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Molecular Characterization of Tunisian Barley (*Hordeum Vulgare* L.) Genotypes using Microsatellites (SSRs) Markers

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Abstract

High level of polymorphism make simple sequence repeats (SSRs) the molecular marker of choice for diversity analysis in plant species. In this study 18 simple sequence repeats (SSRs) markers were used to characterize six Tunisian barley varieties (Faïz, Manel, Martin, Rihane, Roho, and Tej) as well as six landraces from different growing regions in Tunisia (Djerba, Gabes, Jendouba, kairouan, Kebili, Kerkennah). Amplification of SSRs loci were obtained for 17 primer pairs and only 11 among them showed clear polymorphic patterns. These 11 primers produced a total of 31 alleles. The number of alleles per marker ranged from 1 to 5 with an average of 2.81 alleles per locus. The data generated by these 11 primers were sufficient to discriminate the analysed barley genotypes. The UPGMA cluster analysis of the similarity data grouped the 12 studied genotypes into two groups according to their spike type (two-row and six-row barley), except for Manel verity witch is a six row type but it clustered among the two row group. These results will be useful for barley germplasm management in terms of biodiversity protection and design of new crosses.

Keywords: Simple Sequence Repeats (SSRs), barley, varieties, landraces, similarity.

1. Introduction

The barley improving program started with François Boeuf since 1906 (Deghaïs et al., 2007). Up today, 15 new varieties have been officially registered by the National Agronomic Research Institute of Tunisia (INRAT) and released for commercial cultivation.

However, some subsistence farmers still grow traditional landraces. These farmers may have several socio-economic incentives to replace these landraces with modern introduced cultivars and varieties, then these landraces could be lost before they are adequately collected and thoroughly evaluated. While the landraces are the most diverse populations of cultivated plants (Frankel et al., 1995). Having evolved across thousands of years in a multitude of environments and local farming systems, these landraces have developed abundant patterns of variation and would represent a largely untapped reservoir of useful genes for adaptation to biotic and abiotic stresses (Brush, 1995). Effective management and utilization of these resources depends to a large extent on appropriate characterization of their genetic diversity.

The genetic diversity among and within landraces makes them a valuable resource as potential donors of genes for the development and maintenance of modern crop varieties and for direct use by farmers (Soleri et al., 1995). The knowledge and understanding of this genetic diversity serve as a basis for making decisions related to the conservation and the use of the germplasm collection in genetic improvement. Therefore the INRAT collects continuously accessions of barley landraces to investigate their genetic diversity and to compare them to the available germoplasm collection.

The study of the genetic diversity within barley germpalsm has long been based on morphological and physiological traits mainly (Massood et al., 2003). However, morphological variability is limited to some stages of plant growth and might be affected by environment. Recently, studies of the genetic diversity of barley germplasms were based on isozymes (Liu et al., 2000) and seed storage proteins (Yin et al., 2003). The Variability for hordein (prolamin fraction for seed storage proteins) composition has been demonstrated to be a valuable tool for fingerprinting techniques (Pomortsev et al, 2002; Delogu et al, 1990; Cattivelli et al., 1987; Bettaieb-Kaab et al., 2006). Limitations of these descriptors lie in their restricted number and in their narrow variability. Nowadays, a variety of different genetic markers which evaluate diversity at molecular level were proposed to assess genetic variability as a complementary strategy beside traditional approaches in

germplasm conservation and management. DNA-based molecular markers are tools that might help plant breeders to directly evaluate genetic variation among relatives without effect of environmental factors. In addition, DNA techniques allow the assessment of a theoretically unlimited number of polymorphic marker loci (Nguyen et al., 2004). Varieties of molecular markers were used to evaluate the extent of genetic variability. These include restriction fragment length polymorphism (RFLP) (Todorovska et al., 2003), Random Amplified Polymorphic DNA (RAPD) (Zeng et al., 2002), ISSR (Inter Simple Sequence Repeats) (Rashal et al., 2004), SNP (Single Nucleotide Polymorphism) (Soleimani et al., 2007), Amplified Fragment Length Polymorphism (AFLP) (Assefa et al., 2007) and SSRs (Simple Sequence Repeats) (Matus et al., 2002).

SSRs is the marker of choice for many genetic analyses in barley. They are broadly used for four reasons. First, each SSRs locus is genetically well defined and codominant, making SSRs ideal for marker assisted breeding (Deric et al., 2005), genetic mapping (Ramsey et al., 2000) and diversity measurement (Zong-Yun et al., 2006). Second, SSRs are highly variable and therefore able to distinguish closely related plant cultivars (Manifesto et al., 1999). Third, SSRs polymorphism is easily assayed by PCR. Finally SSRs marker is technically efficient, cost-effective to use and are available for barley (Ramsey et al., 2000; Saghai Maaroof, 1994; Becker et al., 1995; Liu et al., 1996; Peterson et al., 1998). Few studies (Hamza et al., 2004; Abdellaoui et al., 2007) have analyzed the pattern of genetic diversity by SSRs markers within Tunisian landraces. In the present research we used the SSRs markers to investigate the genetic diversity among 12 genotypes (six barley varieties and six barley landraces) from the INRAT germplasm collection.

2. Materials and Methods

2.1. Plant Material

The analyses were carried out for six barley varieties (Faïz, Manel, Martin, Rihane, Roho, Tej) and six barley landraces from various growing regions of Tunisia (table 1). They consisted of different local barley lines obtained by mass selection of different local landraces. They were named according to the region of their origin (Djerba, Gabes, Jendouba, Kairouan, Kebili, Kerkennah) from which they were collected. These local landraces were selected among highly tolerant and very sensitive to salt and drought stresses to be used as parents to realize crosses for the linkage analysis and QTL mapping of the mentioned traits.

2.2. DNA Extraction

Total DNA was extracted from young leaves of a single plant per genotype. The extraction buffer (pH 8) was composed of 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 1.44 mM NaCl, 3% CTAB (w/v), 1% ß-mercaptoethanol (v/v). The DNA was purified by a treatment with RNase followed by a phenolic extraction. Quantification of the DNA concentration and the checking of its quality were made by spectrophotometry and gel electrophoresis. The average DNA yield was 15 µg DNA/g of tissue.

2.3. SSRs Analysis

Eighteen microsatellite primer pairs were selected on the basis of their chromosomal location (Von Korff et al., 2004). Their names, sequences and chromosomal locations are listed in table 2. PCR reactions was performed in 25µl of a mixture containing 50-100 ng DNA, 5 µl of 5X Green GoTaq Reaction Buffer (Promega), 1 Unit of GoTaq DNA Polymerase (Promega), 0.2 mM dNTPs and 0.25 µM of each primer. The amplifications were carried out in a Biometra Thermocycler (Germany). The cycling parameters were: one cycle of 95°C for 3 min, 35 cycles of 1 min denaturing step at 94°C, 1 min annealing temperatures between 52 and 56°C (table 2) depending on the different primer combinations and 2 min extension at 72°C, followed by 10 min at 72°C. Amplified PCR products were

separated by electrophoresis using 3% agarose gel "1xTBE", stained by ethidium bromide (0.5 mg/ml) and visualized under UV light. To confirm the results, SSRs products were also separated using 6% non-denaturing polyacrylamide gel electrophoresis in 1x TBE buffer, stained by ethidium bromide and visualized under UV light. A 100bp DNA Ladder (Promega) was used as a molecular size standard.

2.4. Cluster Analysis

Amplified fragments were classified as present (represented by 1) or absent (represented by 0). A data matrix was prepared for the analyses. A pair-wise similarity matrix (table 3) was generated with the software NTSYSpc-2.02j (NTSYS-Numerical Taxonomy and Multivariate Analysis; Rohlf, 1998) using the simple matching coefficient (Sokal et al., 1958). A dendrogram was then constructed based on the similarity matrix data using the UPGMA (Unweighted Pair-Group Method using Arithmetic Averages) cluster analysis of NTSYSpc-2.02j. Bootstrap analysis (Felsenstein, 1985) was also carried out using TREECON 1.3B program (Van de Peer et al. 1994). The SSRs data was bootstrapped by resampling 2000 times.

2.5. Degree of Polymorphism

The data matrix was used to compute the diversity for each SSRs marker. This is equivalent to Polymorphism Information Content (PIC) at each SSRs as described in the following equation by Anderson (1992):

$$PIC = 1 - \sum_{j=1}^{n} P_{ij}^2 \quad (1)$$

Where Pij is the frequency of the Jth SSRs pattern for marker i and the summation covers n patterns.

Table 1: Analysed genotypes, their Cross and/or Pedigree data, the origin, the year of the cross release in the country of origin, the year of inscription in the Tunisian catalogue of varieties (Deghaïs et al., 2007), and the end use.

Six row Vrieties	**Cross and/or Pedigree**	**Origin**	**Year of Cross Release**	**Year of Inscription**	**End use**
Rihane	Atlas 46/Arrivat//Athenais ICB-2L-1AP-3AP-0AP-0Kf	INRAT (Tunisia)/ ICARDA (Syria)	1976	1987	Feed
Manel	L527/5/As54/Tra//2*Cer/TolI/3/Avt/TolI//Bz/4/Vt/Pro//TolI ICB81-607-1Kf-1Bj-12Bj-11Bj-1Bj-1Bj-0Bj	INRAT (Tunisia)/ ICARDA (Syria)	1981	1996	Feed
Martin	Unknown Algerian Population	Pierre Lescure Farmer Lakhouat / Tunisia	1931	1931	Feed
Two Row Varieties	**Pedigree**	**Origin**	**Year of Cross Release**	**Year of Inscription**	**End use**
Faïz	earely Russian / Apam	INRAT (Tunisia)/ ICARDA (Syria)	1979	1985	Feed (NC*)
Roho	Roho 03573	INRAT (Tunisia)/ Riso laboratory (Danemark)	1975	1985	Feed (NC*)
Tej	Bonus/C13576 (WI2198-Australia)	INRAT (Tunisia)/ ICARDA (Syria)	1975	1985	Feed (NC*)
Collected Accessions	**Region of collection**	**Origin**	**Year of Collection**		**End use**
Gabes	Gabes	Tunisia	1983		FF**
Kerkennah	Kerkennah (Island)	Tunisia	1983		FF**
Kairouan	Kairouan	Tunisia	1983		FF**
Djerba	Djerba (Island)	Tunisia	1994		FF**
Kebili 1	Kebili 2	Tunisia	1994		FF**
Jendouba	Jendouba	Tunisia	1994		FF**

* Not yet Cultivated
** Food and Fodder

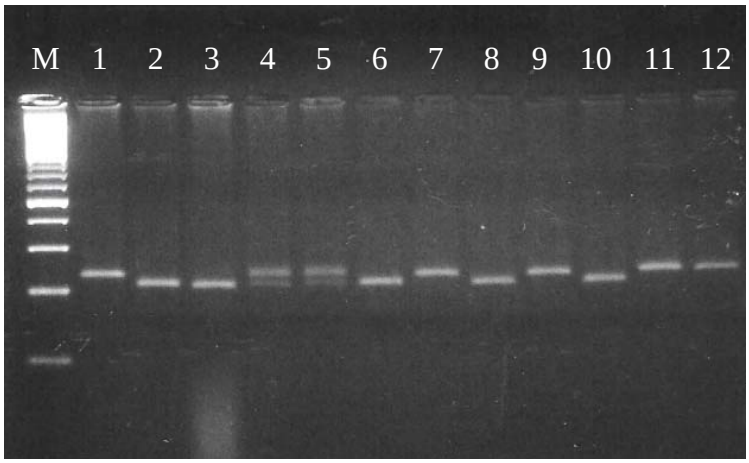
Table 2: Barley SSRs primers, their sequences, the annealing temperature used in the PCR reaction, the chromosomal location (Von Korff et al., 2004) of derived loci, the amplified fragments and the polymorphic information content (PIC).

Primer	Sequence	Annealing temperature (°C)	Chromosome Location	Amplified Fragments		PIC
				Total (T)	Polymorphic	
MGB391	Forward 5'-AgCTCCTTTCCTCCCTTCC-3' Reverse 5'-CCAACATCTCCTCCTCCTgA-3'	54	2 (2H)	2	2	0,50
HVITR1	Forward 5'-CCACTTgCCAAACACTAgACCC-3' Reverse 5'-TTCATgCAgATCgggCCAC-3'	55	3 (3H)	3	3	0,59
Bmag13	Forward 5'-AAggggAATCAAAATgggAg-3' Reverse 5'-TCgAATAggTCTCCgAAgAAA-3'	54	3 (3H)	3	3	0,60
HV13GEIII	Forward 5'-AggAACCCTACgCCTTACgAg3' Reverse 5'-AggACCgAgAgTggTggTgg-3'	56	3 (3H)	2	2	0,49
HVB23D	Forward 5'-ggTAgCAgACCgATggATgT-3' Reverse 5'-ACTCTgACACgCACgAACAC-3'	54	4 (4H)	2	2	0,50
MGB396	Forward 5'-CgCTAgCTTgTTTCTCgTTTg-3' Reverse 5'-TCgCATggCATCAACTACAg-3'	–	4 (4H)	–	–	–
MGB402	Forward 5'-CAAgCAAgCAAgCAgAgAgA-3' Reverse 5'-AACTTgTggCTCTgCgACTC-3'	55	5 (1H)	4	4	0,58
Bmag149	Forward 5'-CAAgCCAACAgggTAgTC-3' Reverse 5'-ATTCggTTTCTAgAggAAgAA-3'	–	5 (1H)	–	–	–
HVGLUEND	Forward 5'-TTCgCCTCCATCCCACAAAg-3' Reverse 5'-gCAgAACgAAAgCgACATgC-3'	–	5 (1H)	–	–	–
MGB371	Forward 5'-CACCAAgTTCACCTCgTCCT-3' Reverse 5'-TTATTCAggCAGCACCATTg-3'	56	6 (6H)	5	4	0,49
MGB356	Forward 5'-TggTCTggAgCTCTCAACAg-3' Reverse 5'-AAgCCACATTgAAggAgCAC-3'	–	6 (6H)	–	–	–
EBmac624	Forward 5'- AAAAgCATTCAACTTCATAAgA-3' Reverse 5'- CAACgCCATCACgTAATA-3'	54	6 (6H)	2	2	0,49
Bmag210	Forward 5'-ACCTACAgTTCAATAgCTAgTACC-3' Reverse 5'-gCACAAAACgATTACATCATA-3'	–	6 (6H)	–	–	–
MGB384	Forward 5'-CTgCTgTTgCTgTTgTCgTT-3' Reverse 5'-ACTCggggTCCTTgAgTATg-3'	–	7 (5H)	–	–	–
BMS02	Forward 5'-AgAgTAgTggAAAgAAAgTT-3' Reverse 5'-TggTAgTgAgATgAggTgAC-3'	–	7 (5H)	–	–	–
MGB318	Forward 5'-CggCTCAAggTCTCTTCTTC-3' Reverse 5'-TATCTCAgATgCCCCTTTCC-3'	55	7 (5H)	2	2	0,37
MGB357	Forward 5'-gCTCCAgggCTCCTCTTC-3' Reverse 5'-AgCTCTCTCTgCACgTCCTT-3'	52	7 (5H)	2	2	0,28
GMS1	Forward 5'-CTgACCCTTTgCTTAACATgC-3' Reverse 5'-TCAgCgTgACAAACAATAAAgg-3'	55	7 (5H)	3	3	0,50

3. Results and Discussion

Among 18 primer pairs used, six showed either monomorphic band profiles (MGB356, MGB384, HVGLUEND) or complex band patterns (MGB396, Bmag149, Bmag210) were discarded. The BMS02 primer did not generate any bands. The remaining 11 primer pairs (EBmac624, MGB318, MGB357, MGB371, MGB391, MGB402, GMS1, Bmag13, HV13GEIII, HVB23D, HVITR1) generated clear patterns with high polymorphism (table 2). The Figure 1 shows two polymorphic bands between 200 and 300 bp generated by EBmac624 primer. The eleven discriminatory primer pairs were used to evaluate the genetic diversity of the 12 barley genotypes. These primer pairs revealed a total of 31 alleles ranging from two to five alleles per locus (table 2) with a mean value of 2.81 alleles per locus. For all genotypes, the highest number of polymorphic bands was developed by the primer pairs MGB371 and MGB402 (table 2).

Figure 1: Agarose gel showing the allelic segregation of the MGB391 SSRs marker in the analysed barley germplasm. 1:Rihane, 2:Manel, 3:Martin, 4:Roho, 5:Faïz, 6:Tej, 7:Gabes, 8:kerkennah, 9:Kairouan, 10:Djerba, 11:Kebili1, 12:Jendouba2. M: Molecular size standard 100 bp DNA ladder.



The PIC (Polymorphism Information Content) value of each SSRs marker measure the marker diversity. In this study the PIC values ranged from 0.28 (MGB357) to 0.60 (Bmag13) with an average value of 0.50 (table 2). The most informative SSRs markers were EBmac624, MGB371, MGB391, MGB402, Bmag13, GMS1, HV13GEIII, HVB23D and HVITR1.

A dendrogram (Fig. 2) of the 12 barley genotypes was constructed by the UPGMA method (Unweighted Paired Group Method Using Arithmetic Averages). The genetic similarity matrix (table 3) was established by Simple Matching coefficient using the data generated by the eleven more expressing primers. These primers enabled us to discriminate all the genotypes and to study the genetic variability within the improved varieties and the local landraces.

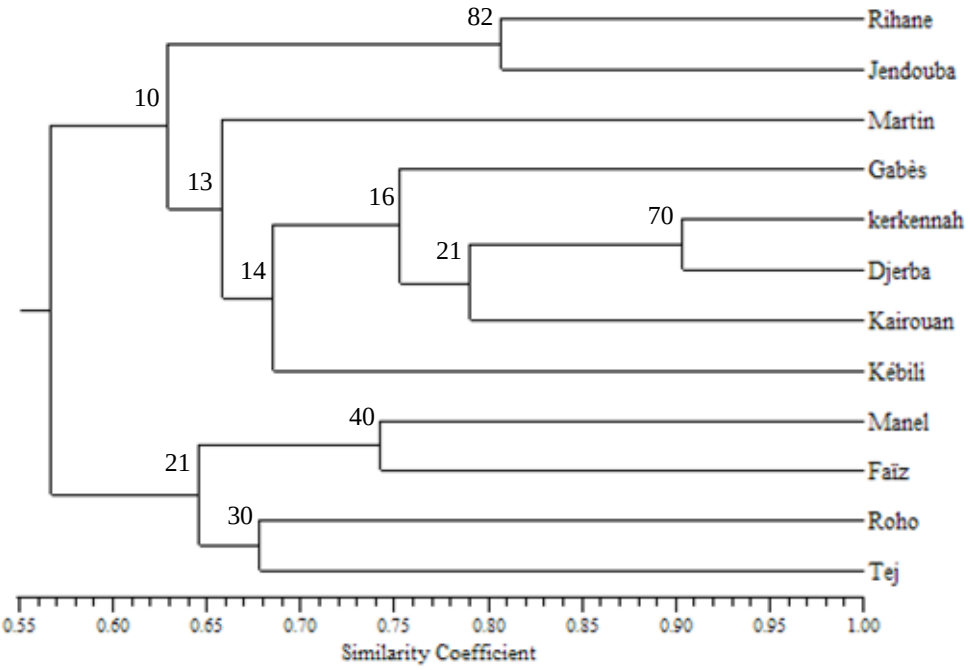
The bootstrap values are reported on the dendrogram (Fig. 2). Only two among the ten nodes had bootstrap value of 70% or higher. Four nodes had bootstrap values between 20% and 40%. The other nodes had bootstrap values between 10 and 20%.

The UPGMA cluster diagram classified the evaluated genotypes in two major clusters corresponding to the number of rows per spike.

Table 3: Similarity matrix among the analysed barley genotypes (Rih: Rihane, Man: Manel, Mart: Martin, Roh: Roho, Faï: Faïz, Tej: Tej, Gab: Gabes, kerk: Kerkennah, Kair: Kairouan, Djer: Djerba, Kéb: Kebili 1, Jend: Jendouba).

	Rih	**Man**	**Mart**	**Roh**	**Faï**	**Tej**	**Gab**	**kerk**	**Kair**	**Djer**	**Kéb**	**Jend**
Rih	1,00											
Man	0,42	1,00										
Mart	0,55	0,48	1,00									
Roh	0,58	0,58	0,39	1,00								
Faï	0,55	0,74	0,48	0,71	1,00							
Tej	0,65	0,65	0,58	0,68	0,65	1,00						
Gab	0,65	0,45	0,65	0,61	0,65	0,61	1,00					
kerk	0,58	0,65	0,71	0,48	0,58	0,74	0,74	1,00				
Kair	0,65	0,65	0,58	0,55	0,77	0,68	0,74	0,74	1,00			
Djer	0,68	0,61	0,74	0,52	0,68	0,71	0,77	0,90	0,84	1,00		
Kéb	0,61	0,48	0,61	0,45	0,48	0,52	0,65	0,77	0,65	0,68	1,00	
Jend	0,81	0,48	0,61	0,45	0,55	0,65	0,65	0,65	0,65	0,68	0,61	1,00

Figure 2: Dendrogram showing similarity and clustering of the 12 barley genotypes included in this study.



The first cluster group is a six-row spike type (Djerba, Gabes, Jendouba, Kairouan, Kebili, Kerkennah, Martin, Rihane). The second is a two row spike type (Faïz, Roho, Tej), except for Manel variety which is a six row type but it clustered in the two row type cluster. A similar clusters types were also identified by Hamza et al. (2004) using molecular and morphological markers.

The first cluster (six-row cluster) groups both local accessions (Djerba, Gabes, Jendouba Kairouan, Kebili, Kerkennah) and two fixed varieties (Martin, Rihane). At genetic similarity GS=65% this cluster could be splitted into two subgroups. The first regroup all the local accessions (Djerba, Gabes, Kairouan, Kebili, Kerkennah) and the Martin variety. The second regroup the Rihane varity and the Jendouba accession. These two genotypes were genetically very similar (GS=81%).

In the first subgroup Djerba and Kerkennah germplasm collections are genetically very similar (GS=90%) even though they are from two different island. Indeed these unique endemic landraces, which are morphologically divergent from continental collection are considered to have evolved in specific environments that differ from the rest of mainland. This demonstrate that gene flow is limited due to spatial isolation. Therefore it can result in rapid fixation of mutations and subsequent speciation (Barton, 1998). In addition, the lack of competition with other species and the possibility for colonization of new habitats may promote speciation on islands (Crawford et al., 1987).

In this subgroup Martin variety was very similar to the local germplasm. The Martin variety is an old genotype derived from a local germplasm collected from Algeria and was cultivated in Tunisia since 1931 (Deghaïs et al., 2007). Being selected from a North African germplasm it should have passed through the same evolutionary pressures and effects as our local germplasm since it has been cultivated in Algeria for long time and then it was introduced and improved in Tunisia. During its evolution Martin variety should have accumulated the same adaptive alleles and characters as our local germplasm. This could explain the morphological and genetic (molecular) resemblance of this variety and our local collection.

In the second subgroup, Rihane variety and the Jendouba accession were genetically very similar (GS=81%). The Jendouba accession is collected from the North west of Tunisia near to Algeria in the neighbourhoods of the vast mountainous system of the Atlas. In this region this accession collectively bears the name of "Djebeli". The word "Djebeli" comes from the Djebel which means

mountain. In fact the local farmers of these regions call the local barley germplasm cultivated in the neighbourhoods of the vast mountainous system of the Atlas as the "Djebeli" barley. The Rihane variety results from a cross implying the Atlas 46 line as a female parent (table 1). The Atlas 46 is a North African line selected in a population "Djebeli" originating from the Atlas of Algeria. Thus both Rihane and Jendouba accessions have Djebeli relatives mainly Atlas46 (table 1). This could explain the morphologic and genetic (molecular) resemblance of the Rihane variety to the local accessions and especially to the jendouba accession. This similarity to the Tunisian local accessions made its diffusion easy in Tunisia.

The second group contained only improved varieties: Faïz, Manel, Roho and Tej. As previously mentioned, the two rows cluster contained three two row spike type (Faïz, Roho, Tej) and a six row spike type (Manel). Faïz and Manel varieties where selected from an ICARDA (International Center for Agricultural Research in the Dry Areas) cross for Tunisian sub humid regions. These two varieties were similar to each other (GS=74%) and were relatively distant to the Roho and Tej varieties which were selected for the semiarid Tunisian regions. By analyzing the pedigree (table 1) of Manel and Faïz, we found, that on the one hand, Manel has a two row relative (Betzes=Bz) and, on the other hand, Faïz has a six row relative (Apam), which could explain the presence of 'Manel' variety within the two row group and its genetic similarity to Faïz variety.

4. Conclusions

The selected set of SSRs has generated clear patterns with high polymorphism. This polymorphism was enough to distinguish all genotypes, to discriminate the two row spike types from the six row spike types and to differentiate the landraces from the new selected varieties with some exceptions. Therefore our SSRs analysis showed that this technique was informative in a range of barley germplasm. It provided us useful information on the level of polymorphism and diversity in barley, showing its utility in the characterization of the barley landraces. These landraces should be a good source of genetic diversity since they had a high polymorphism and they were distinguished from the new varieties. Finally, the present study is useful for the establishment of genetic relatedness and molecular characterization of barley germplasm. This will benefit barley breeding programs to make choice of the genotypes to be used in crosses and will facilitate the germplasm management.

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