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Research Article
Evaluating the Genetic Diversity of *Moringa oleifera* Obtained from South-central and Northern States of India using RAPD Markers Technique

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Abstract

Background and Objective: *M. oleifera* belonging to the monogeneric family Moringaceae. It is drought-tolerant and must be cut back several times to make it branch out more. It tolerates a large range of soils and rainfall conditions. The present study has been aimed to estimate genetic relatedness among the seeds of *M. oleifera* from 3 different states of India i.e., Himachal Pradesh, Maharashtra and Uttarakhand using RAPD markers. **Materials and Methods:** Estimation of genetic relatedness among the seeds done through Isolation of genomic DNA, agarose gel electrophoresis, polymerase chain reaction (PCR) amplification by using RAPD (OPA, TC series) primers and UPGMA dendrogram. **Results:** Initially, nine random decamer primers were used in the study and based on the higher degree of polymorphism obtained only 4 primers were selected for further study which generated reproducible RAPD patterns. The number of bands in the amplified products for different samples which resulted from the selected RAPD primers varied between 40-50, where the size range of various amplicons for different samples varied from 202-3010 bp, approximately. Clustering based on RAPD fingerprints data revealed that the samples from different district of Himachal Pradesh (HP1B and HP2M), Uttarakhand (UK1D and UK2N) and Maharashtra (MH1L and MH2P) got paired into three separate clades at similarity level of 88.5%, showing maximum homology within their respective pairs. **Conclusion:** The results suggest that, high level of polymorphism was observed between the samples from south-central and northern parts of the country which showed that they were distantly related to each other.

Key words: Seeds, random amplified polymorphic DNA, isolation of genomic DNA, agarose gel electrophoresis, polymerase chain reaction (PCR) amplification, RAPD primers and UPGMA dendrogram

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Moringa oleifera tree is the most admired, multipurpose and underutilized tropical tree. *Moringa oleifera* has great economical potential due to several reasons: it's application in multiple industries such as cosmetics, biomedical and fuel, wide range of rich and novel products, distribution throughout many undernourished regions and tolerance to many limiting factors such as environmental or stress factors[1-5]. It is indigenous to Northwest India, Pakistan, Asia Minor, Western and Sub-Himalayan tracts, while it has been naturalized and cultivated in Southeast Asia, Sri Lanka, Caribbean Islands, tropical Africa, Central and South America, the greater of the tropical belt and many other countries[6-10]. *M. oleifera* also known as drumstick tree, is one of the 14 species belonging to the monogeneric family Moringaceae[6]. Seeds can be sown either directly or in containers without any requirement of pre-treatment and sprout readily in 1-2 weeks[11]. In various of its preparations *M. oleifera* is used for exceptionally wide range of properties like antioxidant, antihypertensive, antimicrobial, antidiabetic, antiulcer, antinflammatory, diuretic, antipyretic and antihyperlipidemic[12-17]. The *Moringa* seed extracts (both dry and aqueous) have been successfully used to purify water as it contains cationic polypeptides acting as a natural coagulant[6,2,18-19]. Okun *et al.*[20] suggested that the knowledge of genetic variation as well as geographical and ecological differentiations are important for breeding and sampling strategies. The genetic diversity of the plants is closely related to their geographic distribution. Species with a wide geographic area generally have more genetic diversity[21]. The population structure, genetic diversity and biological factors have all three been shown highly interdependent on each other[22,23]. Analysis of genetic diversity in *Moringa* using RAPD technique has been proven very successful lately and confirms its importance in management of *Moringa*[3,24,25]. Very recently the genetic diversity of commercially grown *Moringa* cultivars from India was investigated in a comparative evaluation of three genetic marker systems including RAPD, ISSR and cytochrome P450-based system and the cultivars have been determined to have high degree of genetic diversity[26]. RAPD technique has been widely used and helps to identify a large number of markers used for estimating genetic variations[27,28]. The present study has been aimed to estimate genetic diversity among the seeds of *M. oleifera* from 3 different states of India i.e., Himachal Pradesh, Maharashtra and Uttarakhand using RAPD markers.

MATERIAL AND METHODS

Study area: Samples were collected from 3 different states of India i.e., Himachal Pradesh, Maharashtra and Uttarakhand in the month of October-November, 2016 and transported to Molecular Plant Microbe Interaction lab, Shoolini University of Biotechnology and Management Sciences, Solan for further study.

Sample collection: For the present study, seed of *M. oleifera* were collected from three different states of India i.e., Himachal Pradesh (Bilaspur and Mandi district), Maharashtra (Latur and Pune district) and Uttarakhand (Dehradun and Nainital district) based on the geographical distribution, morphological differences in color of seed pods and availability of samples. They were authenticated from Y.S Parmar University of Horticulture and Forestry, Solan (Himachal Pradesh). The seeds were sun-dried (to reduce the moisture content) and separated from the membranes. Thereupon, seeds were broken to remove the hard shell and the kernels were used for further studies.

Isolation of total DNA from seeds of *M. oleifera*: Total DNA was extracted using standardized protocol defined by Sambrook *et al.*[29] with slight alterations. Crushing of 100-150 mg of seed content was done in 1-2 mL of extraction buffer (100 mM TrisHCl, 50 mM EDTA, 500 mM NaCl, 0.01% β-mercaptoethanol) using autoclaved pestle-mortar. 130-150 µL of 20 % sodium dodecyl sulphate (SDS) was added to the mixture/mL of extraction buffer for denaturation of proteins and incubated at 65°C for 15 min. Then the mixture was centrifuged in 1.5 mL microfuge tube at 10,000 rpm for 10 min. The resulted supernatant was filtered over a miracloth filtration membrane and transferred to a new microfuge tube. Equal volume of phenol/chloroform (1:1) was added, shaked vigorously for 1 min and further centrifuged at 3,000 rpm for 30 min. Aqueous phase containing nucleic acid was collected and 2.5 volume of absolute ethanol were added to precipitate the DNA in a fresh microfuge tube. Mixed the solution gently and incubated on ice for 1 min. The solution was then centrifuged at 12,000 rpm for 20 min to pellet down the precipitates. The pellet was washed with 70% ethanol, air dried and resuspended in 50 µL of 10X TE buffer (10 mM TrisHCl, 50 mM EDTA). The qualitative and quantitative analysis of DNA was done by agarose gel electrophoresis (using 1% agarose) and spectrophotometry (determining the optical density at 260 and 280 nm).

Genetic diversity analysis of *M. oleifera* seeds using random amplified polymorphic DNA (RAPD) technique: Isolated DNA from seeds of *M. oleifera* was used to study the genetic variation using RAPD technique. RAPD analysis was performed by the method described by Williams *et al.*[30], with some modifications. The standard RAPD reaction was set up by using random decamer primers. Nine decamer primers [OPA-1 (5′-CAGGCCCTTC-3′), OPA-3 (5′-AGTCAGCCAC-3′), OPA-9 (5′-GGGTAACGCC-3′) OPA-13 (5′-CAGCACCCAC-3′), OPA-14 (5′-TCTGTGCTGG-3′), TC-1 (5′-GGAGTACTGG-3′), TC-2 (5′-GGTCTAGAGG-3′), TC-3 (5′-GAGTCTCAGG-3′), TC-4 (5′-GGACTGCAGA-3′)] were used in this study. PCR was conducted in 25 µL mixture containing 1 µL (~100 ng) DNA, 2.5 µL of 2 mM dNTPs, 2 µL (10 pM) of RAPD primer and 0.3 µL (5 U μL^{-1}) of *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd.) with 10X PCR buffer (containing 15 mM $MgCl_2$). Temperature conditions for PCR included initial denaturation at 94°C for 5 min, followed by 40 cycles comprised of 3 stages with denaturation at 94°C for 30 sec, annealing at 36°C for 1 min and elongation at 72°C for 1 min. The additional final elongation step was conducted at 72°C for 7 min. The final PCR products were analyzed by performing electrophoresis using 1% agarose.

RAPD product scoring and data analysis: The RAPD fragments obtained upon agarose gel electrophoresis were scored for presence (1) and absence (0) of amplification bands for each sample. The data of similarity coefficients was generated and used to construct the dendrogram indicating the genetic relatedness or differentiation among the *M. oleifera* seeds. Data was compiled as binary 0-1 matrix, '1' represents the presence of a band and '0' represents the absence of a band in specified size range. All high and low intensity bands were considered in statistical analysis. Dendrogram was produced from the distance matrix by UPGMA (Unweighted Pair-Grouped Method Arithmetic) average[31], constructed using online D-UPGMA program (DendroUPGMA: A dendrogram construction utility), version-2015.

RESULTS AND DISCUSSION

DNA isolation: Total DNA was extracted from 6 samples of *M. oleifera* seeds i.e., a sample each from Bilaspur and Mandi districts of Himachal Pradesh, Latur and Pune district of Maharashtra and also from Dehradun and Nainital district of Uttarakhand. DNA bands of good quality were observed upon agarose gel electrophoresis (Fig. 1). The concentration of DNA determined at 260 nm was found to be 7.5 µg μL^{-1} with OD of 1.5 and dilution factor of 100. Correspondingly, the purity of DNA was confirmed with $A_{260\,nm}/A_{280\,nm}$ ratio which was found to be of range 1.8-2.0.

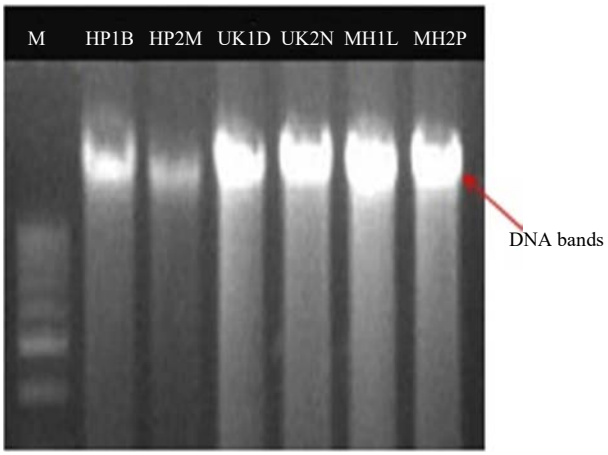

Fig. 1: Isolated DNA of 6 *M. oleifera* samples
HP1B and HP2M from Himachal Pradesh (Bilaspur and Mandi districts, respectively), UK1D and UK2N from Uttarakhand (Dehradun and Nainital districts, respectively), MH1L and MH2P Maharashtra (Latur and Pune districts, respectively), M: 1kb DNA Ladder (TaKaRa)

Table 1: Maximum band size, minimum band size and the total number of amplified fragments obtained for all the 4 selected RAPD primers viz., TC-1, TC-2, TC-3 and TC-4

	Band size (bp)		
Primers	Maximum	Minimum	Amplified fragments
TC-1	1500	295	46
TC-2	961	221	40
TC-3	3010	202	42
TC-4	2250	495	49

RAPD analysis: Isolated DNA from all different samples of *M. oleifera* seeds was used for study of genetic variations using RAPD technique. The standard RAPD reaction was set up by using standard RAPD primers. Initially, nine random decamer primers (OPA-1, OPA-3, OPA-9, OPA-13, OPA-14, TC-1, TC-2, TC-3, TC-4) were used in the study and based on the higher degree of polymorphism obtained only 4 primers viz.,TC-1, TC-2, TC-3 and TC-4 were selected for further study which generated reproducible RAPD patterns (Fig. 2). A total of 46, 40, 42 and 49 bands were scored by using TC-1, TC-2, TC-3 and TC-4 primer, respectively (Table 1). The number of bands in the amplified products for different samples which resulted from the selected RAPD primers varied between 40-50, where the size range of various amplicons for different samples varied from 202-3010 bp, approximately.

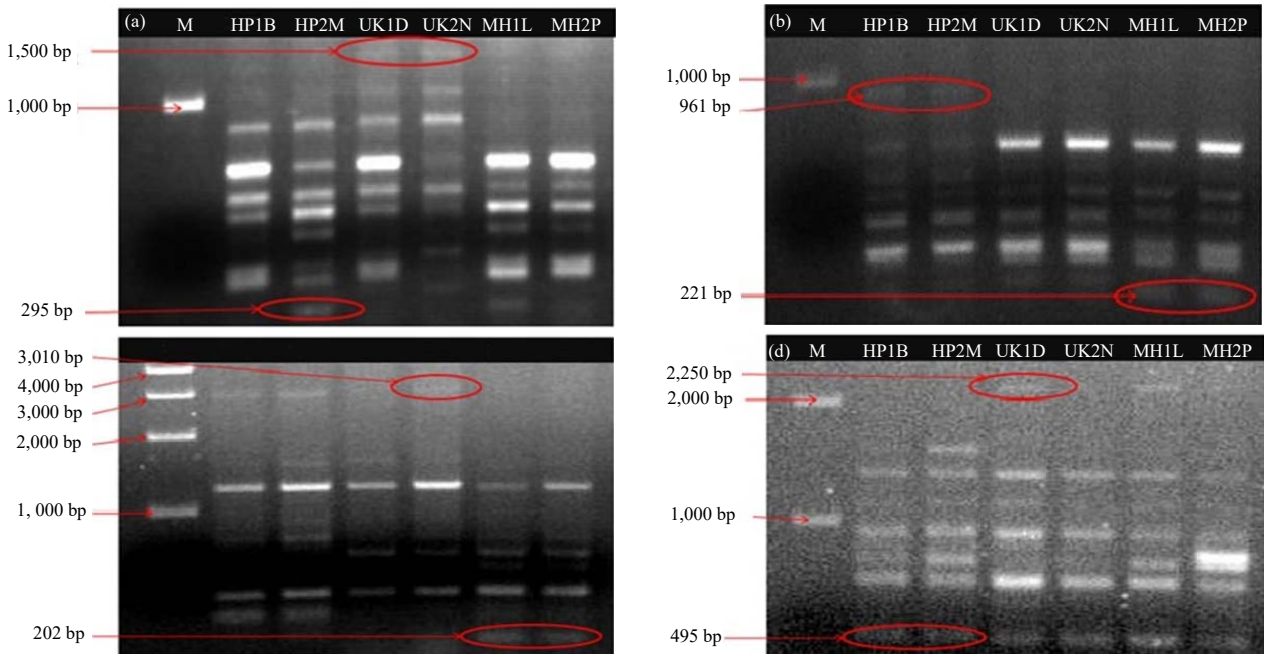

Fig. 2(a-d): RAPD banding patterns of 6 samples of *M. oleifera* using 4 selected random primers which showed higher degree of polymorphism, (a) TC1 primer, (b) TC2 primer, (c) TC3 primer and (d) TC4 primer

HP1B and HP2M samples from Himachal Pradesh, UK1D and UK2N samples from Uttarakhand and MH1L and MH2P samples from Maharashtra, M: 1 kb DNA Ladder (TaKaRa)

Clustering based on RAPD fingerprints data revealed that the samples 'HP1B and HP2M' (from 2 separate districts of Himachal Pradesh), 'UK1D and UK2N' (from 2 separate districts of Uttarakhand) and 'MH1L and MH2P' (from 2 separate districts of Maharashtra) were grouped or got paired-up into 3 separate clades at similarity level of 88.5%, which revealed that they showed maximum homology within their respective pairs and it also favors the fact that the paired samples do belong to the same region or same state of the country. The results stand in support of the generalization that 2 closely related organisms, species or varieties of same species would be expected to yield more similar banding patterns than the ones those are distant in evolutionary terms[32]. The samples 'MH1L and MH2P' from Maharashtra (south-central state of the country) were paired in a very divergent independent group, which immediately separated them from all other 4 samples from the Himachal Pradesh and Uttarakhand states (viz., northern states of the country) which got clustered into a separate clade. This revealed high level of polymorphism between the samples from the south-central and northern parts of the country and showed that the samples were distantly related to each other. A high level of polymorphism detected in the present study indicates that the primers TC-1, TC-2, TC-3 and TC-4 could be employed in future for RAPD based genetic diversity studies of various varieties of *M. oleifera*.

Our results suggest the existence of variations among the samples of *M. oleifera* from different regions of the country as all the 6 samples were divided into 2 main clusters where the pair of Maharashtra samples was found to have a distant relationship with other 2 pairs of samples from Himachal Pradesh and Uttarakhand states of the country as shown in Fig. 3. For future studies, RAPD technique can be used as a more reliable and specific tool for identifying variations occurring within the species. However, RAPD markers are generally cheap, fast, simple to carry out in comparison with different other methods, such as ISSR, AFLP and SNP in detecting polymorphisms, they are nevertheless not easy to reproduce[33]. It will continue to be relevant particularly in studies of genetic diversity in underutilized medicinal species as long as other DNA-based methods remain unavailable in terms of upper hand in cost, time and labor[27,34]. Previously, assessment of genetic diversity in *M. oleifera* has been described using AFLP and RAPD markers[2,35,36]. Similarly, Mgendi *et al.*[36], investigated the genetic diversity by RAPD markers between cultivated and non-cultivated provenances

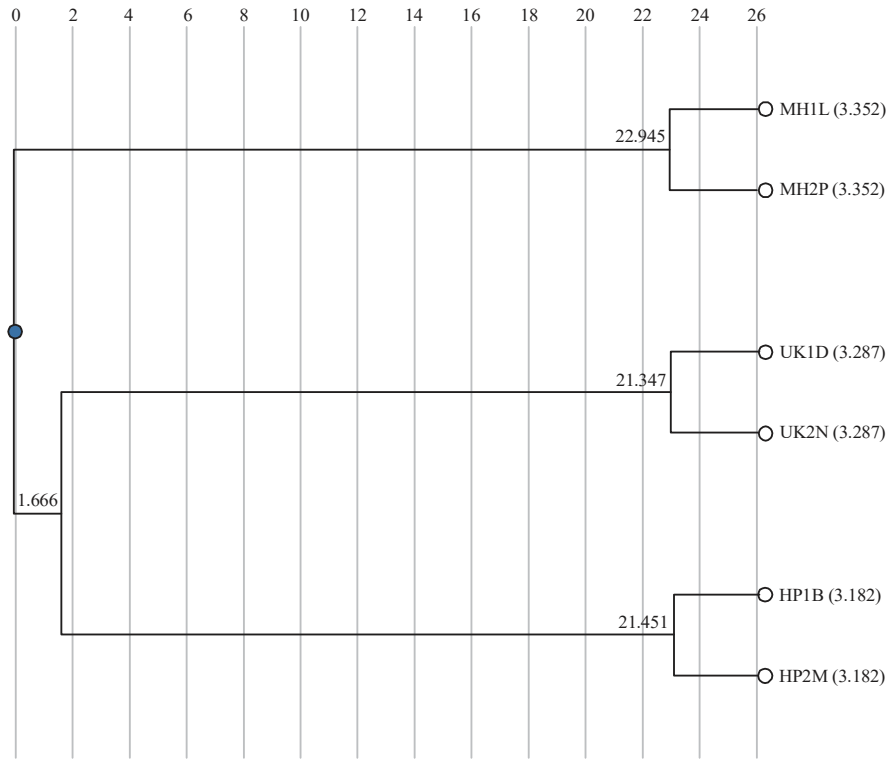

Fig. 3: Dendrogram for samples of *M. oleifera* derived from RAPD fingerprints generated by using 4 different primers produced from the distance matrix based upon UPGMA (Unweighted Pair-Grouped Method Arithmetic) average

HP1B and HP2M samples from Himachal Pradesh, UK1D and UK2N samples from Uttarakhand, MH1L and MH2P samples from Maharashtra

of *M. oleifera* from coastal regions of Tanzania and obtained 89.6% polymorphism using 12 RAPD primers whereas Abubakar *et al.*[24], obtained 75% polymorphism using 24 RAPD primers. In another study to check the genetic diversity in 16 accessions from the germplasm bank of Brazil, RAPD primers generated a total of 95 fragments of which 59 were polymorphic[3]. All of these reports indicate the genetic behavior of *M. oleifera*. The RAPD primers used in this study are effective in generating polymorphisms in *M. oleifera* from three different states of India.

CONCLUSION

In the present study, we have used RAPD to find out genetic diversity of *M. oleifera* between South-Central and Northern states of India. RAPD data revealed that the samples from different regions got paired into 3 separate clades at similarity level of 88.5%, showing maximum homology within their respective pairs and high level of polymorphism was observed between the samples from south-central and northern parts of the country which showed that they were distantly related to each other. RAPD is a fast and sensitive method and provides reproducible fingerprints of complex genomes without prior sequence information and provides a cost-effective method for the precise evaluation of variability.

SIGNIFICANCE STATEMENT

This study has discovered the existence of the considerable genetic variation between the samples of northern and south-central part of the country, which could be beneficial for the scientific community in exploring the possibility for the presence of different types of species of *M. oleifera* existing around the distant regions in India and even across the world. Moreover, this also encourages the researchers in a direction to uncover the critical areas of human interest which may include the exploration of some novel phytochemical, medicinal and nutritional properties which are still undiscovered in the species under study till date. Thus, a new drive in researchers may arrive that could lead to rapid strides in the field of human health care considering the historical cultural background of *M. oleifera* claiming it to be a miracle tree having medicinal properties.

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