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Nucleotide Polymorphism in Ribosomal DNA Gene and Internal Transcribed Spacers of Cultivated and Wild Barley

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Abstract: The internal transcribed spacers (ITS1 and ITS2) and 5.8S ribosomal DNA gene were sequenced from twelve genotypes representing four species of *Hordeum*. Whole sequences of ITS region revealed 87 variable nucleotides. Alignment sequence lengths of (ITS1, ITS2 and 5.8S) regions were 220-222bp, 223bp and 161bp, respectively. Differences in ITS1 lengths are attributed to insertion/deletion, while the sequence length of ITS2 and 5.8S regions was uniformly. GC content was higher in ITS2; number variable sites were higher in ITS1 while transitions/transversions ratio was higher in ITS2.
Phylogenetic analysis of the ITS1, ITS2 and 5.8S regions distinguished clearly the four *Hordeum* genomes (H, I, Xa, and Xu). These results indicated that ITS region was successful marker in confirming species. However, ITS region is not suitable marker for intraspecific level studies in genus *Hordeum.*
Key words: ITS1; ITS2; 5.8S ribosomal DNA;*Hordeum*; phylogeny

Introduction

Barley (*Hordeum vulgare* L.) is an important feed and food cereal crop, ranking fourth among the cereals in worldwide production, and grown in a wide range of environments. *Hordeum*is the largest genus in the tribe *Triticeae* and comprises about 50 taxa[1]. Classification of the genus is mainly based on morphometric data, the capacity of different species to produce fertile progeny in crosses, and the analysis of meiotic pairing of chromosomes in hybrids[1]. Previous studies, based on the meiotic pairing behavior of different interspecific combinations of *Hordeum* species, suggested the presence of four "basic genomes" designated I, Xa, Xu and H [2,3,4], both *H. vulgare* and *H. bulbosum* carried the Igenome, whereas *H. marinum* and *H. murinum* carried the Xa (formerly X) and Xu (formerly Y) genome, respectively, but the remaining species sharedvariants of the H genome.

ITS region has been used to gain insight into DNA sequence evolution[5]. As it belongs to a multigene family with hundreds to thousands of homogenized copies in a single genome[6], it allows for direct sequencing of PCR products for many eukaryotes. Since ITS sequences evolve relatively quickly[7,8], it makes them an interesting subject for evolutionary investigations[9,10,11]as well as biogeographic investigations[12,13,14].

ITS sequence data has been implemented in earlier studies to assess genetic diversity in cultivated barley[15]. Also, sequence comparison of the ITS region was used to determine genetic diversity at intraspecific level in the wild barley *Hordeum spontaneum* and common wheat *Triticum aestivum* L. [16].

The present study was devoted to estimate nucleotide polymorphism of the 5.8S rDNA, ITS1and ITS2, and to demonstrate phylogenetic relationships between *Hordeum* genotypes which belong to groups with different genomes.

Materials and Methods

Plant Material:

Twelve genotypes representing four species of genus Hordeum were used in this study. The grains had been provided by The International Center for Agricultural Research in the Dry Area (ICARDA) and the General Commission for Scientific Agricultural Research in Syria (GCSAR) as shown in table 1. The grains were grown in plastic trays at a glass house.

DNA extraction

Fresh young leaves -during stage of two to three leaves- from three plants at least for each genotype were frozen in the liquid nitrogen, then disrupted and homogenized by Tissue Lyser LT (Qiagen, Germany). Total genomic DNA was isolated using DNeasy Plant Mini Kit (Qiagen, Germany). Its quality was determined by running the DNA on 1% agarose gel. DNA concentration was adjusted to 50 ng/µL by DNase/RNase Free Water after measuring the concentration by spectrophotometer.

PCR reaction and DNA sequencing

The target segments containing the ITS1-5.8S-ITS2 region were amplified using the following universal primer pair: ITS-4(5'-TCCTCCGCTTATTGATATGC-3'),ITS-5(5'-GGAAGTAAAAGT CGTAACAAGG-3')[17].PCR amplification was done using HotStarHiFidelity Polymerase Kit (Qiagen), containing 35.5µl RNase-free water, 10µl of 5x HotStarHiFidelity PCR Buffer containing dNTPs, 1µl of each primer (10 picomole), 1.5µl (50ng) genomic DNA and 1µl HotStarHiFidelity DNA Polymerase. PCRs were performed in peqSTAR 2X Gradient Thermocycler (Germany), as follows: primary denaturing at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min, with a final extension step at 72°C for 7 min. The PCR products were enzymatically purified with ExoSAP-IT kit (GE Healthcare), and then sequenced from both directions using the forward and reverse primers by running on an ABI Prism 310 capillary DNA sequencing instrument. All sequences obtained in this study were submitted to the GenBank database under the accession numbers KP008150, and KP126667 to KP126677.

Sequence alignment and tree construction

The boundaries of ITS-1, ITS-2 and 5.8S sequences presently investigated were determined depending on the published literature [18].Sequence segments were assembled into contigs by CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA),and then were alignend using ClustalW multiple sequence alignment[19]. Sequence characteristics; total nucleotide length (bp), GC content (%), number of deletion/insertion, conserved and variable sites and parsimony informative sites) were calculated with MEGA version 6 software[20]. The proportion of nucleotide differences was calculated among pairs of species using the Kimura two-parameter model[21], with all codon positions weighted equally. Phylogenetic construction was performed using the neighbor-joining method and the Jukes-Cantor method[22].Statistical support for internal nodes was estimated by 100 bootstrap re-sample[23].The investigated samples were analyzed together with four homologous sequences of *Hordeum* species represent*H.Chilense* (AJ607872),*H. brevisubulatum* (AJ607853), *H.marinum* sub sp*marinum* (AJ607984) and *H. marinum* subsp. *gussoneanum*(AJ607976)obtainedfrom GenBank. As well as *Sorghum bicolor*(SBU04789) and *Sorghum nitidum* (U04797.1) that belong to the tribe *Triticeae*were used as an out-group in the construction of the phylogenetic tree.

Table 1: List of the species and sequences used for phylogenetic analysis

Species	Subspecies	Ploidy	Genome group	Accession number(IG)	Origin/Province	Source	Sequence name	Accession numbers
H. vulgare	subsp. *vulgare*	2x=14	**I**	[a]Barley Line	Syria	[b]ICARDA	*vulgarevulg*(1)	**KP008150**
			I	Furat 9 cultivar	Syria	[c]GCSAR	*vulgarevulg*(2)	**KP126677**
			I	Abiad cultivar	Syria	GCSAR	*vulgarevulg*(3)	**KP126676**
	subsp. *spontaneum*	2x=14	**I**	IG39918	Syria /Sweida	ICARDA	*vulgarespon*(1)	**KP126675**
			I	IG38639	Syria/Al Hasakah	ICARDA	*vulgarespon*(2)	**KP126673**
			I	IG39843	Syria /Raqqa	ICARDA	*vulgarespon*(3)	**KP126674**
H .bulbosum	subsp. *bulbosum*	4x=28	**I**	collecting	Syria/Homs	[d]NCBT	*bulbosum.bulb*(1)	**KP126667**
			I	collecting	Syria/Damascus	NCBT	*bulbosum.bulb*(2)	**KP126668**
			I	collecting	Syria/Sweida	NCBT	*bulbosum.bulb*(3)	**KP126669**
H. murinum	subsp. *glaucum*	2x=14	**Xu**	IG39948	Russia	ICARDA	*murinumglauc*	**KP126671**
	subsp. *leporinum*	4x=28	**Xu**	IG48945	Iran/Tehran	ICARDA	*murinumlep*	**KP126672**
H .marinum	subsp. *gussoneanum*	4x=28	**Xa**	IG39887	Cyprus/Kyrenia	ICARDA	*marinumguss*	**KP126670**

[a]**Barley Line**: Man/Huiz//M69-69/3/Apm/Rl//H272/4/CP/Bra/5/Joso'S'/6/Hyb85-6/7/Aths 'ICB95-0224-0AP-9AP-0AP-8TR-5TR-0AP'
[b]**ICARDA**: International Center for Agricultural Research in the Dry Area
[c]**GCSAR**: General Commission for Scientific Agricultural Research in Syria
[d]**NCBT**: National Commission for Biotechnology

Results and Discussion:

A single PCR product ranged approximately from 653 to 655 bp was obtained from each studied genotype. The number of aligned sites used for this analysis was 608bp only, because the two segments;18S Ribosomal RNA at the 5' end of ITS1 and26S Ribosomal RNA at the 3' end of ITS2, were not included in the analysis. The ITS1 varied in length (220and 222 bp), whereas the length of 5.8S and ITS2 sequences were uniformly; 161 and 223 bp, respectively. The length variation observed in the ITS1 in *Hordeum* species was caused by an indels. The deletion within ITS1 believed to interfere with rRNA processing by inhibited production of mature small and large subunit rRNAs[24,25], whereas certain deletions and point mutations in ITS2 prevented or reduced processing of large subunitrRNA[26].

The GC content of ITS1 ranged between 56.81% and 59.9%, for ITS2 ranged between 59.64% and 62.78%, and for 5.8S rDNA ranged between 59.62% and 60.24% (table2). This is somewhat similar to the findings for *Hordeum* and *Triticum*[27,14,15,16,28]. As a result, the GC content of ITS2 is relatively high compared to ITS1, which is compatible with theresult of Zhanget al.[29], who indicated that GC content of tetraploid wheat varied from 59% to 62% for ITS1 and from 60% to 65% for ITS2.

Table 2:Length and GC Content of ITS1,5.8S and ITS2regions

Genotypes	ITS1		ITS2		5.8S	
	Size (bp)	%GC	Size (bp)	%GC	Size (bp)	%GC
H.vulgaresubspvulgare						
vulgarevulg(1)	220	56.81	223	62.33	161	59.62
vulgarevulg(2)	220	56.81	223	62.33	161	59.62
vulgarevulg(3)	220	56.81	223	62.33	161	59.62
H.vulgaresubspspotaneum						
vulgarespon (1)	220	56.81	223	62.33	161	59.62
vulgarespon (2)	220	56.81	223	62.33	161	59.62
vulgarespon (3)	220	56.81	223	62.33	161	59.62
H.bulbosumsubspbulbosum						
bulbosum.bulb(1)	220	58.18	223	60.08	161	60.24
bulbosum.bulb(2)	220	58.18	223	59.64	161	60.24
bulbosum.bulb(3)	220	58.18	223	59.64	161	60.24
H.murinum						
murinumglauc	222	58.55	223	60.98	161	59.62
murinumlep	222	58.55	223	60.98	161	59.62
H.marinum						
marinumguss	222	59.9	223	62.78	161	59

Multiple Sequence Alignment (MSA) of the 12 sequences obtained from the 12 studied genotypes was constructed using ClustalW (Figure 1). Within entire ITS sequence region, out of 608 total nucleotides, 519 nucleotides were constant. On the other hand, 69 variable nucleotides were informative and 18 nucleotides were uninformative. ITS1 provides higher number of variable sites 46 (20.72%) compared with 39 (17.49%) sites of ITS2. In addition, ITS1 contributes a greater number of informative sites(38)than does ITS2 (30) (table 3).This level of divergence in ITS1 comparing with ITS2 is in agreement with previous studies[12,30,31].

Table 3: Sequence characteristics of ITS region of*Hordeum* species in this study

Transversions Pairs	8	3	0
	ITS1	**ITS2**	**5.8S**
No. of variable sites (%)	46 (20.72%)	39 (17.49%)	2 (1.24%)
No. of constant sites (%)	176 (79.28%)	184 (82.51%)	159 (98.75%)

No. of informative sites (%)	38 (16.96%)	30(13.45%)	1(0.62%)
No. of indels	4	0	0
Transitionsal Pairs	10	11	1

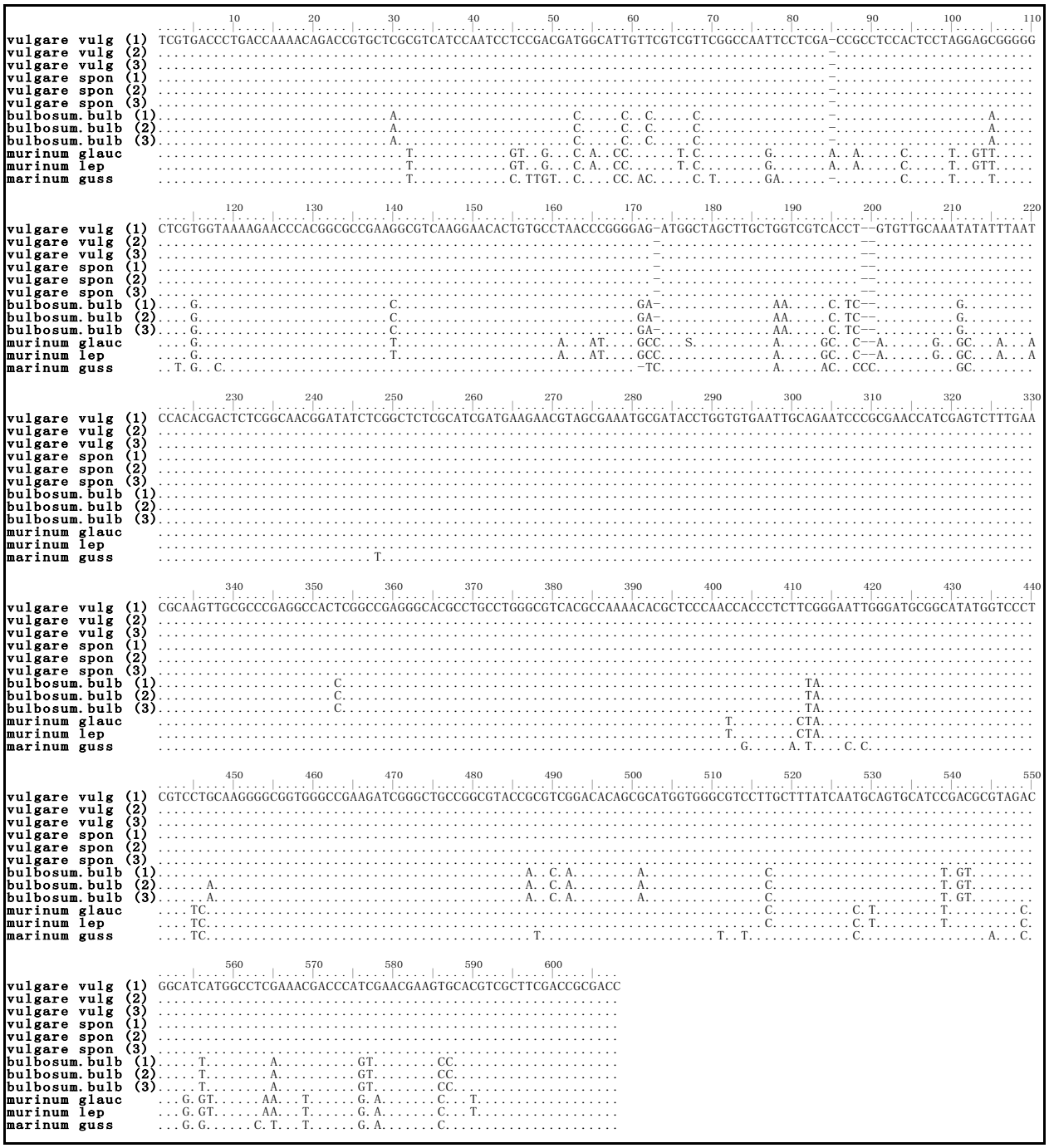

Figure 1 Multiple alignment of ITS sequence for 12 *Hordeum* genotypes constructed using ClustalW system

The transitions/transversions (ti/tv) ratio for a pair of sequences ranged from 1.61 to 3.25 (table 4). Our data are similar to that of Brown *et al*.[32], who found that transitions generally occurring more frequent than transversions. Also Wakeley[33] showed that ratio of the rates of transitional to transversional changes plays a role in evolutionary distance correction methods, and implemented in several evolutionary models.

We report here that 5.8S rDNA region was the most conserved compared to ITS1 and ITS2 regions, as it exhibited only two nucleotide base substitutions (transitions) at position number 24 bp (T↔C) for *H. marnium* and 129bp (T↔C) for *H. bulbosum* species. Pairwise divergence values (0.0 to 0.013) from comparison of 5.8S sequences were much lower than both ITS1 and ITS2 regions, which is in agreement with

other studies[34,35,28] that emphasized that the 5.8S region is identical and less different than both of ITS1 and ITS2 regions in the two related genera *Hordeum* and *Triticum*.

In this study low levels of ITS sequence divergence were observed[16]. The sequence divergence of ITS1 ranged from 0.0 to 0.174, and it is relatively higher than that of ITS2 ranged from 0.0 to 0.152 [12,30,31]. The ITS sequences of three *H. vulgare*, three *H. spontaneum*, three *H. bulbosum*, and two *H. murinum* genotypes were used to investigate the intraspecific genetic variation in ITS region. Overall, sequences of each species were identical except for *bulbosum.bulb*(2), which has one substitution at position number 447 bp (G↔A) sequence divergence ranged from 0.000 to 0.002 (Kimura 2-parameter distances). Generally, ITS sequences of cultivated varieties of barley are identical due to the highly conserved nature of this region[35,15], therefore the internal transcribed spacer can be useful in determining interspecies [36,37,38], and sometimes intraspecies relationships[39].

Table 4: Estimation of evolutionary divergence of entire ITS regions among 12 genotypes of *Hordum*,using Kimura 2-Parameter model (the lower-left matrix) and the numbers of transitions/transversions (upper-right).

No	Sequence name	1	2	3	4	5	6	7	8	9	10	11	12
1	*vulgarevulg*(1)		0	0	0	0	0	25/8	26/8	26/8	33/22	33/22	32/18
2	*vulgarevulg*(2)	0.000		0	0	0	0	25/8	26/8	26/8	33/22	33/22	32/18
3	*vulgarevulg*(3)	0.000	0.000		0	0	0	25/8	26/8	26/8	33/22	33/22	32/18
4	*vulgarespon*(1)	0.000	0.000	0.000		0	0	25/8	26/8	26/8	33/22	33/22	32/18
5	*vulgarespon*(2)	0.000	0.000	0.000	0.000		0	25/8	26/8	26/8	33/22	33/22	32/18
6	*vulgarespon*(3)	0.000	0.000	0.000	0.000	0.000		25/8	26/8	26/8	33/22	33/22	32/18
7	*bulbosum.bulb*(1)	0.057	0.057	0.057	0.057	0.057	0.057		1/0	1/0	33/20	33/20	36/18
8	*bulbosum.bulb*(2)	0.059	0.059	0.059	0.059	0.059	0.059	0.002		0	34/20	34/20	37/18
9	*bulbosum.bulb*(3)	0.059	0.059	0.059	0.059	0.059	0.059	0.002	0.000		34/20	34/20	37/18
10	*murinumglauc*	0.098	0.098	0.098	0.098	0.098	0.098	0.092	0.094	0.094		0	30/16
11	*murinumlep*	0.098	0.098	0.098	0.098	0.098	0.098	0.092	0.094	0.094	0.000		30/16
12	*marinumguss*	0.088	0.088	0.088	0.088	0.088	0.088	0.096	0.098	0.098	0.081	0.081	

Note:*distances in the lower-left matrix*: gaps and missing information were removed from the analysis (pairwise distance computation)

The sequence of the ITS regions did not reveal any variation between *H. vulgare* and *H. spontaneum*. However, 33 variant sites were recorded in the same regions with *H. bulbosum*. This result is highly concordant with that of Shao[40], Zohary and Hopf[41]who postulated that *H. spontaneum* is the oldest ancestor, and the first stage of evolution of cultivated barley.

The sequence divergence between *H. vulgare* and *H. murinum* (0.098) was greater than the divergence between *H. vulagre* and *H. marinum* (0.088) (table 5).These results disagree with the findings of Bothmer[1]who revealed that *H. murinum* as the closest wild species to *H. vulgare*.

Table 5: Conserved motif in ITS1 of some plant species

Species	Sequence
*H.vulgare*subsp*vulgare*	GGCGCCGAA**G**GCGTCAAGGAA
*H.vulgare*subsp*spotaneum*	GGCGCCGAA**G**GCGTCAAGGAA
*H.bulbosum*subsp*bulbosum*	GGCGCCGAA**C**GCGTCAAGGAA
*H.murinum*subsp*glaucum*	GGCGCCGAA**T**GCGTCAAGGAA
*H.murinum*subsp*leporinum*	GGCGCCGAA**T**GCGTCAAGGAA
*H.marinum*subsp*gussoneanum*	GGCGCCGAA**G**GCGTCAAGGAA
Triticumaestivum	GGCGCCGAA**G**GCGTCAAGGAA
Oryza sativa	GGCGCCGA**CG**GCGTCAAGGAA
Arabidopsis thaliana	GGCACG-AAAAG**T**GTCAAGGAA

A conserved motif within ITS1 region was identified, as the conserved sequences GGCGCCGAA (B) GCGTCAAGGAA (where B= C or T or G) were present in ITS1 sequence. In table 5 the variance in this conserved motif among Hordeum genotypes, Oryza sativa and Arabidopsis thaliana were presented. In previous studies on flowering plants, this conserved motif has been reported in the middle of ITS1 which was presumed to work as a recognition site for processing of a primary transcript into the structural rRNA. Identification of such conserved motif will facilitate alignment of sequences for phylogenetic analysis [42].

Phylogenetic tree construction of the *Hordeum* Species:

The phylogenetic relationships within and among *Hordeum* species were studied using substitutions in the sequences of ITS-1, ITS-2 and 5.8S regions. The phylogenetic analysis identified two well-resolved clades, supported by high bootstrap values attached to the out-group clade of *Sorghum*.

The first main clade consistedof two subclades; the first one contained *H. vulgare* subsp. *vulgare* and subsp*spontaneum*that grouped together with bootstrap 100%, while *H. bulbosum* formed the second subclade. This position highly corroborated bythe previous study [18].

The second main clade subdivided into three subclades corresponding to two sublclades including *H. marinum* group,*H. chilense* and *H. brevisubulatum* group, which clustered together with the third subclade*H. murinum* group (bootstrap values 96%).

Phylogenetic analysis based on the ITS sequences of *Hordeum* species distinguished unambiguously the four basic *Hordeum* genomes (Fig. 2), as the groups reflect the taxonomical classification within the genus *Hordeum*[2,43,44].

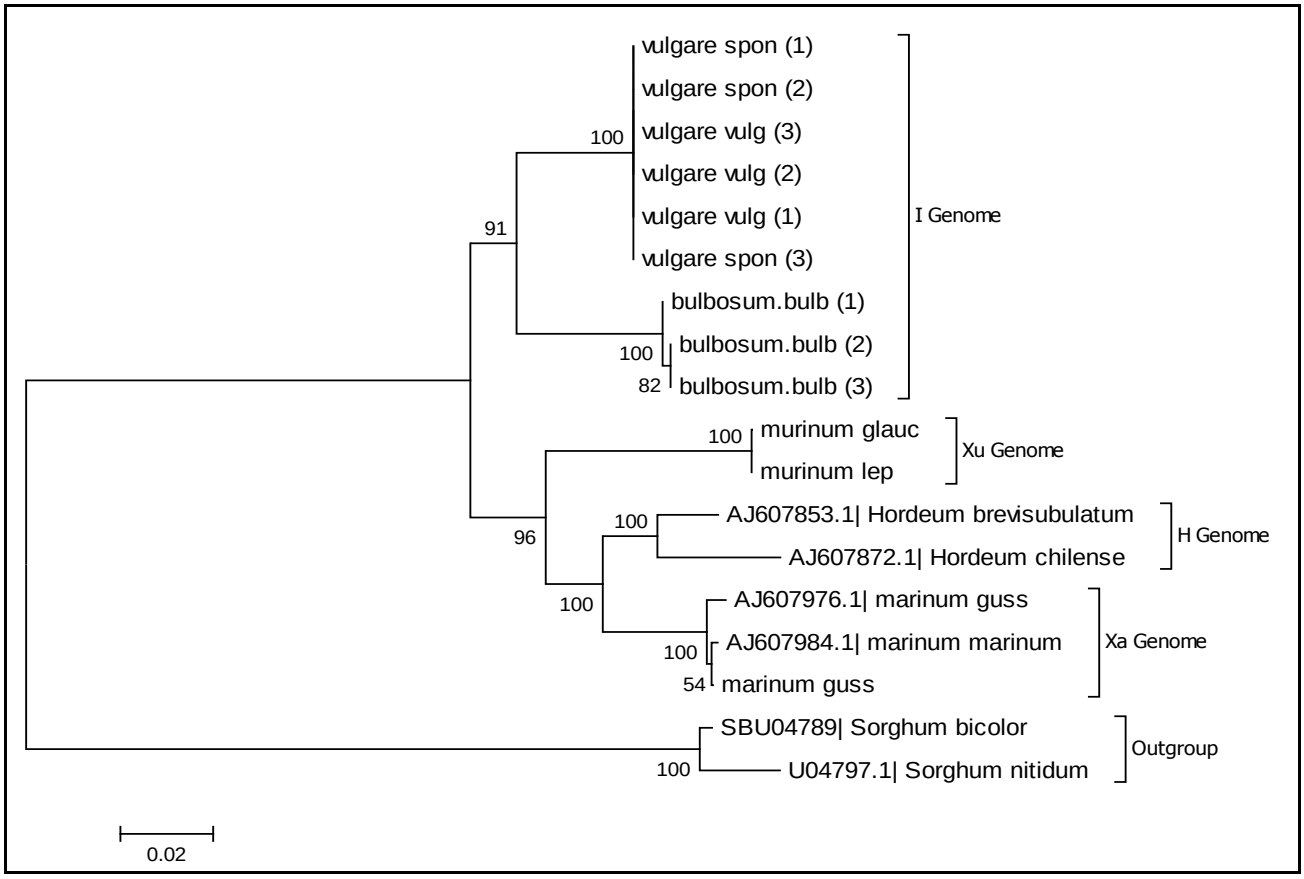

Figure 2 Phylogenetic tree of *Hordeum* based on ITS region sequences using the Neighbor-Joining method was constructed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method.

In conclusion, ITS region was a convenient marker in confirming the species, revealing hidden diversity in *Hordeum* species and its evolutionary construction. Although ITS analysis was sensitive enough to identify the interspecific variation, but it was not suitable for intraspecific level studies, as sequences of ITS region were highly conserved within the same species. Our data of ITS-rDNA have strengthened the view that the *H. bulbosum* is phylogenetically closest to *H. vulgare*. Our findings were in a good agreement with the classification system which suggested dividing the genus into four major groups according to the genomes H, Xa, I, and Xu. Finally, the nucleotide differences may prove to be more useful, especially when sequence data are used either for PCR or probe development.

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