Genetic Diversity of Tunisian Local Barley Accessions Analyzed with Morphological and RAPD Makers: Relationship between the Two Methods

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ABSTRACT

Thirteen Tunisian barley (Hordeum vulgare L.) accessions were collected from different regions. Morphological traits were studied and analyzed using principal components analysis (PCA) and clusters were constrained based on median joining distance. Further, genetic diversity was studied using RAPD markers. The cultivate variety ‘Martin’ was added in these essays. Both methods were used to compare how morphological traits and RAPD molecular markers described accessions relationship. Both analysis results showed a high degree of variation among analyzed accessions, indicating an important source of genetic diversity that can be used in future breeding programs. Morphological PCA traits and cluster indicated geographic information. Indeed, they grouped barley accessions according to genetic criteria such as ear attitude, length of glum and its awn relative to grain. Distances obtained by each of the approaches were compared with special attention to the coincidences and divergences between the two methods. Comparison of morphological and molecular data using the Mantel test indicated a very low correlation (r = 0.12). Therefore, both techniques are not complementary but necessary for barley characterization.

Key words: Barley accessions, Mantel test, morphological traits, RAPD markers, genetic diversity.

INTRODUCTION

Barley (Hordeum vulgare L.) was domesticated for 10,000 years ago from the wild barley (Hordeum spontaneum C. Koch) (Zohary and Hopf, 1998). It is used for human consumption, fodder, brewing and whiskey production.

Barley is a major cereal crop in Tunisia and is of great importance as forage species. It had been the subject of intensive genome mapping and quantitative trait dissection efforts. Barley is raking fourth in the world after rice, wheat and maize (Forster et al., 2000).

Local barley is of six rows ear and presenting a genome of 2n = 2x = 14. In Tunisia, since the beginning
of the cereal improvement program, we have registered only 15 varieties which make a narrow genetic diversity. In the opposite, we have more than 30 varieties of durum and bread wheat officially recorded (El Faleh, 1998).

Genetic barley erosion could be avoided through the establishment of a local genotype resources collection and conservation. Consequently, genetic diversity, identification and maintain of our local resources should be achieved to be used in breeding programs.

In this study, thirteen local barley accessions collected from different region and an improved variety “Martin” were described using some morphological traits. Analysis of principal components (PCA) was also dealt. Morphological distance clustering and phenologic RAPD clustering were compared to estimate correlation between morphological traits and RAPD markers.

**MATERIALS AND METHODS**

**Plant material and experimental design**

Thirteen-six row accessions were collected from different Tunisian regions and named according to their origin. In addition, a cultivated variety ‘Martin’ was used as a control. Climatic stage of each collect region is given on Figure 1. Seeds of each accession were sown in pots in three replications. The experiment was carried out at field capacity in the National Agronomic Research Institute of Tunisia (INRAT). Morphological measurements of 16 traits were assigned (Table 1) using UPOV scale, which attributes ranges for non-quantitative characters. The growth habit stage and the presence of leaf sheaths hairiness of the lowest leaves were noticed at the tillering stage. The other characters were recorded after ear emergence and during ripening period. Analyses of principal components (PCA) were dealt and also were morphological distance clustering and phenologic RAPD markers tree.

**DNA extraction, PCR amplification and electrophoresis**

To extract DNA from barley leaves or seed embryos, many protocols were described in the literature (Murray and Thompson, 1980; Saghai-Maroof et al., 1984; Webb and Knapp, 1990). However, in this study, genomic DNA was extracted and purified from 100 mg seed embryos according to Ben Naceur (1998) protocol, which combines three methods and includes high CTAB concentration to overcome polysaccharide contamination. DNA was then quantified at 260 nm using a spectrophotometer (standard CECIL CE2501 series.

![Figure 1. Collection sites of 13 local barley accessions: Tozeur 1 (a), Tozeur 2 (b), Kébíli 1 (c), Kébíli 2 (d), Kébíli 3 (e), Kasserine (f), Sidi Bouzid (g), Jendouba 1 (h), Jendouba 2 (i), Souihli (j), Kalaâ (l), Kélibia 1 (m) and Kélibia 2 (n).]
Table 1. Morphological and phenological traits in *Hordeum vulgare* L.

<table>
<thead>
<tr>
<th>Characters</th>
<th>Abbreviations</th>
<th>Signification</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>GH</td>
<td>Growth habit</td>
</tr>
<tr>
<td>C2</td>
<td>HLS</td>
<td>Lowest leaves: hairiness of leaf sheaths</td>
</tr>
<tr>
<td>C3</td>
<td>TEE</td>
<td>Time of ear emergence</td>
</tr>
<tr>
<td>C4</td>
<td>EAt</td>
<td>Ear: attitude</td>
</tr>
<tr>
<td>C5</td>
<td>PL</td>
<td>Plant: length cm (stem, ear and awns)</td>
</tr>
<tr>
<td>C6</td>
<td>ES</td>
<td>Ear: shape</td>
</tr>
<tr>
<td>C7</td>
<td>ED</td>
<td>Ear: density</td>
</tr>
<tr>
<td>C8</td>
<td>EL</td>
<td>Ear: length (excluding awns)</td>
</tr>
<tr>
<td>C9</td>
<td>AwL</td>
<td>Awn: length (compared to ear)</td>
</tr>
<tr>
<td>C10</td>
<td>RLfs</td>
<td>Rachis: length of first segment</td>
</tr>
<tr>
<td>C11</td>
<td>RCfs</td>
<td>Rachis: curvature of first segment</td>
</tr>
<tr>
<td>C12</td>
<td>SSAt</td>
<td>Sterile spikelet: attitude (in mid-third of ear)</td>
</tr>
<tr>
<td>C13</td>
<td>MSLG</td>
<td>Median spikelet: length of glume and its awn relative to grain</td>
</tr>
<tr>
<td>C14</td>
<td>GRAT</td>
<td>Grain: rachilla hair type</td>
</tr>
<tr>
<td>C15</td>
<td>HVF</td>
<td>Hairiness of ventral furrow</td>
</tr>
<tr>
<td>C16</td>
<td>GDL</td>
<td>Grain: disposition of lodicules</td>
</tr>
</tbody>
</table>

2000/3000); the OD$_{260}$/OD$_{280}$ ratio was calculated to determine DNA purity. For this fact, 5 µl of each DNA were diluted in 995 µl of buffer TRIS-EDTA (TE). The Concentration (C) of the DNA was calculated using the formula:

$$C \, (\mu g. \, \mu l^{-1}) = OD(260) \times 10$$

Forty RAPD primers (Operon B and E) were tested on DNA samples. Some primers which generate variable amplification, gave poor amplification products or non-repeatable banding patterns were discarded. Only primers that showed bands are used for PCR amplification (Table 2). DNA amplification was carried out in a final volume of 25 µl containing 12 µl of 10× ready mix (buffer with MgCl$_2$, dNTP and Taq polymerase), 20 µM of Operon primer, 20 ng/µl of DNA and adjusted with distilled water. The program of amplification; using a thermocycler (Biometra UNO II); consisted of a pre-denaturation cycle of 2 min at 94°C, 35 cycles of a denaturation for 30 second at 94°C, an hybridization for 30 seconds at 50°C, an extension for 30 seconds at 72°C followed by a post-extension cycle for 5 min at 72°C.

The amplification products of each primer were electrophoresed at 80V for 1 hr in horizontal 1% agarose gel and 1×TAE (TRIS Acetate EDTA) buffer and stained with Ethidium Bromide. Bands were visualized under UV waves on a Polaroid camera system. Only clear and reproducible bands were considered to make binary matrix, study similarity and discuss polymorphism among accessions. RAPD clustering was compared to estimate correlation between morphological traits and RAPD markers.

**Statistical analysis**

The PCA was dealt and the genetic distances between the different accessions were calculated on the centered and standardized variates using measured data. Cluster analysis was conducted on the distances matrix with MVSP 3.13 software (Kovach, 1993). The relationship between the morphological distance matrix and the distance obtained with RAPD markers was analyzed.

The comparison between the dendrograms is not fully adequate in some cases. Thus, the relationship was performed according to the approach developed by Mantel (1967) using MxComp procedure from NTSYS 2.02 program (Rholf, 1993). The principle of this approach is to compare the observed $Z$-value or $r$-value with its permutational distribution according to a null hypothesis (no difference between the distance matrix, $Z$...
### RESULTS

#### Molecular study

Only 10 primers were able to generate visible and reproducible bands (Table 2). The size of bands varies from 300 to 2000 bp. The dendrogram of genetic distances (Fig. 2a) was constructed based on UPGMA Method using midpoint-joining procedure of Nei and Li, (1979) dissimilarity matrix.

According to genetic distances dendrogram obtained by RAPD markers and referring to a similarity rate of 65, we distinguished five groups. The first group is composed of two sub-groups (‘Tozeur 2’, ‘Kébéli 3’, ‘Kalaâ’, ‘Martin’ and ‘Kélibia 2’) and (‘Jendouba 1’,

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**Table 2.** Primer sequences generating polymorphic bands.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primers sequences</th>
<th>Number of total bands</th>
<th>Number of polymorphic bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Op E-03</td>
<td>5'-CCA-GAT-GCA-C-3'</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Op E-07</td>
<td>5'-AGA-TGC-AGC-C-3'</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Op E-12</td>
<td>5'-TTA-TCG-CCC-C-3'</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Op E-15</td>
<td>5'-ACG-CAC-AAC-C-3'</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Op E-20</td>
<td>5'-AAC-GGT-GAC-C-3'</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Op B-1</td>
<td>5'-GTT-TCG-CTC-C-3'</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Op B-5</td>
<td>5'-TGC-GCC-CTT-C-3'</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Op B-8</td>
<td>5'-GTC-CAC-ACG-G-3'</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Op B-10</td>
<td>5'-CTG-CTG-GGA-C-3'</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Op B-18</td>
<td>5'-CCA-CAG-CAG-T-3'</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>50</td>
<td>48</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>5</td>
<td>4.8</td>
</tr>
</tbody>
</table>

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In this comparison, 5000 random permutations were made.

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**Figure 2.** Genetic distances (a) and morphologic classification (b) of barley accessions. a) Phylogenetic dissimilarity distance generated by RAPD markers using UPGMA procedure according to Nei and Li (1979) method. b) Hierarchical classification of the 14 accessions based on median constrained distance using MVSP 3.131.
‘Jendouba 2’ and ‘Sidi Bouzid’). The similarity percentage between accessions of the first sub-group, varies between 77.42 and 90.77; but that of the second sub-group was ranged between 68 and 76. The accessions ‘Kébili 1’, ‘Kébili 2’ and ‘Kasserine’ form the second group. Their similarities vary from 73.17 to 82.93. Finally, the accessions ‘Tozeur 1’, ‘Kébili 1’ and ‘Souihli’ form, respectively, groups III, IV and V. The Souihli’s distance in comparison with ‘Tozeur 1’ and ‘Kébili 1’ is of 93.33 and 75 respectively. Despite being collected from the same region, the accession ‘Tozeur 1’ is distant from ‘Tozeur 2’; ‘Kébili 3’ is distant from ‘Kébili 1’ and ‘Kébili 2’ since they are classified in different groups. The same case is observed for the accessions ‘Kébili 1’ and ‘Kébili 2’. It’s also to be remarked that the group consisted of Souihli is very far away from the others.

**Principal components analysis**

Considering the Eigenvalue of 0.8 (0.77) as suggested by Tomassone et al. (1993), principal components analysis grouped variables in six components which explained up to 90.61% of the total variance. The two first axes were considered as they elucidate the maximum simple variance (respectively 25.4 and 19.29% of the total variation) with a cumulative variance of 44.67%.

The principal components assembly accessions into four groups. The first group consisted of ‘Kébili 1’ and ‘Tozeur 2’ that are positively correlated to both axis one and two. The second one formed by ‘Martin’, ‘Kébili 2 and 3’, which are negatively correlated to the second axis. The third set composed of ‘Sidi Bouzid’, ‘Tozeur 1’ and ‘Jendouba 1 and 2’ are negatively correlated to axis one. Finally, the latest group is negatively correlated to the two axes and gathered ‘Kalaâ’, ‘Souihli’, ‘Kasserine’ and ‘Kébili 1 and 2’ (Fig. 3).

Loadings variables, correlation coefficients between the original data (standardized matrix) and the PCA scores were illustrated (Fig. 4). Each principal axis was interpreted by its correlation with the original variables. Four groups of characters can be distinguished. Two groups are positively correlated to the first axis, the first one is composed of EAT and MSLG and is also positively correlated to the second one. The second group including RCfs, AwL, ED and HLS is negatively correlated to axis two. The two other groups are negatively correlated to the first axis. However, the group composed of GH, ES, SSAT and GRAT is positively correlated to axis two, in contrast of variables EL, GDL, PL, RLfs, and HVF which represent the fourth group negatively correlated to axis two.

We used the percent similarity and median joining method to draw morphological distances (Fig. 2b). The Comparison of morphological characters using similarity percentage gave five accession groups (Fig. 2b). The first group consisted of ‘Tozeur 1’, ‘Tozeur 2’ and ‘Kébili 1’ with a percentage similarity that varies between 64 and 79%. ‘Kébili 2’, ‘Kébili 3’ and ‘Kasserine’ form the second with a similarity that oscillates between 82 and 91%. ‘Sidi Bouzid’ and

![Figure 3. Principal Components Analysis of cultivars’ morphological traits.](image-url)
The PCA of accessions classify them according to climatic regions. In fact, 'Kébíllı 2', 'Kébíllı 3', 'Martin', 'Tozeur 2' and 'Kébíllı 1' are positively correlated to the first component. Except the variety 'Martin' which is widely cultivated by Tunisian farmers from the north to the south, these accessions seem to be adapted to desert climate. However, 'Jendouba 1', 'Jendouba 2', 'Kalaâ', 'Kélibia 1', 'Kélibia 2', 'Kassérine', 'Souihli', 'Sidi Bouzid' and 'Tozeur 1', are negatively correlated to the first component and form a second group. The first five accessions belong to humid and sub-humid climatic stage. The accession 'Kassérine' was collected from a humid microclimate high land providing a sufficient argument for its belonging to the second group. The presence in this second environmental pool, of the accessions 'Souihli' and 'Sidi Bouzid' collected from arid climate, also 'Tozeur 1' that belongs to sahara suggested that they derived from seeds mixture transferred from Nordic field to the center and southern region during seed storage and their distribution for farmers.

Several authors have shown that the geographic origin of the collected material was sufficient to obtain a reasonable structuration in groups (Julier et al., 1995). On the other hand, obtained classification depends on the material involved. In our study, the PCA variable loadings confirmed that characters, which are positively correlated to both axes, are related to genetic rather than...
Generic diversity of Tunisian Local Barley Accessions

The geographic origin of the accessions studied. As a result, the geographic origin did not fit exactly with the dendrogram obtained. This could be due to high exchange of barley seeds between Tunisian cereal farmers of different regions and the spread of cultivated areas on different climatic stage. That is why; our clustering depends mainly on ear attitude (Eat), length of glumes and its awn relative to grain (MSLG). It is also common known that each accession has typical characteristics appreciated by farmers who conserve them every harvesting. The strong attachment of farmers to these accessions contributes to their conservation and consequently preserves the core of our plant germplasm. Further more, farmers are involved in seed dynamic system transfer between local cultivated areas.

RAPD markers seemed to be proficient to discriminate local barleys defined as accessions or populations geographically based (Ben Hmida-Ben Salem, 2000). It is also a valuable tool for assessing genetic diversity levels. Also the dendrogram based on RAPD our dendrogram obtained by RAPD markers study showed that the structuration group depends on the geographical origin. This result is in agreement with that found using PCA variable loadings, PCA accession loadings and the dendrogram of morphological traits. In this study, distance matrices derived from RAPD markers and morphological data showed a low correlation (r = 0.12). This result is in agreement with those of Chia-Szu and Hsiao (1999) working on Lilium longiflorum and reporting very low correlation (r = 0.035; p = 0.389) between RAPD markers and morphological characters. Furthermore, Marić et al. (2004); studying hexaploid wheat cultivars; obtained a non significant correlation between RAPD markers, morphological traits and coefficients of parentage. In the same way, Spooner et al. (2005) have obtained low correlation coefficient between potato genotypes by means of AFLP and morphologic characters. This weak correlation shows that there is no multilocus association between molecular and morphological traits in these accessions. Thus, RAPD markers were not efficient indicators of morphological divergence. In contrary to these authors, Roldán-Ruiz et al. (2001) working on perennial ryegrass found a correlation coefficient of 0.42 between STS markers and morphological traits methods. Moreover, Crochemore et al. (1998) working on 26 alfalfa population genetic structures found a global correlation coefficient of 0.51. While, Duarte et al. (1999) found a correlation of 0.89 between the genetic distances obtained with RAPD and the Mahalanobis’ distances indicating that the markers provide similar estimates of genetic divergence to those obtained using morpho-agronomical data on bean cultivars.

PCA and dendrogram analysis of both morphological traits and RAPD markers showed an important genetic diversity of local Tunisian barleys. In fact, the accessions were distributed in different groups belonging to different bioclimatic location and on some genetic characteristics as ear attitude (Eat) and length of glumes and its awn relative to grain (MSLG).

As a conclusion, we believe that correlation between morphological and RAPD markers could be improved if there were more morphological traits analyzed as reported by other researchers (Martinez de Toda and Sancha, 1997) or more RAPD primers used. In fact, a combination of detailed molecular and morphological analyses proved to be a powerful tool for unravelling intraspecies taxonomy. When these two types of data are interpreted in concert, some of the problems encountered in traditional morphometric analysis can be avoided (Hedrick, 1986). Hence, important consideration should be given to collection and conservation of local material for breeding, in order to maintain and preserve local barley germplasm from genetic erosion.

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