MOLECULAR IDENTIFICATION OF THE CULTIVATED MORINGA OLEIFERA IN SAUDI ARABIA

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ABSTRACT: Seven hundred and nine nucleotides spanning the ITS region (ITS1 and ITS2 and the highly conserved 5.8S rDNA exon located in between) and its flanking regions of small and large ribosomal DNA subunits (18S and 26S genes) were amplified and sequenced for Moringa oleifera seedlings. The obtained data have been analyzed by maximum-parsimony and neighbor-joining methods in order to identify the cultivated plant molecularly. The parsimony could not be discriminated between the cultivated taxon from either M. oleifera or M. peregrina. The tree showed clustering of the three species with 100% bootstrap supports without clear identification of the seedlings sample and the neighbor-joining method showed similar tree topology. The genetic distance obtained from the sequenced data indicated that the smallest distance was found between cultivated and wild M. oleifera and between wild M. oleifera and M. peregrina while the distance between the cultivated M. oleifera and M. peregrina was higher. The current data therefore identified the seedlings sample to be affected by the ecological habitat of Saudi Arabia which is considered as an endemic habitat of M. peregrina. Further molecular study is necessary by collecting more wild and cultivated samples of both species in order to identify with clear resolution the molecular relationship between both wild and cultivated Moringa. 

Key words: Moringa, Plant DNA, Saudi Arabia, plant nurseries

INTRODUCTION

Moringaceae is an angiosperm family consists of 13 [1] species which belong to only one genus called Moringa [2]. Morton [3] reported that the most common species are Moringa peregrina (forsk) fiori and Moringa oleifera. These species are the most widely cultivated of the Moringaceae family and was utilized by the ancient Egyptians, Romans and Greeks. M. peregrina has a wider range, growing from the Dead Sea area sporadically along the Red Sea coasts to northern Somalia and around the Arabian Peninsula to the mouth of the Persian Gulf [2]. In Saudi Arabia, M. peregrina is mainly distributed in South and North Hijaz [4,5,6]. Moringa oleifera is a small to medium sized tree native to the south central Asia from India to Nepal [7,8]. The species is widely cultivated in many parts of the world including Tanzania [9]. The studies on the genetic diversity of M. peregrina or M. oleifera are very limited. However, recent few investigations have tackled this point using RAPD-PCR [10] inter simple sequence repeat (ISSR) and cytochrome b [11,12] and internal transcribed spacer of the nuclear ribosomal DNA [13]. The present study therefore aimed to use the nuclear rDNA at the region spanning 18S gene, internal transcribed spacers (ITS1 and ITS2) and 5.8S gene to infer the molecular features of the cultivated M. oleifera in Saudi Arabia and such study could be a prerequisite for future crop improvement programme.

MATERIALS AND METHODS

Samples and DNA extraction

In this study, three samples of the cultivated M. oleifera species have been collected. The seeds of this cultivated plant were brought from Egypt and be cultivated in plant nurseries in Riyadh of Saudi Arabia. The samples were labeled, sealed in sterilized polythene bags, brought to the laboratory and were stored at -20 °C till their use for DNA isolation. DNA was isolated and purified by DNeasy Plant Mini Kit following the manufacture protocol.

PCR experiments

The following primers were used in this study; forward primer (ITS4) 5’- TCTCTCCGCTATTGATATGC-3’ and the reverse primer (ITS5) 5’- GGAAGTAAAAGTCTGTAACAAGG -3’ [14] were designed for the amplification of M. peregrina.
A total volume of 25 µL PCR mixture (1 µL genomic DNA, 12.5 µL PCR Master Mix, 0.5 µL of each primer and 10.5 µL distilled sterilized H2O) was used. PCR amplification was carried out in a Techne thermocycler. The PCR conditions were 95 °C for 5 min as initial denaturation step followed by 35 cycles of 95 °C for 60 s denaturation, 56 °C for 60 s annealing and 72 °C for 60 s extension, the final extension was at 72.0 °C for 4 min. PCR products were run on 1% agarose gel containing ethidium bromide and visualized. The PCR products of the 3 samples were sent for sequencing using the same two primers used in the PCR amplification.

Data analyses
Seven hundred and nine nucleotides spanning 18S, ITS1, 5.8S and 28S genes from the nuclear DNA for the collected samples were sequenced in this study. Comparisons with sequences in the GenBank database were achieved in BLASTN searches at the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov). The sequenced data were aligned with their counterparts found in the Genbank database for M. peregrina from Yemen (JX092071), M. oleifera (AY845130) from national botanical garden of Belgium, M. ovalifolia (AY461551) collected from national botanical garden of Belgium and M. rivae (JX092072) collected from Kenya. The aligned data were used for phylogenetic analyses and the sequence for M. ovalifolia was used for tree rooting. The gap-containing sites and unambiguous nucleotides were deleted so that 614 bp were left for phylogenetic analyses. The aligned nucleotides can be obtained from Sayed Amer upon request.

The phylogenetic analyses were conducted by maximum-parsimony and neighbor-joining methods packaged in PAUP* 4.0b10 [15]. Both methods were set to heuristic searches with the TBR branch swapping and 10 random taxon additions. Bootstrapping replicates were set to 10000 for both methods. The neighbor-joining was adjusted by Tamura-Nei distance option.

RESULTS
The amplified and sequenced ITS region, in this study, was shown in figure 1. The sequenced fragment was aligned with its counterparts for 3 M. peregrina and 4 M. oleifera samples that represented all published data for both species in the Genbank database. The length of ITS1, 5.8S and ITS2 regions, frequency of different bases and adenine-thymine and cytosine-guanine ratio (AT/CG and CG/AT) for the studied taxa are illustrated in Table 1. The entire ITS region was 629 bp in both cultivated M. oleifera and M. peregrina and was 631 bp in wild M. oleifera. The number of nucleotides in the ITS1 was 248 bp in the three studied taxa. The 5.8S region was 166 bp in both cultivated M. oleifera and M. peregrina and was 167 bp in wild M. oleifera. The ITS2 region was 215 bp in cultivated M. oleifera and M. peregrina and was 216 bp in wild M. oleifera. The AT/CG ratio was 37.67 / 62.33 % in cultivated M. oleifera, 73.36 / 62.64 % in M. peregrina and 37.34 / 62.66 % in M. oleifera.

The ITS2 region appeared more homogenous in all taxa followed by the 5.8S and the ITS1 regions. The aligned nucleotide sequence of the ITS1 region showed 4 base substitutions. The base at position 58 was cytosine in both wild species and it was guanine in the cultivated M. oleifera while bases number 59 and 84 were guanine in both wild species and were adenine in the cultivated taxon. For 5.8S region, only one substitution was found at the base number 283 where cytosine in both wild and cultivated M. oleifera was substituted with thymine in M. peregrina. The base number 124 was cytosine in the cultivated M. oleifera and was thymine in wild M. oleifera. Only two insertions were found one in the 5.8S at base number 278 and the other was in the ITS2 region at base number 621. The data showed base frequencies of A = 19.9%, C = 31.9%, G = 30.7% and T = 17.5%. Of the 614 nucleotides used for tree analyses, 440 were constant and 174 were variables. From the variable sites, 157 were parsimony uninformative and 17 were informative under parsimony criterion. The consensus parsimony tree constructed showed consistency index (CI = 1.00), homology index (HI = 0.00), retention index (RI = 1.00) and rescaled consistency index (RC = 1.00).

![rDNA - repeat](image)

Figure 1. The gene arrangement of the nuclear DNA that spanning the rDNA genes. It consists of 18S, 5.8S, and 26S tracts and forms a tandem repetitive cluster. ETS is the external transcribed spacer while ITSs are the internal transcribed spacers 1 and 2 as numbered from 5' end.
A single parsimony tree (Fig. 2) was found in which the cultivated *M. oleifera* was grouped with both *M. oleifera* and *M. peregrina* in one polyphyletic cluster (bootstrap= 100). Meanwhile, the sequenced fragments executed one neighbor-joining tree that showed a similar tree topology with the maximum-parsimony tree. The pairwise genetic distances among the studied *Moringa* species are listed in Table 2. The genetic distance showed the smallest values between the cultivated *M. oleifera* and the wild *M. oleifera* and between *M. peregrina* and the wild *M. oleifera* (D= 0.005). The distances between either *M. peregrina* and *M. oleifera* and other species were high.

![Tree Diagram](image)

Figure 2. A neighbor-joining tree constructed from 614 bp sequenced fragments from ITS region of *Moringa* genome. Values at nodes refer to the bootstrapping of maximum-parsimony and neighbor-joining methods which were shown when they were over 50%.

Table 1. Base frequency and length of the sequenced region for the studied taxa.

<table>
<thead>
<tr>
<th>Moringa taxa</th>
<th>ITS1</th>
<th>5.8S</th>
<th>ITS2</th>
<th>Total base frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>T</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td><em>M. oleifera</em> (cult.)</td>
<td>54</td>
<td>35</td>
<td>85</td>
<td>74</td>
</tr>
<tr>
<td><em>M. peregrina</em></td>
<td>52</td>
<td>35</td>
<td>86</td>
<td>75</td>
</tr>
<tr>
<td><em>M. oleifera</em> (wild)</td>
<td>52</td>
<td>36</td>
<td>85</td>
<td>75</td>
</tr>
</tbody>
</table>

Table 2. Pair wise genetic distances among the different *Moringa* species as calculated from the sequenced plant genome fragments in this study

<table>
<thead>
<tr>
<th>Cultivated <em>M. oleifera</em></th>
<th>0.005</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. oleifera</em></td>
<td>0.005</td>
</tr>
<tr>
<td><em>M. peregrina</em></td>
<td>0.083</td>
</tr>
<tr>
<td><em>M. oleifera</em> (wild)</td>
<td>0.297</td>
</tr>
<tr>
<td><em>M. ovaleifolia</em></td>
<td>0.303</td>
</tr>
</tbody>
</table>

**DISCUSSION**

ITS region is intervening spacers found between the 18S-5.8S-26S region separating the elements of the rDNA locus. It consists of ITS1 and ITS2 flanking from both sides the highly conserved 5.8S rDNA exon [16]. In angiosperms, the total length of this region varies between 500 and 750 bp [17] and in *Moringa*, it is approximately 630 bp. Unlike the coding regions, the ITS spacer exhibits more changes and therefore, is extensively used as a marker for systematic reconstruction at different taxonomic levels. After its first application in this regard [18], it has become widely used for phylogeny. This region is biparental inheritance, easy to be amplified and sequenced with several universal primers and suitable for evolutionary studies at the species or generic level [16,19, 20]. The spacer therefore, varies in its length from species to species. It length is 629 bp in the current sample and in *M. oleifera* while it is 631 bp in *M. peregrina*.

The biogeographic distribution of *M. peregrina* is North Africa and Arabia while that of *M. oleifera* is the Indian continent [1]. The genetic diversity within *M. peregrina* is very limited [13], while that within *M. oleifera* indicated a high level of population differentiation [21]. The genetic distance between the cultivated *M. oleifera* and *M. peregrina* was higher than that found between this taxon and both wild species. This result could indicate that the genetic structure of the cultivated *M. oleifera* did not altered by the new habitats of Saudi Arabia.
However, the small genetic variability could be induced by some genetic separation between cultivated and non-cultivated individuals. This possibly could be attributed to the genetic changes taking place in escapees’ individuals as they adapt to the natural environment. Similar findings were revealed in Tanzanian *M. oleifera* [22]. These results may also be supported by recent findings [23] in which the authors revealed a large genetic diversity among the Indian populations of *M. oleifera* which can be used for crop improvement programme.

REFERENCES


