GENETIC DIVERSITY OF NATURAL POPULATIONS OF *Moringa peregrina* IN EGYPT

Sabha S.S. Moustafa1*, Ghada A. Hegazi1, Mahdia F. Gabr1, M.A. El-Mekawy1 and A.H. Belal1


**ABSTRACT**

In order to examine the genetic diversity of two population of the *Moringa peregrina* in South Sinai, research was performed using ten primers of ISSR molecule marker. The investigation was carried out as a primary step towards developing effective conservation strategies for the protection of *Moringa* germplasm. The aim of this study was to assess the genetic diversity within and among twenty individuals *M. peregrina* were collected from different regions Wady Zaghra and Wady Feiran. Primer 807 showed the highest number of bands (31 bands), but primers 17899B, showed the lowest number of bands. ISSR marker analysis showed significant level of genetic variation within the populations as revealed by number of bands moderately high average values of PIC (0.53) that the highest amount of PIC related to HB4, HB1 and 844B, while the lowest pic was related to primer 17899B. Cluster analysis was performed to construct dendogram using UPGMA. A dendogram clustered the individuals into two major clusters, first one cluster Wady Zaghra individuals and the second one is Wady Feiran individuals. Among all the individuals a total of percentage about 78.95 - 100% polymorphism was observed. According to the genetic diversity results, the conservation and improvement strategies should be taking in consideration the provenance factor. So, this study should be made appropriately the large sampling for the better representing of all diversity existing on the scale of the natural distribution of the moringa.

**Kay words:** *Moringa peregrina*, ISSR, genetic diversity, primers, polymorphism.

**INTRODUCTION**

*Moringa peregrina* (Forsk) Fiori is a green deciduous tree with sweet and oil rich white seeds. It belongs to the Moringaceae family that has only one genus called *Moringa* with 13 species (*Steinitz et al.*, 2009). The most famous Moringa species are *M. oleifera* and *M. peregrina* (*Lalas and Tsaknis, 2002*). *M. peregrina* is distributed in tropical and non-tropical areas. Egypt, Ethiopia to Somalia, Sudan, the Red Sea region, Palestine and Jordan are the main centers of distribution (*Lalas and Tsaknis, 2002*). It has been shown that *M. peregrina* has high aridity adaptation with short germination time after irrigation.

Traditionally *M. peregrina* has both medicinal and industrial uses. In Greece, Egypt and Romania its seeds' oil is used to make perfume and its wood is used in building because of its resistance to termites. In addition, Moringa is used as medicine for abdominal pain, headache, and skin protection as well as alaxative (*Steinitz et al.*, 2009). *M. peregrina* is becoming an endangered plant species all over the world as a result of unmanaged grazing and slow regeneration rate after
browsing (Gomma and Picó, 2011; Steinitz et al., 2009). Many recent studies are aiming to find efficient cultivation methods to save M. peregrina from extinction (Zaghloul et al., 2010).

Allam (2009) ben (Moringa) is a subtropical plant grown in upper Egypt and Sinai from many decades and the most known species are M. oleifera and M. peregrina. Nowadays, much attention was given to the nutritional value of Moringa plant and there is an increasing interest in spreading these species in the newly reclaimed land in an attempt to decrease the gap in oil production. Recently, developed modification of SSR-based marker systems, i.e. ISSR analysis, consolidates this requirement for flanking sequence information, and thus has found wide applicability in a variety of plants. Liu and Wendel (2001) reported that ISSR analysis involves PCR amplification of genomic DNA using a single primer that targets the repeat per sec., with 1-3 bases that anchor the primer at 3′ or 5′ end. In addition to freedom from the necessity of obtaining flanking genomic sequence information, ISSR analysis is technically simpler than many other marker systems. The method provides highly reproducible results and generates abundant polymorphisms in many systems. Also, ISSR markers show high level of repeatability and have been used as useful molecular markers in studying genetic diversity and species relationships (Pharmawati et al., 2004; Dogan et al., 2007).

ISSR is a simple, fast, and efficient technique that produces amplified products of 200–2000 bp in length. The technique is highly reproducible due to the use of longer primers, which allow for high annealing temperatures (Reddy et al., 2002). Alternative markers for species delimitation may be needed. Recently, ISSR markers have been widely applied in population genetics and bryophyte taxonomy (Hassel and Gunnarsson, 2003; Vanderpoorten et al., 2003; Werner et al., 2003; Natcheva and Cronberg, 2007; Buczkowska, 2010; Pla’s’ek and Sawicki, 2010). ISSR markers, which require no a priori knowledge of the DNA sequence, provide a genome-wide screening of a high number of loci (Ziętkiewicz et al., 1994). Also, Resmi et al. (2007) studied the genetic variability among twenty five distinct drumstick M. oleifera Lam. landraces using thirty five RAPD primers. The primers produced 34 bands, of which 58% (20 bands) were polymorphic. The study showed the use of RAPD markers in detecting genetic variability present among the drumstick landraces studied. In the same trend, Mgendi et al. (2010). Analyzed M. oleifera species growing in coastal regions of Tanzania using 12 RAPD primers. They found similarity ranging from 54% to 96% between cultivated and non-cultivated individuals. In vitro Al Khateeb et al. (2012) studied genetic stability of in vitro micropropagated M. pergrina plants using ISSR. However, Saini et al. (2013) detected that a genetic variability between eight cultivars of M. oleifera, the results suggested that the ISSR markers are the most effective for assessment of genetic diversity. Also, based on the three types of marker data, the eight cultivars of M. oleifera were grouped into four sub-clusters in a dendogram, but without any distinct geographical pattern. This suggests spread of planting material and high rates of gene flow through cross pollination. Also, Rufai et al. (2013) studied the genetic diversity and analyzed of genetic relationship among 20 M. oleifera were carried out with the aid of twelve primers from, RAPD marker. M. peregrina and M. oleifera are the only Moringa (Kelor) species found in Saudi Arabia. Robiansyah et al. (2015) studied to characterize a new biotype Moringa observed in Al Bahah Region, Saudi
Arabia. We used 11 RAPD and 15 ISSR primers to characterize and compare the new biotype with *M. peregrina* and *M. oleifera*. Level of polymorphism generated by each marker was calculated. Recently, Al Dhaberi (2016) studied the genetic fidelity of the clones produced from the *in vitro* protocol was assessed using ISSR molecular markers. The aim of this study was to conserve *Moringa peregrina* as a natural and endangered Egyptian plant with economic importance.

**MATERIALS AND METHODS**

This study was carried out in Molecular Genetics Laboratories, North Sinai Research Station (El-Sheikh Zuwayed), Desert Research Center (DRC), El Matarya, Cairo, Egypt.

**Plant Collection**

Genetic diversity was studied within and among twenty individuals of *M. peregrina* collected twice from two locations at South Sinai (Wady Zaghra and Wady Feiran, 10-15 km apart). Leaf samples were collected from ten individuals from each site. Wady Zaghra has 800 ft altitude and located at 28° 39' 338" N latitude and 34° 17' 623" E longitude, and Wady Feiran has 800 ft altitude also, and located at 28° 42' 214" N latitude and 33° 39' 556" E longitude (Fig. 1).

DNA fingerprinting of collected samples was carried out using inter simple sequence repeat polymerase chain reaction (ISSR-PCR) technique. Fresh leaves from the new twigs of *M. peregrina* individuals were packed in plastic zipper bags and stored in ice cooler, then finally stored in a -80°C freezer for DNA extraction.

Fig. (1): Map showing geographic location of *Moringa peregrina* studied populations at Wady Zaghra and Wady Feiran.
Extraction of genomic DNA and PCR amplification

Genomic DNA extraction from fresh leaves of the twenty *M. peregrina* individuals was done by employing the CTAB methodology as described by Doyle and Doyle (1987) with slight modifications.

DNA concentration and DNA quality assessed by gel electrophoresis. Ten ISSR primers were used for preliminary screening. PCR amplifications were carried out in a total volume of 20 µl containing 1X PCR buffer (75 mM Tris /HCl, 50 mM KCl, 2.0 mM MgCl₂, and 20 mM (NH₄)₂SO₄); 0.3 mM each of dATP, dTTP, dCTP, and dGTP; 0.6 mM primer in Table (1); 1 ml Taq DNA polymerase (Ampliqon); and 25 ng template DNA and water. Amplification was carried out in Stratgene Robocycler Gradient 96, which was programmed for 45 cycles as follows: denaturation (one cycle) 94°C for 2 min, followed by 30 cycles as follows; 94°C for 30 sec., 44°C for 45 sec., annealing at 72°C for 1 min and 30 sec., and finally one cycle of extension at 72°C for 20 min, then 4°C (infinite). Amplified products were resolved by electrophoresis on 1.5% agarose gels in 1X TBE buffer at 90 V for 2 hr. then stained with ethidium bromide and the DNA fragments were visualized under UV light using a gel documentation system. PCR reactions were repeated twice to check the reproducibility of the banding patterns. A 1 kbp DNA ladder was used as the molecular standard in order to confirm the appropriate ISSR markers.

Data analysis

The genetic similarity between individual pairs of genotypes was analyzed by using the NTSYS pc 2.1 Software (Rohlf, 2000). The binary data score was used to construct a dendogram. The pairwise genetic relationship between accessions were determined by calculating Jaccard’s similarity coefficient. The similarity coefficients were used for cluster analysis and a dendrogram was constructed by the Unweighted Pair-Group Method (UPGMA) Sneath and Sokal (1973). Polymorphism information content (PIC) values were calculated according to Smith (1989) using the following formula as follows:

\[ PIC = 1 - \sum \pi_i^2 \]

\( \pi_i^2 \) is the frequency of the allele. PIC provides an estimate of the discriminatory power of a locus by taking into account, not only the number of alleles but also the relative frequencies of those alleles. PIC values vary from 0 (monomorphic) to 1 (very highly discriminative, with many alleles in equal frequencies). To compare the efficiency of the one markers among *Moringa peregrine*, we estimated following parameters for each assay unit.

Table (1): ISSR primers’ names and their sequences.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>807</td>
<td>(AG)₈ T</td>
<td>HB4</td>
<td>(GACA)₄</td>
</tr>
<tr>
<td>814</td>
<td>(CT)₃ TG</td>
<td>HB15</td>
<td>(GTCG)₃GC</td>
</tr>
<tr>
<td>844A</td>
<td>(CT)₈ AC</td>
<td>17898A</td>
<td>(CA)₈ AC</td>
</tr>
<tr>
<td>844B</td>
<td>(CT)₈ GC</td>
<td>17898B</td>
<td>(CA)₈ GT</td>
</tr>
<tr>
<td>HB1</td>
<td>(CAA)₅</td>
<td>17899B</td>
<td>(CA)₈ GG</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

Assessment of genetic diversity is very important for the conservation of plant genetic resources in their natural habitat. Ten ISSR primers were used to produce DNA fingerprint profiles. The primers generated a total of 198 loci, 189 loci were polymorphic about 95.54%. Total band in Wady Zaghra were 167 bands, number of polymorphic bands was 133 but the number of non-polymorphic bands was 34. While in Wady Feiran the total bands were 132 divide to number of polymorphic bands (102) and number of non-polymorphic bands (30) showed in Table (2).

The level of polymorphism from each primer represented by PIC value was ranged between 0.397 and 0.667 (Table 3). The results clearly showed that both premier 807, 844A, HB1, HB15, 17898A, 17898B and 17899B completely polymorphism 100%, and premier 814 was a highly monomorphic with about 21.05% in all individuals.

Completely polymorphism 100%, and primer 17898A was a highly monomorphic about 69% and primer 844B,17898 B with percentage about 50% in Wady Feiran individuals. While, primer HB1 was height percentage about 93.33% and primer 844B was a highly monomorphic about 56% in Wady Zaghra individuals.

The obtained results from the genetic polymorphism analysis in different populations of M. peregrina, such as effective number of alleles (ne), polymorphic information content (PIC), marker index (MI) and number of polymorphic loci (L) are presented in Table 3. The results showed that within the two studied populations, the maximum number of effective alleles (22.23) was found in all individuals and the lowest number (15.5) was detected in population Wady Feiran. In the present study analysis of the genetic polymorphism obtained with ISSR markers demonstrated that the highest percentage of polymorphic loci (95.54%), moreover, the largest number of polymorphic loci (189) and the highest polymorphic information content 0.595 and marker index (3.763) were found in Population Wady Zaghra region. The lowest values were observed for Population from Wady Feiran.

Cluster analysis of moringa genotypes based on ISSR marker

The dendogram in present study showed that relationship between the examined Moringa peregrina individuals based on the data obtained by the ISSR technique. All ISSR alleles from ten markers scored were used for genetic diversity analysis. Dice's similarity coefficients were calculated to assess the genetic resembleances among the genotypes and the similarity coefficients matrix was used for UPGMA cluster analysis Fig. (3).

The dendogram based on genetic similarities showed that the 20 moringa Individuals a percentage about 39% to 90%. The cluster divided two major cluster first one cluster Wady Feiran and the second one Wady Zaghra.

The results of Jaccard’s similarity matrix indicated that the amount of similarity among the genotypes studied ranged from 0.39 to 0.90 representing high genetic variation among the genotypes in this study. The genetic variation may be caused by nature of cross pollination, high heterozygosis and genetic recombination in the studied genotypes. The results of this study of genetic distances of genotypes in Jaccard’s similarity matrix showed that the individual 16 and 17 had the highest amount of similarity and the individuals 3 and 20 had the lowest amount of similarity (0.39) (Fig. 4).
M: 1Kbp DNA ladder, Lanes 1-10: individuals from Wady Zaghra, lanes 11-20: individuals from Wady Feiran.

Fig. (2): ISSR profile of *Moringa peregrina* individuals DNA generated by primer 807.

M: 1Kbp DNA ladder, Lanes 1-10: individuals from Wady Zaghra, lanes 11-20: individuals from Wady Feiran.

Fig. (3): ISSR profile of *Moringa peregrina* individuals DNA generated by primer HB4.

Table (2): ISSR analysis of the DNA of all studied *Moringa peregrina* individuals via 10 primers.

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer name</th>
<th>Total number of bands amplified</th>
<th>Bands length range (bp)</th>
<th>Number of polymorphic bands</th>
<th>Percentage of polymorphism (%)</th>
<th>PIC Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>807</td>
<td>31</td>
<td>4000-300</td>
<td>31</td>
<td>100</td>
<td>0.623</td>
</tr>
<tr>
<td>2</td>
<td>814</td>
<td>19</td>
<td>2750 -300</td>
<td>15</td>
<td>78.95</td>
<td>0.471</td>
</tr>
<tr>
<td>3</td>
<td>844A</td>
<td>19</td>
<td>3500 -600</td>
<td>19</td>
<td>100</td>
<td>0.667</td>
</tr>
<tr>
<td>4</td>
<td>844B</td>
<td>17</td>
<td>2500 -500</td>
<td>15</td>
<td>88.24</td>
<td>0.524</td>
</tr>
<tr>
<td>5</td>
<td>HB1</td>
<td>16</td>
<td>3500 -350</td>
<td>16</td>
<td>100</td>
<td>0.518</td>
</tr>
<tr>
<td>6</td>
<td>HB4</td>
<td>24</td>
<td>3500-500</td>
<td>21</td>
<td>87.5</td>
<td>0.535</td>
</tr>
<tr>
<td>7</td>
<td>HB15</td>
<td>19</td>
<td>2370 – 400</td>
<td>19</td>
<td>100</td>
<td>0.582</td>
</tr>
<tr>
<td>8</td>
<td>17898A</td>
<td>19</td>
<td>2800-380</td>
<td>19</td>
<td>100</td>
<td>0.397</td>
</tr>
<tr>
<td>9</td>
<td>17898B</td>
<td>20</td>
<td>3000-350</td>
<td>20</td>
<td>100</td>
<td>0.568</td>
</tr>
<tr>
<td>10</td>
<td>17899B</td>
<td>14</td>
<td>2000 -500</td>
<td>14</td>
<td>100</td>
<td>0.473</td>
</tr>
<tr>
<td></td>
<td>Total band</td>
<td>198</td>
<td></td>
<td>189</td>
<td>95.45</td>
<td></td>
</tr>
</tbody>
</table>

**PIC**: Polymorphism information content
Table (3): Average value of total number of scored bands, number of polymorphic bands, percentage of polymorphism, and total fragments amplified by using ISSR primers among the clones of *Moringa peregrina*.

<table>
<thead>
<tr>
<th>Properties of markers</th>
<th>Wady Zaghra individuals</th>
<th>Wady Feiran individuals</th>
<th>All individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of markers</td>
<td>$U$</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Number of non-polymorphic bands</td>
<td>$n_{np}$</td>
<td>34</td>
<td>30</td>
</tr>
<tr>
<td>Number of polymorphic bands</td>
<td>$n_p$</td>
<td>133</td>
<td>102</td>
</tr>
<tr>
<td>Average number of polymorphic bands/assay unit</td>
<td>$n_p/U$</td>
<td>13.3</td>
<td>10.2</td>
</tr>
<tr>
<td>Number of loci</td>
<td>$L$</td>
<td>167</td>
<td>132</td>
</tr>
<tr>
<td>Number of loci/assay unit</td>
<td>$n_u$</td>
<td>8.35</td>
<td>6.6</td>
</tr>
<tr>
<td>Total number of effective alleles</td>
<td>$N_e$</td>
<td>17.29</td>
<td>15.59</td>
</tr>
<tr>
<td>Min of PIC</td>
<td>$PIC$</td>
<td>0.157</td>
<td>0.031</td>
</tr>
<tr>
<td>Max of PIC</td>
<td>$PIC$</td>
<td>0.567</td>
<td>0.548</td>
</tr>
<tr>
<td>PIC value</td>
<td>$PIC$</td>
<td>0.359</td>
<td>0.361</td>
</tr>
<tr>
<td>Fraction of polymorphic loci</td>
<td>$B$</td>
<td>0.7964</td>
<td>0.7727</td>
</tr>
<tr>
<td>Assay efficiency index</td>
<td>$A_i$</td>
<td>1.729</td>
<td>1.559</td>
</tr>
<tr>
<td>Effective multiples ratio</td>
<td>$E$</td>
<td>10.484</td>
<td>8.54</td>
</tr>
<tr>
<td>Marker Index</td>
<td>$MI$</td>
<td>3.763</td>
<td>3.08</td>
</tr>
<tr>
<td>Total Banding pattern</td>
<td>$Bp$</td>
<td>109</td>
<td>81</td>
</tr>
<tr>
<td>Effective number of patterns/ assay unit</td>
<td>$P$</td>
<td>10.9</td>
<td>8.1</td>
</tr>
</tbody>
</table>

Fig. (4): A dendogram showed the relationship between the examined moringa individuals based on the data obtained by the ISSR technique.
Generally the results of the study of genetic variation, geographical distance and genotypes farness and closeness in the study showed that geographical distance is not the reason for away and close genetics of individuals. Situate of genotypes related to different ecological conditions in the same cluster may be due to the existence of an identical genetic basis in them, which had been transferred to different regions of the world by scholars collecting germplasm.

ISSR is a simple, fast, and efficient technique that produces amplified products of 200–2000 bp in length. The technique is highly reproducible due to the use of longer primers, which allow for high annealing temperatures (Reddy et al., 2002). Alternative markers for species delimitation may be needed. Recently, inter-simple sequence repeat (ISSR) markers have been widely applied in population genetics and bryophyte taxonomy (Hassel and Gunnarsson, 2003; Vanderpoorten et al., 2003; Werner et al., 2003; Natcheva and Cronberg, 2007; Buczkowska, 2010; Pla’scek and Sawicki, 2010).

Lei et al. (2006) The ISSR primers were able to detect 184 polymorphic loc. Analysis of molecular variance (AMOVA) indicated that species level genetic diversity was relatively high (p=97.83%, and Ho=0.464). The Yunnan populations from three locations were further divided into three corresponding groups, indicating that genetic differentiation was correlated to geographic distribution. Understanding the genetic structure of R. crenulata provides insight for the conservation and management of this endangered species. Resmi et al. (2007) They found that primers produced 34 bands, of which 58% (20 bands) were polymorphic. Generally the amount of polymorphism observed in the study was higher than those obtained by Pivoriene and Pasakinskiene (2008) who reported that 6 out of 33 ISSR markers showed the highest amount polymorphism (100%) and 2 markers showed the least amount of polymorphism (20%). In the same trend, Mgendi et al. (2010). They found similarity ranging from 54% to 96% between cultivated and non-cultivated individuals. In addition, Ana et al. (2012) found similarity ranging from 27% to 69%. The results are important in designing strategies for conservation. Abubakar et al. (2012) found that the high degrees of polymorphism (74%) among the accessions were observed in terms of genetic relationship and were grouped into five clusters. This high variability can be utilized for mapping out breeding strategies in the production of cultivars with better yield of M. oleifera to meet the pressing needs of these multi-purpose crops to our growing populations. Finally, Pakseresht et al. (2013) found that using 7 primers of ISSR molecule marker. Using this primer, 32 bonds were scored, primer 807 showed the highest number of bands and primers 878, showed the lowest number.

This rate of polymorphism is low compared to values obtained with other sets of ISSR primers in Larix gmelinii (Rupr.) (98.8%) (Zhang et al., 2013) and Erythrina velutina Willd (98.0%)

Rufai et al. (2013) studied the genetic diversity and analyzed of genetic relationship among 20 M. oleifera were carried out with the aid of twelve primers from, RAPD marker. One the other hand, Al Dhaberi (2016) found that similarity index between the progenies and parent was above 94.9%.This shows that in vitro developed plantlets are identical with its mother plant. This will facilitate standardizing future conservation programs to limit the erosion of the genetic biodiversity in M. peregrina and similar species. since ISSR primers classified as moderately informative have been used successfully in other species such as Lupinus albus. Oumer et al. (2015). Panax stipuleanatus. Trieua et al. (2016), Cipadessa baccifera. Jebarubi

Conclusion: We conclude that the markers generated by the primers 807, 814, 844A, 844B, HB1, HB4, HB15, 17898A, 17898B, 17899B allow us to detect the genetic polymorphism among individuals of M. peregrina, being useful to determine the genetic diversity of this species.

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Moustafa, et al.


المختصر العربي

التنوع الوراثي الحيوي في عشائر نبات اليسار النامي طبيعياً في مصر

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تم اختيار التنوع الوراثي لعشائرين من نبات اليسار النامي طبيعيًا في جنوب سيئاء حيث استخدم عشارة بانت وتعتبر هذه خطوة في اتجاه الحفاظ على المصادر الوراثية لهذا النبات، والهدف من هذا البحث هو دراسة التنوع الحيوي لنبات اليسار بين أفراد الموقع الواحد وبين أفراد الموقعين، حيث تم تجميع العينات من عشارة أفراد من وادي زغة وأخرى من وادي فيران، وأوضحت النتائج أن باند 8499 أظهر أعلى عدد من البندات كانت مع باند 8278 ونسبة الاختلاف في وادي زغة حوالي 74% وفي وادي فيران كانت 78% بين الوالدين كانت 95% وكانت 844 وحيث هذه الدراسة هامة للتعرف على أهمية المناطق التي يتم فيها نبات اليسار وتحمل النبات لتغير الظروف البيئية.

الكلمات الإرشادية: التنوع الوراثي الحيوي، العشائر، نبات اليسار.

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