Addis Ababa University
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GENETIC VARIATION IN *MORINGA STENOPETALA* GERMPLASM OF ETHIOPIA BY USING RAPD AS GENETIC MARKER

BY
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Declaration

I, the undersigned, declare that this thesis is my own work and has not been presented in other universities, colleges or institutions, seeking for similar degree or other purposes. All sources of materials used for the thesis have been duly acknowledged.

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Abstract

The germplasm of *Moringa stenopetala* were assessed for genetic divergence within and among populations. Nineteen accessions that were represented by seventy-one individuals were studied. A total of 251 unambiguous bands were scored from OPA-13 primers. No common band was found for the entire populations. The range of mean diversity estimates of Shannon's index varies from 0.00 for Lasho and Berber to 0.3623 for Konso Gamolle within populations and the entire population was found to have the index of 0.3124. Similarly, Nie's mean estimates of diversity within populations range from 0.00 for Lasho and Berber to 0.2440 for Konso Gamolle and its entire population mean was 0.1818. The phenetic tree (dedrogram) constructed to evaluate relationships within populations was unable to discriminate 42% of the analyzed accessions. On the other hand, the range of the genetic distance was 0.00 to 0.41 and with a mean of 0.09.The genetic variability among the accessions was found to be low.

1 Introduction

Moringa stenopetala (Bak.f.) Cuf. belongs to the family Moringaceae. The family consists of a single genus with about fourteen species, which follows the distribution pathway from Rajasthan (India) through South West Africa (Verdcourt, 2000). Verdcourt further suggested that there are nine species endemic to the Somali Masai floristic region, one of which extends through Arabia to Israel, one species is confined to SW Africa (Angola), two to Madagascar and two to India including the widely cultivated *M. oleifera*. The family consists of trees and shrubs and inhabits the dry habitats of Old World Tropics (Olson, 2002). *M. stenopetala* is a native tree in Southern Ethiopia, North Kenya and Eastern Somalia. That is why; it is often named as the African Moringa. In Ethiopia, *M. stenopetala* is found in many arid areas of the Southern regions most extensively between Arbaminch and Konso at altitude of up to 1800m a. s. l. (Stenz and Mayer, 1990). *M. stenopetala* propagates from seeds unlike *M. oleifera* which propagates both from seeds and stem cuttings. It has a very low regeneration from stem cuttings (Jahn, 1991). The propagation of *M. stenopetala* has been established and maintained by local farmers. Information about its genetic diversity is scarce and pollen biology and patterns of mating is not documented. Such information would facilitate improvement programs, *ex situ* and *in situ* conservations and exploitation of Moringa genetic resources wisely (Muluvi *et al.,* 1999; Muluvi *et. al*., 2004).

The taxonomic position of the family is not clear although it has some morphological similarity to those of Bracicaceae and Capperidaceae (Verdcourt, 2000; Olson, 2002). A study made by Olson (2002) on both morphological features such as leaf form, leaf glands, life form, woody anatomy, gum duct (articulated laticifers), flowers and fruit and test anatomy and molecular data on chloroplast genome (cpDNA), gene sequence of ribulose -1-5-

bisphosphate carboxylase / oxygenase (rbcL) revealed that Caricaceae and Morinagaceae are sister taxa.

The plant is the major vegetable crop in areas surrounding Arbaminch and Konso (Lindtjørn, 1983; Mekonnen and Gessesse, 1998; Demeulenaere, 2001). It is also used as bee forage (Tessema *et al*., 1993). In Konso area, *M. stenopetala* is cultivated in terraced fields, gardens and small towns, and also naturally grows in riverin and Acacia Commiphora woodlands and on rocky grounds (Verdcourt, 2000). Jiru (1995) further stated that the plant grows in dry climates in which most of the native trees hardly survive. Therefore, it may be an important vegetable crop recommended in harsh environments, which are frequently affected by drought. The food chemistry of *M. oleifera* worked out by Makkar and Becker (1997) revealed that the leaf nutritional value is attractive because of its protein content of about 23%, favorable amino acid pattern and low level of anti nutritional factors. *M. stenopetala* may also have equivalent or more potential than that of its close relative Indian Moringa (*M. oleifera*). Unfortunately, there is no work on nutritional contents of the leaves and other parts of this plant. Xerophthalmia, the deficiency disease of vitamin A was found to be a major health problem in Gardula localities (Former Gamo Gofa Administrative region in South-West Ethiopia) as suggested by Lindtjørn (1983). It is a preventable disease through feeding foods, which are rich in vitamin A. It is advantageous to study the food chemistry of *M. stenopetala*, which is the staple dietary in the study area. It is valuable to study the nutritional contents of *M. stenopetala* instead of introducing less practiced indigenous feeding habits to the area. Costwise, it is also more economical and acceptable by local people because they eat cooked Moringa leaves.

There is no molecular data, which assess the genetic diversity that exists in the species. The Moringa plants collected from different parts of the country show variation in growth rate, nutritive value of leaves, bitterness of leaves (due to the antinutritive glucosinolate compound), insect pest and disease and drought resistance (Personal communication). The assessment of genetic diversity and relatedness in germplasm collection is important for effective utilization of the germplasm, for breeding programs and identification of conservation priorities (Bekele, 1983; Demissie and Bojørnstand, 1996).

The information about the extent and distribution of genetic variability within species is an important requirement for effective collection, characterization, *ex situ* and *in situ* conservations as well as for utilization in crop improvement (Bretting and Widrlechner, 1995). This study is an attempt to assess the genetic variability that exists in the selected collected accessions. The collected accessions are planted at Konso, Adami Tulu, Debrezit and Arbaminch. The genetic variability was assessed using Random Amplified Polymorphic DNA (RAPD). The main advantages of the RAPD technology are suitability for the work on anonymous genome, application to the problem where only limited amount of DNA is needed and its efficiency and cost effectiveness (Hadrys *et al*., 1992, Tingey and Tufo, 1993; Rabouam *et al*., 1999). Therefore, the method is chosen to assess genetic variability.

2 Objective of the Study

- ❖ To assess the genetic divergence within the accessions.
- ❖ To assess the genetic distance between the accessions.
- ❖ To suggest conservation strategies for the management of the accessions.

3 Literature review

3.1 Description of *Moringa stenopetala*

Kingdome *Plantae*

Subkingdome *Tracheobionta*

Division *Magnoliophyta*

Class *Magnoliopsida - Dicotyledons*

Subclass *Dilleniidae*

SuperOrder *Violanae*

Order *Moringales*

Family *Moringaceae*

Genus *Moringa*

Species *stenopetala*

The taxonomy is extracted (Kertesz, 2004). The family Moringaceae consists of only one genus called Moringa. The genus holds fourteen species (Verdcourt, 2000). *M. stenopetala* is one of the species which belongs to the genus, Moringa. It is distributed in tropical Africa (Eilert *et al.,* 1981; Jahn, 1991; Mekonnen, 1999).

Moringa stenopetala has the following features: It is a tree 6-10 m tall; trunk up to 60 cm in diameter at breast height; crown strongly branched; sometimes with several trunks, thick at base; bark white to pale grey or silvery, wood soft, branches with leafy scarces 6-10 mm across; young stems and leaves densely velvetly pubescent. Leaves up to 55 cm long, 2-3 pinnate with five pairs of pinnae and 3-9 leaflets elliptic to ovate, 3.3 – 6.3 cm long, 1.8 – 3.3 cm wide, acute, with thickened apiculus at apex, rounded to cuneate at the base, pubescent or glabrous; petiole 10 – 15 mm long. Inflorescence pubscent, dense many-flowered panicles up to 60 cm long, flowers strongly scented, regular, hypogynous, petals up to 10 mm long, very

densly villous inside. Pods elongate, raddish with greysh bloom, at first twisted, later straight, sometimes torulose, 19.7 – 50 cm long, (1.8-) 2.3 – 4 cm wide, the valve grooved. Seeds creame and brown, spongy, smooth, elliptic – trigonous, the body 25 – 35 cm long, 14 – 20 mm wide, the wings 60 – 90 mm long, 25 – 32 mm wide over all with sinuses. These features of the species are adopted from Verdcourt (2000) and specimens deposited at the National Herbarium of the Addis Ababa University. The photo of the plant is shown in the Fig. 3.1.

Fig. 3.1 *Moringa stenopetala* (Photo K. Sonder).

3.2 Konso, Selection of *Moringa stenopetala*

Konso people have a well organized and adaptable agro forestry farming to the existing dry climatic condition. A plot of moringa farmer is shown in the Fig. 3.2. There are a variety of plant species cultivated in Konso plot such as trees (Moringa, Terminalia and Balanites species) and shrubs (pigeon pea, cotton, coffee and others). This diversity of plant species per

plot boosts the ecological service plants gains in the integrated manner. Moreover, the tree species remains in the plot after the annual and biennial crops collected are used as a windbreak to prevent erosion. It is a sustainable agriculture.

The local people maintain the selection and propagation of the plant. Basically, the gene pool of *M. stenopetala* has been shaped and maintained predominantly by long time selection and domestication experience of Moringa farmers. Frankel (1974) mentioned those cultivars restored in the traditional agriculture are the center of genetic diversity. Frankel (1974) further added that this variability of cultivated plants is important reservoir for future improvement of cultivars in the modern agriculture. Konso can be considered as the area where the tree was first domesticated (Engels and Göttsch, 1991). They further suggested that from there the domestication has been spread into neighboring areas where it is being used intensively as well. Konso, moringa farmers have the way of selecting the mother tree to collect seeds, for propagation. The optimal temperature for germination of *M. stenopetala* seeds is $25^{o}C$ (Teketay, 1995). The criterion adopted for the selection is associated with the taste of leaves; the farmers favor the less bitter varieties (Jahn, 1991; Demeulenaere, 2001). Jahn (1991) also further added that in addition to the taste of leaves, they also use the less disintegrating leaf variety while cooking. Actually, the leaves taste bitter. Moreover, the Konso farmers transplant spontaneously germinated seedlings (Jahn, 1991; Demeulenaere, 2001). Demeulenaere (2001) further mentioned that selection is also made up on transplanted seedlings based on the character noted under the tree (the suspected mother) they were collected; small seedlings that are collected at the foot of bitter leaved trees are get rid of. Demeulenaere (2001) added that some dwellers try to recognize the quality of leaves based on the colour of root: that of ‘good’ tree is red, that of ‘bad’ ones white. The local people cook repeatedly at least three times by pouring the former water and fill it with new water to reduce

the bitter taste of the leaves. This could have a negative effect on the amount of water soluble vitamins remains after cooking. Unfortunately, konso farmers cannot select better tasting and easily disintegrating leaf varieties. In addition to these traits the plant shows variability for traits such as drought and pest resistance, growth rate, nutritive values of leaves and others (personal communication). The farmers do not recognized them.

Its close relatives *M. oleifera* is adapted for mixed patterns of mating, that are 26% selfing and 74% out crossing rates that revealed from AFLP (Amplified Fragment Length Polymorphism) markers (Muluvi *et al*., 2004). Therefore, it is very difficult to fix additive genetic variance with agronomic importance through allowing the same individual to propagate. The pattern of mating of *M. stenopetala* is unknown to forward a reliable suggestion for the failure of domestication of better tasting and disintegrating varieties. The local people trying to propagate *M. stenopetala* from its stem cuttings unlike to that of Indian moringa, it has slow regeneration (Jahn, 1991). This method of propagation helps them to fix the desired variety, however it is less successful due to its slow regeneration.

The basic problem of the Moringa farmers is associated with the attack of caterpillar of *Noorda trimaculalis* a photo of defoliated plant is shown in the Fig. 3.3. Demeulenaere (2001) has reported that the pest will destroy the leaves of the entire plant population of village within a week. This pest could be a drawback for conservation of the plant. Severely affected plants were observed during the field trips to collect samples at Arbaminch and Konso germplasm plantation sites.

Fig. 3.2 The plot of a typical Konso farmer that consists of a variety of plants (Photo K. Sonder).

Fig. 3.3 Severely defoliated *M. stenopetala* at Konso by *Noorda trimaculalis* (Photo K. Sonder).

3.3 Use of *Moringa stenopetala*

Moringa stenopetala is a multipurpose tree that is cultivated as agro forestry in Southern Ethiopia. The plant is used as a living hedges and wind breaks to reduce the rate of erosion. It is grown as a vegetable tree and medicinal plant in Southern Ethiopia and Njemb tribe, living in Kenya (Berger *et al*., 1984; Jahn, 1991). The major growing regions in Ethiopia are Arbaminch and surrounding areas, Negelle and Wellayta sodo (Mekonnen and Gessesse, 1998). *M. stenopetala* is commonly called Shiferaw in Amharic and Aleko in Wellayeta and Gamo languages. The members of its family (Moringaceae) such as *M. oleifera* and *M. stenopetala* their leaves, young fruits and roots are eaten (Cornquist, 1981). The leaves of *M. stenopetala* are eaten after boiling like that of cabbage (Brassica sp.) (Hallpike, 1970; Liditjørn, 1983; Göttsch, 1984; Mokonnen and Gessesse, 1998). Thus, it could play a much more important role in nourishment of people and in the sustainable use of the environment with limited rainfall in tropical belt between 1400m and 1900m a. s. l. (Engels and Göttsch, 1999). Mokonnen and Gessesse (1998) have also reported that local peoples use boiled leaves as tea or chopped and mixed it with water to treat malaria, hypertension, stomach problems, expulsion of retained placenta and in some other problems like asthma and diabetes. The expulsion of retained placenta was proved on test animals by Mekonnen (1999). Mekonnen confirmed that traditional use of the juice of leaves of *M. stenopetala* to expel retained placenta in experimental animal might be due to the presence of oxytocic like substance in the leaves. Oxytocic is a hormone produced by posterior lobe of pituitary gland which causes spontaneous contraction of the uterus wall and ejection of milk from the mammary gland (Ramarao and Suryalakshmi, 1994).

People inhabiting around Lake Turkana use the leaves for treatment of Leprosy, and also fodder for their livestock (Jahn, 1991). Jahn further suggested that in Ethiopia rift valley, and

in settlement of the Konso and Burji minorities in Kenya*, M.stenopetala* leaves are sold in the vegetable markets. Therefore, it serves them as a source of income for Moringa farmers. Usually, it is frequently consumed during famine time, it's cost also yet not encouraging for the farmers to produce the vegetable crop as cash crop. This observation is further confirmed by Jahn (1991) who mentioned that some farmer cooperatives which were chosen at North of Arbaminch in 1988 to receive *Moringa stenopetala* seedlings grown in a government nursery asked for Mango seedlings for future marketing. It is a high time for intellectuals in the field and government in particular to promote Moringa in order to bring the desired behavioral change with respect to the plant potential uses and to create an interest among local farmers to use the plant for nutrition on daily bases, fodder and sustainable agriculture (Agro forestry) and others. The flower is good nectar sources for honey bees (Tessema *et al*., 1993).

Dried and crushed seeds of the plant are important to clarify muddy and turbid water, to suit it for drinking analogous to the chemical coagulant Aluminum sulphate (alum) (Göttsch, 1984; Eilert *et al*., 1981 and Hundie and Abebe, 1991). Eilert *et al*. (1981) and Hundie and Abebe (1991) have also further proved that it lowers the concentration of bacteria (it has bactericidal effect) and fungi (it has fungicidal effect). Eilert *et al*. (1981) have also reported that water purification and its bactericidal and fungicidal effect over weigh that of *M. oleifera*. However, the local people in Ethiopia do not use the plant for water clarification (Göttsch, 1984). Göttsch (1984) further added that only the roots of *Maerua subcordata* (Amaharic: Wiha matariya) is used for water purification by people living on both sides of the lower Omo river in Gamo Gofa, South-West Ethiopia. Unfortunately, this species is toxic and not very efficient coagulant but it is on use else where in Africa (Jahn, 1991). Whereas, water purified using crushed seeds of *M. oleifera* and *M. stenopetala* is safe for human consumption (Berger *et al.,* 1984). Most Ethiopians living in rural areas are dependant on surface water, and this situation

is not likely to change in the near future. Even if locally treated water is not perfect hygienically, at least it would be important to have safe water by clarifying it with cheap and easily accessible method. Therefore, the seeds have potential for water purification. Ben oil (mustard oil) that is extracted from seeds of *M. oleifera* is used in perfume and lubricant (Cornquist, 1981; Makkar and Becker, 1997). Makkar and Becker (1997) further added that the oil could also be used for human consumption and healing of wounds by smearing over it. Therefore, *M. stenopetala* also has potential to extract the ben oil. One of the major limitations of *M. stenopetala* is that it is much slower to fruiting than that of *M. oleifera. M. oleifera* requires only ten months whereas *M. stenopetala* at least two and half years to produce the first fruit (Jahn, 1991). In general, the plant has vast potential to benefit the local people and the country economy through generating foreign currency and / or saving it.

3.4 Population structure

Population can be defined as a group of individuals of the same species who share a common gene pool, inhabit restricted geographical area and have the potential to interbreed. A population that has long been established in particular selective regime will have many genes adapted for the prevailing environmental conditions suggested by Heywood and Watson (1995). Ultimately, it may give rise to locally differentiated populations.

Populations could be structured in to individual organisms, subpopulaion and total populations (Escudeor *et al*., 2003; Devicente *et al*., 2004). Population structure affects the extent of genetic variation and its pattern of distribution in prevailing mating systems (Pirchner, 1964). In other words, understanding of the structure of population is important for devising a rational approach for *in situ* and *ex situ* conservations and planning of breeding programs. . Population is the primary unit of evolution in which change in allelic frequency

occur (Strickberger, 1985; Crandall, 2000). They further reasoned out that it is there the gene frequency change takes place. That is why different researchers study the genetic structure of populations to investigate its organization with respect to allelic frequencies at different hierarchical levels such as between cultivars and wild (Ayana *et al*., 2000; Birmeta *et al*., 2004), regions (Muluvi *et al*., 1999; Ayana *et al*., 2000; Offei *et al*., 2004), ecological sub grouping (Semagn *et al*., 2000), individuals and other relevant categories.

3.5 Conservation genetics

The threats of biological diversity appear in different angles. These are habitat fragmentation, range destruction, inappropriate land use practices, subsistence agriculture in the third world tropics and others. Lande (1988) argues that the preservation of rare and endangered species may require an expansion of their habitat. Because, their effective management and conservation is hampered due to habitat fragmentation. For sustainable use of biodiversity, one may demand assessment of genetic variability. Diversity implies a range of varieties or differences that exist among alleles, populations, ecotypes, taxa, communities and ecosystems. The restoration of endangered species requires assessment of genetic variability and interpreting the data generated in population genetics context (Hedrick, 2004). According to Heywood and Watson (1995), genetically based conservation of rare and endangered species has to fulfill the following three objectives in the identification of their conservation priorities. These are:

- To ensure that individuals of representatives of genetic variability within species are included in conservation program.
- To help and to determine which population(s) contain the genetic variation and
- To conserve populations across their geographic range that means the ecotype in which they are found. This ensures that co adapted gene complexes (that is group of

alleles on one or more genes that adapt to the same selective pressures experienced in a particular environment) are conserved and not just representatives of the same alleles.

Conservation genetics tries to identify and prioritize more genetically variable representative population(s), which bears the variability. This variability is the evolutionary adaptability potential which guarantee its continuity in future. Moreover, it might become more resilience to the adverse effects of the environment that occurs in space and/or time (Luck, 2003). Conservation genetics design a rational approach to identify priority units of conservation activities that may involve offsite collection, rehabilitation, habitat expansion, reintroduction of living organisms in germplasm and others to rescue rare and endangered species (Falk, 1990; Heywood and Watson, 1995).

3.6 Molecular markers

Studies of genetic diversity in geographically isolated populations are designed to identify the process of micro evolutionary changes. The availability of easily accessible, reproducible, polymorphic, less time consuming, cost effective and locus specific markers have been available more than thirty-five years ago; but since the mid 1960's several major technological advances have given rise to a variety of new methods for measuring genetic diversity. Morphological and ecological characterization in the field is also essential not only for the preliminary evaluation of germplasm but also for seed production. However, most non molecular methods suffer from a lack of supportive genetic information and weak discriminatory power (Lee, 1998). Lee (1998) further argued that the methods shall remain important, but they have been, could be, addressed by techniques derived from plant genomics. The use of molecular markers (RAPD and others) is controversial; the reason is

that the markers are selectively neutral (King and Burke, 1999; Heywood and Iriondo, 2003). The correlation between these traits and the adaptive traits of a species is not straight forward (Heywood and Iriondo, 2003). However, they are important in providing relevant information for identification of units of conservation suggested by themselves and others. Nowadays, it is shifted from assessing of protein polymorphism in to DNA based assessment to generate data. Molecular markers have been proven crucial for shaping of *in situ* and *ex situ* management strategies and for successful identification of units of conservation (King and Burke, 1999; Gaudeul *et al*., 2000; Heywood and Iriondo, 2003) and to trace parental sources of favorable alleles (Lee, 1998). Because of the advancement of the technology a lot of studies have been exponentially grown in the use of molecular markers for the evaluation of germplasm, managements, breeding programmes, genome mapping, patterns of matings, migration (gene flow) and others. They provide unambiguous power to analysis of genetic relationships between organisms (Hedrick, 1992).

Molecular markers provide the amount of genetic differentiation among populations that are isolated by distance. These information would provide us useful data for planning actions such as reinforcement of the existing populations, reintroduction, or *ex situ* conservation or seed collection and others (Lee, 1998; Gaudeul *et al*., 2000). Therefore, assessment of genetic diversity is important before management decisions have been taken. King and Burke (1999) suggested that the greater the number of independent markers' class used, the more the likelihood that useful genetic variation will be reflected in identifying the management unit(s).

3.6.1 Random Amplified Polymorphic DNA (RAPD)

RAPD reactions amplify segments of DNA that are essentially unknown to scientists in the field that is why it is prefixed with a word random. The technique samples the genomic region

more randomly without discriminating coding and non coding regions (Lynch and Milligan, 1994). Here, PCR is used to amplify a segment of DNA that is flanked by unknown sequence region of DNA at which the forward and backward primers can anneal. In this reaction, a single species of primers binds to the genomic DNA that are small inverted repeats scattered throughout the genome. If these priming sites are within an amplification distance, discrete DNA products are produced through thermo cyclic amplification (Hadrys *et al.,* 1992; Tingey and Tufo, 1993). Then after, they are identified and scored as gel phenotypes (bands) through gel electrophoresis. The inherent limitation of the technique is a dominant marker, which cannot distinguish between the homozygote and heterozygote dominants. In other words it is monoallelic, meaning that only one allele can be detected (amplified) and the corresponding allele is not detected (null allele). Therefore, it under estimates the genetic variability. This does not prevent the estimation of allelic frequencies necessary for population genetic structure studies, but it does reduces the accuracy of such estimation relative to analysis with codominant markers such as RFLP (Restriction Fragment Length Polymorphism), Isozyme and STS (Sequence Tagged Sites) as suggested by Lynch and Milligan (1994). Its further drawback is the lack of guarantee of homology between bands of similar mobility (molecular weight), thus creates difficulties for some type of studies such as phylogenetic studies. The advantage of using the technique for molecular characterization of a species is that, it does not require sequence information, thereby avoiding much labour intensive training work, it can be used in which only small quantities of DNA are available, and also it is inexpensive compared to other DNA markers (Hadrys *et al.,* 1992; Tingey and Tufo, 1993; Rabouam *et al.,* 1999). Therefore, it is a valuable molecular marker to assess and manipulate gene pool for various reasons such as breeding program, evaluation of exotic gene pool, diversity assessment, germplasm management and others. Lee (1998) suggested that inexpensive and rapid diagnostic markers are valuable to do these things. Thus, the RAPD marker fulfills these

requirements. Moreover, the technique creates the potential to employ large number of primers facilitates in the identification of useful marker(s) that are important for clone identification, genome mapping, study of sexual differentiation and identification of self incompatibility alleles (Arnold and Emms, 1998).

RAPD method is based on PCR by using usually decamer primers (Yu and Pauls, 1992) which consists of 60% to 80% G–C content with no self-incompatible ends (Perez *et al.,* 1998) which are commercially available with arbitrary sequences. Polymorphisms of amplified fragments are due to:

- Base substitution or deletion in the priming sites or
- Insertion that renders priming sites too distant to support amplification or
- Insertion or deletion that changes the size of amplification fragments.

These preconditions for polymorphism are suggested by Hadrys *et al.,* (1992), Hedrick (1992), Tingey and Tufo (1993), Rabouam *at al.,* (1999). On the other hand the number of primer mixing is also accountable for the number of bands seen at each RAPD reactions (Sall *et al.,* 2000). The molecular marker is frequently employed in assessing genetic diversity, germplasm management, micro evolutionary history and phylogenetic studies and others (Hadrys *et al.,* 1992; Ayana *et al*., 2000; Semagn *et al*., 2000; and Masumbuko *et al*., 2003)

4 Materials and Methods

4.1 Sample collection and the study area

The *Moringa stenopetala* leaves were collected from Arbaminch and Konso germplasm plantation sites by using zip locked plastic bag containing silica gel as used by Muluvi *et al.,* (1999) in *M. oleifera* and Olson (2000) in Moringa species. Leaf tissues of seventy-one individuals of nineteen accessions were collected randomly from plantation sites (Table 4.1 and Fig. 4.1).

4.2 DNA extraction from leaf samples

Nuclear genomic DNA was extracted from silica gel dried leaf tissues following the CTAB protocol procedure (Wang *et al*., 1996) with slight modification. Thirty to forty mg of dried leaves were weighed and then grounded into powder by pouring liquid nitrogen on it. The powder was collected in eppendrof tube and 750 µl of extraction buffer (0.1 M Tris acetate pH 8, 0.05 M EDTA and 0.5 M sodium chloride) and 100µl of SDS (10 % w/v) were added. The mixture was incubated for 20 minutes at 65 ^{0}C then after 250µl of 5 M sodium acetate was added and mixed by shaking gently. The mix was kept on ice for at least 30 minutes before centrifugations for about 15 minutes at 14000 rpm. The supernatant was collected and precipitated by adding the same volume of –20^{0}C cold isopropanol as of the supernatant and centrifuged about 10 minutes at 14000 rpm. Then, the pellet was air dried after disposing the supernatant about 20 minutes. The collected pellet was dissolved in 250µl TE (0.01 M Tris HCl pH 8 and 0.01 M EDTA) and then 250µl CTAB buffer (0.2 M Tris HCl pH 8, 0.05 M EDTA, 2 M NaCl and 2% CTAB) was added, and incubated for about 15 minutes at 65^{0}C. Genomic DNA was extracted by centrifugation for 5 minutes at 14000rpm following the addition of the same volume that is 500µl of chloroform. After centrifugation, there were three layers: top aqueous phase, middle: debris and protein and bottom: chloroform. The

aqueous phase contains the genomic DNA, which was collected gently without disturbing the lower layers. The chloroform step was repeated by adding the same volume of the collected top layer then after the top layer was collected after centrifugation for about 5 minutes at 14000rpm the same manner as mentioned above. Finally, the genomic DNA was precipitated by adding the same volume of -20^{0}C cold isopropanol as the collected top layer and then centrifuged at 14000rpm for about 15 minutes. The supernatant was decanted, the pellet was air dried at room temperature about 20 minutes. Then the pellet was diluted in 100µl TE and kept over night at 4 ^{0}C. RNA was digested by adding 5µl RNase (0.05 mg/ml in sample) and then incubated at 37 ^{0}C for 30 minutes. The DNA was kept at 4 ^{0}C for later use.

4.3 DNA Quantification and Quality test

The DNA concentration and its purity were determined by measuring UV absorbance. The intactness of DNA was checked through running 0.8 % (w/v) agarose gel electrophoresis that contained 0.5µl/ml of 0.5µg/ml ethidium bromide. The intensity of orange florescence was also used for rough estimation of the concentration of DNA together with UV absorbance.

4.4 DNA PCR mix for RAPD analysis

The DNA amplification was performed in total volume of 20µl containing half of its volume i.e. 10µl hotstar master mix (purchased from QIAGEN company). The master mix consists of 2.5 units of taq polymerase, 1XPCR buffer (10mM Tris HCl pH 8.7, 50mM HCl, 1.5mM $MgCl_2$ and 0.1% Triton) and 200µM or 0.2mM of each of the precursor molecules (i.e. dATP,dTTP, dGTP and dCTP). Moreover, 12ng of Operon technology primer and 20-30ng of template DNA. A DNA free sterilized distil water was added to make the final volume 20µl. while picking up of each of the components, mixing was done by tabbing and pipetting up and down to effect thorough mixing. A master mix was prepared for each RAPD reaction to

minimize measurement deviations. This drawback usually happens while measuring small volumes. All of the work was done on ice bed until they were placed in the thermocycler (eppendrof company built). The thermocycler lid was preheated before the RAPD PCR conducted.

4.5 RAPD amplification

The amplification was carried out in thermocycler (eppendrof company built) programmed for initial melting temperature step at 94^0C for 15 minutes according QIAGEN company which is optimal initiation temperature for their taq polymerase, following 45 cycle of each at denaturing temperature of 94^0C for 1 minute, annealing temperature of 36^0C for 1 minute, and extension temperature of 72^0C for 2 minutes. They were conducted at the fastest transition time that is ramp time. The last cycle was followed by an additional extension at 72^0C for about 10 minutes to allow unfinished extensions to complete.

4.6 Primer selection

Six decamer Operon technology produced random primers were used to screen a suitable primer for the study. From kit A, four primers were screened (OPA-08, OPA-09, OPA-10 and OPA-13). Out of the four primers screened, OPA-08 and OPA-10 were found monomorphic but reproducible, OPA-09 did not produced a band at all and OPA-13 was found reproducible, polymorphic and produced enough bands to suit it for the study. Therefore, OPA-13 was chosen for further study to assess the accessions of *M. stenopetala*. From kit C, two primers were screened (OPC-02 and OPC-20). OPC-02 was found polymorphic and reproducible where as OPC-20 did not produced a band at all. However, OPC-02 was not selected because it produced few bands while screening (Table 4.2). Out of the six primers screened only OPA-13 was found suitable to conduct the study.

Table 4.1 Population ID, accession localities, accession codes, sample size and geographic Information of the 19 accessions studied.

Population ID	Accession Localities	Sample Size	Accession Code	Latitude NS (dec. and deg.)	Longitude EW (dec. and deg.)	Altitude a.s.l. m
1	Lasho	4	1	6.6541	37.8961	1273
2	Arbaminch W.	4	7	6.3088	37.7408	1267
3	Berber	4	4	6.4861	37.7828	1245
4	Anata M.	3	26	5.7817	37.6467	1171
5	Dokatu-B	4	12	5.4286	37.5728	1638
6	Dera	4	21	5.5736	37.6275	1416
7	Aselle	3	17	4.9608	36.9117	727
8	Wacha	4	34	6.5183	37.3831	1245
9	Kube	4	20	5.3819	37.6306	1295
10	Korashana	4	32	6.2061	37.8097	1246
11	Yayke	4	3	6.6114	37.9758	1266
12	Skele-B	3	29	5.1058	37.6581	1129
13	Dokatu-A	4	10	5.4264	37.5767	1538
14	Wajetto	4	2	6.6850	37.9858	1224
15	Gerssale	3	23	5.4300	37.4719	1285
16	Lante	5	5	6.1911	37.6850	1223
17	Skele-A	3	28	6.1058	37.6581	1129
18	Konso G.	4	13	5.3819	37.5442	1609
19	Secha	3	30	6.1686	37.6072	1451

Where:

- Dec. and deg. Stands for Decimal and Degree respectively.
- Arbaminch W., Anata M. and Konso G. stands for the accessions collected from Arbaminch water Technology University, Anata Mieder and Konso Gamolle, respectively.

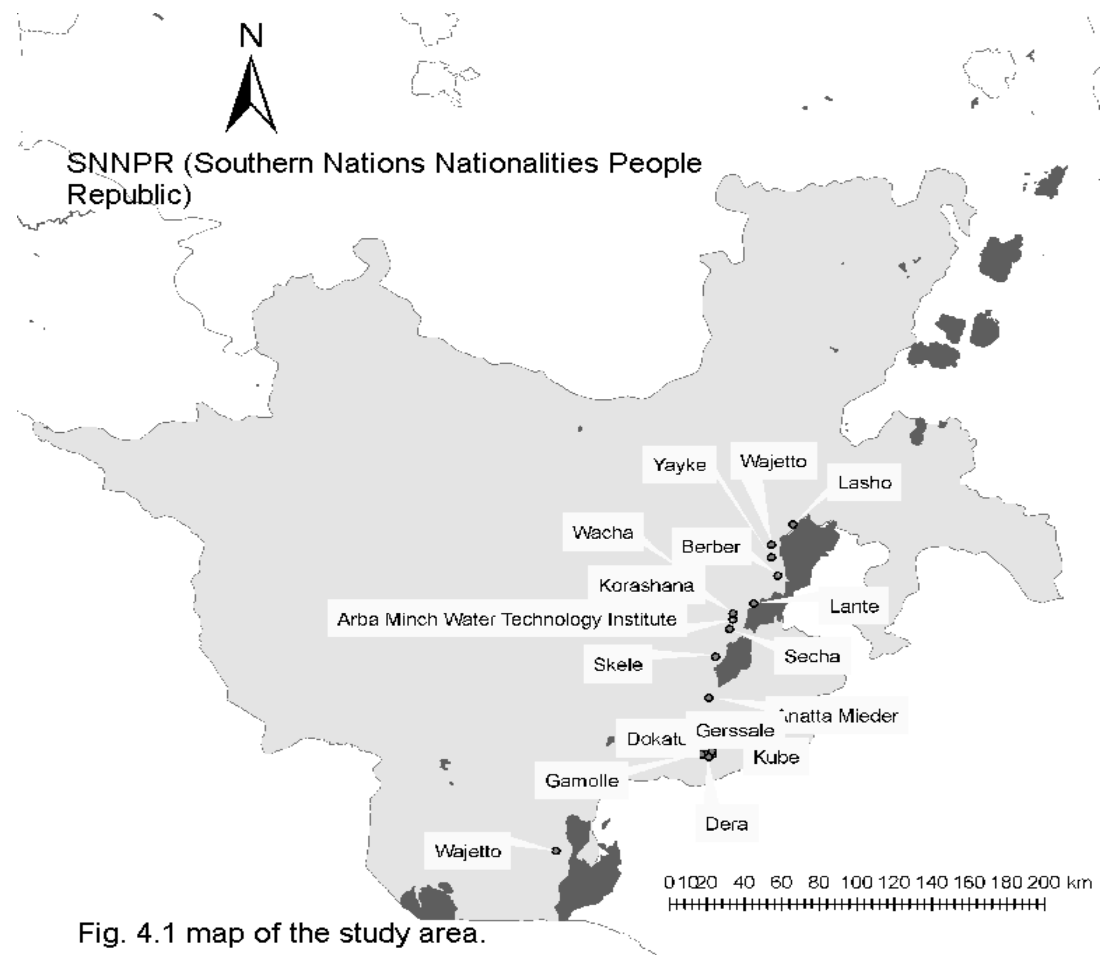

Fig. 4.1 map of the study area.

Table 4.2 Primers screened to select them for RAPD study and their details.

Primer	Sequence 5'-3'	% G-C	No. Bands	Pattern	Remark
OPA-08	GTGACGTAGG	60	1	Monomorphic	Rejected
OPA-09	GGGTAACGCC	70	0	-----	Rejected
OPA-10	GTGATCGCAG	60	1	Monomorphic	Rejected
OPA-13	CAGCACCCAC	70	Min.1and max.6	Polymorphic	Accepted
OPC-02	GTGAGGCGTC	70	Min.2 and max.4	Polymorphic	Rejected
OPC-20	ACTTCGCCAC	60	0	----	Rejected

N.B: Min. and max. Stands for minimum and maximum number of bands observed respectively.

4.7 PCR Product examinations

The PCR amplification products were electrophoresed on 1.2 % (w/v) agarose gel which consists of 0.5µl/ml of 0.5mg/ml ethidium bromide. TAE buffer (40mM Tris acetate pH 8 and 1mM EDTA) was used as running buffer and also in the gel preparation. The PCR products were loaded into wells after 5µl of loading buffer (0.041% bromophenol blue, 0.041% xylene cynol and 6% glycerol) were mixed to them. The electrophoresis was done at 90volts for about 2 and half hour. The gel phenotypes (bands) were scored through UV light illuminator and photographed by SensiCam digital camera. Each RAPD bands was considered as independent character or locus and assigned numbers in order of decreasing molecular weight. The position of each gel phenotypes was estimated against the DNA molecular ladder to avoid error while scoring. The bands were scored as either presence (1) or absence (0).

4.8 RAPD data analysis

The Operon technology OPA-13 primer has generated six unambiguous RAPD bands. They were scored from gel photograph captured by SeniCam digital camera as a binary model either present (1) and absent (0) in descending order of their molecular weights. They were also scored without considering their intensity of fluorescence. Nineteen accessions

(subpopulations) were analyzed (Table 4.1). The data generated were used to calculate: the number of polymorphic loci, percent of polymorphism, estimates of Shannon's index (Lewontin; 1972) and gene diversities (Nei; 1973) for each population and the entire data set using the POPGENE (version 1.31) Microsoft window-based software (Yeh *et al.*, 1999). The summary of the result is given in the table 5.1. The genetic diversity estimates was analyzed based on Shannon's diversity estimates by using the formula suggested by Hutcheson (1970). The formula is:

$$H = -\sum P_i \ln P_i$$

Where P_i is the proportion of amplified bands among genotypes of accessions (treating each RAPD as a single locus according to the approach of Lewontin (1972) for Isozymes). Estimates of Nie's gene diversity were also calculated following Nei (1973).

The pair wise genetic distances and identities for each subpopulations and entire data set were computed using unbiased estimates as suggested by Nei (1978). The formula employed for the estimation of these variables is given as:

$$D = -\ln \frac{\sum_m \sum_i P_{1mi} P_{2mi}}{\left[\sum_m \sum_i P_{1mi}^2\right]^{1/2} \left[\sum_m \sum_i P_{2mi}^2\right]^{1/2}}$$

Where,

Σ_m = Summed over all loci

Σ_i = Summed over all alleles at m^{th} locus

P_{1mi} = The frequency of ith allele at m^{th} locus in P_1 population and

P_{2mi} = The frequency of ith allele at m^{th} locus in P_2 population

D = Nei's Genetic distance

The corresponding genetic matrix was subjected to cluster analysis using Unweighted Pair Grouping Method with Arithmetic Average (UPGMA) according to Sneath and Sokal (1973). The dendrogram was generated using program NEIGHBOR of PHYLIP version 3.5c by Felsentien (1993).

5 Results

5.1 Variation of RAPD Loci

Out of six decamer Operon technology screened primers, only one primer (OPA-13) was selected to conduct the study, see table 4.2. It generated six unambiguous bands. There is no any monomorphic band(s) common to all of them. Some of the samples was repeated to assess the reproducibility of RAPD profiles, they produced identical pattern of banding. A total of 251 bands were scored from 71 individuals of the 19 accessions investigated. An example of RAPD gel obtained during the study is shown in Fig. 5.1.

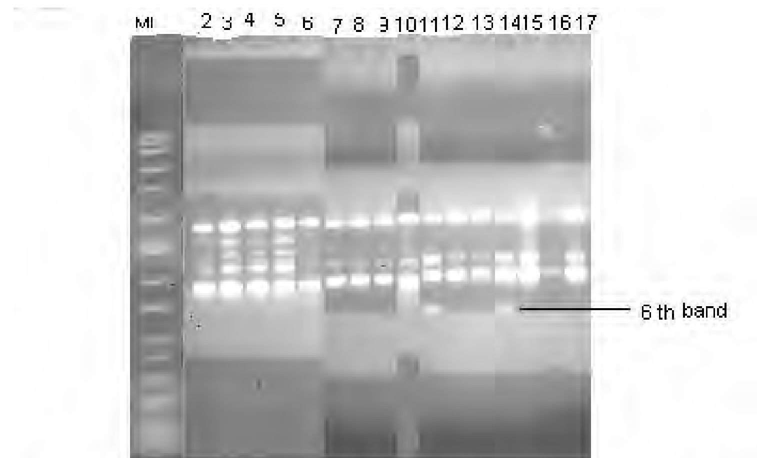

Fig.5.1 .RAPD for genomic DNA of 17 samples using OPA-13.

No population specific band(s) were observed at all. However, three accessions (Konso Gamolle, Gerssale and Yayke) had shown the sixth band (Fig 5.1). The percentage of polymorphic bands among populations was found varied from 0.00 for Lasho and Berber to 66.67 for Konso Gamolle populations as shown (Table 5.1).

5.2 Estimation of genetic variation

The amount of genetic variation for 19 accessions was determined from band frequencies by applying Shannon's index and Nei (1973) gene diversity estimates (Table 5.1)**.** The means of Shannon's index within populations ranged from 0.00 for Lasho and Berber to 0.3623 for Konso Gamolle populations. For entire data, the mean was found to be 0.3124 and standard error of 0.0206 (Table 5.1). The means of Nei (1973) estimates within populations ranged from 0.00 for Lasho and Berber populations to 0.2440 for Konso Gamolle population (Table 5.1). The entire population had shown the Nei's gene diversity estimates with a mean of 0.1818 and a standard error of 0.0150 as shown in table 5.1.

Table 5.1 Percentage of polymorphic bands (%P) and mean estimates of Shannon's Weaver diversity index and generic diversity estimates (Nei, 1973) with their standard error (SE) for 19 populations and entire data set.

Category	**Accessions**	**% P**	**Gene diversity Mean ± SE**	**Sannon's index Mean ± SE**
Populaton ID 1	Lansho	0.00	0.00 ± 0.00	0.00 ± 0.00
2	Arbaminch. W	33.33	0.1524 ± 0.1188	0.2163 ± 0.1682
3	Berber	0.00	0.00 ± 0.00	0.00 ± 0.00
4	Anata Mieder	33.33	0.1313 ± 0.1223	0.1930 ± 0.1766
5	Docatu-B	33.33	0.1667 ± 0.1291	0.2310 ± 0.1789
6	Dera	33.33	0.1077 ± 0.0883	0.1664 ± 0.1332
7	Aselle	16.67	0.0499 ± 0.0706	0.0794 ± 0.1134
8	Wacha	50	0.2053 ± 0.1227	0.2967 ± 0.1715
9	Kube	16.67	0.0387 ± 0.0474	0.0656 ± 0.0804
10	Korashana	33.33	0.0774 ± 0.0599	0.1313 ± 0.1017
11	Yayke	16.67	0.0387 ± 0.0474	0.0656 ± 0.0804
12	Skele-B	50.00	0.1498 ± 0.0947	0.2383 ± 0.1507
13	Dokatu-A	16.67	0.0387 ± 0.0474	0.0656 ± 0.0804
14	Wajetto	33.33	0.0774 ± 0.0599	0.1331 ± 0.1017
15	Gerssale	33.33	0.0999 ± 0.0893	0.1589 ± 0.1421
16	Lante	50.00	0.2374 ± 0.1165	0.3338 ± 0.1634
17	Skele-A	16.67	0.0813 ± 0.1150	0.1135 ± 0.1606
18	Konso Gamolle	66.67	0.2440 ± 0.1119	0.3623 ± 0.1555
19	Secha	33.33	0.0999 ± 0.0893	0.1589 ± 0.1421
Entire data		---	0.1818 ± 0.0150	0.3124 ± 0.0206

Table 2.5 Nei's unbiased measure Genetic identity (similarity) and distance (dissimilarity) Based on Nei (1978).

pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	***	0.95	1.00	0.95	0.92	0.99	1.00	0.92	1.00	1.00	0.81	1.00	0.87	1.00	1.00	0.93	0.77	0.93	1.00
2	0.05	***	0.95	0.91	0.85	1.00	0.98	0.97	0.97	0.99	0.86	1.00	0.88	0.99	0.97	0.98	0.97	0.97	1.00
3	0.00	0.05	***	0.95	0.92	0.99	1.00	0.92	1.00	1.00	0.81	1.00	0.87	1.00	1.00	0.93	0.78	0.93	1.00
4	0.05	0.09	0.05	***	0.84	0.94	0.94	0.98	0.96	0.95	0.83	0.95	0.99	0.95	0.95	0.99	0.77	0.85	0.95
5	0.08	0.15	0.08	0.16	***	0.90	0.92	0.80	0.92	0.91	0.69	0.91	0.76	0.91	0.91	0.81	0.64	0.92	0.91
6	0.01	0.00	0.01	0.06	0.10	***	1.00	0.97	1.00	1.00	0.85	1.00	0.85	1.00	1.00	0.98	0.90	0.97	1.00
7	0.00	0.02	0.00	0.06	0.08	0.00	***	0.95	0.99	1.00	0.81	1.00	0.86	1.00	1.00	0.95	0.85	0.96	1.00
8	0.08	0.03	0.08	0.02	0.20	0.03	0.05	***	0.93	0.95	0.75	0.96	0.95	0.95	0.92	1.00	0.91	0.92	0.96
9	0.00	0.03	0.00	0.04	0.08	0.00	0.01	0.07	***	1.00	0.86	1.00	0.86	1.00	1.00	0.95	0.80	0.93	1.00
10	0.00	0.01	0.00	0.05	0.09	0.00	0.00	0.05	0.00	***	0.86	1.00	0.86	1.00	1.00	0.96	0.85	0.95	1.00
11	0.19	0.14	0.19	0.17	0.31	0.15	0.19	0.25	0.14	0.14	***	0.88	0.64	0.86	0.89	0.87	0.74	0.76	0.88
12	0.00	0.00	0.00	0.05	0.09	0.00	0.00	0.04	0.00	0.00	0.12	***	0.85	1.00	1.00	0.97	0.87	0.97	1.00
13	0.13	0.21	0.13	0.01	0.24	0.15	0.14	0.05	0.14	0.14	0.36	0.15	***	0.86	0.86	0.94	0.59	0.75	0.86
14	0.00	0.01	0.00	0.05	0.09	0.00	0.00	0.05	0.00	0.00	0.14	0.00	0.14	***	1.00	0.96	0.85	0.95	1.00
15	0.00	0.03	0.00	0.05	0.09	0.00	0.00	0.08	0.00	0.00	0.11	0.00	0.14	0.00	***	0.95	0.80	0.93	1.00
16	0.07	0.02	0.07	0.01	0.19	0.02	0.05	0.00	0.05	0.04	0.13	0.03	0.06	0.04	0.05	***	0.90	0.91	0.98
17	0.23	0.03	0.22	0.23	0.36	0.10	0.15	0.09	0.20	0.15	0.26	0.13	0.41	0.15	0.20	0.10	***	0.91	0.88
18	0.07	0.03	0.07	0.15	0.08	0.03	0.04	0.08	0.07	0.05	0.24	0.03	0.25	0.05	0.07	0.09	0.09	***	0.96
19	0.00	0.00	0.00	0.05	0.09	0.00	0.00	0.04	0.00	0.00	0.12	0.00	0.14	0.00	0.00	0.02	0.12	0.04	***

Where: Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

5.3 Cluster Analysis

The pair wise comparison of genetic distances generated based on Nei (1978) among the 19 accessions collected revealed that there is low genetic distance among them as shown (Table 5.2**)**. This also parallel with the pair wise genetic identity as depicted in the table based on Nei (1978). For accessions tested, the lowest genetic distance is zero in 45 pairs, out of the possible 171 pair comparisons generated. As a remark the genetic distances, which were observed below zero, were also changed into zero. The maximum distance was 0.41 between populations of Sekele-A (17) and Dokatu-A (13). The mean genetic distance was 0.09. This reveals that there is low genetic divergence among the accessions.

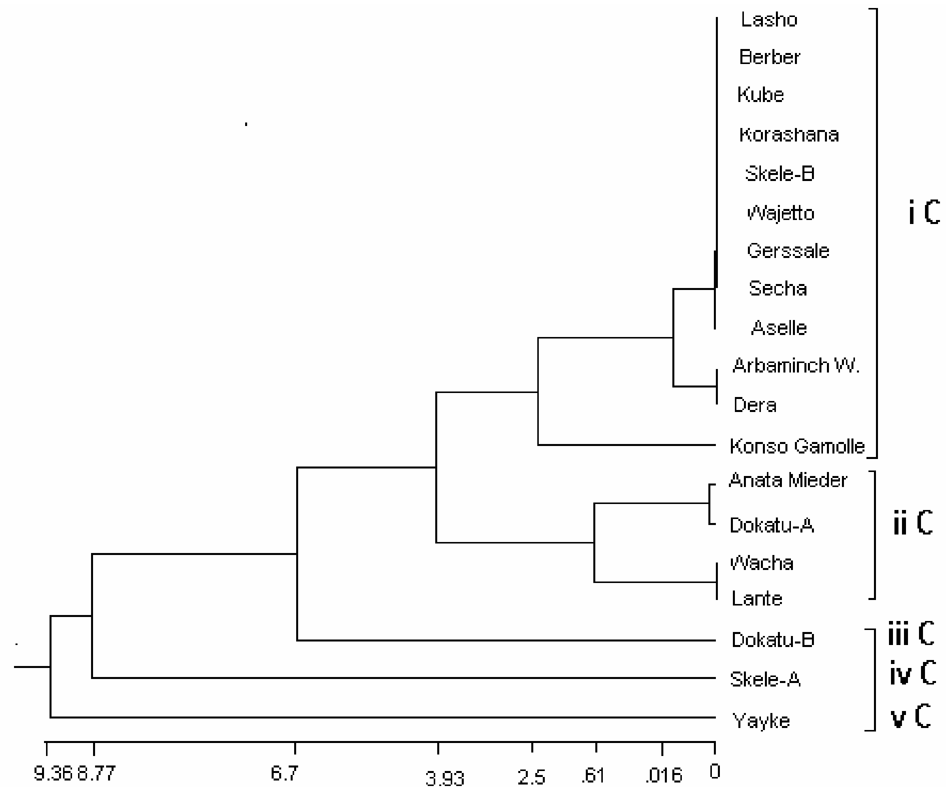

Fig. 5.2 Dedrogram using UPGMA analysis showing relationships among 19 accessions of M. stenopetala from a single primer OPA-13 based on (Nei, 1978).

The UPGMA dendrogram was constructed using NEIGHBOR of PHYLIP version 3.5c by Felsentein (1993). The accessions tested were branched in to five clusters as depicted in the Fig. 5.2**.** The cluster (iC) is made up of the first branch that comprises the clumped eight accessions flanking by Lasho and Secha and then Aselle. Furthermore, this cluster (iC) was sub branched into Arbaminch Water technology and Dera. The next sub branch of the first cluster bears only Konso Gamolle. The second cluster (iiC) is made up of two sub branches. The first branch bears Anata Mieder and Dokatu-A. Whereas, the next one bears Wacha and Lante. Cluster iiiC, ivC and vC are sub branched each other one after the other respectively. They also bear accessions, which stand-alone; they are Dokatu-B, Skele-A and Yayke respectively.

6 Discussion

In this study, the extent of genetic variability in *M. stenopetala* accessions collected in the Southern parts of Ethiopia was determined by using RAPD multilocus marker. The OPA-13 primer generated six unambiguous bands, there were no any single common band(s) found to all of individuals subjected to the study. Here, we used single primer to assess the existing genetic variation in the collected accessions. Other researchers in such studies use pooled data to evaluate similar problems for instance Ayana *et al.* (2000), Masumbuko *et al*. (2003), Offei *et al.* (2004) and others. Therefore, the generated data may be insufficient to forward a valuable conservation measures and priority unit(s). Moreover, the sample size and the number of accessions are also the real limiting factors as suggested by Bonin *et al*. (2004). They further stated that genotyping errors affect both the allelic frequency estimates and the accuracy of discrimination of different demes. In contrast of these limitations, we can see variability from mean estimates of genetic diversities. From comparative evaluation of the samples, the Konso Gamolle accessions had shown higher values both in the percentage of polymorphic loci and the estimated means of Shannon's index and Nei's diversities. This result may support the idea that Konso district could be the area at which the first domestication of the wild relatives occurred and the cultivation was expanded to the rest part of the country (Engels and Gottsch, 1991; Jahn, 1991). Similar demes may be taken and propagated to the rest part by taking the Konso district as the center of genetic diversity. After expansion to the rest localities the allelic frequency may be frequently affected by genetic drift, which ultimately increases homozygosity. This may be because of founder effect such that small populations were established at each locality. The low genetic distances revealed by pair wise comparison further supported this assumption. Population specific marker was not found which is important for population identification and gene flow studies. However, the

sixth band (the lowest molecular size) was observed only in three accessions (Konso Gamolle, Gerrsale and Yayke). This phenotype may be useful in the identification of these accessions.

The constructed dendrogram demonstrate that there is a considerable differentiation among the accessions. However, eight of the demes were indiscriminate and found clumped together in the first cluster (iC) of the first branch. It covers around 42% of the subjects studied. One of the advantages of the RAPD marker is to avoid repetitions in the collected accessions (demes). All in all, this could happen due to a close tie of populations through continual gene flow and / or similar selection pressure that might operate in their respective geographic sites. These evolutionary forces retard local differentiations of the populations into distinct demes. Schaal *et al.* (1998) suggested that historical relationships have been found to contribute populations to have similar genetic structure like that of sharing common ancestry. Therefore, there may be similar gene pools initially established in the demes during their establishment and then similar pattern of selection pressure operates on them. The present data supports eight of the populations can be represented by a single population for the purpose of seed collection, planting and management. However, with one RAPD primer study is not conclusive. Therefore, a further study is important by increasing the number of primers, accessions and sample size. In case of conservation of genetic resources, seed collection should be done across the species range to all populations so as to ensure a more representative sampling of the genetic variation, which is adapted to different geographic ranges.

The genetic similarity (identity) was found to be very high. For most of them it is very close to one in pair wise comparison. This suggests the existence of little genetic differentiations among the demes. Repeated targeted selection and domestication for a better taste and

palatable cooking of leaves varieties by Konso Moringa farmers was done through traditional selection (Jahn, 1991; Demenlenaere, 2001). Unfortunately, they failed to select the desired variety. This implies that the plant is outbreeder. Its close relative *M. oleifera* was reported to be more than 70% outbreeder (Muluvi *et al*. 2004). Outbreeding encourages genetic variability than inbreeding. Most of the zero values of the genetic distances were the transformed values of those genetic distances found below zero as suggested by Nei (1978).

7 conclusions

In this study, the genetic diversity of 19 *M. stenopetala* accessions collected in Southern Ethiopia was analyzed using RAPD markers. The OPA-13 primer was used for 71 individuals subjected for the analysis. From the result of this investigation, the following conclusions were drawn:

- Population as well as individual genotype specific marker was not found.

- The mean diversity estimates of Shannon's and Nei's had shown the existence of genetic variability within, among and entire populations.

- No common band was found to all of the subjects studied.

- The pair wise comparison of genetic distances between the accessions was low.

- The constructed dendrogram was unable to discriminate about 42% of the accessions studied. They were found lumped together in the first cluster (iC) of the first branch. Further genotyping with more primers and increasing the sample size are needed to discriminate the accessions. Therefore, seed collection should be done in the range of the entire species to catch genetic variability's adapted at different ecological settings.

- RAPD markers are cost effective and less time consuming though with limitation of dominancy and reproducibility can be used for further genotyping of the collected accessions.

8 Recommendations

- To make more effective the out come of the genotyping the researchers have to increase the number of accessions, sample size and primer number. Studies using other molecular markers such as Isozymes, RFLP (Resriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism) and SNP (Single Nucleotide Polymorphism) to assess genetic diversity are needed.

- So far the pollination biology and pattern of matings of the plant is not documented. The documentation of these biological phenomenons is important to devise a rational approach for breeding programs and germplasm managements. Therefore, researchers in the field have to address the problem to generate valuable benefits from the genetic diversity of the plant.

- The plant is mainly restricted in semiarid areas of Southern part of the country. Researchers should test the performance of the plant for various important agronomic traits (growth rate, productivity, resistant to pest and drought and others) at different agro ecological zones to promote the expansion of the plant in the rest part of the country and to guarantee the utilization of the plant by signifying the potential of the plant.

- The local peoples perceive that its vegetable leaves as poor family diet; mainly it is consumed during famine time. This is due to the plant remains evergreen after harsh weather conditions. Therefore, it becomes the sole food source to

sustain the lives of the local peoples. Here, I echo that the government, local agricultural offices, non governmental organizations and volunteers have to do Moringa promotion to change the attitude of the local peoples and to establish positive attitudes for the exploitation of the multipurpose tree in its real potential it has to be exploited to come out of poverty.

- The unexploited uses of the plant include such as water clarification abilities, bactericidal, fungicidal and source of ben oil of the seeds of the plant. This benefit of the plant is important to promote the use of the plant and its conservation. Therefore, the promotion part should include the introduction of the unexploited potential. Researchers in the field have to devise methods that can be accessible by indigenous peoples to exploit its potential at maximum extent.

- Different researchers report its close relative *M. oleifera*, to be rich in nutritional quality and quantity, anti oxidant and others. For instance, the eye disease caused by deficiency of vitamin A was reported the most serious health problem in Gardula region, it is the area at which the plant is grown. However, *M. oleifera* its close relative reported to be rich in vitamin A. This observation signifies the importance of *M. stenopetala* to alleviate the problem, which is a vegetable food in the area. Therefore, researchers in the field have to do the food chemistry of the plant.

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