temperate zones of both northern and southern hemispheres (Morrell *et al*., 2003). *H. spontaneum* C. Koch, the only recognized wild progenitor of cultivated barley (*Hordeum vulgare*), is a self-pollinating diploid (2n = 14) originates from the Fertile Crescent areas of southwest Asia (Morrell *et al*., 2003).

Different approaches were used to assay genetic diversity in crop plants including morphological traits and isozyme electrophoresis, however, these techniques are insufficient to serve as accurate markers due to environmental influences on morphological traits and insufficient polymoprhism produced among closely related genotypes (Matus and Hayes, 2002).

Certain properties are desirable for a molecular marker such as highly polymorphic behavior, co dominant inheritance and frequent occurrence in the genome, even distribution throughout the genome, selectively neutral behavior, easy access, easy and fast assay and high reproducibility (Weising *et al*., 1995). Examples of such DNA molecular markers are: Random Amplified Polymorphic DNA (RAPDs) (Welsh and McCelland, 1990; Williams *et al*., 1990) amplified fragment length polymorphisms (AFLPs). RAPD and AFLP markers had proved to be good genetic markers to assay and evaluate the genetic diversity between and within the same species, populations and individuals (Warburton *et al*., 1996). RAPD marker depends on the amplification of DNA sequence by polymerase chain reaction using only a single primer of arbitrary nucleotide sequence. The technique has proved to be fast and simple needs small quantities of template DNA, beside its ability to detect relatively small amounts of genetic variation (Warburton *et al*., 1996).

AFLP is a highly sensitive method for detecting polymorphisms throughout the genome. It is based on the selective amplification of subsets of genomic fragments generated by digestion by restriction enzymes then ligation of complementary adapters and PCR amplification with primers consisting of the adapter sequences, extended with variable numbers of nucleotides (1-3). It is reliable, reproducible marker assay and large number of AFLP loci can be detected in a single experiment providing large numbers of informative markers (Breyne *et al*., 1999).

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A series of studies have been conducted on *H. spontaneum* as a model organism for regional and local diversity of allozymes (Nevo, 1992), RAPDs (Dawson *et al*., 1993; Weinings and Henry, 1995; Nevo, 1998a,b; Owuor *et al*., 1999), RFLPs (Zhang *et al*., 1993; Saghai-Maroof *et al*., 1995), SSRs (Saghai-Maroof *et al*., 1994; Turpeinen *et al*., 2001), AFLPs (Pakniyat *et al*., 1997), rDNA (Zhang *et al*., 1990; Saghai-Maroof *et al*., 1990) and hordein (Chalmers *et al*., 1992).

The objectives of this study were to locate and collect wild barley accessions of the species (*Hordeum spontaneum*) from certain regions in northern Jordan (Ajloun area) to characterize the molecular diversity of the collected barley accessions by analyzing the DNA amplification products using AFLP and RAPD molecular marker methodologies; evaluate the AFLP and RAPD methods to be used in barley as genetic markers and improve such techniques as suitable strategies for barley germplasm characterization.

MATERIALS AND METHODS

Plant material and DNA extraction: This study was conducted between 2000-2002 in northern Jordan (Ajloun area). Grains, represented five populations of *H. spontaneum* C. Koch, were collected from various locations in the northern Jordan (Ajloun area which situated between 32°20′ North to 35°45′ East with average 760 m above sea level) on August 18, 2000, which covered five locations (representing five different populations). These locations were Zubya, Barkash, Wadi Rayan, Wadi Baun and Samtah (Table 1).

DNA was extracted from the young leaves of green house planted seedlings (4 week old) following the CTAB method as described by (Doyle and Doyle, 1990).

RAPD amplification: Forty random primers (10 m) from two kits (A and B) (Operon Technologies, Alameda, CA, USA) of arbitrary sequence were used in this study (Table 2).

RAPD reactions were done in a total volume of 25 μL containing 60 ng of primer, 200 μM each of dATP, dCTP, dGTP and dTTP, 100 ng of DNA template, 2 mM $MgCL_2$ and 1.5 u of Taq DNA polymerase in 1X PCR buffer. DNA amplification was performed in a Perkin Elmer Cetus DNA Thermal Cycler (model 480) in 0.5 mL PCR tubes programmed for initial denaturation at 95°C for 2 min followed by 43 cycles for 1 min at 95°C, at annealing temperature of 37°C for 1 min and at 72°C for 2 min as an extension step. The final extension step was done for 5 min at 72°C and the reactions were kept at soak file at 4°C.

The RAPD-PCR amplified products were analyzed by gel electrophoresis in 1.5% ultrapure agarose in 0.5X TBE buffer stained with ethidium bromide (0.5 μg mL^{-1}) at 125 volts for 5 h using horizantal gel electrophoresis apparatus (Sigma Chemical Co. Louis, MO, USA). The amplified products were visulalized under UV light and photographed with black and white Polaroid films (Polaroid type 667). 1 kb ladder was used as a DNA standard to estimate the molecular weights of the amplified products.

AFLP amplification: AFLP analysis was performed according to Vos *et al*. (1995). AFLP starter primer kit was purchased from Gibco-BRL life technologies (Gaithersburg, MD. USA).

Total genomic DNA was digested with *Mse*I and *Eco*RI. AFLP fingerprints were generated using primer combinations with varying selective nucleotides. Three *Eco*RI primers with three selective nucleotides (AAG, ACA and ACC) were combined with *Mse*I primers carrying three nucleotides (CAA and CAC).

For each individual primer (for RAPD) and primers combination (for AFLP), PCR amplified products were designated. Data were scored on the basis of the presence or absence of the amplified products. If the product is present in a genotype, it was scored as 1, if absent, it was designated as 0. Using the SPSS statistical computer program, genetic similarities between and within the *H. spontaneum* genotypes were calculated using the simple matching coefficient and clustered by unweighted pairs group method with arthmetic average (UPGMA) (average linkage) to construct dendrograms (Rohlf, 1993).

Table 1: Sites of collection (Coordinates and description)

Site	LON	LAT	ALT (m)	Site description
Zubya	35° 44′ 11″ E	32° 26′ 04″ N	785	Medium degradation of environment, high erosion, soil in pockets, vegetation mainly fruit trees and few shrubs.
Barkash	35° 44′ 13″ E	32° 26′ 07″ N	805	Grazed over land, vegetation mainly annuals with trees of *Quercus calliprinos* and *Pistacia*, highly degraded.
Wadi Rayan	35° 41′ 18″ E	32° 24′ 12″ N	336	Cultivated area with orchard and vinyards, the slopes of the valley medium degraded.
Wadi Baun	35° 42′ 30″ E	32° 23′ 18″ N	576	High degradation, high erosion, soil in pockets, vegetation mainly herbaceous and shrubby with few scattered trees, the slope is about 65 degree.
Samtah	35° 50′ 13″ E	32° 23′ 38″ N	1040	Grazed over land, vegetation mainly annuals with few scattered trees like *Quercus calliprinos* and *Pistacia*, highly degraded.

LON: Longtitude, LAT: Latitude, ALT: Altitude. E: East, N: North

Table 2: Random primers used to screen *H. spontaneum* germplasm for RAPDs

Primer	Sequence 5` to 3`
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG
OPA-05	AGGGGTCTTG
OPA-06	GGTCCCTGAC
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG
OPA-09	GGGTAACGCC
OPA-10	GTGATCGCAG
OPA-11	CAATCGCCGT
OPA-12	TCGGCGATAG
OPA-13	CAGCACCCAC
OPA-14	TCTGTGCTGG
OPA-15	TTCCGAACCC
OPA-16	AGCCAGCGAA
OPA-17	GACCGCTTGT
OPA-18	AGGTGACCGT
OPA-19	CAAACGTCGG
OPA-20	GTTGCGATCC
OPB-01	GTTTCGCTCC
OPB-02	TGATCCCTGG
OPB-03	CATCCCCCTG
OPB-04	GGACTGGAGT
OPB-05	TGCGCCCTTC
OPB-06	TGCTCTGCCC
OPB-07	GGTGACGCAG
OPB-08	GTCCACACGG
OPB-09	TGGGGGACTC
OPB-10	CTGCTGGGAC
OPB-11	GTAGACCCGT
OPB-12	CCTTGACGCA
OPB-13	TTCCCCCGCT
OPB-14	TCCGCTCTGG
OPB-15	GGAGGGTGTT
OPB-16	TTTGCCCGGA
OPB-17	AGGGAACGAG
OPB-18	CCACAGCAGT
OPB-19	ACCCCCGAAG
OPB-20	GGACCCTTAC

RESULTS

RAPD assay: Out of a total of 288 amplification products (0.2 to 4 kilo base pairs) using twenty-seven primers, 90 (31.3%) were polymorphic (Table 3) and 198 products were shared among all genotypes (not polymorphic). The twenty-seven primers (OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, OPA-07, OPA-09, OPA-10, OPA-11, OPA-12, OPA-13, OPA-14, OPA-15, OPA-16, OPA-17, OPA-18, OPA-19, OPA-20, OPB-03, OPB-04, OPB-05, OPB-06, OPB-07, OPB-10, OPB-11, OPB-12, OPB-14) produced different banding patterns for all genotypes. While thirteen primers (OPA-06, OPA-08, OPB-01, OPB-02, OPB-08, OPB-09, OPB-13, OPB-15, OPB-16, OPB-17, OPB-18, OPB-19 and OPB-20) detected no polymorphism although they did successfully amplify a range of monomorphic bands. The average number of RAPDs that was detected per polymorphic primer was 3.3 (Table 3). Figure 1 indicates a repersentive example of RAPD markers detected in

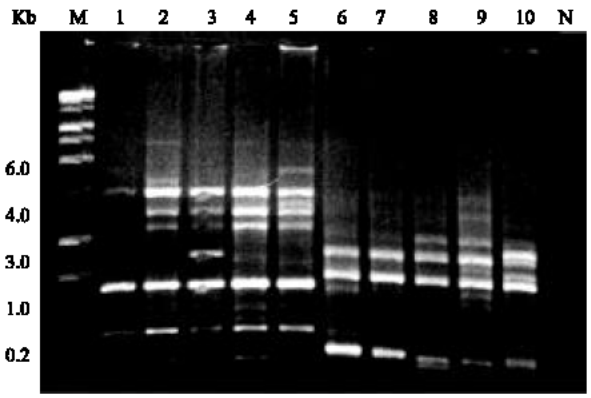

Fig. 1: DNA amplification products of individual samples of wild barley populations. Lanes (1-5): RAPD patterns of DNA from individual plants of populations Zubya, Barkash, W. Rayan, W. Baun and Samtah population respectively using the primer OPA-04. Lanes (6-10): RAPD patterns of DNA from individual plants of populations Zubya, Barkash, W. Rayan, W. Baun and Samtah population respectively using the primer OPA-12. Lane M is a 1 kb molecular weight marker, lane N is a negative control: amplification product lacking DNA template sample

Table 3: Comparison between RAPD and AFLP assay in *H. spontaneum* germplasm

	RAPD	AFLP
Total No. of primers (in RAPD) or primer combinations (in AFLP)	40	6
Total No. of polymorphic primers or primer combinations	27	6
Total No. of bands amplified from polymorphic primers or primer combinations	288	300
Size range of amplification products	0.2-4 kb	0.1-4 kb
Average No. of bands per polymorphic primer or primer combination	10.7	50
Total No. of polymorphic bands identified	90	155
Average No. of RAPDs or AFLPs per polymorphic primer or primer combination	3.3	25.8
Percentage of total bands which were polymorphic	31.3%	51.7%

H. spontaneum germplasm represented by five different populations. In an attempt to estimate the relatedness of individual plants within each barley population, eight 10-mer selected primers OPA-05, OPA-07, OPA-11, OPA-15, OPA-17, OPA-19, OPB-07 and OPB-10) were used in RAPD reaction. Polymorphism was detected; it was significant but was much less than between populations.

AFLP assay: The AFLP assay showed that out of a total of the 300 amplification products of (0.1 to 4 kilo base pairs) scored, 155 (51.7%) were polymorphic (AFLPs) (Table 3). One hundred and fifty four products were common among all genotypes. All six primers

combinations produced a sufficient number of polymorphic bands for all genotypes. The average number of AFLP products detected per polymorphic primers combination was 25.8 (Table 3).

Figure 2 shows a representative silver stained AFLP gel with AFLP fingerprints detected in the five studied populations of the *H. spontaneum*.

Polymorphism was detected also among individual plants within each wild barley populations using AFLP using 4 primer combinations (E-AAG * M-CAA, E-AAG * M-CAC, E-ACA * M-CAA and E-ACA * M-CAC14).

A number of few polymorphic bands of different sizes were detected in many plants of each population, especially in W. Rayan and Samtah populations. This detected polymorphism was much less than between populations but the polymorphism that was detected by AFLP was much informative than does RAPD.

The results in Table 3 indicated clearly that the AFLP analysis reveals more polymorphism at interpopulational level than do RAPD analysis.

The RAPD and AFLP amplified products resulted in the same dendrogram as shown in Fig.3a and b. Two major clusters resulted: One cluster included Wadi Rayan population (represented by the unique individual plant Rayan 4) by itself and the second cluster included the rest of the four populations (Zubya, Barkash, Wadi Baun and Samtah). This second cluster was subdivided into two sub clusters (Samtah sub cluster and the other sub cluster included W. Baun, Zubya and Barkash).

Also cluster analysis of each RAPD and AFLP profiles (Fig. 3a and b) showed that strong genetic variation was detected in population of W. Rayan (Rayan 4) that was obviously different from the other studied populations (Rayan 4 showed 2% similarity in both RAPD and AFLP assay) which could reflect different genetic background, Samtah population which showed 56% similarity (in RAPD) and 40% similarity (in AFLP) indicated a significant genetic variation in this population. Moreover, cluster analysis showed that Zubya and Barkash populations. were genetically close (they showed 96% similarity in RAPD and AFLP). Also W. Baun showed 90% (in RAPD) and 88% similarity (in AFLP) to Zubya and Barkash populations but less than Samtah and Wadi Rayan populations.

The correlation between the two genetic similarity matrices constructed from RAPDs and AFLPs was tested using the Mantel test (Mantel, 1967). The comparison yielded a correlation coefficient of $r = 0.885$ ($p<0.05$), which indicated that the two sets of markers provided related estimates of genetic relationships.

The results in the dendrograms (Fig. 3a and b) and Table 3 showed that the AFLP methodology was sensitive enough to detect low levels of variation and to discriminate between highly related genotypes than does RAPD.

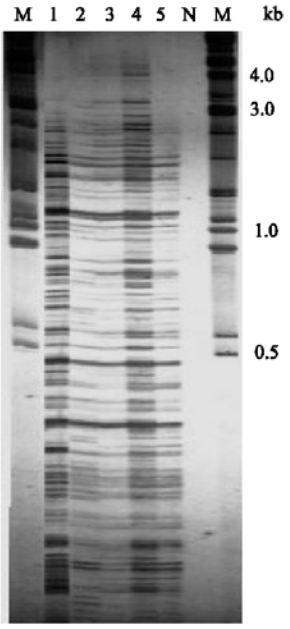

Fig. 2: DNA amplification products of individual plant samples of the wild barley population. Lanes (1-5) represent AFLP patterns of DNA from individual plants of populations Zubya, Barkash, W. Rayan, W. Baun and Samtah population respectively. Lane M is a 1 kb molecular weight marker, lane N is a negative control: Amplification product lacking DNA template. The PCR amplification was done with the primer combination (E-AAG*M-CAA)

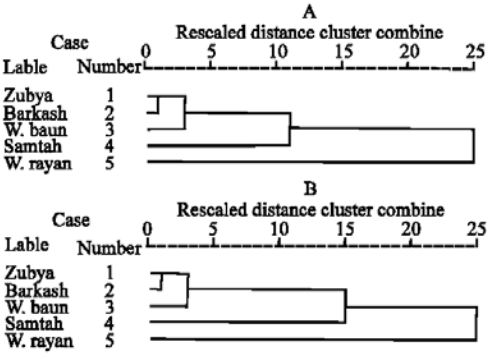

Fig. 3: a): Dendrogram of the five collected *Hordeum spontaneum* C. Koch populations based on all RAPD data, b): Dendrogram of the five collected *Hordeum spontaneum* and c): Koch populations based on all AFLP data

DISCUSSION

The current study aimed on using the PCR-based protocols to assess genetic variability and to fingerprint genotypes of the *Hordeum* genus (*H. spontaneum*) in Jordan. Both random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers had been used effectively to assess the amount of genetic diversity in germplasm collections. Using wheat, barley, rye and wheat-barley addition lines, Weining and Langridge (1991) detected polymorphism using conserved, semi random and random primers. With different combinations of primers, they were able to detect both inter and intra specific diversity. Using 12 primer combinations (through AFLP methodology), Pakinyat *et al.* (1997) were able to reveal 204 polymorphic bands (76% polymorphism) in 39 genotypes of *H. spontaneum*.

In this study it was possible to show that the amplification products from 27 random primers (RAPD assay) and 6 primer combinations (AFLP assay) were sufficient to discriminate among and within individual genotypes in each accession of wild barley (*H. spontaneum*) for each location. Also, the assay was useful in discriminating among plants of the same accession of the same population. The ability to distinguish between closely related individuals was simply a function of the observed number of AFLP and RAPD bands.

The results of the AFLP and RAPD markers were compared in a genetic diversity assessment of *H. spontaneum* in order to investigate the extent of genetic diversity of *H. spontaneum* germplasm, the differences in the level of polymorphism detected by the markers and evaluating the potential of these markers in assessing the genetic variation in other *Hordeum* species.

In recent studies, fingerprints based on different markers were compared using genotypes from different species. There were both agreements and disagreements in findings based on different markers and species: The study of *Brassica oleracea* by Lanner-Herrera *et al.* (1996), a moderate spearman's rank correlation (r = 0.38) between RAPD and isozyme distances was found. Russell *et al.* (1997) compared the levels of genetic variation among barley accessions revealed by RFLP, AFLP, SSR and RAPD and reported that when the spearman's rank correlation was used, the correlation between SSR and RAPD was 0.235, the highest correlation was found between RFLP and AFLP (0.708).

A very different situation was observed by Thormann *et al.* (1994), who reported correlations of r = 0.969 between RFLP and RAPD for a group of 18 accessions from different *Brassica* species and Sun *et al.* (1997) reported a correlation of r = 0.80 between RAPD and wheat micro satellite-PCR for 20 accessions of different *Elymus* species.

In present study, the Mantel test comparison of RAPD and AFLP matrices gave high correlation value (r = 0.885) which indicated that the two sets of markers provided related estimates of genetic relationships.

In this study, variation within populations was studied with RAPD and AFLP markers. The intrapopulation variation was detected by these two markers. AFLP markers detected higher intrapopulation polymorphism than did RAPD (Table 3 and Fig. 3b). Different populations revealed varying degrees of genetic polymorphism in each marker profile. The W. Rayan (Rayan 4) and Samtah populations showed higher variation than the other three studied populations at inter and intrapopulational level. Moreover, such polymorphism, that was observed in barley plants, could lead to variation that could be useful in the improvement of the barley crop. This could be achieved by crossing the resulted barley genotypes with the cultivated genotypes and the products of crossing could be monitored for few generations. The resulting variation from new combination of characters in the hybrids might represent the future improvement of barley crop in Jordan.

In conclusion, the two molecular markers (AFLP and RAPD) gave different views (though complementary) on the amount of genetic diversity in *H. spontaneum* in Jordan. This study reported the successful fingerprinting of *H. spontaneum* accessions using RAPD and AFLP and demonstrated the usefulness of these markers in estimating the extent of genetic variation in *H. spontaneum* germplasm; high interpopulation and intrapopulation genetic variation existed in W. Rayan and Samtah populations and the degree of genetic variation among accessions was notably high (compared to within accessions) as revealed by the RAPD and AFLP markers.

Finally, the use of RAPD and AFLP markers in *Hordeum* must be further continued in order to drive specific linkage between RAPD and AFLP markers and genes controlling agronomically important characters. These diagnostic molecular tools will greatly assist in the identification of new and different sources of diversity which may help breeders to decide what genotypes to cross for making new genetic combinations and to determine which genetic resources should be retained in a collection in order to conserve maximum genetic diversity in the gene bank.

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