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RESEARCH ARTICLE

GENTIC DIVERSITY ANALYSIS ON MORINGA OLIEFERA BY USING DIFFERENT MOLECULAR MARKERS: A REVIEW

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ABSTRACT

Moringa Oliefera is a multipurpose tree introduced to Africa from India at the turn of this century. Despite limited knowledge of the levels of genetic diversity and relatedness of introduced populations, their utilization as a source of seed for planting is widespread. In order to facilitate reasoned scientific on its management and conservation and prepare for a selective breeding programme genetic analysis of different population of *Moringa oliefera* DNA marker techniques, i.e., random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), Amplified fragment length polymorphism (AFLP-PCR), inter simple sequence repeat (ISSR) and Cytochrome P450 gene-based marker were used. This study reveals a huge genetic diversity among the cultivars and this can be utilised for conservation and cultivar development in breeding programmes to produce high yielding, nutritionally superior cultivars of *M. oliefera.*

INTRODUCTION

Moringa oleifera Lam. (synonym: *M. ptreygosperma* Gaertn.), an economically important multipurpose tree indigenous to northwest India, is the most widely cultivated, applied, and well-known one of all 13 species in the monogeneric family Moringaceae (Olson, 2002).

The plants have always been vital for mankind irrespective of the era and area all over the globe since the beginning of life. Popularly known as ‘‘Drumstick’’ tree, horseradish tree, or Ben tree, *M. oleifera* is a deciduous-to-evergreen shrub or small tree with a height of 5 to 10 m (Morton, 1991). Its seedlings are fast-growing with early sexual maturity and a height up to 4.5min 9 months and flowering in half a year (Von Maydell, 1986). *M. oleifera* used to distribute wildly in the forests of Western Himalaya (Hooker, 1879), and then throughout India by cultivation (Selvam, 2005).

Featured by richness in proteins, minerals, and vitamins, the leaves of M. oleifera are used as a highly nutrient vegetable and as cattle fodder (Mughal *et al*., 1999). In addition, the seed powder is used in water purification (Ndabigengesere and Narasiah, 1998), and the seed oil is acquired for edibles, lubrication, and cosmetics (Anwar and Bhanger, 2003). Because of its multiple applications and commercial benefits, *M. oleifera* has been broadly introduced and cultivated around the world, and has been identified as important in agri-horti-silviculture programs (Morton, 1991). It is commonly planted in hedges and house yards, especially intercropped in agroforestry systems, and it thrives in various subtropical and tropical regions (Selvam, 2005). Nevertheless, there is a deficient understanding of its detailed gene flow pattern and population genetic structure, which causes uncertainty in designing and managing seed orchards (Muluvi *et al*., 2004). Thus, the development of efficient molecular markers for *M. oleifera* is needed. However substantial variation in quantitatively inherited trait has been documented in natural populations of Moringa in India (Ramchandran *et al*., 1980). Moreover limited studies have been conducted using DNA-based markers to identify and assess diversity among various genotypes of *M. oliefera* (Muluvi *et al*., 1999).

Molecular Assessment of Genetic Diversity

Analyses of genetic diversity are usually based on assessing the diversity of an individual using either allozymes (i.e., variant forms of an enzyme that are coded for by different alleles at the same locus) or molecular markers, which tend to be selectively neutral. It has been argued that the rate of loss of diversity of these neutral markers will be higher than those which are associated with fitness. In order to verify this (Reed and Frankham, 2003) conducted a meta-analysis of fitness components in three or more populations and in which heterozygosity, and/or heritability, and/or population size were measured. Their findings, based on 34 datasets, concluded that heterozygosity, population size, and quantitative genetic variation, which are all used as indicators of fitness, were all positively correlated significantly with population fitness.

Genetic variability within a population can be assessed through:

1. The number (and percentage) of polymorphic genes in the population.
2. The number of alleles for each polymorphic gene.
3. The proportion of heterozygous loci per individual (Primack, 1993)

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Proteic methods, such as allozyme electrophoresis, and molecular methods, such as DNA analysis, directly measure genetic variation, giving a clear indication of the levels of genetic variation present in a species or population (Karp *et al*., 1996) without direct interference from environmental factors. Given below is an overview of the different types of molecular markers used for assessing genetic diversity (Spooner *et al*., 2005).

Molecular Marker

Molecular markers work by highlighting differences (polymorphisms) within a nucleic sequence between different individuals. These differences include insertions, deletions, translocations, duplications and point mutations.

In addition to being relatively impervious to environmental factor, molecular markers have the advantage of: (i) being applicable to any part of the genome (introns, exons and regulation regions); (ii) not possessing pleiotrophic or epistatic effects; (iii) being able to distinguish polymorphisms which not produce phenotypic variation and finally, (iv) being some of them co-dominant. The different techniques employed are based either on restriction-hybridization of nucleic acids or techniques based on Polymerase Chain Reaction (PCR), or both (Table 1).

Table 1 Acronyms commonly used for different molecular markers

AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily primed PCR
ARMS	Amplification Refractory Mutation System
ASAP	Arbitrary Signatures from Amplification
ASH	Allele-Specific Hybridization
ASLP	Amplified Sequence Length Polymorphism
ASO	Allele Specific Oligonucleotide
CAPS	Cleaved Amplification Polymorphic Sequence
CAS	Coupled Amplification and Sequencing
DAF	DNA Amplification Fingerprint
DGGE	Denaturing Gradient Gel Electrophoresis
GBA	Genetic Bit Analysis
IARO	In Inter-Simple Sequence Repeats ter-Retrotrasposon Amplified Polymorphism
ISSR	Inverse Sequence-Tagged Repeats
ISTR	Microsatellite-Primed PCR
MP- PCR	Oligonucleotide Ligation Assay
OLA	Randomly Amplified Hybridizing Microsatellites
RAHM	Randomly Amplified Microsatellite Polymorphisms
RAMPs	Randomly Amplified Polymorphic DNA
RAPD	Retrotrasposon-Based Insertion Polymorphism
RBIP	Restriction Endonuclease Fingerprinting
REF	Retrotrasposon-Microsatellite Amplified Polymorphism
REMAP	Restriction Fragment Length Polymorphism
	Selective Amplification of Polymorphic Loci
RFLP	Sequence Characterised Amplification Regions
SAMPL	Single Nucleotide Polymorphism
SCAR	Single Primer Amplification Reaction
SNP	Single Polymorphic Amplification Test
SPAR	Sequence-Specific Amplification Polymorphisms
SPLAT	Single Strand Conformation Polymorphism
S-SAP	Single Sequence Length Polymorphism
SSCP	Simple Sequence Repeats
SSLP	Sequence-Tagged Microsatellite Site
SSR	Sequence-Tagged-Site
STMS	Thermal Gradient Gel Electrophoresis
STS	Variable Number Tandem Repeats
TGGE	Randomly Amplified Microsatellites
VNTR	
RAM	

In addition, the different techniques can assess either multi-locus or single-locus markers. Multi-locus markers allow simultaneous analyses of several genomic loci, which are based on the amplification of casual chromosomic traits through oligonucleic primers with arbitrary sequences. These types of markers are also defined as dominant since it is possible to observe the presence or the absence of a band for any locus, but it is not possible to distinguish between heterozygote (a/-) conditions and homozygote for the same allele (a/a). By contrast, single-locus markers employ probes or primers specific to genomic loci, and are able to hybridize or amplify chromosome traits with well-known sequences. They are defined as co-dominant since they allow discrimination between homozygote and heterozygote loci.

Used molecular markers in assessment of *M. olifera*

Limited studies have been conducted using DNA-based markers to identify and assess diversity among various genotypes of *M. oleifera*. Muluvi *et al*. (1999) used amplified fragment length polymorphism (AFLPs) to investigate *M. oliefera* populations present in Kenya, revealing significant differences between regions and populations.

Assessment of genetic diversity in *M. oleifera* has been done using AFLP (Muluvi *et al*. 1999; Muluvi *et al*., 2004), Cytochrome P450 gene-based marker (Schalk *et al*., 1999) and RAPD (Mgendi *et al*., 2010) in accessions from Kenya and Tanzania, respectively. A total of 17 RAPD, 6 ISSR and 7 pairs of cytochrome P450-based markers generated 48.68, 48.57 and 40.00 % polymorphisms, respectively (Saini *et al*., 2013). Cytochrome P450 genes are widely found in microorganisms, animals and plants (Schalk *et al*., 1999) and play important roles in oxidative detoxification and the

secondarymetabolite production (Ohkawa *et al*., 1998). Cytochrome P450 gene-analogues have been successfully used as new genetic markers for genetic diversity studies in plants, which reflects both functional and genome-wide regions (Yamanaka *et al*., 2003b).

Recently, Wu *et al*. (2010) have developed 20 microsatellite or simple sequence repeat (SSR) markers for *M. oleifera* that can serve as useful markers for pursuing additional genetic diversity studies.

The availability of information on the genetic variation within populations and the differentiation between populations plays a significant role in the formulation of appropriate management strategies for conservation of genetic resources (Milligan *et al*., 1994). ISSR

(Inter-Simple Sequence Repeats) are dominant markers and detect polymorphisms in microsatellite and inter-microsatellite loci and do not require prior information of DNA

Sequences (Zietkiewicz *et al*., 1994). These markers have been widely used to assess genetic diversity and population structure and require comparatively small amount of DNA

(Wolfe *et al*., 1998a, b; Esselman *et al*., 1999). Each ISSR primer is composed of not only 2-3 repeats complementary to microsatellite region of the genome, but also 1-3 additional arbitrary nucleotides at the 5' or 3' end. The later serve as anchors against reverting strand slippage during amplification (Gupta *et al*., 1994). Further, the anchoring nucleotides facilitate attachment of primers to specific inter microsatellite regions, resulting in diverse banding patterns from identical repeat sequences with varying anchoring nucleotide(s) (Wolfe *et al*.,1998a, b). In contrast to other molecular markers, the target sequences for ISSR primers are abundant throughout the eukaryotic genome and evolve rapidly. Consequently, ISSR markers help in revealing a much higher number of polymorphic fragments than RAPD (Random Amplifi ed Polymorphic DNA) markers. In addition, the ISSR reaction is more specific than RAPD reaction (Williams *et al*., 1990; Fang and Roose, 1997; Wolfe *et al*., 1998).

Random Amplefied Polymorphic DNA (RAPD): RAPD is based on the amplification of genomic DNA with single primers of arbitrary nucleotide sequence. These primers detect polymorphisms in the absence of specific nucleotide sequence information and the polymorphisms function as genetic markers and can be used to construct genetic maps. Since most of the RAPD markers are dominant, it is not possible to distinguish whether the amplified DNA segment is heterozygous (two different copies) or homozygous (two identical copies) at a particular locus. In rare cases, co-dominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, may be detected.

The basic technique of RAPD involves (i) extraction of highly pure DNA, (ii) addition of single arbitrary primer, (iii) polymerase chain reaction (PCR), (iv) separation of fragments by gel electrophoresis, (v) visualization of RAPD-PCR fragments after ethidium bromide staining under UV light and (vi) determination of fragment size comparing with known molecular marker with the help of gel analysis software. It is important to note that RAPD technique requires maintaining strictly consistent reaction conditions in order to achieve reproducible profiles. In practice, band profiles can be difficult to reproduce between (and even within) laboratories, if personnel, equipment or conditions are changed. Despite these limitations, the enormous attraction of this technique is that there is no requirement for DNA probes or sequence information for primer design. The procedure involves no blotting or hybridizing steps. The technique is quick, simple and efficient and requires only the purchase of a thermocycling machine and agarose gel apparatus and relevant chemicals, which are available as commercial kits (e.g., Ready-To-Go RAPD analysis beads; GE Healthcare, Buckinghamshire, UK). Another advantage is the requirement for only small amounts of DNA (10-100 ng per reaction). Mgendi *et al*. (2010) identified genetic variations between cultivated and non-cultivated populations of *M. oleifera* present in Tanzania using 12 random amplified polymorphic DNA (RAPD) primers, and similarly Da Silva *et al*. (2012) have used RAPD markers to assess the genetic diversity of 16 Moringa accessions present in Brazil, concluding that conservation strategies should be adopted for these plants.

Amplified fragment length polymorphism AFLP-PCR: It is a highly sensitive method for detecting polymorphism in DNA. The technique was originally described by Zabeau and Vos (1993). In detail, the procedure of this technique is divided into three steps: Digestion of total cellular DNA with one or more restriction enzymes and ligation of restriction half-site specific adaptors to all restriction fragments. Selective amplification of some of these fragments with two PCR primers that have corresponding adaptor and restriction site specific sequences. Electrophoretic separation of amplicons on a gel matrix, followed by visualization of the band pattern.

Along the *Moringa olifera*, amplified fragment length polymorphism (Vos *et al*., 1995) technique has previously been used in successfully in diversity studies in other species (Maughan *et al*., 1996; Sharma *et al*., 1996; paul *et al*., 1997; Arens *et al*., 1998). AFLP assays require no previous sequence knowledge and can detect 20-100 loci per assay (Maughan *et al*., 1996; Powell *et al*., 1996). AFLP has been used to examine the genetic distinct units and genetic differentiation between natural's populations of *Moringa oliefera* from India an introduced populations in Kenya for conservation purpose (Muluvi *et al*., 1999).

The highest level of genetic diversity was found within the Indian populations. Among the Kenyan populations, the highest level of genetic variation was observed among the widely dispersed Kibwezi population while the more geographically restricted Kitui populations had the lowest level of diversity. Distribution range and population size have been identified as major correlates of within-population genetic variation in tropical tree species with restricted populations showing significantly less variation than those with a broader distribution (loveless, 1992), while genetic variation varies directly with population size (Travis *et al*., 1996). The relatively low levels of genetic variation in the introduced populations may suggest that these populations were based on a small number of genetically related accessions at the time of introduction (Muluvi *et al*., 1999).

Simple sequence repeats (SSRs) Microsatellite: Short tandem repeats (STRs) or simple sequence length polymorphisms (SSLPs), are tandem repeats of sequence units generally less than 5 bp in length (Bruford and Wayne, 1993).

These markers appear to be hypervariable, in addition to which their codominance and reproducibility make them ideal for genome mapping, as well as for population genetic studies. Based on codominant features and high allelic polymorphism, microsatellites [simple sequence repeats (SSRs)] have become a useful marker system in genetic diversity studies (Walter and Epperson, 2001) and paternity analysis (Chaix *et al*., 2003).

One common example of a microsatellite is a (CA)n repeat, (Fig. 1) where n is variable between alleles. These markers often present high levels of inter and intra specific polymorphism, particularly when tandem repeats number ten or greater. CA nucleotide repeats are very frequent in human and other genomes, and present every few thousand base pairs. Inter SSRs are a variant of the RAPD technique, although the higher annealing temperatures probably mean that they are more rigorous than RAPDs. Chloroplast microsatellites (cpSSRs) are similar to nuclear microsatellites but the repeat is usually only 1 bp, i.e. (T)n (Provan *et al*., 1999).

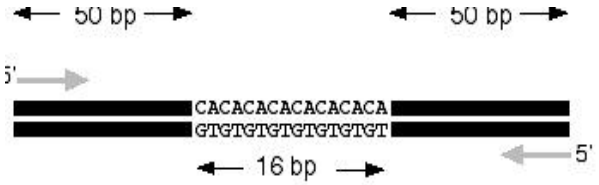

Fig.1 (CA) n repeats in microsatellite.

Microsatellites owe their variability to an increased rate of mutation compared to other neutral regions of DNA. These high rates of mutation can be explained most frequently by slipped strand mispairing (slippage) during DNA replication on a single DNA double helix. Mutation may also occur during recombination during meiosis. Some errors in slippage are rectified by proofreading mechanisms within the nucleus, but some mutations can escape repair. The size of the repeat unit, the number of repeats, the presence of variant repeats and the frequency of transcription in the area of the DNA repeat are the factors responsible for generating polymorphism.

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The microsatellite protocol is simple, once primers for SSRs have been designed. The first stage is a PCR, depending upon the method of detection one of the primers is fluorescently or radioactively labelled. The PCR products are separated on high resolution polyacrylamide gels, and the products detected with a fluorescence detector (e.g. automated sequencer) or an X-ray film. The investigator can determine the size of the PCR product and thus how many times the short nucleotide was repeated for each allele.

According to Wu *et al*. (2010), a total of 20 polymorphic microsatellite markers were developed from *Moringa oleifera* to screen polymorphism. Out of 20 SSRs used in assessing genetic diversity and population structure 19 provided good amplification, and were used to assess genetic diversity in 161 accessions of Moringa .

The availability of these microsatellite primers would provide a powerful tool for aspects of detailed population genetic studies of *M. oleifera*. With these microsatellite primers, paternity analysis of *M. oleifera*, involving pollen dispersal distance and gene flow pattern, would accumulate fundamental data for design and management of *M. oleifera* seed orchards. Moreover, these SSR primers would be helpful in revealing introduction traces and genetic components of introduced populations.

Future aspects and conclusion

Information on genetic variability of wild and cultivated plants is important for identification, conservation and cultivar development (Demir *et al*., 2010) and DNA markers are the most appropriate to study the genetic diversity among a set of genotypes or cultivars.

However, there is lack of information for Indian cultivated genotypes since India is the centre of origin for *M. oleifera*. Similarly, there is a need to evaluate the efficiency of other molecular markers for proper assessment of genetic diversity in *M*. oleifera particularly markers based on Cyt P450 genes to be exploited for assessing the intra-species diversity.

In the present study, the combination of non-functional (RAPD and ISSR) and functional (Cyt P450 based) marker systems were used to provide wider genome coverage and, therefore, will be a better indicator of the genetic relationships among the different cultivars of *M. Oliefera.*

The genetic diversity (variability) found in different study is directly linked with higher levels of biodiversity, which is useful for food security, productivity and ecological sustainability. Significant variability can be utilised in breeding programmes to produce high-yielding nutritionally superior cultivars with better adaptations to different climatic conditions. In India, major breeding programmes are on-going for the development of fast growing, disease resistant and high pod yielding cultivars of *M. oleifera.*

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