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Molecular fingerprinting and assessment of genetic variations among advanced breeding lines of *Moringa oleifera* L. by using seed protein, RAPD and Cytochrome P_{450} based markers

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ABSTRACT

Moringa oleifera L. is one of the important vegetable crop having huge commercial and nutraceutical value. Understanding the genetic diversity among advanced breeding lines is prerequisite to any genetic improvement and development of superior cultivars. In the current study, genetic variation and relationships among seven advanced breeding lines were investigated using seed protein profile, 31 RAPD and 10 Cytochrome P_{450} gene based markers. Seed protein exhibited variation in breeding lines G1 and G5 by lacking the protein bands of 21 kDa and 35 kDa. Selected RAPD and Cytochrome P_{450} gene markers showed an average of 79.68 and 86.44% polymorphism respectively. Marker index RAPD (1.65), Cytochrome P_{450} (4.81) suggests that Cytochrome P_{450} gene markers are most efficient for variability analysis. Dendrogram constructed for genetic similarity using UPGMA clustered genotypes into two main clusters and two sub-clusters, indicates variation within the lines and a major cultivar. High bootstrap values (100 and 85) were obtained at major nodes of the dendrogram by means of WINBOOT software. Principal coordinate analysis plots of the two markers were found highly concordant with each other. Mantel test was employed to determine cophenetic correlation and value above 0.90 for both marker systems was obtained. Distinct bands observed in KDM-01 and Dhanraj can be utilized for marker assisted selection. Comparing these functional and non-functional markers suggested that these marker systems are promising in assessment of genetic diversity among advance breeding lines of *Moringa oleifera* L. and could be exploited for breeding program, DNA fingerprinting, marker assisted selection and cultivar development.

1. Introduction:

Moringa oleifera L. commonly known as drumstick or horseradish is a small to medium-sized tree, native to Northern India of South Asia and tropics (Ramachandran et al. 1980). *M. oleifera* has diploid chromosome ($2n = 28$) and it is the most widely cultivated species of the genus *Moringa*. Drumstick is an important food source (Nautiyal and Venkataraman 1987) containing proteins, vitamins, minerals and antioxidant properties, where it helps to protect cells from damage. Nutritionally, pods are good sources of calcium, phosphorous and vitamin C (Fuglie 2005). It is also used in feeding programs to fight malnutrition in many third world nations. Many phytochemicals present in *M. oleifera* have potent anticancer and hypotensive activity reported to use in ancient Siddha medicine (Soller and Beckmann 1983). Immature pods, fresh/dried leaves, and flowers are generally used for cookery purpose (Jonathan and Weden 1990) and are on high demand in the modern dietary system. The edible oil extracted from *M. oleifera* is highly nutritious with high concentration of oleic acid (>73%) along with low polyunsaturated fatty acids. Despite such immense importance, the crop is still unexploited (Pandey et al., 2011). There is a need to understand genetic diversity pattern to facilitate improved breeding program.

Due to the fast-growing nature and adaptability to variable climatic conditions, drumstick has been popularly cultivated across India. Genetic variability assessment of breeding lines based on morphological and cytological approaches are not only time consuming but can also lead into an error at multiple levels (Monica et al. 2010). Understanding of genetic diversity using desired markers can aid classical crop breeding activities for various traits (Bretting and Widrelechner 1995). In plant breeding program, assessment of genetic relation is necessary for determination of uniqueness, distinctness of the phenotype and to understand genetic constitution of genotype (Davierwala et al. 2000).

Abbreviations: PCR, polymerase chain reaction; PIC, polymorphism information content; RAPD, random amplified polymorphic DNA; UPGMA, unweighted pair group method with arithmetic averages; RFLP, restriction fragment length polymorphism; MR, multiplex ratio; PIC, polymorphic information content; MI, marker index.

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Table 1
M. oleifera accessions used for genetic diversity analysis.

Sl. no.	Name	Pedigree	Year of release
G1	Drumstick (KDM-01) Bhagya	6/4 LP-98KLP-99-KDM-01	2010 By UHS, Bagalkot
G2	Drumstick	HUB-DRM-10/1	Germplasm line under testing
G3	Drumstick	HUB-DLM-10/2/1	Germplasm line under testing
G4	Drumstick	HUB-DRP-10/LP-1	Germplasm line under testing
G5	Drumstick (Dhanraj)	S-6/4	1994, by UHS Dharwad
G6	Drumstick	HUB-/LP-1/3	Germplasm line under testing
G7	Drumstick	HUB-DRP-10/LP-1/4	Germplasm line under testing

Use of marker assisted selection of germplasm for the desired trait will benefit faster-breeding program. Identification of polymorphism at genome level reveals the fact of diversity and polymorphism which can easily detect genetic diversity within a population (O'Neill et al. 2003: Wu et al. 2004). Thus, in recent years, uses of such genetic markers for crop improvement approaches are widely employed (Garcial et al. 2004).

Most commonly used PCR-based molecular markers are restriction fragment length polymorphism (RFLP) (Thomas et al. 2000), amplified fragment length polymorphism (AFLP) (Kim et al. 2002), microsatellites and mini satellites (Saini et al. 2013), inter-simple sequence repeats (ISSR) (Zietkiewicz et al. 1994) and RAPD (Jacobson and Hedren 2007). Among these RAPD is the most popular, rapid and inexpensive method followed to ascertain the genetic diversity of plant. Cytochrome P_{450} gene based markers are also one of the most effective markers for wide genome diversity analysis (Yamanaka et al. 2003: Ohkawa et al. 1999). These are the terminal oxidase enzyme in electron transfer chain called hemoproteins, involved in biosynthetic pathway of major phytoalexins, alkaloids, terpenoids, lipids, cyanogenic glycosides, and glucosinolates, as well as plant hormones (Masaharu and Daisaku 2010), hence these markers can successfully contribute to large genomic approach for diversity analysis.

These Cytochrome P_{450} gene based markers are known to produce high level of polymorphism which covers wide genome space and help to understand complete genetic diversity. Apart from use of arbitrary markers, protein markers also provide greater precision for genetic diversity analysis (Nelson 1999: Panwar et al. 2010). Seed storage proteins are primary product of structural genes and are coded by a huge number of gene families and a sub-family of genes which code for particular protein. These proteins are stable product of genes and cannot be denatured during seed formation (Wei-dong et al. 2006). Seed protein analysis is a comparatively simpler approach for bulk protein analysis. These stable products of genes reflect genetic diversity of plant systematics. Polymorphism developed by seed protein has widely applied in plant classification, varietal identification, screening of mutants for seed storage proteins and genetic diversity analysis. Seed protein marker for genetic diversity has been used in several crops such as, rice, wheat and maize but not successfully employed in drumstick.

Assessment of genetic diversity among commercially cultivated varieties of *M. oleifera* has been carried by AFLP (Muluvi et al. 2004), RAPD (Mgendi et al. 2010: Silva et al. 2012) and ISSR (Saini et al. 2013). However, the present research was carried out to understand the molecular pattern of genetic similarity between sib-mated lines of *M. oleifera* using seed protein, RAPD and Cytochrome P_{450} gene based marker. Understanding genetic variation among advanced breeding lines could be exploited for varietal selection and cross breeding program.

2. Materials and methods

2.1. Collection of plant material

Seven advanced breeding lines of *M. oleifera* developed at different locations were used for the study. Pure seeds were collected from different places of Karnataka, India. Accession details of each breeding line are given in Table 1.

2.2. Seed protein analysis

Single seed from each breeding line was grounded into fine powder form and protein was extracted by adding 1000 μl 0.25 M Tris- HCl buffer (pH 8.8) containing 0.01% 2-mercaptoethanol for overnight. Samples were centrifuged at 10,000g for 15 min, supernatant was collected and ice cold acetone was added to obtain protein pellet. The protein pellet was dissolved in working buffer (0.15 M Tris–HCl pH 6.8 containing 3% SDS, 5% 2-mercaptaethanol and 7% glycerol) and used for vertical electrophoresis. The medium range marker was used to assess the size of the studied protein profile in electrograms. Electrophoresis was performed until the tracking dye reaches the bottom of resolving gel. Gel was stained using staining solution 9% methanol, 2% acetic acid and 0.05% coomassie brilliant R-250 (Hames and Rickwood 1990) for 3 h and de-staining was performed using 9% methanol, 2% acetic acid until protein bands were clearly observed, gels were documented.

2.3. DNA extraction, purification and quantification

Standard CTAB method (Sambrook and Russell 2001) was followed for isolation of genomic DNA with minor modification to remove phenolic content. Freshly harvested leaves (200 mg) were grounded to fine powder with liquid nitrogen in pre-chilled mortar and transferred into 2 ml centrifuge tubes containing 750 μl extraction buffer (0.1 M Tris Hcl (pH 8.0), 0.02 M EDTA (pH 8.0), 2% CTAB (*w*/*v*), 1.4 M NaCl, 2% polyvinyl pyrrolidone (*w*/*v*) and 0.2% β-mercaptoethanol (*v*/*v*)) and incubated in water bath at 65 °C for 30 min, for DNA purification

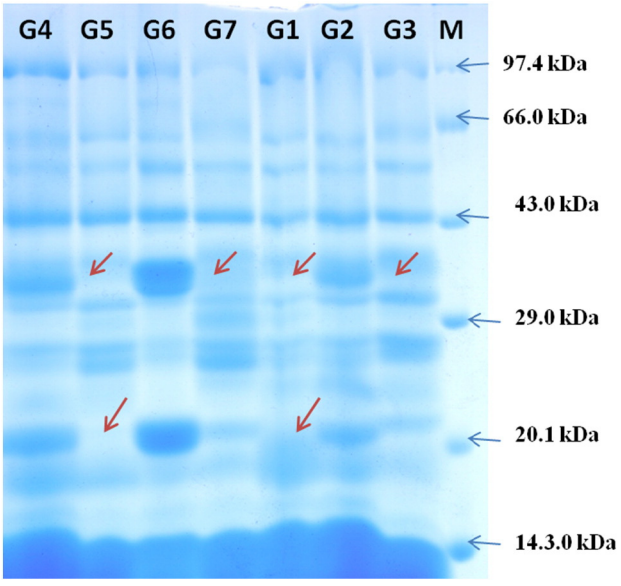

Fig. 1. Seed protein profile of seven lines of *M. oleifera* showing variations, arrows represents polymorphic bands in a particular line.