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The use of RAPD and ISSR markers for genetic diversity among some barley cultivars

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Abstract

The success of a breeding program depended on the genetic variability available into the germoplasm of the crop. The objectiveof the present study were to assess molecular variation among different Romanian and foreign barley cultivars, and to determine the level of genetic similarity among them. The results demonstrated that RAPD analysis are useful for evaluation of genetic diversity between different barley cultivars, considering the fact that the average polymorphic rate was 91,17 %, and 7,5 polimorphyc bands /primer. Total polymorphism generated by a certain primer (PIC), presented values between 0,147 to P27 and 0,438 to P-1. The discrimination index (PI), registered values among 0,787 for the primer E6 and 2,626 for P-1 primer, which had the highest capacity to generate polymorphic bands to barley cultivars studied. Among the tested ISSR primers, only 5 amplified polymorphic ISSR loci with an average number of 9.2 bands/primer and the mean percentage of ISSR polymorphism was 89,13%. The total of polymorphism (PIC) presented values contained among 0,245 to 811 and 0,391 to 810. The (PI) registered values among 1,562 for primer 810 and 2,803 for HB15 primer. RAPD-PCR and ISSR analysis through two categories of markers, attested the existence of a high genetic variability among studied cultivars, which can be efficiently exploited in the breeding programs of barley. The use of cultivars from various clusters and sub clusters offer the possibility of obtaining an appropriate genetic variability in hybrid population.

Key words: RAPDs and ISSRs, Bulked analyses, barley, genetic diversity,

Introduction

Barley (*Hordeum vulgare L.*) was one of the most important crop species in the World and had been subject to considerable genetic studies. It was a diploid (2n-2x-14), largely self-fertilizing species with a large genome [3].

The identification of varieties of crop plants had become increasingly important to the documentation of genetic resources and to the protection of the breeders' interests. To the malting and brewing industries, that was especially important because different varieties of barley (*Hordeum vulgare L. ssp. vulgare*) had widely different qualities and use characteristics. Farmers needed positive identification for the protection of their proprietary rights on varieties. The grower needed assurance that the seed lot was of the variety he intended to use. Processors might be assured of varietal identity and that it was free from mixtures. Examination of grain morphological characteristics was the standard method of identifying barley cultivars, but not all of them could be distinguished on that basis. Several biochemical techniques had been used to complement morphological examination of barley cultivars, and most of them rely on variations among isoenzymes [16] and seed storage proteins [5]. Nevertheless, characterization with those kinds of markers was not very efficient for barley varieties due to the low levels of allelic variation in many isoenzymatic loci, the

high degree of genetic relationship among the different varieties, and the high degree of polymorphism within barley varieties.

Molecular markers had been proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. The advent of the polymerase chain reaction (PCR) favored the development of different molecular techniques such as random amplified of polymorphic DNA (RAPD) [1,31], simple sequence repeats (SSR or microsatellite) [17], the sequence-tagged sites (STS), random amplified microsatellite polymorphism (RAMP), and inter-simple sequence repeat polymorphic DNA (ISSR) [24]. Those molecular markers had been used in barley for detecting genetic diversity (Ciulca, 2010), genotype identification, and genetic mapping [10, 17, 27].

Genetic analysis with RAPD markers is relatively easy, fast and efficient, therefore it has been extensively used to determine genetic diversity among barley varieties and to identify the best quality ones for malting [9, 10, 27]. Nevertheless, there are few studies about genetic characterization of hull-less barley [32]. Determination of genetic variability among genotypes is useful for both practical applications in a breeding program and cultivar protection. A variety of approaches, from morphological data to molecular markers including RAPD [4, 13, 33], AFLP [1], SSR [14, 15] and ISSR [13, 22] markers have been applied for assessment of genetic variability among genotypes.

ISSR markers, which involve PCR amplifications of DNA using a primer, composed of a microsatellite sequence anchored at 3′ or 5′ end by 2–4 arbitrary, could be used to assess genetic diversity [28]. ISSRs had been used for cultivar identification for potatoes [23], wheat [21], bean [18], and barley [10, 26]. The understanding and knowledge of genetic variation and genetic similarities present within individuals or populations are useful for the efficient use of genetic resources in a breeding program. The breeder can use the genetic similarity information to complement phenotypic information in the development of breeding populations [11].

In this study, we evaluated genetic diversity using RAPD and ISSR markers, in order to establish a base line to assist future conservation and breeding programs of this species. Also we aimed to report the usefulness of RAPD and ISSR for the assessment of genetic diversity.

Materials and methods

Plant material. Ten leaves from each of the 19 cultivars were collected separately and stored immediately at-80^{o} for DNA extraction. The cultivars numbers and country of origin are listed in (Tab.1).

Table 1. Studied barley cultivars and their geographical origins

code.	Genotypes	Locatian	code.	Genotypes	Location
1	**Orizont**	Romania	11	**Plaisant**	Hungary
2	**Dana**	Romania	12	**Tas**	Hungary
3	**Precoce**	Romania	13	**Secura**	Austria
4	**Adi**	Romania	14	**Dina**	Germany
5	**Mădălin**	Romania	15	**DH19/1**	Romania
6	**Andrei**	Romania	16	**DH 254/10**	Romania
7	**Regal**	Romania	17	**DH 260/18**	Romania
8	**Compact**	Romania	18	**DH 260/12**	Romania
9	**Gerbel**	Germany	19	**DH 261/22**	Romania
10	**Lyric**	France			

DNA extraction Total genomic DNA from fresh leaves was extracted using cetyltrimethyl ammonium bromide (CTAB protocol). DNA concentration was determined by both spectrophotometry at 260 nm and by 2% agarose gel electrophoresis.

Primers

A set of 13 RAPD primers was screened using ten DNA samples from each cultivar and on the basis of that preliminary data, 9 polymorphic RAPD were chosen, and 7 primers were tested for ISSR, and 5 were chosen.

In table 2 were presented the sequences of the RAPD and ISSR primers used for detection of the genetic polymorphism.

Table 2. Primers sequences used for PCR analysis

Primers RAPD code	Nucleotide sequences (5'-3')	Primers ISSR code	Nucleotide sequences (5'-3')
P-1	ACACAGAGGG	HB-12	$(CAC)_3GC$
P-16	TCGGCGGTTC	HB-14	$(CTC)_3GC$
P-17	CTGCATCGTG	HB-15	$(GTG)_3GC$
P-27	CCGTGCAGTA	810	$(GA)_8T$
OPA-05	AGG GGT CTT G	811	$(GA)_8C$
OPA-11	CAA TCG CCG T		
OPG-02	GGCACTGAGG		
E-6	AAGACCCCTC		
A-16	AGCCAGCGAA		

RAPD- PCR reaction

The amplifications were carried out in a 25 µl PCR buffer containing: GoTaq® Green Master Mix ready-to-use solutions (GoTaq® DNA Polymerase, dNTP, MgCl2, and reactions buffers at optimal concentrations for efficient amplification of DNA templates), RAPD primers, DNA template and Nuclease-Free Water. GoTaq® Green Master Mix contained two dyes (blue and yellow) that allow monitoring of progress during eletrophoresis. DNA amplification was carried out using a Thermalcycler by Corbett and reactions were submitted to the following PCR program: preliminary DNA denaturation step at 94°C for 4 min, followed by 35 cycles at 94°Cfor 1 min., 35°C for 1 min. and 72°C for 2 min. A final extension for 4 min at 72°C was included. The RAPD products were separated by 2% agarose gels electrophoresis (3V cm-1), which run with 1xTAE buffer. The PCR marker (1000-50bp) was also run on each gel as a molecular weight standard. Photo documentation was performed under UV light using a photo imaging system.

ISSR-PCR reaction

The amplifications were carried out in a 25 µl PCR buffer containing: Go Taq® Green Master Mix ready-to-use solutions (GoTaq® DNA Polymerase, dNTP, MgCl2, and reactions buffers at optimal concentrations for efficient amplification of ADN templates), ISSR primers, DNA template and Nuclease-Free Water. GoTaq® Green Master Mix contains two dyes (blue and yellow) that allow monitoring of progress during electrophoresis. DNA amplification was carried out using a Thermalcycler by Corbett and reactions were submitted to the following PCR program: preliminary DNA denaturation step at 94°C for 5 min, followed by 40 cycles at 94°C for 30s, a primer annealing step at 52°C for 45 s. and an extension at 72°C for 2 min, then a final extension was carried out at 72°C for 6 min. The ISSR products were separated by 2% agarose gels electrophoresis (3V cm-1), which run with 1xTAE buffer. The standard marker used as marker of ADN quantity was PCR marker. Photo documentation was performed under UV light using a photo imaging system.

Data analysis

To make calculs after the analysis made through different primers, were selected as being present (1) only the clear bands, while the bands with a very reduced resolution were marked as absent (0). Further, the respectively bands were introduced in a binar matrix. Concerning the potential characterisation of different systems of molecular markers to evaluate the interpopulational variability to the studied genotypes, were calculated different parameters:

-total polymorphism generated by a certain primer (PIC) which indicated its discriminatory power: $PIC = 1 - \sum_{i=1}^{n} P_{ij}^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2$;

Pi- allele' frequencyi; Pj- allele' frequency; Pij- allele' frequency i for locus j;
n- total number of loci.

-discrimination index (Pi), which attested the efficiency of a certain primer in polymorphism detection [8].

$$PI = \sum PIC .$$

Genetic similarity among genotypes studied calculated through coefficient Jaccard, which was recommended to be used for dominant markers ISSR, RAPD, taking in view that the absence of a bands was associated to a homozygous loci. JC= a / (a+b+c), where a, b, c, represented the commons and un-commons of those genotypes [29].

On base of genetic similarity matrix among genotypes, it was made the dendrogram using the method of clusters average. Two separate dendrograms for ISSR and RAPD data were generated. The distance matrices obtained in RAPD and ISSR analyses were compared using correlation analysis.

Results and Discussions

RAPD and ISSR analysis

Studies conducted on different primers with arbitrary sequence have established that to produce detectable levels of amplification, the minimum length of primers might be of nine bases, while in case of decamer primers guanine cytosine should be at least 40% [30]. Each primer, generally determines the amplification of sequences from several genome loci this being the reason why this technique is an effective means of assessing DNA polymorphism in different genotypes [19].

Table 3. Polymorphism rate of barley cultivars through RAPD primers

No.	Primer code	No. bands		Polimor-phism (%)	Range of fragment size (pb)	PIC $\bar{x} \pm s_{\bar{x}}$	PI
		Total No of fragments	Polymorphic fragments				
1	P-1	7	6	85.71	300-1250	0,438±0,074	2,626
2	P-16	9	6	66.67	120-1000	0,235±0,069	1,407
3	P-17	9	9	100	100-1000	0,228±0,058	2,050
4	P-27	11	10	90.91	75-1250	0,147±0,043	2,526
5	OPA-05	8	8	100	150-1200	0,298±0,046	2,382
6	OPA-11	7	7	100	200-1000	0,334±0,053	2,338
7	OPG-02	7	6	85.71	200-1000	0,192±0,065	1,341
8	E-6	3	3	100	1400-2000	0,262±0,121	0,787
9	A-16	7	7	100	1000-3500	0,258±0,062	1,806

Among the tested RAPD primers, only nine generated polymorphic bands, resulting a polymorphic rate with values between 66,67% in case of P-16 and 100% for oligonucleotides OPA05, OPA11, P-17, A16, E6, (Table.3). The average number of polymorphic bands per primer was 7,5 with an average rate of polymorphism being 91,17%.

Total polymorphism generated by a certain primer (PIC), presented values between 0,147 to P27 and 0,438 to P-1. The discrimination index (PI), registered values among 0,787 for the primer E6 and 2,626 for P-1 primer, which had the highest capacity to generate polymorphic bands to barley genotypes studied.

The dendrogram of genetic distances among all tested genotypes based on band polymorphism generated by RAPD –PCR after using the primers is shown in fig.1. The dendrogram separated all cultivars in five clusters. First clusters divided in two sub clusters; first sub cluster included Romanian cultivar Orizont together with foreign Dina and Secura cultivars and second subcluster is composed of Adi, Madalin, Regal, Andrei, Lyric, Tas cultivars, showing an average genetic diversity of approximately 8%.

The second cluster consist of cultivars Precoce, Plaisant and double haploid lines DH 19/1, DH260/18, DH254/10, DH260/12 recording a genetic similarity of 60% between them, and an average diversity of approximately 13% toward the first group. Genotypes Dana and Gerbel, similar between them in proportion of 54% made the third cluster. The forth cluster contained only genotype Compact, it was similar in proportion of 63% with cultivars Andrei , Regal and presented the highest genetic difference (62%) face to double haploid line DH261/22. Line DH261/22 contained the fifth cluster, manifesting the highest diversity face to cultivars Madalin (63,64%), Compact (61,9%) and (57,2%) Regal and Andrei.

In consequence the usage of some genitors from different groups and subgroups presented had offered the possibility to obtain a genetic variability corresponding in hybrid populations.

According to data presented in table 4 it observed the existence of some correlations statistically assured among the majority of primers studied which attested them as useful with the same efficiency for polymorphism identification and genetic diversity establishment to barley. The strongest correlation existed among oligonucleotides OPA 05 and P27, which had capacities very nearby to polymorphism detection at barley genotypes analyzed.

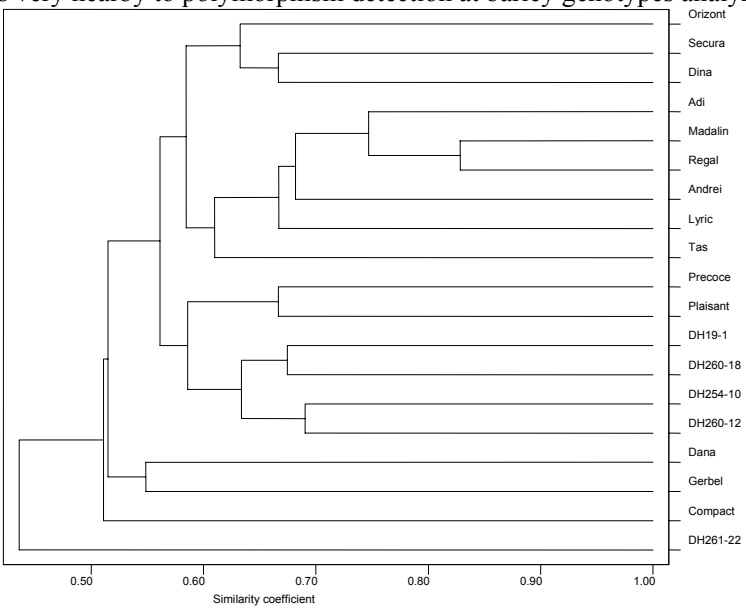

Fig 1. UPGMA clustering of barley cultivars using the RAPD primers

Table 4. The coefficient values of correlation among RAPD primers under the genetic similarity aspect of the barley cultivars studied

Primers	P-1	P-16	P-17	P-27	OPA5	OPA11	OPG2	E-6	A-16
P-1	-	0,072	0,178***	0,334***	0,246***	0,205***	0,170**	0,027	0,182***
P-16		-	0,323***	0,168**	0,129*	0,317***	0,101	0,149**	0,090
P-17			-	0,383***	0,270***	0,170**	0,355***	0,392***	0,192***
P-27				-	0,536***	0,267***	0,338***	0,177***	0,210***
OPA5					-	0,107*	0,251***	0,137*	0,269***
OPA11						-	0,152**	0,137*	0,079
OPG2							-	0,387***	0,183***
E-6								-	0,171**
A-16									-

$r_{5\%} = 0,105$; $r_{1\%} = 0,138$; $r_{0,1\%} = 0,176$;

The six alleles common identified for to all the 19th genotypes studied (Table 5) could be considered specific markers RAPD for the barley.

Table 5. Specific RAPD alleles common for all the barley cultivars studied

No.	Specific alleles
1	OPG2-400pb
2.	P1-400pb
3.	P27-600pb
4.	P16-530pb
5.	P16-650pb
6.	P16-850pb

Inter simple sequence repeat (ISSR)-PCR is a technique, which involves the use of microsatellite sequences as primers in a polymerase chain reaction to generate multilocus markers. It is a simple and quick method that combines most of the advantages of microsatellites (SSRs) and amplified fragment length polymorphism (AFLP) to the universality of random amplified polymorphic DNA (RAPD). ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology [25] .ISSR markers have been used for studies of hybridization and hybrid speciation [2], population and conservation genetics [7], and systematic investigations in natural populations [20].

Among the tested ISSR primers, only five amplified polymorphic ISSR loci, resulting a rate of polymorphism with values between 57,14% in case of primer 810 and 100% for the first 811, HB12, HB15 (Table.6). The amplified fragments had size among 150 and 600 pb for HB-15 primer, while 810 primer registered the highest amplitude of amplified fragments size (950- 5100 pb). The average number of polymorphic bands per primer was 9.2 with an average rate of polymorphism being 89,13 %.

The total of polymorphism generated by a certain primer (PIC) presented values among 0,245 to 811 and 0,391 to 810. The discrimination index (PI) registered values among 1,562 for primer 810 and 2,803 for HB15 primer.

Table 6. Polymorphism rate of barley cultivars through ISSR primers

No..	Primers	Nucleotide sequence (5'-3')	No. bands		Polymorphism (%)	Range of size bands (pb)	PIC $\bar{x} \pm s_{\bar{x}}$	PI
			Total No of fragments	Polymorphic fragments				
1	HB-12	(CAC)3GC	9	9	100	175-850	0,309±0,044	2,781
2	HB-14	(CTC)3GC	12	10	83.33	200-1400	0,264±0,053	1,806
3	HB-15	(GTG)3GC	9	9	100	150-600	0,311±0,059	2,803
4	810	$(GA)_8T$	7	4	57.14	950-5100	0,391±0,111	1,562
5	811	$(GA)_8C$	9	9	100	950-2000	0,245±0,053	2,205

According the ISSR data, a dendrogram was developed for 19 cultivars and indicates four main clusters; the first cluster divided into two sub clusters, first sub cluster included Romanian cultivars Orizont and Dana presenting a genetic similarity of 75% for ISSR alleles and second sub cluster formed a separate sub cluster with Precoce cultivar, that presenting a difference of 10% face to the cultivars from the first sub cluster. Second sub cluster was further divided in two sub clusters. Among the two sub clusters, first sub cluster formed a separate sub cluster with Romanian cultivars Adi, Regal, Andrei DH 254/10, DH260/18, Madalin, and second sub cluster included foreign cultivars Gerbel, Lyric, Tas, Plaisant, Dina, and Romanian duble haploide lines DH19/1.Cultivar Compact made second cluster manifesting an average genetic similarity of approximate 46% with cultivars Orizont and 55% with Regal and Andrei. Cultivar Secura presented a genetic similarity of 52% with Orizont, making the third cluster. Double haploid lines DH260/12, DH261/22 made a fourth cluster, presenting a genetic difference of approximate 53% face to the genotypes from the first cluster; the highest genetic difference registering face to cultivars Compact (75%), Regal and Secura.

ISSR analysis through the 5 primers, attested the existence of a high genetic variability among cultivars involved in this study, which could be efficiently exploited in breeding programs of barley.

The results presented in table 7 attested the existence of some significant correlations among ISSR primers, with exception 811 primer. The highest correlation obtained among HB15 and HB14 primers which had capacities very nearby of polymorphism detection to barley genotypes analyzed.

Table 7. The coefficient values of correlation among ISSR primers under genetic similarity aspect of barley genotypes studied

Primers	HB-12	HB-14	HB-15	810	811
HB-12	-	0,338***	0,331***	0,333***	0,192***
HB-14		-	0,401***	0,219***	0,383***
HB-15			-	0,258***	0,063
810				-	0,179***
811					-

$r_{5\%} = 0,105$; $r_{1\%} = 0,138$; $r_{0,1\%} = 0,176$;

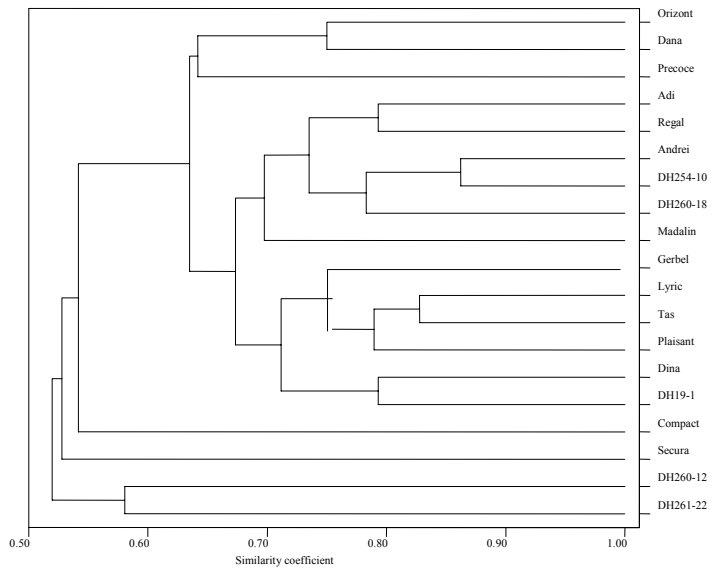

Fig. 2. UPGMA clustering of barley cultivars using the ISSR primers

At the same time were identified 5 alleles presented to all the 19th genotypes studied (Table.8). Those alleles could be considered specific markers for the barley.

Table 8. Specific alleles common for all the barley genotypes studied

No.	Specific alleles
1	HB14-1000pb
2.	HB14-450pb
3.	810-950pb
4.	810-1100pb
5.	810-1300pb

The analysis results made through two categories of primers had emphasized the fact that to some cultivar studied it were identified unique bands (Table.9). Those bands could be used as potential DNA markers to identify the barley genotypes.

Table 9. Specific alleles for certain barley genotypes

No.	**Genotype**	**Specific alleles**
1.	Lyric	HB15-150pb
2.	Secura	811-1000pb
3.	Dina	811-1500pb
4.	Compact	HB14- 200pb
5	Precoce	OPA-05-450pb

Molecular characterization revealed 91,17% polymorphism of RAPD markers and 89.13% polymorphism of ISSR markers among cultivars. The difference is perhaps explained by the difference in the DNA segments targeted by the two methods. The similar results were observed by [10,13, 26]. Markers ISSR proved more efficient than RAPD markers thanks to the higher number of bands/primer (9,2) and of efficiency index (8,2). Fernandez *et al*. (2002) have studied 16 barley cultivars form different countries and they have found high similarity index by ISSRs than by RAPDs. It may be due to highly polymorphic, abundant nature of the microsattelites due to slippage in DNA replication.

The variation amplitude about the sizes bands was significant superior in case of ISSR primers (4950pb), comparative with RAPD primers (3425pb). The differences found among the dendrograms generated by RAPDs and ISSRs could be partially explained by the different number of PCR products analyzed (68 for RAPDs and 46 for ISSRs) reinforcing again the importance of the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships among barley cultivars. Similar results had been observed by [10] also in barley.

The microsatellites or intersimple sequence repeat (ISSR) markers and randomly amplified polymorphic DNA (RAPD) markers have proved to be the most polymorphic markers in barley and hence are highly useful markers for various applications in barley [10]. These markers can be used for selection of important agronomic traits which would increase the efficiency and precision of breeding. In a previous study [12], some traits were used to evaluate the agronomical potentiality of barely specimens in south Tunisia. It has been noted that although the importance of agronomic parameters, it is necessary to use other markers to study diversity and select genotypes with high potential.

With that study we could conclude that the bulk analyses of RAPD and ISSR markers were useful for study the genetic relationships between barley cultivars, providing the ISSR

markers a powerful tool for the generation of potential fingerprinting diagnostic markers for cultivars.

Table 10. Comparative analysis of bands characteristics generated by RAPD and ISSR markers

Parameter	RAPD	ISSR
Number of primers	**9**	**5**
Total number of bands	**68**	**46**
Number bands/primer	**7,55**	**9,2**
Number polymorphic bands	**62**	**41**
Rate of polymorphism	**91,17**	**89,13**
Bands size	**75-3500**	**150-5100**
Efficiency index	**6,88**	**8,2**

Conclusions

The results demonstrated that RAPD and ISSR analyses are useful for evaluation of genetic diversity between different barley cultivars, considering the fact that the average polymorphic rate was 91.17 % respectively 89.13% and number of polymorphic bands / primer.

The use of cultivars from various clusters and sub clusters offers the possibility of obtaining an appropriate genetic variability in hybrid populations. The strongest correlation existed among oligonucleotides OPA 05 and P27; HB15 and HB14 which had capacities very nearby to polymorphism detection at barley genotypes analyzed. The analysis results made through two categories of primers had emphasized the fact that to some cultivar studied it were identified unique bands. Those bands could be used as potential DNA markers to identify the barley genotypes.

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