African Journal of Biotechnology Vol. 10(61), pp. 13064-13070, 10 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1515
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Application of randomly amplified polymorphic DNA (RAPD) markers and polyphenol oxidases (PPO) genes for distinguishing between the diploid (*glaucum*) and the tetraploid (*leporinum*) accessions in *Hordeum murinum* complex

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Accepted 26 August, 2011

The genome of 12 wild barley accessions belonging to *Hordeum murinum* complex (eight out of them belong to *H. m. glaucum* and four belong to *H. m. leporinum*) was characterized using RAPD-PCR markers and polyphenol oxidases genes (PPO). A total of 123 RAPD markers with 22 arbitrary decamer primers were used. 56 out of the RAPD markers were common to all accessions, and the other 67 markers were polymorphic. 35 out of the polymorphic markers are distinguishable between the two taxa but not among all accessions, 15 RAPD markers are specific to *H. m. glaucum* and 17 are specific to *H. m. leporinum*. The PCR amplification of PPO genes revealed that amplified fragments from these genes are randomly distributed among the studied accessions regardless of their taxonomy. All data were analyzed using NT-SYS-pc (numerical taxonomy and multivariate analysis system) program to address the phylogenetic relationships between the studied taxa. The examined accessions were clustered into two main groups; the first one consists of 4 accessions representing the *H. m. leporinum* (diploid) and the other one consists of eight accessions representing the *H. m. leporinum* (tetraploid).

Key words: *Hordeum, glaucum*, *murinum, leporinum*, random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), polyphenol oxidases (PPO), numerical taxonomy and multivariate analysis system (NTSYS-pc).

INTRODUCTION

Wall barley (*Hordeum murinum*) consists of three subspecies, naturally distributed from southern Central Asia through the Mediterranean region to northwestern Europe, but now an invasive weed in many parts of the world (Jakoba and Blattner, 2010). Polymerase chain reaction (PCR) based molecular markers have become increasingly popular for fingerprinting and cultivars identification since the development of PCR technology (Saiki et al., 1988). RAPD-PCR (randomly amplified polymorphic DNA) was first conducted by Williams et al. (1990). It is based on amplification using arbitrarily 10-mer primers, and has been successfully used for *Hordeum* genome characterization, for major quantitative trait loci mapping, facilitating positional cloning of genes, studying genetic diversity and genetic fingerprinting for phylogeny studies (Marillia and Scoles, 1996; Lainé et al., 2000; Waldron et al., 2002; Blattner, 2004).

The *Hordeum murinum* complex is a group of in-breeding annual plants, belonging to section *Campestria* (Åberg, 1940) or section *Hordeum* (Bothmer et al., 1987) of the genus *Hordeum*, family *Poaceae*. Taxa of the complex are distributed in central Europe, Mediterranean region, North Africa, southwestern Asia, Caucasus, Southern Uzbekistan, Tadzjikistan, Iran, and Afghanistan, and as weeds, have colonized most parts of the world (Bothmer et al., 1995). Morphologically, size of seed triplets and the relatively large inflated lateral spikelets,

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Table 1. The scientific name and the site of collection for the studied *H. murinum* complex accessions.

Number	species	Site of collection
1	*H. glaucum* 1	25 Km West Matrouh
2	*H. glaucum* 2	20 Km East Matrouh
3	*H. glaucum* 3	35 Km East Matrouh
4	*H. glaucum* 4	110 Km West Alexandria
5	*H. leporinum* 1	Marriott
6	*H. glaucum* 5	42 Km West Alexandria
7	*H. glaucum* 6	30 Km West Alexandria
8	*H. leporinum* 2	30 Km East Alex
9	*H. glaucum* 7	Cairo, Alex. Desert Road, 60 km South Alex
10	*H. leporinum* 3	10 Km West Rosetta
11	*H. leporinum* 4	Rosetta
12	*H. glaucum* 8	28 Km West Arish

set the *H. murinum* complex aside from all other *Hordeum* species except *H. bulbosum* and *H. vulgare.* Today, it is widely agreed that the complex contains three major morphodemes, namely *H. glaucum*, *H. murinum*, and *H. leporinum* (Nevski, 1941; Bowden, 1962; Täckholm, 1974; Löve, 1984, Melderis, 1985; Bothmer and Jacobsen, 1985; Jaaska, 1992; Jacobsen and Bothmer, 1992, 1995; Bothmer et al., 1987, 1995). Tzvelev (1976), Humphries (1980), Bothmer and Jacobsen (1985), Melderis (1985), Jacobsen and Bothmer (1995) and Bothmer et al. (1995) treated the *H. murinum* complex as one species consisting of three subspecies, *H. m. murinum*, *H. m. leporinum*, and *H. m. glaucum*.

Blattner (2004) studied the phylogenetic relationships among diploid and polyploid taxa of the genus *Hordeum* by analyzing the nuclear rDNA internal transcribed spacer region (ITS) for 91 accessions, representing all *Hordeum* species.The phylogenetic analysis revealed four major clades that concurred with the four genome groups in *Hordeum* (H, I, Xa, and Xu). Jakoba and Blattner (2010) also analyzed the phylogeny of the *H. murinum* group using amplified fragment length polymorphisms (AFLP) and sequences of cloned PCR products of the nuclear ribosomal DNA internal transcribed spacer region (ITS), a part of the nuclear single-copy gene topoisomerase 6 (Topo6) spanning two introns, and sequences of the chloroplast trnL-F region together with length variation at six chloroplast microsatellite loci, including multiple individuals of each subspecies and cytotype, covering the entire natural distribution area of the species.

Polyphenol oxidase (PPO) activity is highly related to the undesirable browning of wheat-based end products, especially Asian noodles. Characterization of PPO genes and the development of their functional markers are of great importance for marker-assisted selection in wheat breeding. He et al. (2007) characterized two genomic DNA sequences of two PPO genes, each one located on chromosomes 2A and 2D and characterized their allelic variants by means of in silico cloning and experimental validation. He et al. (2009) also examined six wheat relative species corresponding to common wheat varieties and identified seven new alleles from these species. They discussed the relationships between these allelic variants and grain PPO activities. More also, Taketa et al. (2010) studied the duplication of polyphenol oxidase genes on barley chromosome 2H and their functional differentiation in the phenol reaction of spikes and grains.

In the current study, the genome of 12 wild barley accessions (eight belong to *H. murinum glaucum* and four belong to *H. murinum leporinum*) was characterized using 22 arbitrary RAPD-PCR primers and the presence of polyphenol oxidases genes through PCR amplification of the polyphenol oxidases genes using two specific primers.

MATERIALS AND METHODS

In total, twelve *Hordeum murinum* accessions were used in this study and sites of their collection are shown in Table 1.

Genomic DNA extraction for RAPD markers development

Genomic DNA was extracted from young leaves of actively growing 15-days old seedlings of the twelve barley accessions. The leaves were collected in sterilized 50 ml polypropylene tubes and immediately frozen in liquid nitrogen. 0.3 g of the frozen plant materials were ground in liquid nitrogen using mortar and pestle. Genomic DNA was immediately extracted using DNeasy Plant DNA Mini kit Cat # 69104 (Qiagen).

RAPD-PCR reaction

22 decamer oligonucleotide primers (Invitrogen GmbH, Karlsruhe,

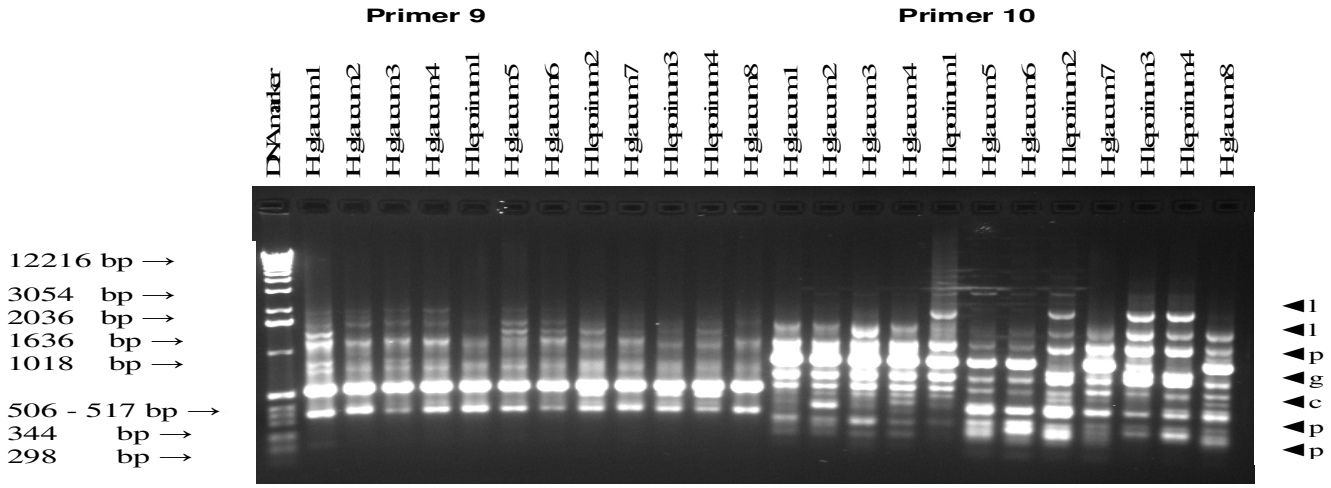

Figure 1. Examples of randomly amplified DNA of the studied *Hordeum murinum* complex accessions using Primers 9 (the first twelve lanes after marker and primer 10 (the other twelve lanes). The sequence of these markers are as aforementioned. The letters **c** refers to bands common to all accessions of the two taxa, **g** bands characteristic to *H. glaucum*, **l** bands characteristic to *H. leporinum* and **p** bands polymorphic in both taxa but not in all accessions.

Germany) were used to generate RAPD markers using Biorad thermocycler unit. PCR reactions were performed in a 25 µL mixture, containing 10 to 25 ng DNA in 5 µL, 2.5 µL 10 x PCR buffer, 1.5 µL 25 mM $MgCl_2$, 2.5 2 mM dNTPs, 2.5 RAPD 10-mer primer 1.5 to 2 U of *Taq* polymerase in 0.3 and 10.7 µL ddH_2O. For DNA amplification, the DNA thermocycler was programmed as follows: incubation at 93°C for 3 min; 45 cycles at 93°C for 1 min., 50°C for 1 min and 72°C for 2 min, followed by one final extension cycle of 5 min at 72°C. The amplification products were separated by electrophoresis in 1.5% (w/v) agarose gels. An Invitrogen ready load 1 kb DNA ladder (Cat. No. 10381-010) was used as DNA sizing tool for estimating the molecular weight of the RAPD markers.

The sequence of the 22 RAPD primers (5' to 3')

1- **5'**- CAGGCCCTTC -**3'** 2- **5'**- AGGGGTCTTG -**3'** 3- **5'**- GAAACGGGTG -**3'** 4- **5'**- GTGACGTAGG -**3'** 5- **5'**- GGGTAACGCC -**3'** 6- **5'**- GTGATCGCAG -**3'** 7- **5'**- CAATCGCCGT -**3'** 8- **5'**- TCTGTGCTGG -**3'** 9- **5'**- TTCCGAACCC -**3'** 10- **5'**- AGCCAGCGAA -**3'** 11- **5'**- GTTTCGCTCC -**3'** 12- **5'**- TGATCCCTGG -**3'** 13- **5'**- GGACTGGAGT -**3'** 14- **5'**- TGCGCCCTTC -**3'** 15- **5'**- TGCTCTGCCC -**3'** 16- **5'**- GGTGACGCAG -**3'** 17- **5'**- GTCCACACGG -**3'** 18- **5'**- CTGCTGGGAC -**3'** 19- **5'**- GTAGACCCGT -**3'** 20- **5'**- TTCGAGCCAG -**3'** 21- **5'**- GATGACCGCC -**3'** 22- **5'**- GAACGGACTC -**3'**

PPO amplification

The presence of polyphenol oxidases genes was investigated in the two wild barley taxa by PCR amplification of the genomic DNA using two 36 bp oligonucleotide specific primers, namely T3-001 and T7-010 based on functional markers according to He et al. (2007). These primers were constructed by Invitrogen GmbH, Karlsruhe, Germany, and used to amplify the polyphenol oxidases genes. The sequences of these primers were as follows:

T3-001: 5`-CCA TTA ACC CTC ACT AAA GGG ACC GTA GTT TCA AGC-3`
T7-010: 5`-CCT AAT ACG ACT CAC TAT AGG GTC ATT GAT TAA TTT-3`

Amplification was performed using Biorad thermocycler unit in a 25 µL mixture, containing the following: 25 ng genomic DNA in 3 µL, 2.5 µl PCR buffer, 2 µL $MgCl_2$, 2 µL T3-001 primer, 2 µL T7-010 primer, 0.5 µL Taq polymerase (2.5 units) and 13 µL didistilled H_2O. For PPO genes amplification, the thermocycler was programmed as follows: 93°C for 2.5 min, then 30 cycles at 93°C for 30 s, 50°C for 60 s and 68°C for 60 s, and a final step for 5 min at 72°C.

Scoring and analysis of data

Both RAPD markers and PPO genes data were scored as binary system where 1 and 0 indicated the presence or absence of a particular band respectively. Data were analyzed using NT-SYS-pc (Numerical taxonomy and multivariate analysis system) program. Both AFLP and protein gels were scored as 0/1 for absence/presence of the bands, respectively. Number and percentage of the polymorphic bands were calculated. Similarity coefficient matrices were calculated using Dice similarity algorithm (Dice, 1945) for both markers (AFLP and protein). Phenograms were constructed using the UPGMA method (Unweighted Pair-Group Method with arithmetical algorithms Averages; Sneath and Sokal (1973)) and the correlation cophenetic coefficients were calculated. For the aforementioned analyses, the NTSYS PC2.0 software was used (Rohlf, 1998).

RESULTS

The results of RAPD-PCR fingerprinting using 22 arbitrary primers produced 123 bands distributed in the 12 *H. murinum* accessions. Figure 1 shows an example of the RAPD amplification patterns using primer 9, 10. Fifty-six of them were common to both species and 67

Table 2. Statistics of RAPD fingerprinting results.

Primer number	Monomorphic band	Polymorphic band	Band characteristic to glaucum	Band characteristic to leporinum	Approximate bp range of bands	Total number of band
1*	5	0	0	0	500 - 1450	5
2*	5	0	0	0	350 - 1350	5
3	2	3	0	3 (1000, 1700 and 2100 bp)	500 - 2100	5
4	1	7	1 (300 bp)	2 (350 and 700 bp)	300 - 1000	8
5	2	2	2 (350,500 bp)	0	350 - 1000	4
6	1	3	0	0	300 - 800	4
7	2	2	1 (950 bp)	0	550 - 2200	4
8	1	3	1 (850 bp)	1 (750 bp)	350 - 950	4
9	4	6	3 (1100, 1600, 2000 bp)	2 (450 and 1050 bp)	350 - 2000	10
10	2	9	0	2 (850 and 2000 bp)	250 - 2500	11
11	2	5	1 (300 bp)	2 (450 and 550 bp)	400 - 2200	7
12	0	1	1 (1600 bp)		1600	1
13	0	1			1200	1
14	4	6	1 (1500 bp)	3 (1100, 1250 and 1300 bp)	250 - 1700	10
15	4	4	0	1 (1000 bp)	350 - 1600	8
16	0	4	2 (400, 1700 bp)		400 - 1700	4
17	2	2	0	0	300 - 1200	4
18*	11	0	0	0	300 - 1700	11
19	1	3	0	0	350 - 1000	4
20*	3	0	0	0	300 - 500	3
21	1	3	0	0	300 - 1300	4
22	3	3	2 (600, 800 bp)	1 (700 bp)	300 - 800	6
Total	56	67	15	17		123

*Primers showed no polymorphism.

were polymorphic. 15 bands were specific to *H. glaucum*, whereas 17 were characteristic to *H. leporinum* and 33 markers were distributed in both species. Table 2 illustrates the results of RAPD data and gives some statistics concerning occurrence of the different markers in the two taxa of the complex.

18 RAPD primers succeeded to differentiate between the two taxa. The number of polymorphic bands varied from one band (in primer no. 12 and 13), two bands (in primers 5, 7 and 17), three bands (in primers 3, 6, 8, 19, 21 and 22), four bands (in primer 15 and 16), five bands (in primer 11), six bands (in primer 9 and 14), seven bands (in primer 4) and nine bands (in primer 10). In contrast, the other four RAPD primers (primers 1, 2, 18 and 20) failed to differentiate between the two species, but gave rise to 24 bands common to both species that can be used in fingerprinting them to be distinguished from other plant species.

PPO amplification

The PCR product (Figure 2) appeared as a ~ 400 bp marker that represents the polyphenol oxidases genes. This marker appeared in six accessions out of the 12 accessions, five of which belong to *H. m. glaucum (H. m. glaucum* 1, 4, 5, 6 and 7) and only one accession belongs to *H. m. leporinum* (*H. m. leporinum* 3) as shown in Figure 2.

RAPD and PPO data analysis

All the 123 RAPD markers and the PPO data were analyzed using NTSYS-pc program to investigate the phylogenetic relationships among the studied taxa (Figure 3). Out of the two main clusters, the first one contained 4 accessions representing *H. m. leporinum* and the other group includes 8 accessions representing *H. m. glaucum*. The subgroups of both groups are more or less consistent with the geographical areas from which they were collected. In *H. m. leporinum* group, *H. lporinum* 1 that was collected from Marriott is partially distant from the other samples, followed by *H. m. leporinum* 2 which was collected from 25 km East of Alexandria. *H. m. leporinum* 3 and *H. m. leporinum* 4 are closely related to each other and were collected from close locations; 10 km West Rosetta and Rosetta, respectively. In *H. m. glaucum* group, sample *H. m. glaucum* 7 that was collected from 60 km south Alexandria location was found

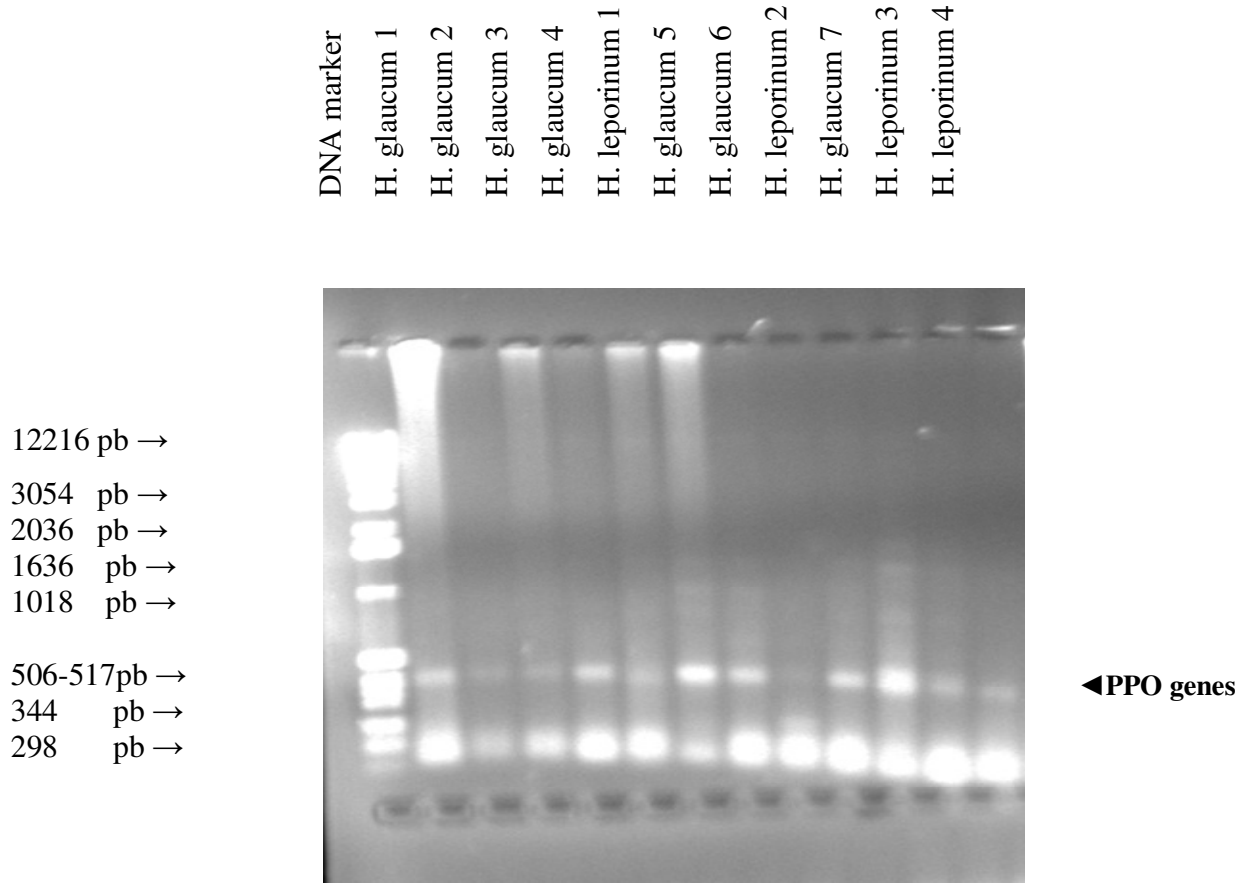

Figure 2. The amplified polyphenol oxidases genes (~400 bp band) in the studied *H. murinum* accessions.

distant from all other Mediterranean coast samples, followed by sample *H. m. glaucum* 8 which was collected from 28 km West of El Arish, and *H. m. glaucum* 6 and 5 that were collected from 30 and 42 km West of Alexandria respectively. A subgroup containing 4 samples collected from East and West of Matrouh was separated according to the geographic distribution of the samples. *H. m. glaucum* 3 that was collected from 35 E Matrouh is closely related to *H. m. glaucum* 4 that was collected from 110 km West of Alexandria and similarly the other two samples *H. m. glaucum* 1 and 2 that were collected from 25 West and 20 km East of Matrouh respectively are closely related to each other.

DISCUSSION

The wild barley called *H. murinum* complex is easily distinguished from other species of the same *Hordeum* section based on leaf blades characters (Bowden, 1962). Within the complex, the taxon *H. m. murinum* (which is not represented in the Egyptian flora) shares with *H. m. leporinum* several characters, more than it does with the *H. m. glaucum*. This latter taxon is characteristic for its poor vigor, shorter spikes and the shorter red spotted central spikelet anthers different from those of the lateral spikelets. While the taxon *H. m. murinum* is unique in having sessile or sub-sessile central spikelets and the glabrous lateral spikelet palea, which is pilose in *H. m. glaucum* and scabrid in *H. m. leporinum* (Bothmer et al., 1995).

The problem left unsolved by the morphological analysis is to understand if what was obtained are species or subspecies. Giles (1984) and Giles and Lefkovitch (1986) considered the complex as consisting of three groups with equal status.

These groups could be considered as species based on low inter-fertility. However, they can be defined also as subspecies based on certain natural opportunity to cross. Bor (1970), Täckholm (1974) and Baum and Bailey (1984a, b) favor separate species. Studies of interspecific hybrids (Bothmer et al., 1987) assign to *H. m. glaucum* the genome indicated with the symbol Y, different from the ones of *H. m. murinum* and *H. m. leporinum* which are more similar to each other. Bolous (2005) treated them as subspecies. However, they can be defined also

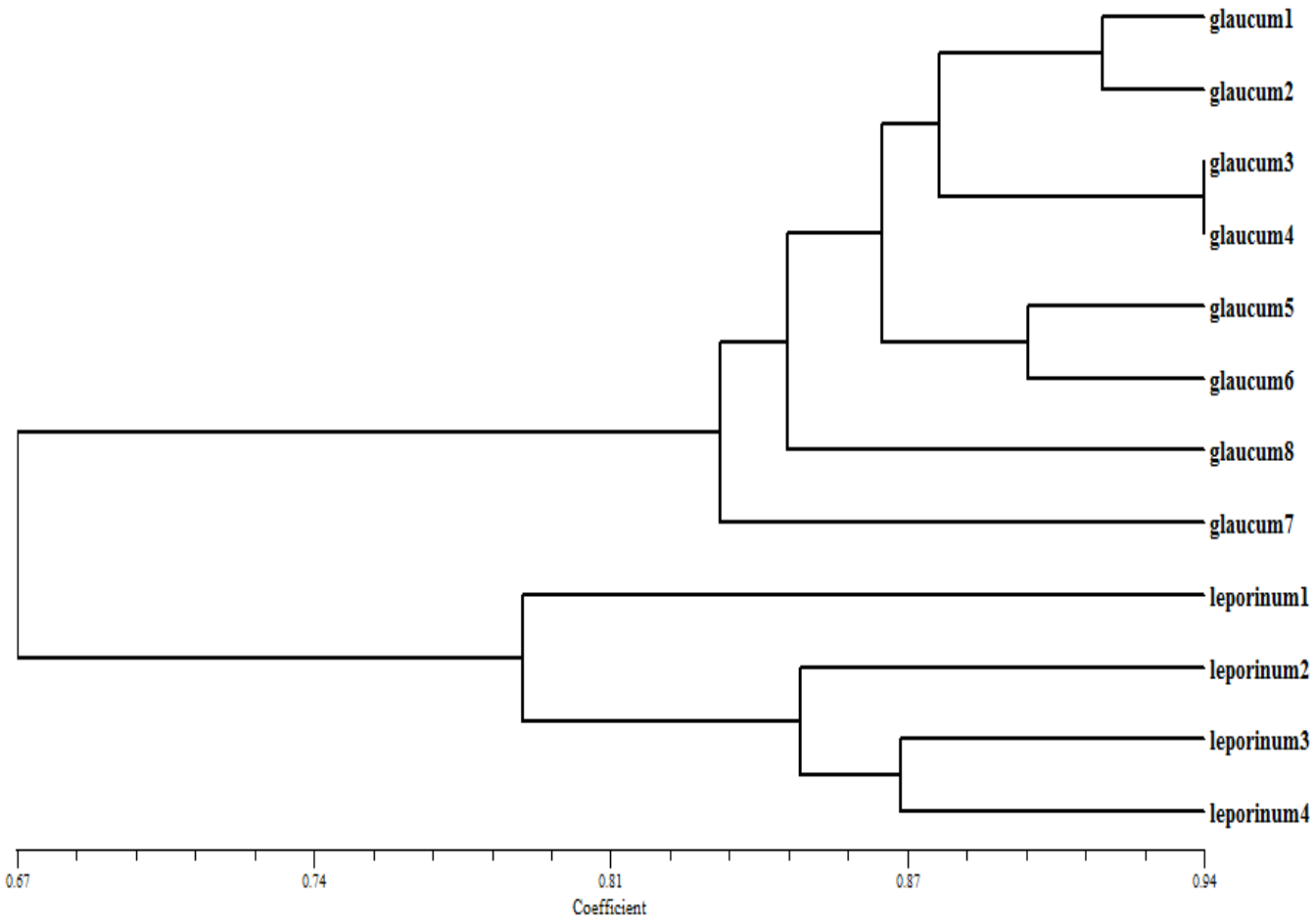

Figure 3. Dendrogram shows the clustering of the two taxa of the studied *H. murinum* complex accessions into two distinct groups. The dendrogram is based on RAPD and polyphenol oxidases data and analyzed using NTSYS-pc program.

as subspecies based on certain natural opportunity to cross. El Rabey (1999) based on cytological investigations, supported the separation of the complex into two ploidy groups; a diploid *H. glaucum* with 2n=2x=14 chromosomes and polyploid *H. murinum murinum* and *H. murinum leporinum* (the former is tetraploid with 2n=4x=28 chromosomes and the latter is tetraploid with 2n=4x=28 chromosomes or hexaploid with 2n=6x=42 chromosomes).

The RAPD-PCR results of the current study showed that 18 RAPD-PCR primers succeeded in distinguishing between *H. m. glaucum* and *H. m. leporinum* accessions. This result integrates the previously published cytological, morphological, biochemical and molecular evidences (Baum and Bailey, 1989; El Rabey et al., 2002; Jakoba and Blattner, 2010). This conclusion is also consistent with molecular results based on chloroplast DNA restriction patterns (Baum and Bailey, 1989) and *in situ* fluorescence hybridization (Bustos et al., 1996). The molecular evidences provided in this study, due to their congruence with both published data based on morphological, cytological, biochemical and various molecular data (Baum and Bailey, 1989; Bustos et al., 1996; El Rabey et al., 2002; Blattner, 2004 and Jakoba and Blattner, 2010) are in favor of the existence of two genomes within the complex; suggesting the separation of the *H. murinum* complex into two independent species, a diploid *H. glaucum* with 2n=2x=14 chromosomes and polyploid *H. m. murinum* and *H. m. leporinum* (the former is tetraploid with 2n=4x=28 chromosomes and the latter is tetraploid with 2n=4x=28 chromosomes but rarely also hexaploid with 2n=6x=42 chromosomes).

Jakoba and Blattner (2010) emphasized that tetraploids of the *H. murinum* complex arose via allopolyploid formation involving subsp. *glaucum* and its sister group, a now extinct diploid taxon belonging like subsp. *glaucum* to the Xu-genome group. The lineage leading to this extinct species split from the *glaucum* lineage about 4 to 5 million years ago. A close diploid relative of this extinct species hybridized again with tetraploid subsp. *leporinum* resulting in the formation of the hexaploid cytotype. The AFLP and ITS phylogenies indicate that both extinct taxa were genetically quite distinct, which is the reason for us to assume that they were already distinct evolutionary

lineages (species) when hexaploid formation took place. While Jakoba and Blattner (2010) stated that none of the molecular methods was able to separate tetraploid subsp. *leporinum* and *murinum*, in contrast however, El Rabey et al. (2002) using AFLP and ITS region analysis could distinguish between the polyploid and the tetraploid *Hordeum murinum* taxa. Moreover, the current study using RAPD PCR markers could efficiently distinguish between *H. m. glaucum* (the diploid taxa) and *H. m. leporinum* (the tetraploid taxa).

On the other hand, it is worthy to mention that four RAPD primers (primer 1, 2, 18 and 20) failed to differentiate between the two species, hence, they gave rise to 24 band common to both taxa, but they can be used in fingerprinting them to be distinguished from other plant species. Similarly, polyphenol oxidases genes showed polymorphic distributed in both taxa without differentiation, thus indicating that the PPO genes are not useful in distinguishing between *H. m. glaucum* and *H. m. murinum*.

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