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Study of Genetic Diversity among Rainfed Barley Genotypes Using ISJ Markers and Morphological Traits

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Abstract

Barley ranks below wheat, corn and rice in total world production. Barley is one of the most important crops in Iran. In this study, genetic diversity of 20 rainfed barley genotypes were assessed using morphological traits as well as 20 primers of ISJ semi-random markers. There were significant differences among genotypes for the all traits, indicating high genetic variation among barley germplasm. Based on molecular data, 133 bands were detected and 89 bands were polymorph. The mean number of bands was 6.65 per primer. According to cluster analysis of similarity matrix of molecular and Euclidean distances of morphological data, similarities ranged between 0.42-0.85 and 1.44-43.22, respectively. Based on molecular data and morphological traits, the highest similarities were belonged to genotypes number 2, 8 (0.85), and 5, 8 (1.44) respectively. The results showed that intron-exon splice junction (ISJ) markers and morphological traits rather could distinct two and six-rowed and also hulless and hulled barley genotypes. Distinction of two clusters did not follow the same pattern.

Keywords: rainfed barley, morphological traits, genetic diversity, ISJ

1. Introduction

Barley (*Hordeum vul*gare L. subsp. *vulgare*) is one of the earliest domesticated crop plants, this crop is a high value crop in large parts of arid and drought inflicted regions. In recent years, barley is becoming an important food grain for human consumption due to its nutritional and clinical values (Bathy, 1999; Gill et al., 2002). The development of high-yielding cultivars with improved quality and resistance/tolerance to biotic and abiotic stresses is the main aim of modern barley breeding. The genetic structure and phenotypic expression of a quantitative trait are highly influenced by environmental factors (Breese, 1969). During the last two decades the development of wide range of DNA markers (RFLP, RAPDs, AFLPs, SSRs, STSs and SNPs) and their use in genome analysis has provided unprecedented insight into structural features of the barley genome (Graner et al., 2004). Strelchenko et al. (1999) studied the genetic differentiation of barley from principal cultivation regions of the world using RAPDs, and reported three distinct groups that can be related to the evolutionary directions and geographical distribution of the crop. The first group indicated the westward distribution of barley from west Asia to Europe and New world across Ethiopia and then Mediterranean region. The second group was associated with eastward distribution of the crop and comprised the East Asian and central Asian accessions, and the third group represented the evolution and dissemination of hulless barley in central Asia and Caucasus region. The genetic diversity of *H. spontaneum* in the Australian germplasm collection was investigated using the polymerase chain reaction with random and semi-random primers. The results indicate that the genetic diversity of the wild barleys is broadly correlated with geographic distribution (Weining and Henry, 1995). Vahabi et al. (2008) assessed diversity of 22 populations of *plantago ovata* using RAPD, ISJ and morphological markers. Cluster analysis based on RAPD showed that a closely association exists among morphological and RAPD dendrograms, while there is no accordance between ISJ-genetic similarity with RAPD and morphological variation. Manjunatha et al. (2007) used morphological and RAPD descriptors of a collection of 70 barley landraces from the higher Himalayan ranges of Uttaranchal state to analyze levels of genetic diversity. A wide range of variation was recorded for various morphological characters in univariate analysis. Clustering based on qualitative traits, however, clearly distinguished naked and hulled forms. RAPD profiles efficiently differentiated

naked barleys from hulled forms, but could not differentiate between oriental and occidental hulled forms. More diversity was observed in hulled barleys than the naked forms, both for morphological and RAPD markers. Eshghi and Akhundova (2010) investigated genetic diversity among 63 hulless barley accessions originating from ICARDA using agro morphological traits, seed storage protein and RAPD variation. The RAPD-based genetic similarity ranged from 0.22 to 0.81, with the mean of 0.48. Cluster analysis based on morphological traits divided genotypes into eight different groups, genotypes with high grain yield were clustered in same group. The average of genetic diversity index for RAPDs and storage proteins were compared and showed that mean of genetic diversity index was less for RAPDs than storage proteins. Hamza et al. (2004) assessed the genetic diversity of 26 Tunisian winter barley cultivars/landraces with 17 SSRs, and reported a moderate level of diversity in this sample (DI=0.45). Moreover, the UPGMA cluster analysis based on SSRs data and morphological data clearly differentiated the local landraces from modern varieties. A good correspondence was found between the clusters based on SSRs and morphological data. Russell et al. (1997) compared the level of polymorphism detected by RFLP, AFLP, SSRs and RAPDs markers, by analyzing 18 barley cultivars. The SSRs detected the highest level of polymorphism compared to other three assays. Some approaches aimed at improving the RAPD analysis, such as the selection of large number of primers and the time and cost of the PCR analysis. PCR based system with semi-random primers targeting the Intron-exon Splice Junction (ISJ), proposed by Weining and Langridge (1991) and developed by Rafalski et al. (2001). The sequences of primers were based on the consensus sequences of the ISJ, 7 and 9 bases in length, common for plants and necessary for effective splicing (Brown, 1986). The additional bases were added at random to extend the length of the primers (Rafalski et al., 1997). The present study was carried out in order to estimate the extent of the genetic diversity in barley advanced lines and cultivars using morphological and ISJ molecular characterization data and comparing them.

2. Materials and Methods

2.1 Plant Materials and Field Experiments

Twenty rainfed barley genotypes were used in this study obtained from the Lorestan Agriculture and Natural Resources Research Center (Table 1). Genotypes were evaluated using a randomized complete block design with three replications under rainfed conditions. Each plot contained 4 rows with 20cm apart and 6m in length, and planted at rate of 300 seeds m^{-2}. Ten competitive random plants from the middle rows of the experimental plots were taken for recording the observations on plant height, number of grains $spike^{-1}$, awn length, peduncle length, spike length, 1000-kernel weight and grain yield trait.

Table 1. Name or pedigree and number of row, hulled and hulless of rainfed barley genotypes

No	Name or pedigree		
1	Mahor Wi2291/Emir	2 row	hulled
2	Viringas/3/h. Sponta.21-3/ Arar 84/w12269/4h	2 row	hulled
3	DD-21/4/Aliso/ C130.9.2// Hb602/3/Nala / So/ ya	2 row	hulless
4	Atahualpa/Tarida	2 row	hulled
5	W13159/6/Anca/2469/Toji/3/Shyri/4/Ataco/5/A	2 row	hulled
6	Izeh Lignee527/Savuson	6 row	hulled
7	W131180/4/Aliso/C139.902//Hb602/3/Mol/Shy	2 row	hulled
8	Atahualpa/DD-21	2 row	hulled
9	Caimr/ F6NB2/Khomes	6 row	hulled
10	Bkfmaguelone1604/3/Apro//SV.02109/Mari/U/Giza	6 row	hulled
11	Petunia1/8/Post/Copal/5/Gloria	6 row	hulless
12	DD-21	2 row	hulless
13	BF891M-59//Acc#116131-Coii#8901-44-Gizo	2 row	hulled
14	Atahvalpa/5/Alger/Ceres//SIS/3/ER/APM/4/W12197	6 row	hulled
15	Atahulpa/Barque	2 row	hulled
16	Soufara-o2/RM1508/POR/W12269/41/AML-O2/Arabi/Abiader/APM	2 row	hulled
17	Alanda/Zafara/Athualpa/5/Lignee527/Chno1//GU/Store/4/RHNO8/3/Deiiallon/106/PL71/Strain205	6 row	hulled
18	Alger/Ceres/SIS/3/En/APM/4/WI2197/Mazurka	2 row	hulled
19	Atahulpa/IPA99	6 row	hulled
20	2Hedarii2/Ndbii2//Mora/5/B1-BAR//Mari	2 row	hulled

2.2 DNA Isolation and ISJ Method

For each genotype, a number of seeds were grown in the greenhouse. Young leaves were collected from two-week old plants. The samples were stored at −80°C. DNA was isolated from 100–200mg of frozen leaves according to the CTAB protocol (Ausubel et al 1999). The DNA concentration of all samples was determined in 0.8% agarose gels. Primers were used in this study listed in table 2. The details of the intron-splice-junction primers were previously described by Weining and Langridge (1991). PCR was carried out in a 25 μ1 volume containing 10x PCR buffer, 2 μl of genomic DNA template, 0.8 μM of each primer (IT or ET), 200 μM each of dATP, dCTP, dGTP and dTTP, 50 mM KC1, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgC1_2$, and 1 units of Taq polymerase (Bioron). The PCR was performed in a Thermal Cycler (Bio-Rad Model thermal cycler). The first 7 cycles were at 94^0C for 1 min, 40^0C 1.8 min and 72^0C 2 min. This was followed by a further 28 cycles of 94^0C 1 min, 58 ^{0}C 1.5 min and 72^0C 2 min. PCR products were analyzed on 1.5% agarose gels and stained with Gel-Red, visualized under UV and pictured with Gel Documentation.

Table 2. ISJ primers used in the study

Primer	Sequence (5'-3')	P%*	Primer	Sequence (5'-3')	P%*
ET 2-18	ACTTACCTGCTGGCCGGA	42.86	IT 31-15	GAAGCCGCAGGTAAG	100
ET 4-18	ACTTACCTGCCTGCCGAG	66.67	IT 34-15	ACCTACCTGGCCGAG	60
ET 26-12	AGCAGGTGGACT	22.22	IT 35-15	CGAAGCCAGGTAAG	55.56
ET 27-12	AGCAGGTCCTAG	100	IT 10-1	ACGTCCAGAC	60
ET 28-12	AGCAGGTCGAAG	66.67	IT 10-2	ACGTCCAGGT	66.67
ET 29-12	AGCAGGTCGTGA	33.33	IT 10-3	ACGTCCAGCA	77.78
ET 30-12	AGCAGGTGGTAC	83.33	IT 10-4	ACGTCCACCA	75
ET 35-15	ACTTACCTGCCGCAG	75	IT 10-5	ACGTCCAGAG	85.71
ET 31-15	ACTTACCTGGGCCAG	100	IT 10-6	ACGTCCATCC	80
ET 10-18	ACTTACCTGAGGGCGAC	100	IT 36-15	ACCTACCTGGGGCTC	75

*: Polymorphism percentage

2.3 Statistical Analysis

Morphological data were analyzed using SAS (SAS Institute, 2002), and means were compared by Duncan's multiple range test at 0.05 probability level. Cluster and principal component analysis were also employed using SPSS (version 11.5) software. The ISJ markers were scored, depending on the presence (1) or absence (0) of the bands. It was also used in obtaining other results as well as similarity coefficient matrix of Jaccard (Jaccard, 1908). Based on similarity matrix, a dendrogram showing the genetic relationships between genotypes was constructed using the un-weighted pair group method with arithmetic average (UPGMA) through the software NTSYS-pc program (Rohlf, 1992).

3. Results and Discussion

3.1 Morphological Traits

Significant differences were observed among wheat genotypes for all morphological traits (Table 3). These results indicate the presence of high genetic variation among genotypes. Comparison means of traits are shown in Table 4. As it is shown, hulless barley genotypes have lower grain yield than hulled genotypes. Genotypes 4 and 16 with 3.70 and 3.51 grain yield (t ha^{-1}) scored the highest yield among genotypes under study. The hulless barleys were generally poor yielding than hulled barley; yields of current hulless barley are generally 10 to 20 percent lower than those of hulled barley genotypes, (Manjunatha et al., 2007). Since the hulless barley easily lose their husk cover of caryopses, its grain yield was less than hulled barley. Other trait means are shown in Table 3. Principal component analysis was performed for all traits under study (Table 5). The first two components could justify more than 62.46% of the whole variance in the original data. The first component could justify the most amount of variance among genotypes (37.28%). Rotate component matrix showed that awn length, plant height, and grain yield were in the first group (component1) and number of grains $spike^{-1}$, peduncle length, spike length and 1000-kernel weight were in the second group (component2). It can be

concluded that the traits of each group are correlated. According to similarity matrix of Euclidean distances, similarities ranged between 1.44-43.22, highest similarities were belonged to genotypes numbers 5 and 8 (1.44), these are two-rowed and have the most similarity especially in grain yield (Table 4). The least similarity belonged to genotypes 3 (hulless) and 16 (hulled), these genotypes are also two-rowed. Cluster analysis based on morphologic traits was carried out using Ward method (Figure 1) if the cutting is done on the distance 16; genotypes are divided into five major groups. Twelve genotypes were grouped in cluster-I, which are two-rowed, except No, 17. These genotypes had high grain yield. Cluster-II comprised two genotypes, which were low grain yield. Cluster-III contained two six-rowed genotypes. Two hulless barley fall in cluster-IV and one of them falls in cluster-V. It can be concluded that cluster analysis based on morphological traits could rather distinct genotypes. Although morphological traits are influenced by environment, in some cases they can be used for plant genetic diversity investigation.

Table 3. ANOVAs of morphological traits in rainfed barley genotypes

S.O.V.	df	Plant height	Number of grains spike^{-1}	Awn length	Peduncle length	Spike length	1000-kernel weight	Grain yield
Replication	2	166.86	3738.81	8.22	46.50	1.59	113.21	9.99
Genotype	19	253**	8455.09**	10.43**	26.84**	2.20**	39.30**	1.06**
Error	38	53.59	1685.09	1.62	7.97	0.30	5.81	0.42
CV%		12.37	24.93	9.49	22.11	8.14	5.16	25

** :significant at 0.01 probability level.

Table 4. Mean comparison of morphological traits in rainfed barley genotypes

No	Plant height (cm)	Number of grain spike^{-1}	Awn length (cm)	Peduncle length (cm)	Spike length (cm)	1000-kernel weight (g)	Grain yield (kg h^{-1})
1	42.33 h	135.33 cd	14.51 bcde	9.23 cd	6.13 defg	46.33 bcd	3.05 abcd
2	50.40 fgh	104.67 d	10.91 h	7.03 d	7.01 cde	47.33 abcd	2.28 bcde
3	66.50 abcd	149.33 cd	15.51 bcd	6.81 d	9.05 a	47.66 abcd	1.8 de
4	63.06abcdef	140.67 cd	12.71 efgh	10.65 bcd	6.78 def	49 abc	3.7 a
5	56.16 cdfghe	108 d	11.65 gh	8.81 cd	6.58 def	51.66 a	2.88 abcde
6	50.56 fgh	194.67 bc	118.28 fgh	15.51 ab	5.40 g	47.33 abcd	3.41 abc
7	61.16 bcdef	134 cd	14.18 bcdef	13.55 abc	7.98 bc	50.66 ab	2.43 abcde
8	52.13 efgh	128.67 cd	12.63 efgh	11.16 bcd	6.46 def	51 ab	2.88 abcde
9	57.16 bcdeg	245 ab	11.61 gh	12.73 abc	5.78 bg	51.66 a	2.31bcde
10	67.76 abcd	253 ab	14 bcdefg	15.88 ab	6.28 defg	38.33 f	2.59 abcd
11	70.83 ab	287.33 a	15.80 bc	13.35 abc	6.99 cd	43.33 de	1.96 de
12	68.96 abc	248.33 ab	18.36 a	15.38 ab	8.28 ab	43.66 de	1.88 de
13	61.96 bcdef	162.67 dc	13.28 defgh	15.28 ab	5.93 efg	47.66 abcd	2.46 abcde
14	51.73 fgh	128.33 a	15.91 b	15.93 ab	7.19 cd	49.33 abc	2.15 cde
15	76.70 a	145 cd	11.71 fgh	17.01 a	6.65 def	49.66 abc	2.81 abcde
16	50.46 fgh	138.33 cd	12.70 efgh	148 ab	6.43 defg	43.66 de	3.51 ab
17	54.20 defgh	129 cd	12.45 efgh	13.98 abc	6.09 ed	47.66 abcd	2.76 abcde
18	47.46 gh	207 abc	13.15 defgh	12.6 abc	6.83 def	45.33 cde	3.08 abcd
19	68.33 abcd	134 cd	13.43 cdefg	11.94 abcd	7.01 cde	41 ed	1.62 e
20	65.06 abcde	128 cd	11.98 fgh	13.68 abc	6.31 defg	44.33 de	2.03 de

Means followed by same letters in each column are not significantly different at 0.05 levels of probability.

Table 5. The principal component analysis for morphological traits

Component	Eigen value	Cumulative percentage	Plant height	Number of grains spike^{-1}	Awn length	Peduncle length	Spike length	1000-kernel weight	Grain yield
1	2.61	37.28	0.69	0.56	0.80	0.26	0.60	-0.51	-0.68
2	1.76	62.46	0.08	0.57	-0.12	0.76	-0.68	-0.42	0.41

3.2 ISJ Data Analyses

In the present study, 20 ISJ primers belonged to two groups of Intron Targeting (IT) and Exon Targeting (ET) that showed a high level of polymorphism and a high number of clearly amplified bands (Figure 2). Primers generated 133 bands (89 polymorphic bands) that their sizes were varied from 100–1500 bp, the mean number of bands was 6.8 per primer. Rafalski et al. (2002) used semi-random primers in rye genotypes and reported that average number of bands per primer is 8.9. The highest number of polymorph band was obtained with primers IT10-3 and IT10-1, while the lowest number was obtained with primer ET26-12. Generally, IT primers in comparison with the ET primers generated polymorphic fragments with higher resolution; these findings are in accordance with the results of Vahabi et al. (2008) in Plantago. Polymorphism percentage ranged from 22.22 to 100% (Table 2). ISJ data were utilized to calculate the genetic similarity matrix using Jaccard similarity coefficient and UPGMA method. Similarities ranged between 0.42-0.85, the lowest genetic similarity (42%) was found between genotypes numbers 12 (hulless) and 18 (hulled), while the highest (85%) was observed between genotypes number 2 and 8, both genotypes are hulled and two-rowed barley. Genetic similarities among the genotypes are presented as a dendrogram (Figure 3). If the cutting is done on the 70% similarity coefficient, genotypes will divided into five major groups. Cluster-I comprised two hulled barleys, cluster-II contained maximum number (14) of genotypes that were mainly two-rowed and hulled barleys.

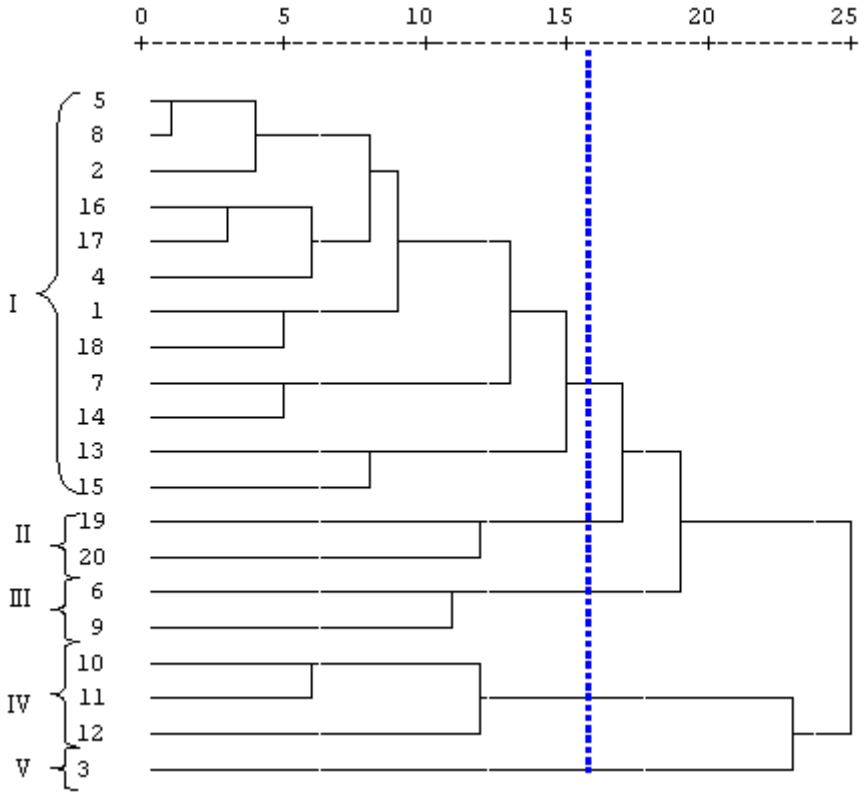

Figure 1. Dendrogram of rainfed barley genotypes using ward method based on Euclidian distance for morphological traits

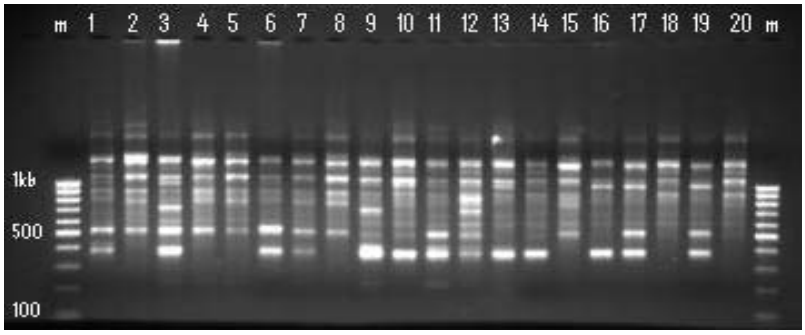

Figure 2. Banding pattern of rainfed barley genotypes using IT10-1primer in agaros gel

Only one genotype grouped in each of the cluster-III and IV that were two-rowed and hulled barley. Cluster-V comprised two hulless and two-rowed barleys. Thus, the marker can well separate hulless and two-rowed genotypes, also hulled and two-rowed genotypes groups in one cluster. So ISJ markers almost succeed in separation of genotypes.

3.3 Comparison between Morphological Traits and ISJ Polymorphisms

The morphological and ISJ data gives two dendograms with five groups that were not completely corresponding; however there were some concordances between them. Two dendrograms distinct hulless and two-rowed barley efficiently (No 3 and 12) similarity in both denrograms majority of two-rowed barley were in one group. Although morphological traits are strongly associated with environmental variation and the morphological similarities observed may be due to the under laying genetic differences, the present study showed that some morphological similarities may have molecular basis. Manjunatha et al (2007) in barley found that clustering based on qualitative traits and RAPD marker, clearly distinguished naked and hulled forms. Eshghi and Akhundova (2010) in hulless barley reported that there is no correlation between RAPD marker and morphological traits. Hamza et al. (2004) showed that the correlation between SSR markers and morphological traits was highly significant, and the correspondence between the clustering based on DNA markers and morphological data was relatively good. In some cases, two clusters do not match. For example, both markers could not well separated six-rowed genotypes, although in this case, cluster analysis based on morphological traits had better result, as genotypes number 3, 11 and 12 were closely related in the morphological based analysis, in ISJ analysis only genotypes number 3 and 12 were related. One reason probably is that genotypes number 3 and 12 are two-rowed, while genotypes number 11is six-rowed. Semagn (2002) suggested two reasons for low correlation between DNA markers and morphological data: (1) DNA markers cover a larger proportion of the genome, including coding and noncoding regions, than the morphological markers. (2) DNA markers are less subjected to artificial selection compared with morphological markers. Martinez et al. (2003) and Salem et al. (2008) believed that the correspondence between different methods might be improved by analyzing more morphological characters, and DNA markers. The present study showed a large amount of genetic variation which exists between rainfed barley genotypes can be used efficiently to select parent for improved genotypes. Genotypes that have the greatest genetic distance can be used as the parent in breeding programs.

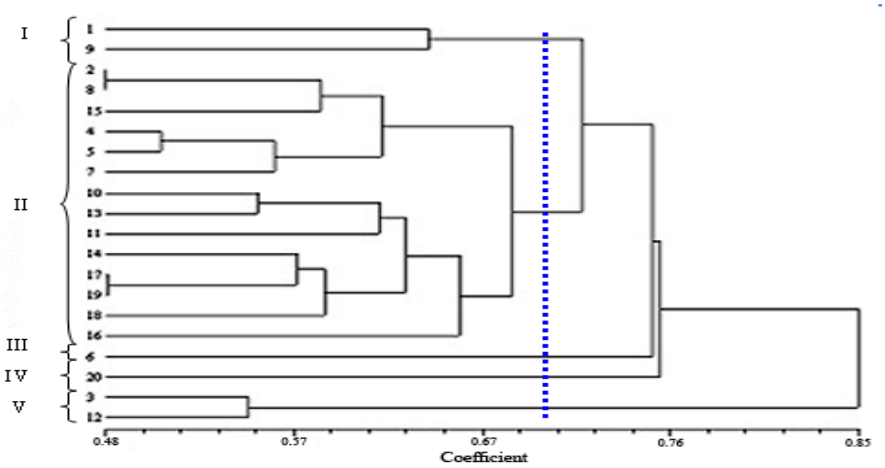

Figure 3. Dendrogram of rainfed barley genotypes using UPGMA method based on Jaccard's coefficient for ISJ data

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