American-Eurasian J. Agric. & Environ. Sci., 2 (1): 68-74, 2007
ISSN 1818-6769

Assessment of Genetic Variation among Jordanian Barely Landraces (*Hordeum vulgare* L.) as Reveled by Molecular Markers

[1]*Aida Al-Nashash,* [2]*Huseain Migdadi,* [3]*Mohamad A. Shatnawi,* [4]*Hani. Saoub and* [5]*Sameer Masoud*

[1,2]National Center for Agricultural Research and Technology Transfer (NCARTT)
[3]Al-Balqa Applied University, Faculty of Agriculture and Technology, Al-Salt 19117, Jordan
[4]University of Muta, [5]University of Jordan NCARTT, Baqa (19381), Jordan

Abstract: DNA genetic variation among eleven selected Jordanian Barley landraces that showed significant morphological differences and the three long-term checks, in addition to two improved varieties (Rum and Acsad-176) was estimated utilizing DNA marker-based Random Amplified Polymorphic DNA (RAPD). Using a set of 5 primers, a total of 349 data points were scored over all of the landraces. The scored data points corresponded to a total of 40 RAPD markers of which 32 markers were polymorphic with a percentage of 80%. A genetic similarity matrix based on Jaccard coefficient was constructed using the generated RAPD data to assess the genetic relatedness. The mean similarity indices ranged from 0.92 to 0.30 with an average of 0.60, which indicated a high DNA polymorphism occurrence among the landraces. Clustering based on genetic similarity indices basically showed clustering of the same row type regardless to collection sites, which indicated that there was agreement between classical classification based on agronomic traits and those generated by RAPD analysis. Of the 16 bulked samples used, there was an average of 96% reproducibility in the two to three replications using five primers. This analysis demonstrated that the RAPD-PCR has proved to be a useful tool to determine the extent of genetic diversity among barely landraces.

Key words: Barley land races · genetic diversity · polymorphism · RAPD-PCR · reproducibility

INTRODUCTION

Evaluation of genetic diversity based on morphological and agronomic characteristics do not usually provide accurate estimates of genetic differences as they are highly influenced by environmental factors. In recent years, DNA based molecular techniques (RFLP, PCR-based markers) have increasingly been used to detect genetic variation either among cultivars or even within closely related individuals [1]. Among these, the Random Amplified Ploymorphic DNA technique (RAPD) gives reasonably reproducible fingerprints of any complex genome.

Traditionally morphological markers, isozymes and protein markers were used for evaluation of genetic diversity [2, 3]. Estimation of genetic diversity based on morphological characters, could be misleading particularly for quantitative traits, which are controlled by multigenes [4] that are highly influenced by environmental factors, developmental stages and management practices [5] Therefore, comparisons should be made between materials measured in different years and different locations, which consuming time and resources [6]. Even if they are used as markers in local races and populations, they may not be appropriate for elite breeding germplasm [7]. Gerdes and Tracy [2] considered morphological markers as poor indicators of genetic distances. Although biological markers, such as isozyme and protein analysis, give better resolution for genetic diversity than morphology [8], they are also subject to developmental and environmental variation [3]. Also they cannot be used to distinguish between closely related accessions because they give low levels of polymorphism and limited number of loci [9]. These limitations in both morphology and traditional biochemical markers have led researchers to adopt other biochemical techniques for reliable identification and evaluation of genetic diversity in germplasm [8]. Among these techniques, the advent of modern molecular marker technology is proved to be a valuable tool in demonstrating genetic diversity at the DNA level [10]. Various techniques have been used to mark individual characters in segregated populations

Correspondence Author: Dr. Mohamad A. Shatnawi, Faculty of Agriculture and Technology, Al-Balqa Applied University, Al-Salt 19117, Jordan

and detect polymorphism at the DNA level [11]. One of the most commonly used PCR techniques are Random Amplified Polymorphic DNA (RAPD) analysis which involves visualization of DNA fragments of different sizes generated by PCR amplification of template DNA using short (usually 10-mer) of primers of arbitrary sequences, that can be separated on agrose gel in the presence of ethidium bromide and visualized under ultraviolet light.

RAPD markers revealed genetic variation and relationship between 19 Hordeum species and subspecies. High levels of variation in fragment patterns were observed both within and among species with most of the primers used and a high reproducibility was observed in all cases [12]. Marillia and Scoles [10] demonstrated that RAPD technology represents a useful and reliable tool for detecting polymorphism for phylogenetic relationship among 39 wild *Hordeum* species, subspecies and cultivated barley. The potential of RAPD markers as tools in barley breeding and pedigree relationships was investigated by Tinker *et al.* [13]. The procedure used in the study was relatively simple and the polymorphism detected was repeatable and stably inherited.

In Jordan, few attempts have yet been made to evaluate and exploit in a systematic way [14], the genetic diversity available in landraces used in the areas that capture maximum diversity of the target crop [15]. The target areas were, therefore, selected to cover the possible range of topography, climate and species concerned. Both Ajlun and Muwaqqar are agro-biodiversity rich in both wild species and primitive forms of cultivated species and both areas are threatened by replacement of improved varieties.

This study aims at determining the extent of genetic variation among the collected Barley landraces at the DNA level utilizing Random Amplified Polymorphic DNA (RAPD) technique.

MATERIALS AND METHODS

Eleven landraces of barley that showed significant morphological differences on field evaluation, three long-term checks (Harmal, Zanbaka and Arta) and two additional improved cultivars (Rum and Acsad-176) were used for RAPD analysis. Of these, nine were six-row type and seven were two-row type. Ten different plants from each landrace and the controls were selected randomly and used in the molecular analysis of variation. Genomic DNA was extracted from 10-day-old etiolated seedling germinated in the growth chamber maintained at 21°C for 10 days. Wizard genomic DNA purification kit was used for DNA isolation according to the instructions provided by the manufacturer (Promega, USA). The DNA concentration and quality was determined with Pharmacia Biotech Gene Quant spectrophotometer and agarose gel electrophoresis.

The standard RAPD protocol recommended by Williams *et al.* [16] was performed with some modifications. RAPD reaction was carried out in reaction volume of 25 μl containing 2.5 μl of 10X already prepared PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% (W/V) gelatin), 2.5 μl (2 mM) of deoxynucleotides triphosphate (dNTPs) solution, five picomoles from each arbitrary single 10-base primer (Operon technologies, USA), 0.2 μl of (5 u μl^{-1}) *Taq* DNA polymerase (Promega, USA), one μl (10 ng) of genomic DNA template. Nuclease-free water (Promega, USA) was added to the final volume of 25 μl. Negative control; the reaction mixture without genomic DNA was used for each primer in each PCR reaction to check for DNA contamination. DNA amplification was carried out with MJ-Research Programmable Thermal Cycler (model PTC-100). The PCR program was set as Five minutes at 94°C (initial denaturing step), 42 cycles each one consists of: One minute 94°C (denaturing) One minute 33°C (annealing) Three minute 72°C (extension) and Five minute 72°C (final elongation step). Following amplification, samples were separated by agarose gel electrophoresis. Utilizing 1 kb and 100 bp DNA ladder estimated molecular sizes of the amplification products. The gel was examined and photographed using the gel documentation system (Vilber Lourmat, France).

The presence or absence of DNA bands for each sample was scored by visual inspection of the gel photographs. The data was transformed into a matrix using 1 (present) or 0 (absent) for all tested landraces and fragment sizes. The presence or absence of an amplified fragment was treated as an independent character without consideration of the qualitative aspects of the results i.e. band intensity. Pair-wise comparisons of landraces, based on both unique and shared polymorphic products, were used to generate Jaccard similarity coefficients employing the following formula [17]: JC=N1/(N1+N2+N3), where; N1: number of bands commonly present in individual a and b; N2: numbers of bands present in individual a but not in b and N3: numbers of bands present in individual b but not in a. The similarity coefficients were then used to construct a dendrogram using the SPSS-11.0 PC software for Window computer program.

RESULTS

Quality of extracted DNA has been determined by using 0.7% agarose gel electrophoresis stained by ethidium bromide. The high intensities of DNA bands with minor smears indicate the high molecular weight of extracted genomic DNA with high purity for RAPD analysis.

The purity of extracted DNA was determined spectrophotometically by the ratio of absorbency at 260/280 nm. The ratios at 260/280 nm were mostly 1.8 and this is an acceptable ratio for further analysis [18, 19]. For RAPD analysis, polymorphic marker was defined as an amplified DNA fragment that was present in the bulked DNA sample of at least one individual and was absent in the bulked DNA sample of all individuals. In contrast, markers appearing at random among individuals in all bulk samples will fail to show polymorphism when the bulk samples are compared. Based on this method, RAPD analysis was performed on genomic DNA bulked by Landraces in order to identify RAPD markers that were unique to each of the landrace. Preliminary experiments were conducted to optimize the amounts of template DNA in each PCR reaction using 50, 25 and 10 ng of genomic DNA.

Fifty-eight primers were initially screened using five landraces, which were selected randomly, to determine the suitability of each primer for PCR amplification. Out of 58 primers, five primers (OPA-04, OPA-13, OPF-01, OPN-04 and OPT-13) showed consistently reproducible polymorphic bands and gave repeatable pattern when tested two to three times with the same landraces. These primers were used for further analysis. The fragment sizes of amplified products were estimated using standard curves for bands in the molecular weight marker in each gel. The five primers, which were selected, produced a total of 40 markers, 32 of which are polymorphic using the 16 bulked samples (Table 1). The number of scorable RAPD fragments, generated per primer varied from 4 to 13 with an average of 8 per primer and the number of polymorphic bands per primer ranged from 3 to 12 with an average of 6.4 polymorphic bands per primer. It was found that 20% of the total 40 fragments were shared by all the 16 bulked samples using the five selected primers; therefore, the percentage of polymorphic fragments was 80%.

The size of the scored amplified DNA fragments ranged from 300 to 2000 bp. However, two markers (produced by OPA-04 and OPA-13) had bands with molecular sizes other than the upper limit and were not scored. The matrix of average genetic similarity [17], computed among the studied landraces based on band sharing values is presented in Table 2. The mean similarity indices ranged from 0.30 between samples to 0.92. All samples showed an average of 0.60, which could mean hat the landraces share an average of 60% of their RAPD fragments.

Table 1: Primers used to generate RAPD fragments in barely landraces with data about the total number of amplified bands, polymorphic and monomorphic band identified by primer and size range of scored products

Primer	Total bands	Monomorphic bands	Polymorphic bands	Scored fragments size
OPA-04	13	1.0	12.0	300-2,000 bp
OPA-13	6	3.0	3.0	700-2,000 bp
OPF- 01	7	1.0	6.0	500-2,000 bp
OPN-04	10	2.0	8.0	470-2,000 bp
OPT-13	4	1.0	3.0	900-2,000 bp
Total	40	8.0	32.0	
Mean	80	1.6	6.4	

The relatedness of the different landraces was estimated by a matrix containing digitized scorable bands and analyzed for each accession using the SPSS program. The genetic similarity was calculated using pairwise comparisons of the landraces and the two varieties. The application of this program resulted in the generation of dendrogram clustering of the landraces based on genetic similarity using Jaccard coefficient (Fig. 1).

The results based on the dendrogram showed that the samples were divided into two main clusters at the highest level of hierarchy in the dendrogram (Fig. 1), except (landrace 23) which was separated in its own group. The first cluster consists from all the six row landraces and the two check varieties (Rum and Acsad-176). Within the six-row groups, other qualitative similarities were observed, five sub-clusters, containing nine of six-row barely, with a similarity ranging from 0.92 to 0.72 were formed. This range indicated that landraces of the six-row type are related to each other with high similarity. The second cluster consists of landraces of two-row type only, including the three long-term checks. The similarity between the sub-clusters ranged from 0.82 to 0.52.

In the second cluster, each one of the check-improved landraces made its own cluster, except Arta landrace, which showed high similarity value with (landrace 20). Arta is a single line selection from Syrian barely landrace "Arabi Abiad" [20]. Although a problem in the use of RAPD technique has been DNA pattern

Table 2. Similarity matrix for 11 samples of barely landraces and the checks based on Jaccard Coefficient

Landrace no.	3	4	8	9	11	13	14	20	23	26	32	Harmal	Zanbaka	Arta	Rum
4	0.80														
8	0.89	0.82													
9	0.83	0.76	0.92												
11	0.74	0.73	0.82	0.82											
13	0.66	0.77	0.73	0.73	0.77										
14	0.62	0.61	0.69	0.69	0.73	0.70									
20	0.64	0.69	0.65	0.59	0.63	0.73	0.76								
23	0.39	0.38	0.34	0.30	0.38	0.36	0.42	0.38							
26	0.58	0.52	0.53	0.53	0.56	0.47	0.48	0.45	0.50						
32	0.63	0.61	0.58	0.53	0.56	0.57	0.54	0.62	0.56	0.71					
Harmal	0.58	0.61	0.53	0.48	0.52	0.57	0.43	0.50	0.56	0.60	0.71				
Zanbaka	0.50	0.49	0.50	0.46	0.41	0.35	0.36	0.33	0.47	0.56	0.56	0.61			
Arta	0.62	0.61	0.58	0.53	0.47	0.47	0.44	0.50	0.50	0.65	0.82	0.70	0.71		
Rum	0.67	0.85	0.74	0.68	0.66	0.75	0.58	0.67	0.43	0.48	0.59	0.64	0.50	0.58	
Acsad-176	0.77	0.82	0.67	0.61	0.59	0.61	0.47	0.54	0.48	0.53	0.58	0.69	0.55	0.63	0.74
Mean	0.66	0.66	0.64	0.58	0.57	0.54	0.46	0.50	0.50	0.59	0.65	0.66	0.59	0.60	0.74
Overall mean					0.60										

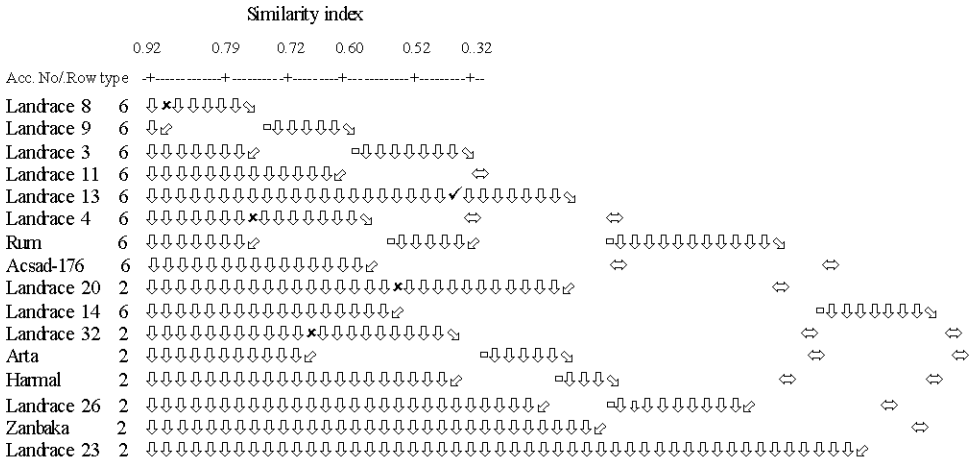

Fig 1: Dendrogram of barely accessions and the controls based on similarity for the 32 RAPD markers produced by five primers

reproducibility, our results demonstrate that RAPD assay can be reproducible with sufficient repetitions (two to three times) in the same laboratory. The 16 bulked samples used showed an average of 96% reproducibility. In the case of reaction failure in one of the three replicates, the scoring was based on the replicate where amplification was successful.

DISCUSSION

The optimum concentration used the lowest amount of template DNA (10 ng) that resulted in the largest number of intense and reproducible bands. In contrast, higher concentrations resulted in non-reproducible or faint bands. This result was in agreement with Weising *et al.* [19] who indicated that for ordinary PCR reaction to be computed, very little amount of DNA was needed. The same results were reported by Abo-elwafa *et al.* [21] and Ko *et al.* [22] who found that the optimum conditions of template DNA concentration were 10 ng/reaction that showed clear banding pattern. However, Hoelzel [18] found that using 100 ng of template DNA per reaction is suitable for RAPD. On the other hand, other concentrations were used for other crop species such as 5-30 ng for pigeonpea [6], one ng for *Aegilops* species [23] and 10-100 ng for wild emmer wheat [24]. In contrast, Schnell *et al.* [24] showed that concentration of template DNA did not affect reproducibility of RAPD markers. The amplified products detected genetic variation among barely landraces in the form of variable number

of different bands [9]. These markers were arbitrarily assumed to amplify only the dominant allele per locus [12, 13]. So, amplification products of any distinct size were assumed to refer to that allele. A locus was considered to be polymorphic if the presence and absence of bands were observed among landraces and monomorphic if the bands were present in all landraces. Since amplified products with large sizes tend to show low reproducibility [9], both faint as well as densely stained RAPD fragments, which were shown in some primers, i.e. OPA-04 and OPA-13, could arise from amplifying two or more products of similar sizes [25]. Variation in the intensity of some bands was also observed in few samples. The possible causes include differences in template sequence copy numbers and varying degrees of mismatch between the primer and the binding sites [6, 26]. The wide range of similarity indices indicated that a high polymorphism at the DNA level among the barely landraces and so, a large amount of genetic variation exists among them.

The genetic similarity was calculated using pairwise comparisons of the landraces and the two varieties. Different authors have used different coefficients to make these estimations: Simple Matching Coefficient [21] Jaccard Similarity Coefficient [9], Nei and Li Coefficient [26], Dice Coefficient [21] or other methods. The most important criterion for the choice of suitable coefficient to apply to RAPD data is that the method does not consider the absence of bands as a similarity. This premise is important in RAPD data analysis because the absence of one band can be due to different mutations which alter the priming site [27]. Therefore, Jaccard Similarity Coefficient, which does not consider absence of bands as a similarity, was used in the present analysis. The clustering was corresponding mainly to the row types of barely. These results agree with that obtained from the cluster analysis of agronomic data (data not shown), which divided the barely landraces into two main clusters according to the row types. Also, the agronomic classification showed that the two-row type harbors more variability than the six-row type.

This study showed that clustering of different landraces did not tend to be clustered according to location of collection, but tend to cluster only according to row-types. This finding is in agreement with our classical classification and that reported by Tinker *et al.* [13]. Whereas Song and Henry [28] demonstrated that the DNA polymorphism of the wild barely correlated with geographical distribution. However, based on RAPD data, Vierling and Nguyen [29] showed that landraces from the same locality tend to cluster together.

Tree topology based on RAPD assay was generally consistent with those based on agronomic treatments, which also divided barely accession into two main groups based on row types. The general good agreement found between classical classification and those produced using RAPDs favors the applicability of the RAPD assay for taxonomic purposes and genetic diversity studies [5, 10, 26, 30]. However, Gonzaler and Ferrer [11] reported RAPD analysis is more accurate in investigating relationships among populations of a single species or very closely related species than between less related species. Although a problem in the use of RAPD technique has been DNA pattern reproducibility, which is greatly influenced by the PCR reaction and amplification conditions in different laboratories [9, 30]. This results demonstrate that RAPD assay can be reproducible with sufficient repetitions (two to three times) in the same laboratory. In the case of reaction failure in one of the three replicates, the scoring was based on the replicate where amplification was successful. Thus under stringent reaction conditions, RAPD pattern could be highly reproducible [27].This demonstrated that RAPD-PCR analysis has proved to be useful in distinguishing among barely landraces that share a high degree of similarity and so, to determine the extent of genetic diversity among them and can provide information for the management of genetic diversity collection and identification [9]. In conclusion, the wide range of similarity indices obtained from RAPD data indicated that high phenotypic and genetic polymorphisms occur among the barely landraces collected in this study, there was an agreement between the classical classification and those produced using RAPD analysis, in which the accession of the six-row type were grouped together in one cluster and clearly separated from accession of the two-row type. The results showed that clustering of different accessions were not based on locations of collection, but mainly according to the row types of and RAPD technique as one of the PCR applications, which should be simple, considered as a useful tool to determine the extent of genetic diversity among barely landraces.

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