ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
DEPARTMENT OF BIOLOGY
APPLIED GENETICS STREAM

ANALYSIS OF GENETIC DIVERSITY AMONG CULTIVATED ENSET (*ENSETE VENTRICOSUM)* POPULATIONS FROM ESSERA AND KEFFICHO, SOUTHWESTERN PART OF ETHIOPIA USING ISSR MARKER

BY

Dagmawit Chombe

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Dagmawit Chombe

A Thesis Submitted to School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology (Applied Genetics)

June 2008

Declaration

I, the under signed, declare that this thesis is my original work; it has not been presented in other university, college or institutions, seeking for similar or other purposes. All sources of the materials used in the thesis have been dully acknowledged.

Dagmawit Chombe

Signature_____

Date _____

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List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
GD	Genetic Diversity
H	Shannon's Diversity Index
ISSR	Inter Simple Sequence Repeat
NJ	Neighbor Joining Analysis
PCO	Principal Coordinated Analysis
RFLP	Restriction Fragment Length Polymorphism
RAPD	Random Amplified Polymorphic DNA
SNPs	Single Nucleotide Polymorphisms
UPGMA	Unweighted Pair Group Method with Arithmetic mean

ABSTRACT

This study was conducted with the objective of investigation of genetic variation between populations of cultivated enset and provides information for conservation and improvement of enset. Leaf samples for seventy-one enset plants were collected from two areas of SW Ethiopia (Kefficho and Essera) and genomic DNA was extracted from the samples CTAB extraction method. Inter simple sequence repeats (ISSRs) as a molecular marker was used to assess genetic diversity both within and between populations using two ISSR primers (834 and 826). A total of 26 clear and reproducible bands were amplified from the two primers. Both UPGMA and neighbor joining trees were constructed for each individual and population using Jaccard's similarity coefficient. The trees indicated two separate clusters which are based on the origin of populations and few intermixing of samples between regions was observed. The PCO analysis also recovered the UPGMA and neighbor joining trees groups. All parameters indicated that the clones of enset from Kefficho are more diverse than clones from Essera. Moreover, partioning of Shannon's diversity index showed that the majority of the variations were observed within populations of enset than between the two populations. In conclusion, the present study using ISSR analysis, all the diversity parameters confirm that there is high diversity in cultivated *Ensete ventricosum* populations of SW Ethiopia and that ISSR markers can be successfully applied for the assessment of genetic diversity of this species. Therefore further research on analysis of genetic diversity of cultivated enset population including divergent production areas has to be done and the conservation capacity of institutes both for in-situ and ex-situ should be strengthen.

Key Words: - Cultivated *Ensete ventricosum*, Essera, Ethiopia, Genetic Diversity, ISSR, Kefficho,

underground corm and the aerial pseudostem made up of overlapping leaf sheaths are edible after some processes. Starchy food is produced by pulverizing the corm or scraping the pseudostem, followed by a short fermentation period. Unlike banana, enset is monocarpic and fruits only once in its life cycle (Shank, 1994). The fruits contain several seeds which are hard and about 1-2 cm long. Sprouting occurs only when the main shoot with the meristem, is artificially decapitated at the junction between the pseudostem and corm at soil surface, while in banana sprouting occurs spontaneously (Brandt *et al.*, 1997). Enset is a diploid plant with the haploid chromosome number n = 9, whereas *Musa* species, including edible banana, have different ploidy levels and chromosome numbers (diploid, triploid or tetraploid), with n=7, n=9, n = 10, and n = 11 (Birmeta *et al.,* 2004).

Enset is used as a staple and co-staple food for millions of Ethiopian people. Products from enset are used in different forms in traditional medicine. A starch for textile, adhesive and paper industries is being produced (Diro and Staden, 2005). However, due to unsatisfactory research attention given to enset crop, its production system is still traditional and tiresome. Different management practices starting from propagation to harvesting and processing demand high labor (Bobosha, 2003). Furthermore, cultivation of enset is constrained by various diseases such as enset wilt, caused by *Xanthomonas campestris* pv. *musacearum,* and by insect pests and abiotic factors. Enset germplasm is currently maintained in a field gene bank but is at risk of diseases, pests and adverse environmental conditions (Diro and Staden, 2005).

Ensete ventricosum was previously cultivated only in the south and southwestern parts of Ethiopia, but the recurrent droughts have led to the expansion of enset cultivation to other parts of the country (Brandt *et al.*, 1997). A wide adaptation within the species to altitude, soil and climate has allowed widespread cultivation in western Bale, southwestern Oromia, including south and east Shewa, Jima, Illubabor and Welega (Shank, 1994).

Human beings, in their struggle for survival, have selected and domesticated crops to fulfill their immediate needs. Nowadays, many people have become aware of the importance of preserving genetic diversity for the survival and continuation of any form of life that exists on earth (Birmeta *et al.,* 2004). Most of the genetic diversity of enset is traditionally maintained *in situ* by farmers. Unfortunately, many valuable clones have been lost due to various human and environmental factors (Gebremariam, 1996 as cited by Negash *et al,* 2002), which may have reduced the total available genetic diversity of the crop. Lack of knowledge about the genetic diversity of this crop species complicates the conservation, improvement, and utilization of enset by farmers, conservationists, and breeders (Negash *et al*, 2002).

As noted in Negash *et al.* (2002) a more extensive investigation including divergent production areas not yet covered would extend the current overview of enset genetic diversity in Ethiopia and allows its effective conservation.

Several modern molecular techniques are now being applied together with morphological studies to investigate genetic diversity and relatedness in crops (Birmeta *et al.,* 2004). Although assessment of morphological variation present in enset is feasible, its use is rather limited due to the small number of phenotypic markers and the fact that they are influenced by environmental conditions (Negash *et al*., 2002). Therefore, in this study, the molecular marker (ISSR) was used to characterize germplasm diversity in cultivated enset as a complementary approach.

2. Literature Review

2.1. Taxonomy of Enset

Enset (*Ensete ventricosum* (Welw) Cheesman) is a perennial monocarpic crop belonging to family *Musaceae* and genus *Ensete* (Lye and Edwards, 1997). However, enset was considered as a member of the genus *Musa* as it strongly resembles banana morphologically and because of this some of the species names formally given to enset were *Musa ensete* and *Musa ventricusa* (Lye and Edwards, 1997 as cited by Bobosha, 2003). Then Chessman (1947) characterized the basic difference between *Musa* and *Ensete* and separated enset from banana on the basis of differences in pseudostem morphology and chromosome numbers.

Enset is diploid with n=9 (Chessman, 1947), while species of *Musa* have different ploidy levels (diploid, triploid and tetraploid) with n=7, 9, 10, 11 (Birmeta, 2004). Both enset and banana have an underground corm, a bundle of leaf sheath that form pseadostem and large paddle-shaped leaves (Birmeta *et al.,* 2004 and Addis, 2005). Enset, however, is usually larger than banana, reaching up to 11-12m in height and with a pseudostem up to one meter in diameter (Addis, 2005). Although enset is thicker and larger than banana, both are herbaceous perennial monocarpic crops; they produce flowers only once at the end of their life cycle (Shank, 1994). When a banana plant dies it is spontaneously replaced by new suckers sprouting from preexisting buds in the corm. However, in enset, sucker production is induced only when the apical meristem is removed (Birmeta *et al.,* 2004). The fruit of enset, unlike banana, are not edible and, therefore, enset is often called false banana (Brandt *et al.,* 1997).

Simmonds (1960) as cited by Brandt *et al.* (1997), summarized and listed only six commonly recognized species of *Ensete: E. superbum* and *E. glaucum* grow wild in Asia, *E. perrieri* in Madagascar, *E. gilletii, and E. homblei in* West Africa and *E. ventricosum* in Eastern Africa. From the six species, *Ensete ventricosum* is

considered to be the only wild species growing in Ethiopia (Birmeta *et al.,* 2004). Recently, the number of species of *Ensete* has increased to seven as the Flora of China has, not entirely convincingly, reinstated *Ensete wilsonii.* There is one species in Thailand, somewhat resembling *E. superbum,* that has not been formally described, and possibly other Asian species. This shows that further research is needed on the taxonomy and distribution of *Ensete* species (Brandt *et al.*, 1997).

Different hypotheses are proposed on the origin of enset agriculture, given the restricted geographic distribution of domesticated enset and the degrees of complexity and variability in contemporary enset agricultural systems, agronomists and biographers have long considered the Ethiopian highlands to be the primary center of origin for enset agriculture (Harlan, 1969 and 1992; Saver, 1952; Vavilov, 1951 as cited in Brandt *et al.*, 1997). In relation with this, anthropologists, archaeologists, historians and other scholars have also developed hypotheses that argue for the domestication of enset in Ethiopia as early as 10,000 years ago (Brandt *et al.*,1997).

It is widely known that enset is an indigenous plant in Ethiopia. Shack, (1996) indicated that some historians and botanists had earlier attempted to trace the origin of enset to ancient Egypt. Later writers, such as Smeds (1955) suggested enset to be indigenous to Ethiopia. According to Smeds (1955) enset cultivation originated in highland Ethiopia because this area was the only part of Africa to possess a more developed agricultural and pastoral economy. Although evidence is lacking to locate the exact place of origin, Smeds (1955) speculated that the highlands along the western edge of the rift valley, particularly, the present Wolaita- Kambata- Gurage regions, is the original center of enset cultivation.

Currently, in Ethiopia cultivation of enset is restricted to south, southwest and central part of Ethiopia because vast majority of enset farmers live in southern Ethiopia. However, historical evidence suggests that enset may have once played a much more important role in the agricultural practices of central and northern Ethiopia (Brandt *et al.,* 1997).Virtually all of the 10 million plus people who now depend upon domesticated enset for food are situated in the highlands of South-central and southwestern Ethiopia. However, enset is also found in the highlands of northern Ethiopia (Simmonds, 1976). These plants appear to be wild or are used mainly, if not exclusively, for ornamental purposes.

2.2. Enset Morphology

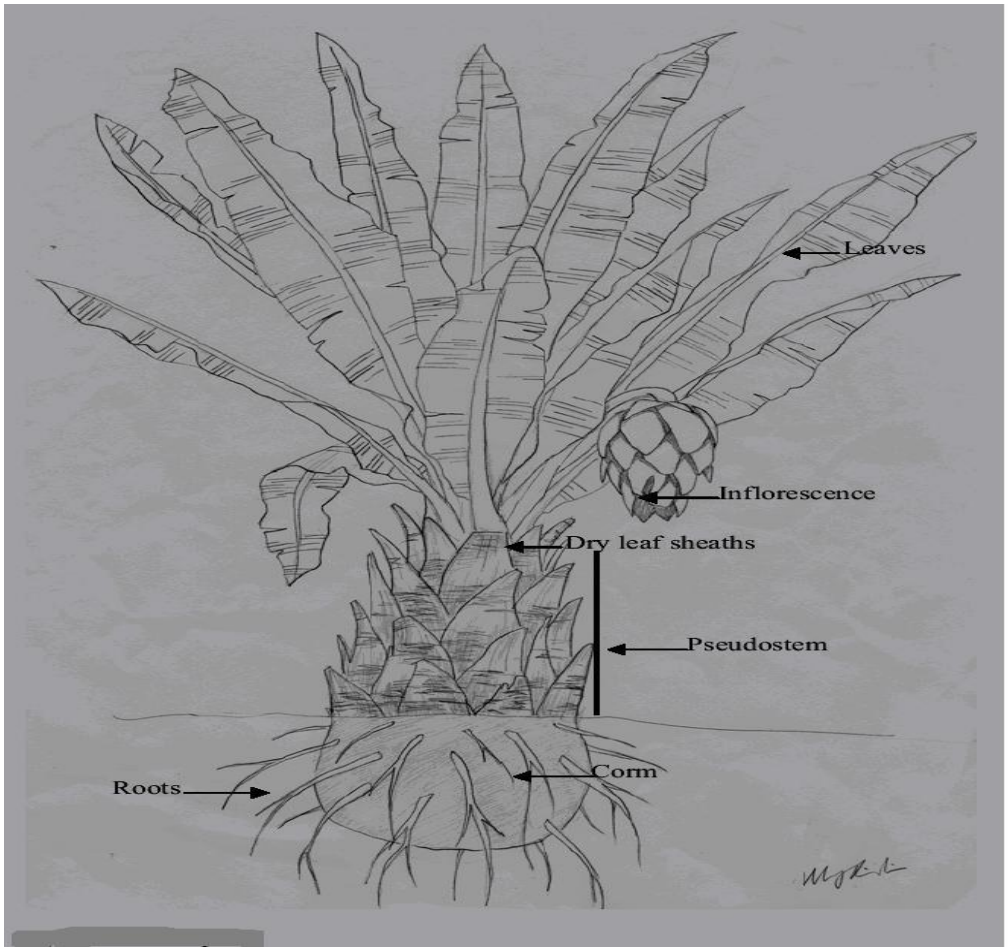


Figure 1: - An enset plant (Adopted from Birmeta, 2004)

2.3. Ecology and distribution of Enset

Wild enset grows at altitudes of 1200–1600 m above sea level while domesticated enset is cultivated at altitudes of 1100–3100 m above sea level. The optimal conditions for enset cultivation occur at 2000–2750 m. Most enset growing areas receive annual rainfall of about 1,100 to 1,500 millimeters, the majority of enset growing areas are between 10 and 21 degree centigrade and the relative humidity is 63 to 80% (Brandt *et al.,* 1997). Soil types in the enset growing areas of Ethiopia are moderately acidic to slightly basic with a pH reaction ranging from 5.6-7.3 heavy clay soils that retain high levels of organic matter when manured (Shank, 1994). These soils contain 0.10 to 0.15 % total Nitrogen and 2 to 3 % organic matters (Taye and Asrat, 1996 as cited by Bobosha, 2003).

Enset is not tolerant to freezing. Frost damages on upper leaves are commonly observed at altitude above 2,800 meters above sea level and serious stunting is seen above 3,000 meters. For a certain range below 1,500 meters, the constraint to enset plant growth probably is more related to available water than high temperatures (Bobosha, 2003).

Shigeta (1990), has noted that enset tolerates short season drought than have seriously damaged annual crops, especially cereals. During the dry spell, only the edge of older leaves and the outer leaf sheath are visibly affected, and the plant resumes normal growth after the onset of the rainy season. Characterization of enset drought tolerance is a vital issue in clarifying the role of enset in Ethiopian food security. Observed drought tolerance and its attributes must be carefully interpreted.

Enset is one of the potential indigenous crops for food production (Taye, 1984) and can be grown in most parts of Ethiopia. According to several authors (Smeds, 1955; Taye, 1984), the enset cultivation system is economically viable and is one

of the few successful indigenous and sustainable agricultural systems. It is sustainable because it has been providing food for humans for generations from the same plot and maintains the quality of life of the people.

Enset does not grow well in the lowlands where the rainfall is erratic and in the high altitude areas where the temperature is extremely cold. It is believed that plants on higher altitudes have desirable characters and farmers tend to get their planting materials from such areas (Brandt *et al.,* 1997).

Enste Ventricosum grows wild in a number of countries in central and eastern Africa including Congo, Mozanbique, Uganda, Tanzania, and Zambia (Biremeta *et al.,* 2004).

2.4. Uses of Enset

Enset is a multipurpose crop with all plant parts being utilized for human food, animal forage, medicinal or ornamental uses. Enset has high significance in day-today-life of the peasant households cultivating this crop as staple food. The peasants indicate that enset is their food, their cloth, their house, their bed, their cattle's feed and their plate (Brandt *et al,* 1997). The uses of enset can be classified into two, the food uses of enset and the non-food use of enset.

Enset is a multipurpose crop of which every part is thoroughly utilized (Shigeta, 1990). It is a good source of starch. The corm and the pseudostem are the most important sources of food (Kefale and Sandford, 1991). The types of food from these parts are known as 'Kocho', 'Bulla' and 'Amicho' (Spring *et al.,* 1996). Kocho is the main food product from enset and is obtained by fermenting the mixure of the scraped pulp of the pseudostem, pulverized corm and stalk of inflorescence (Tsegaye and Stuik, 2001).

Kocho can be stored for long periods of time without spoiling. The quality of Kocho depends on the age of the harvested enset plant, the type of clone (variety), and the harvesting season. Moreover, within one plant, the quality is influenced by the part of leaf sheath and corm processed. The preferred type is white in color and is obtained from the innermost leaf sheath and inner part of the corm, while the lowest grade is blackish and is obtained from the outer leaf sheath and corm (Brandt *et al,* 1997).

Bulla is obtained by squeezing out the liquid containing starch from scraped leaf sheathes and grated corm and allowing the resultant starch to concentrate into white powder. It is considered the best quality enset food and is obtained mainly from fully matured enset plants. Bulla can be prepared as a pancake, porridge, or dumpling (Tsegaye and Stuik, 2001).

Amicho is the underground enset corm, usually of younger plant; the corm is eaten boiled like potato.

Enset flour “bulla” is also mixed with flour from cereal crops such as tef (*Eragrostics tef)* to make the traditional Ethiopian bread inijera. Although rich in carbohydrate, enset foods are low in protein and vitaimin a (Brandt *et al,* 1997).

Enste is rich in carbohydrate and mineral substances like calcium and iron (Bobosha, 2003). The energy yield of enset is by far higher than that of several cereals. Enset energy yield was reported to be higher than barely, potato, sweet potato and banana. This shows that cultivation of enset can significantly improve food security at household and at national level (Taye and Asrat, 1996 as cited by Bobosha, 2003)

Enset provides fiber as a by product of decorticating the leaf sheath. Enset fiber has excellent structure and its strength is equivalent to the fiber of Abaca, a world- class fiber crop. The fiber obtained from enset is used to make bags, ropes, cordage and mats (Brandt *et al.,* 1997).

Enset leaves and dried leaf sheath are also used for wrapping materials. Wrapping is the most common way of using fresh enset leaves but the dried pseudostem is also utilized for the same purpose (Spring *et al.,* 1996). Fresh enset leaves from which midribs are removed can be used for wrapping several kinds of objects. Leaves are indispensable for women in the local periodical market, in order to wrap up dough in “Dabo” making (traditional bread), the commercial goods such as: fermented enset, butter, coffee, beans and several other agricultural products and Clean and fresh leaves are very important when serving cooked foods. Guests are offered fresh, enset leaf as mats to sit on the ground. Furthermore, the leaves are also used for making mats and rope (in place of nail) in house construction and as fuels. Enset leaves are used as cattle feed especially during the dry seasons when feed is scarce (Brandt *et al.,* 1997).

Some clones and parts of enset plants are reported to have medicinal value for both human and animals. These clones are claimed to heal bone fractures, used for treatment of diarrhea and delivery problems i.e. assisting to discharge the placenta (Spring *et al.,* 1996; Brandt *et al.,* 1997). Even some clones protect the liver from the side effect of the medicine used for treating tape worm. Bulla supplemented with milk and milk products is also known as important food for quick recovery of women after child delivery (Brandt *et al.,* 1997).

Furthermore, enset contributes to higher reduction of losses of plant nutrients particularly nitrogen loss through leaching as compared to annual crops (Brandt et al., 1997). Research conducted on fields where enset is continuously cultivated has revealed that there is a higher soil nutrient status in the enset fields than in other crops (Addis, 2005). Asnaketch, (1997) Eyasu, (1998) have also confirmed that soil fertility is maintained and even increased around enset and taro fields.

Enset farming could attain sustainable food production because it is relatively tolerant to drought compared to cereals. Generally, because of its multipurpose uses, its production is highly intermingled with the economic, culture and social life of the people in enset growing regions (Addis, 2005).

2.5. Problems associated with enset cultivation

The cultivation practices of enset that encompasses field preparation and propagation to harvesting and processing are laborious and time consuming. It is mainly women who carry out harvesting and processing using local tools. The sustainability of enset agriculture is threatened by a number of factors, including diseases. These are brought about by the continuous cultivation of enset in the same location, changing patterns of crop cultivation and intensification of the cropping phase in the enset agroecosystem which stem from the ever increasing population pressure (Tsegaye and Stuik, 2001).

Diseases are collectively the most severe biological problem facing enset. The damage that diseases can cause and the lack of knowledge about or implementation of preventive strategies contribute to the severity of enset plant diseases. Diseases are caused by several bacteria, nematodes, fungi, and viruses (Bobosha, 2003).

Although enset foods are known to have high starch content, they are highly deficient in proteins and vitamin A. This has caused some malnutrition-related diseases in regions where the crop is used as the staple food (Birmeta *et al.,* 2004).

2.6. Marker systems and their applications in genetic diversity analysis

Plant characteristics can be divided into four arbitrary groups: agronomic characters, morphological characters (used to distinguish between varieties), biochemical characters, and molecular (DNA) markers (Schut *et al.,* 1997). Divergences between genotypes with regard to any of these characteristics are either indirect or direct representations of differences at the DNA level and are therefore expected to provide information about genetic relationships.

2.6.1. Morphological Markers

Morphological markers have obvious impact on morphology of plant. Examples of this type of marker may include the presence or absence of awn, leaf sheath coloration, height, grain color, aroma of rice etc. In well-characterized crops like maize, tomato, pea, barley or wheat, tens or even hundreds of such genes have been assigned to different chromosomes (Wikipedia, the free encyclopedia: http://en. Wikipedia.org/wiki/Marker assisted selection).

Morphological assays generally require neither sophisticated equipment nor preparatory procedures. So their prime advantages are simplicity and low expense to score even from preserved specimens. The main disadvantage of this approach is that possession of normal phenotype is no guarantee that cryptic changes may be recessive and consequently when in heterozygous forms, do not appear until plants have been selfed and progeny examined (Brown *et al.,* 1993).

2.6.2. Biochemical (enzyme) markers

Biochemical markers are based on genes that encode proteins that can be extracted and observed; Proteins and isozyme variants that migrate at different rates under electrophoresis have been the most widely employed biochemical genetic markers (Coke, 1984).

Isozymes are direct gene products or proteins. They are one step removed from the controlling genes (Vithanage *et al.,* 1995). During the last 20 years, isozyme analysis through starch gel electrophoresis has been the genetic markers most frequently employed in many species. These were used for the identification of cultivars as well as characterization of somaclonal variation (Damiano *et al.,* 1995). They are generally but not always governed by single Mendelian genes. They can be assayed from a wide variety of organs and tissues, and analytical procedure is not exceptionally complicated. In studies of genetic diversity and divergence, isozymes with similar enzymatic activity and electrophoretic migration rates are presumed to be homologous although this assumption cannot be validated without amino acid sequencing (Vithanage *et al.,* 1995).

The main disadvantage of the isozyme system is their dependence on the histochemical staining. It requires the presence of optimal amounts of enzymes from the tissue sampled. Therefore, down-regulation of a particular enzyme in the sampled tissue can lead to negative results (Vithanage *et al.,* 1995). Generally the main disadvantage of biochemical markers is: it is age dependent, influenced by environment and it covers less than 10% of genome.

2.6.3. Molecular markers

To avoid problems specific to morphological markers, the DNA-based markers have been developed (Tanksley *et al.* 1995). Molecular markers are based on naturally occurring polymorphisms in DNA sequences (i.e: base pair deletions, substitutions, additions or patterns) (Gupta *et al.,* 1999). The greater utility of molecular markers arises from the following inherent properties that distinguish them from morphological markers (Powell *et al.,* 1994)

1. Moderately to highly polymorphic
2. Co dominant inheritance (which allows the discrimination of homo- and hetro- zygous states in diploid organisms).
3. Unambiguous assignment of alleles.
4. Frequent occurrence in the genome.
5. Even distribution throughout the genome.
6. Selectively neutral behavior.
7. Easy access (i.e. by purchasing or fast procedure).
8. Easy and fast assay (e.g. by automated procedures).
9. High reproducibility.
10. Easy exchange of data between laboratories.
11. Low cost for both marker development and assay.

Molecular markers are important tools for breeding selection, genotype identification, and studying the organization and evolution of plant genomes. Genome mapping is one of the most important applications of molecular markers. The advent of DNA-based markers has dramatically facilitated the mapping of several plant genomes in the last decade (Tanksley *et al.,* 1995).

Numerous markers have been mapped to different chromosomes in several crops including rice, wheat, maize, soybean and several others (Tanksley *et al.* 1995). Those markers have been used in diversity analysis, parentage detection, DNA fingerprinting, and prediction of hybrid performance. Molecular markers are

useful in indirect selection processes, enabling manual selection of individuals for further propagation.

Molecular markers have provided a powerful tool for breeders to search for new sources of genetic diversity of the plants and to investigate genetic factors controlling quantitatively inherited traits. Many types of molecular markers with dominant or codominant character have been used already such as: Random Amplified polymorphic DNAs (RAPD), Inter Simple Sequence Repeats (ISSR), Restriction Fragment Length Polymorphisms (RFLP), Amplified Fragement Length Polymorphisms (AFLP), Single Nucleotide Polymorphisms (SNPs), Microsattelites and others. The relative advantages and disadvantages of these techniques are summarized below.

Table 1: Comparison and summary of the most commonly used molecular marker systems in cereals (adapted from FAO, 2003).

Features	RFLP	AFLP	RAPD	Microsatellite	SNP
Detection method	Hybridization	PCR	PCR	PCR	PCR
Type of probe/primer used	g DNA/ cDNA sequence of structural genes	Sequence specific primers	Arbitrarily design primer	Sequence specific primers	Sequence specific primers
Requirement of radioactivity	Yes	No/Yes	No/Yes	No/Yes	No/Yes
Extent of genomic coverage	Limited	Limited	Extensive	Extensive	Extensive
Degree of polymorphisms	Low	High	Medium to High	High	High
Phenotype expression	Co dominant	Dominant	Dominant	Dominant	Co dominant
Possibility of automation	No	Yes	Yes	Yes	Yes
DNA required (µg)	10	0.5-1.0	0.02	0.05	0.05
DNA quality	high	moderate	high	moderate	high

2.7. Inter simple sequence repeat (ISSR) markers in genetic analysis

ISSR markers, like any other PCR-based markers, are rapid and require only small amount of the template DNA. Each marker system has its own advantage and disadvantages. ISSR markers with low cost and low labor requirement but with high reliability have been developed since 1994 (Lagercr. antz *et al.,* 1993). It does not require genome sequence information but produce highly polymorphic patterns. ISSR is a technique that is gaining wide acceptance in the area of plant improvement by plant breeding makes use of the fact that certain DNA markers are closely linked to important agronomic traits. Thus it has been widely used to identify markers associated with different qualities in crop plants such as disease tolerance and seed size (Zietkiewicz *et al*., 1994).

ISSR, PCR-based DNA marker system relies on the abundance of simple sequence repeats (SSRs) or microsatellites in the eukaryotic genomes (Lagercr. antz *et al.,* 1993).The method involves PCR amplification of regions between two adjacent and inversely oriented microsatellites using a single, usually 16-25 base pair long (Reddy *et al.,* 2002) SSR-containing primer anchored at the 3 ^{1}or 5 ^{1}end by two to four arbitrary, often degenerate, nucleotides (Fig. 2). The primer can be based on any of the SSR motifs (di-,tri-,tetera- or penta-nucleotides) found at microsatellite loci. The techinique combines the advantages of AFLP and microsatellite (SSR) analysis to the taxonomic universality of RAPD (Zietkiewicz *et al.,* 1994). Unlike SSR analysis, it does not require prior sequence information for primer design but like SSR it is reproducible, and it can overcome some of the technical limitations of RFLP and RAPD (Assefa *et al.,* 2003).

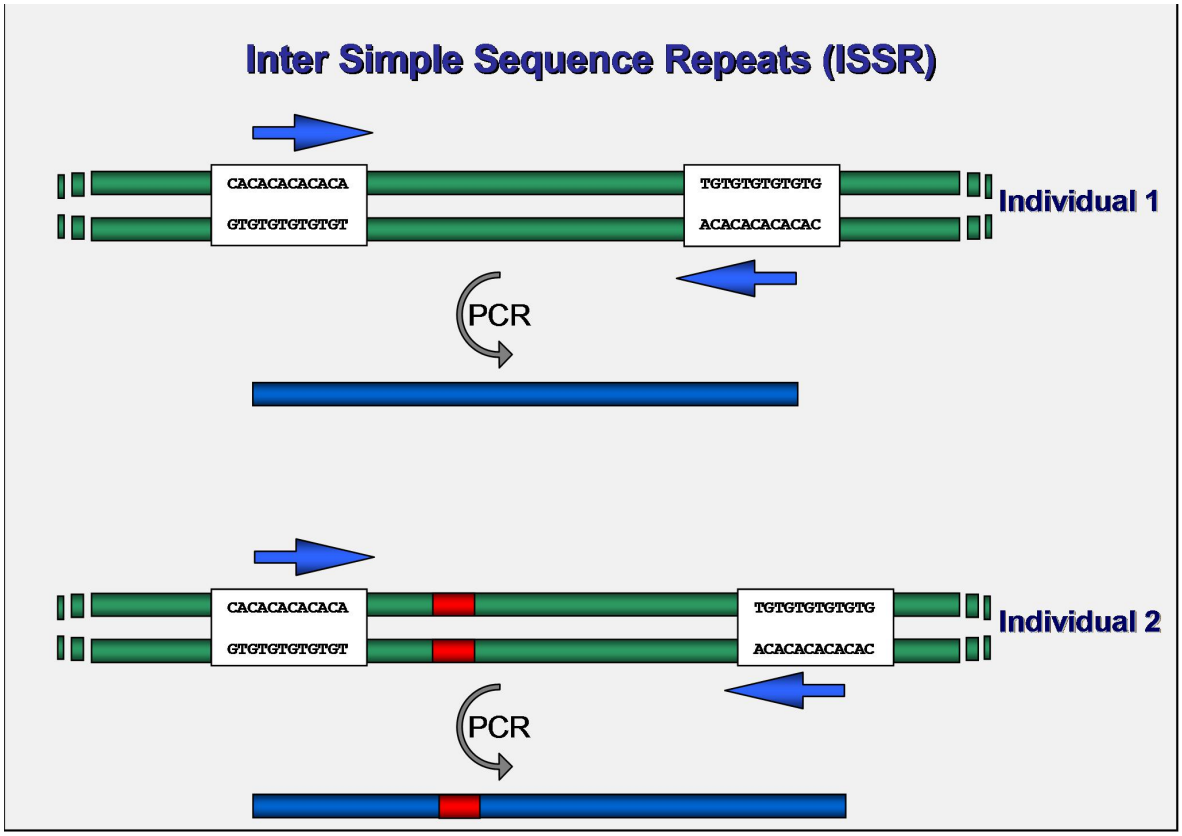


Figure 2:- Principles of the amplification of DNA with a single oligo-nucleotide primer in ISSR marker system. DNA segments delimited by the inverted simple sequence repeats (SSR) (Individual 1 and 2) are amplified with a single ISSR primer (green). ISSR variation that may result from insertion or deletion (Red mark) in different individuals produces PCR fragments of different sizes (blue) of the segment (Tesfaye *et al.,* 2005)

3. Objectives of the study

3.1 General Objective

The general objective of the study is to provide information on genetic diversity of cultivated enset in Kefficho and Essera area that could be used to maintain and conserve enset.

3.2 Specific Objectives

- To perform ISSR analysis and to estimate the magnitude and pattern of genetic diversity in cultivated enset using ISSR marker.

- To identify the relative genetic diversity within and among enset population of Kefficho and Essera area.

4. Materials and methods

4.1. Study Area

The study area chosen are Kefficho and Essera. Both Kefficho and Essera are found in the Southern Nations, Nationalities and Peoples Region (SNNPR). Essera is one of the three weredas found in Dawro zone and shares boundaries with Wolaita and Kambata in the east, Kafa in the west, Gamo and Gofa to the south, and Jimma to the northwest.

Kefficho Shekicho is bordered on the south by Debub Omo, on the southwest by Bench Maji, on the west and north by the Oromia Region, and on the east by Semien Omo (Fig. 3)

The people In these two selected areas live in villages which are organized around homesteads surrounded by dense plantations of enset (*Ensete ventricosum*). And, hence the study site is suitable to accomplish the objective of the study.

Table 2:- Collection site and their Kilometer from a reference town and number of individuals collected from each site.

Population	Collection site	No of individual	Remark
Essera	Essera area	49	About 160km from Wolaita Sodo to SW
Kefficho	Kefficho area	22	About 300km from Wolaita Sodo to the West

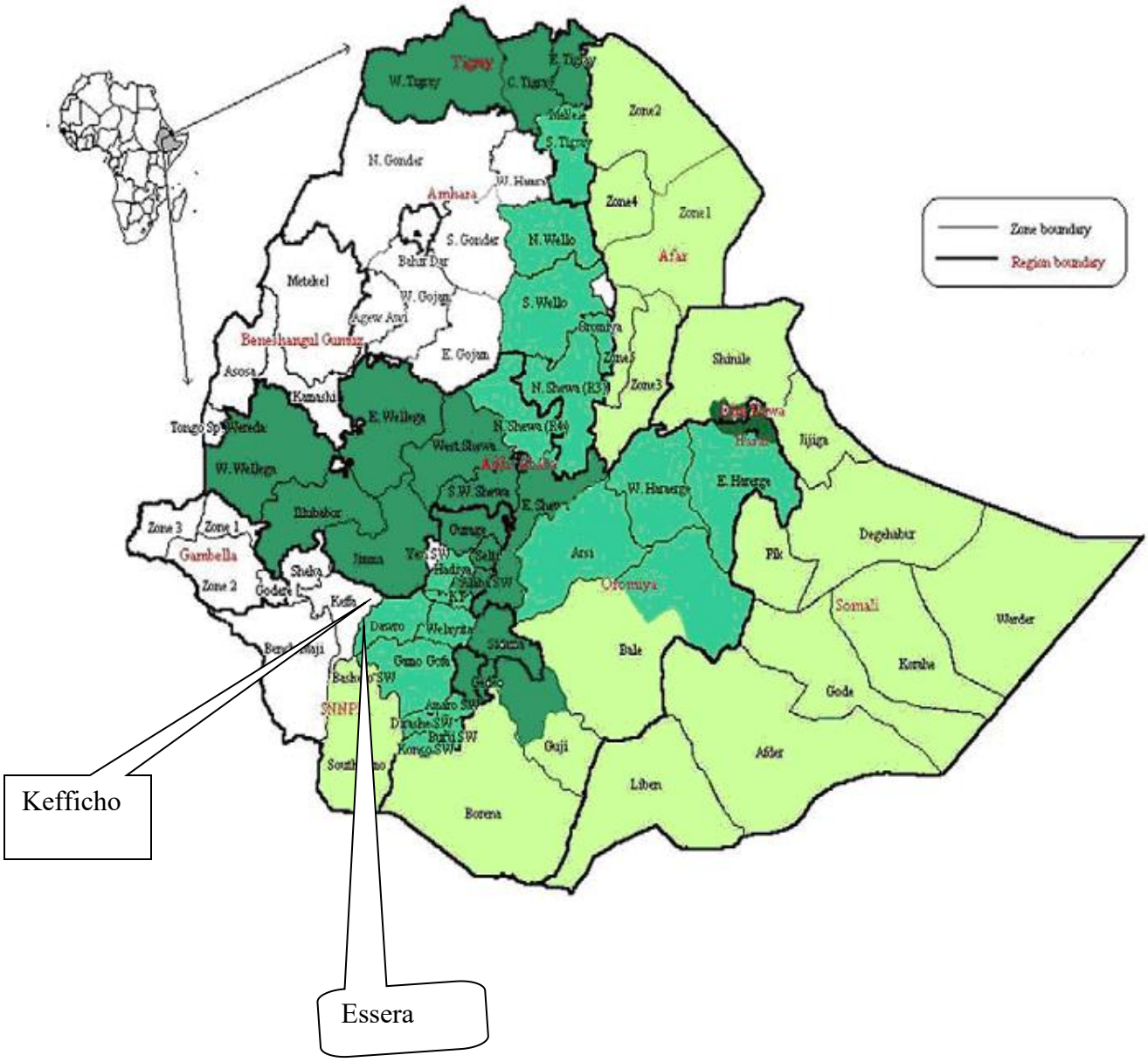


Figure 3:- A map of Ethiopia and the arrows indicate the two weredas, Essera and Kefficho, from where samples were collected (Adopted from Assefa *et al.,* 2003)

4.2. Sampling Technique

A representative leaf sample of cultivated enset population was collected based on farmers definition of clonal cultivars from the two sites that is farmers gave different names for enset because of the different value they obtained from different enset clones. Therefore 49 enset plants from Essera and 22 enset plants from Kefficho, all with different name were collected. Totally 71 cultivated enset clonal varieties were collected.

4.3. Plant material

Young leaves were collected separately from the above seventy one selected individual plants of each population and the leaves were stored in a plastic bag containing silica gel to be dried and preserved until extraction of genomic DNA was done in the laboratory.

4.4. DNA Extraction

The dried leaf samples were ground with pistle and mortar. Total genomic DNA from all the 71 samples of *Enset Ventricosum* Population was isolated from about 0.2g of each pulverized leaf sample using tripple cetyltrimethyl-ammoniumbromide (CTAB) extraction technique (Borsch *et al.,* 2003).

4.5. Loading genomic DNA sample

2 l of genomic DNA sample was added to 6 l 2 loading dye (Bromophenol blue) and mixed very well. The mixture was loaded to the well of the gel that was subjected to electrophoresis for about one hour.

4.6. Primer selection and ISSR-PCR amplification

A total of six ISSR primers listed in Table 3 were used for the initial testing of variability and reproducibility test. Four individual samples were selected from each population to screen the primers with 1:5 dilutions. Each primer was tested for reproducibility for different PCR products of DNA samples of the same population and separate runs on agarose and the one producing consistent DNA fragments across the different samples and PCR runs were selected and finally two primers were selected based on their high discriminatory potential, extended and clear banding pattern and reproducible as compared to other selected primers for the analysis.

Table 3: - List of primers, sequence and annealing temperature

No	**Primers**	**Sequence (5 ^{1}-3 ^{1})**	**Annealing temperature**
1	UBC-834	$(AG)_8YT$	45 ^{0}C
2	818-H818	$(CA)_8G$	48 ^{0}C
3	UBC-811	$(GA)_8C$	48 ^{0}C
4	UBC-817	$(CA)_8A$	45 ^{0}C
5	UBC-820	$(GT)_8T$	48 ^{0}C
6	UBC-826	$(AC)_8C$	48 ^{0}C

The polymerase chain reaction was conducted in Biometera 2000 T3 thermo cycler. PCR amplification was carried out in a 25 l reaction mixture containing 1 l template DNA, 13.2 l H2O, 5.6 l dNTP (1.25mm), 2.6 l Tag buffer (10 × Thermopol reaction buffer), 2.0 l $MgCl_2$, 0.4 l primer (20 pmol/ml) and 0.2 l Tag polymerase (5 u/ l). The amplification program was 4 minutes preheating and initial denaturation at 94 ^{0}C, then 39 × 15 seconds at 94 ^{0}C, 1 minute primer annealing at (45^{0}C /48^{0}C) based on primers used, 1.30 minutes extension at 72 ^{0}C. The final extension for 7 minutes at 72 ^{0}C followed. The PCR reactions were also stored at 4 ^{0}C until loading on gel for electrophoresis.

4.7. Agarose gel electrophoresis

An agarose gel (1.67 % agarose with 100ml 1 × TBE) prepared and 8 l amplification product of each sample with 2 l loading dye was mixed and loaded on gel. DNA ladder or DNA marker 100 bp was also loaded together with the other samples and used to estimate molecular weight. The electrophoresis was done for 2 hrs at constant voltage of 100v. To allow visualization of bands, agarose gel was stained with Ethedium bromide. This was done first by mixing 450ml distilled water with 50 l of 10mg/ml Ethidium bromide in an automatic staining apparatus and then the gel was put in the solution for 30 minutes for staining followed by distaining of the gel in 450ml distilled water for another 30 minutes. Finally gel picture was taken and visualized under UV light, photographed with digital camera mounted on BioDoc Analyze and connected to PC with Biometra software, and saved for later data scoring.

4.8. Data Scoring and Analysis

Since ISSRs are dominant markers only the presence or absence of an allele can be determined. Each ISSR band was considered as an independent character or locus, and polymorphic bands were scored visually as either absent (“0”) or present (“1”) or missing data “?” for each of the 71 genotypes.

Different software’s were employed to calculate standard indices of genetic diversity to identify the amount of genetic variation in each enset population. These are listed as follow.

-POPGENE version 1.32 software (Yeh *et al.,* 1997) is used to see the genetic variation among and within enset population and compute summary statistics like: Allele frequency, Gene diversity, Genetic distance, F-statistics.

- The Shannon- diversity index scores were calculated for each sample as:

$$D = \sum_{i=1}^{S} p_i \times \log_2(p_i)$$

Where

D = the Shannon diversity index

S = the number of alleles in the sample

p_i = the relative abundance of the ith allele in the sample

- Areliquin version 3.01 (Excoffier *et al.,* 2001) was used to calculate Analysis of molecular variance (AMOVA) to estimate population differentiation directly from molecular data and test hypotheses about such differentiation.

- NTSYS_pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek *et al.,* 1999) software’s were used to calculate Jaccard’s coefficient of similarity which is calculated with the formula:-

$$S_{ij} = \frac{a}{a + b + c}$$

Where,

'a' is the total number of bands shared between individuals I and j,

'b' is the total number of bands present in individual I but not in individual j and

'c' is the total number of bands present in individual j but not in individual i.

- From the similarity matrix, a sequential, agglomerative, hierarchical, and nested (SAHN) cluster analysis was performed using the unweighted pair group method with arithmetic means (UPGMA) algorithm computed using NTSYS-pc version 2.02 (Rohlf, 1997). A dendrogram was generated using NTSYS-pc version 2.02 (Rohlf, 1997) to show the genetic relationships and distances of each accession

- The neighbor joining (NJ) method (Saitou and Nei 1987; Studier and Keppler, 1988) was used to compare individual genotypes and evaluate patterns of genotype clustering using Free Tree 0.9.1.50 software (Pavlicek *et al.,* 1999). The major difference between the two algorithms is that UPGMA assumes equal rates of evolution (molecular clock assumption) along all branches, whereas neighbor joining assume variations in the rate of change (Studier and Keppler, 1988; Nei and Kumar 2000).

- To further examine the patterns of variation among individual samples, a principal coordinated analsis (PCO) was performed based on Jaccard's coefficient (Jaccard, 1908). The calculation of Jaccard's coefficient was made with PAST software version 1.18 (Hammer *et al.,* 2001). The three axes were later used to plot with STATISTICA version 6.0 software (Hammer *et al.,* 2001; statistica soft, Inc.2001).Principal-coordinate analysis was used to obtain a graphic representation of the relationship structure of the 71 genotypes. Computations were performed using the MDS procedure in SAS (SAS Institute Inc 1992).

5. Results

5.1. Banding pattern and ISSR primers

The DNA amplification pattern using ISSR primers in this study was reproducible across gels based on the results from 71 DNA samples. Out of six primers tested initially, four of them gave relatively clear banding pattern and two primers were selected from the four for this study. ISSR analysis using two primers produced a total of 26 scorable bands 12 polymorphic bands generated by primer 834 and 14 bands with 826 (Table 4) The size of the bands amplified using the two primers ranged from 250bp to 2.5 kb (Fig. 4).

Table 4: - Fingerprint patterns generated using six ISSR primers; two of them are selected for this study.

Primers used	**Repeat motif**	**Amplification pattern**	**Amplification of specific bands**	**Number of scorable bands**
UBC-834	$(AG)_8YT$	Good	Good	12
818_H	$(CA)_8G$	Good but not all samples are amplified		-
UBC-811	$(GA)_8C$	Good but with smear		-
UBC-817	$(CA)_8A$	Smeared		-
UBC-820	$(GT)_8T$	All samples are not amplified		-
UBC-826	$(AC)_8C$	Good	Good	14

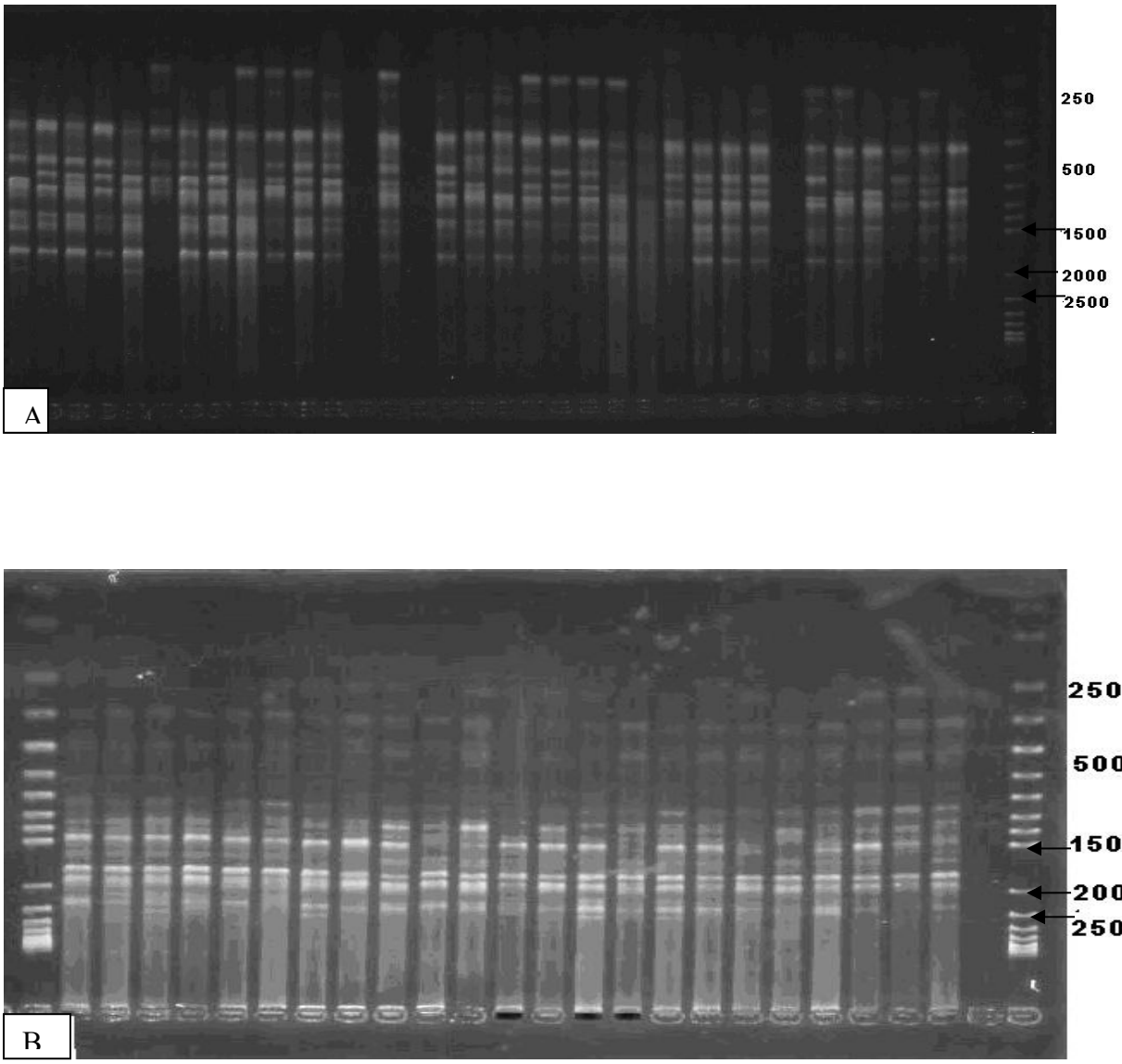


Figure 4:- ISSR fingerprint generated from 71 cultivated enset plants using primer 834 (A) and primer 826 (B) .The arrows indicated the estimate of the size of 100 bp ladder.

5.2. Polymorphism and genetic diversity

The number of polymorphic loci is 12 for primer 834 and 14 for primer 826. Out of the total loci scored, 86.54% is observed to be polymorphic. Among all the populations studied in this study Essera population have a higher percentage polymorphism (92.31%) as compared to Kefficho population (80.7%) (Table 5).

Among the total cultivated enset populations considered in this study, Kefficho population has higher gene diversity (0.32) than Essera population (0.22). The mean overall genetic diversity of enset population was found to be 0.27 (Table 5).

5.3. Genetic differentiation and gene flow

The coefficient of gene differentiation (Gst) for all loci was 0.329, while the estimate of the total genetic diversity (Ht) was found to be 0.402. The among population genetic diversity (Dst) was 0.133, and the within population genetic diversity (Hs) was 0.269. The within population diversity is greater than the between population diversity. The amount of gene flow that is brought by enset plant material exchange among the peoples of the two area, estimated as Nm= 0.5(1- Gst)/Gst was found to be 1.02 (Table 6).

Table 5:- Number of polymorphic loci, percent polymorphism, genetic diversity of cultivated enset as revealed with the two primers (834 and 826)

Population	NPL	PP (%)	GD
Essera	24	92.31%	0.22
Kefficho	21	80.7%	0.32
Average		**86.54**	**0.27**

NPL= Number of polymorphic loci

PP= Percent polymorphism

GD= Genetic diversity

Table 6:- Nei's Analysis of Gene diversity, genetic differentiation and gene flow of cultivated enset with the two primers (834 and 826)

Locus	Sample Size	Ht	Hs	Gst	Nm*
834-1	62	0.4894	0.3660	0.2521	1.4830
834-2	62	0.4511	0.2307	0.4885	0.5236
834-3	62	0.4511	0.2307	0.4885	0.5236
834-4	62	0.4999	0.0116	0.9769	0.0118
834-5	62	0.4953	0.4894	0.0119	41.6358
834-6	62	0.4155	0.2937	0.2931	1.2058
834-7	62	0.3158	0.2386	0.2446	1.5444
834-8	62	0.4212	0.4108	0.0247	19.7755
834-9	62	0.2882	0.2861	0.0076	65.6898
834-10	62	0.4711	0.3678	0.2192	1.7807
834-11	62	0.4978	0.3939	0.2087	1.8953
834-12	62	0.4884	0.1292	0.7354	0.1799
826-1	63	0.0455	0.0444	0.0238	20.4881
826-2	63	0.3511	0.3244	0.0762	6.0579
826-3	63	0.2848	0.2829	0.0065	76.8583
826-4	63	0.3605	0.3296	0.0859	5.3188
826-5	63	0.4408	0.4214	0.0440	10.8590
826-6	63	0.4871	0.1348	0.7232	0.1914
826-7	63	0.3295	0.2610	0.2078	1.9063
826-8	63	0.4758	0.4569	0.0396	12.1263
826-9	63	0.4546	0.2416	0.4684	0.5674
826-10	63	0.4507	0.4457	0.0111	44.4508
826-11	63	0.1579	0.1430	0.0946	4.7860
826-12	63	0.4762	0.1881	0.6051	0.3264
826-13	63	0.4997	0.0225	0.9551	0.0235
826-14	63	0.3485	0.2575	0.2612	1.4146
Mean	63	0.4018	0.2693	0.3298	1.0162
St. Dev		0.0128	0.0177		

Nm = estimate of gene flow, Nm = 0.5(1 - Gst)/Gst

5.4. Shannon's diversity index

The overall Shannon diversity index analysis of the total population with the two primers shows that enset samples collected from Keficho was found to be more diverse than those samples collected from Essera with a value of 0.67 and 0.59 respectively (Table 7). The proportion of genetic variation within enset population (73.5%) was found to be higher than the proportion of genetic variation between enset populations (26.5%).

Table 7: - Shannon's diversity index within and among enset populations

Popn/spps	**Shannon's diversity index (H)**
Essera	0.594
Kefficho	0.673
Hpopn	**0.633**
Hsp	**0.878**
Hpopn/Hsp	**0.735**
1-Hpopn/Hsps	**0.265**

Hpopn= mean variation for popn,
Hsp= mean genetic variation for the entire data,
Hpopn/Hsp= proportion of genetic variations within enset populations and
1-Hpopn/Hsp= proportion of genetic variations between enset population

5.5. AMOVA

Analysis of molecular variance indicated that higher percentage of variation is attributed to between the two populations of enset than the within population variation with a high fixation index (0.591), that means variation among populations of enset (59.12 %) is greater than the within population variation (40.881 %). (Table 8).

Table 8: - Analysis of molecular variance (AMOVA) of enset populations of Essera and Kefficho.

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation Indices
Between Populations	1	122.479	4.51446	59.119	0.591
Within Populations	69	189.192	3.12176	40.881	
Total	70	311.671	7.6362		

5.6. Clustering Analysis

UPGMA and neighbor joining analysis was used to construct dendrogram for 71 individuals based on 26 bands obtained with two dinicluleotide primers, accordingly, in both dendrogram the two populations (Essera and Kefficho) appeared to have their own cluster separately. Individuals collected from Essera region were grouped together in one cluster and populations from Kefficho region formed a separate cluster (Fig. 5 and 6). However, some clones from both groups have escaped from the major cluster in both NJ and UPGMA dendrogram. And the neighbor joining tree shows that these few groups of enset have a longer branches than the other clones (Fig. 6). Both trees recovered almost the same tree topology with similar groupings.

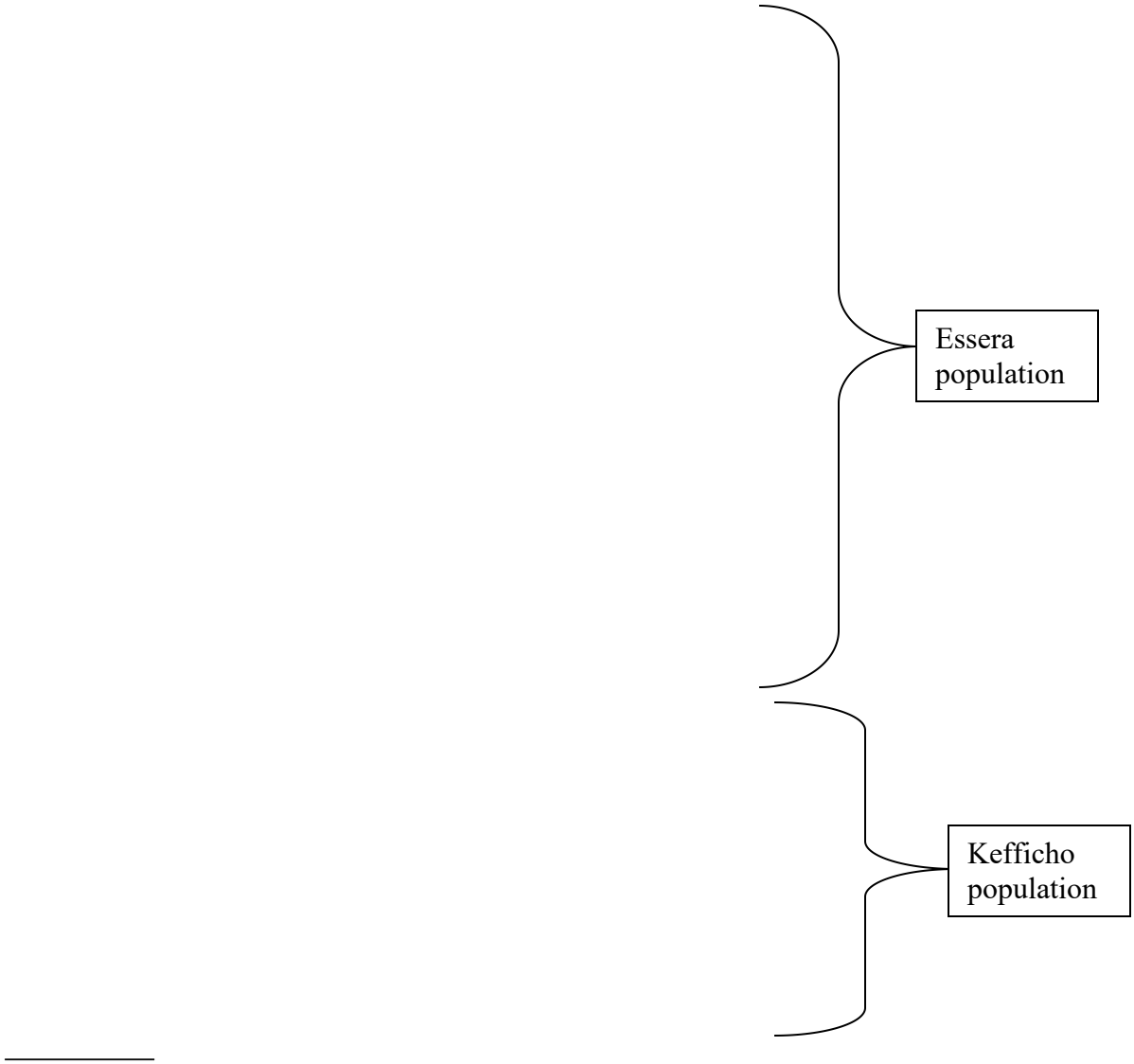


Figure 5: - Dendrogram for 71 individuals of two enset populations (namely K, representing Kefficho and E, representing Essera) obtained using the Unweighted pair group method with arithmetic average (UPGMA) of 26 PCR bands amplified (presence absence data) by two(834 and 826) primers. The UPGMA algorithm is based on Jaccard's coefficients obtained after pair wise comparison of the presence-absence fingerprint.

Figure 6: - Neighbor-joining analysis of 71 individuals based on 26 PCR bands amplified by two di-nucleotide (834 and 826) primers. The neighbor joining algorithm is based on Jaccard's coefficients obtained after pair wise comparison of the presence-absence fingerprint.

5.7. PCO Analysis

All the data obtained using the two ISSR primers were used in PCO analysis using Jaccard's coefficients of similarity. The first three coordinates of the PCO having **eigenvalues** of 8.01, 7.57 and 4.39 with variance of 23.9%, 22.6% and 13.1 respectively (Fig. 8) used to show the grouping of individuals using three coordinates. The two populations observed to form two separate clusters and another third cluster of individuals from both populations. The first two coordinates of the PCO was used to represent the two population of enset in two dimensions (Fig. 7) and similar result was observed like that of three dimensional representations.

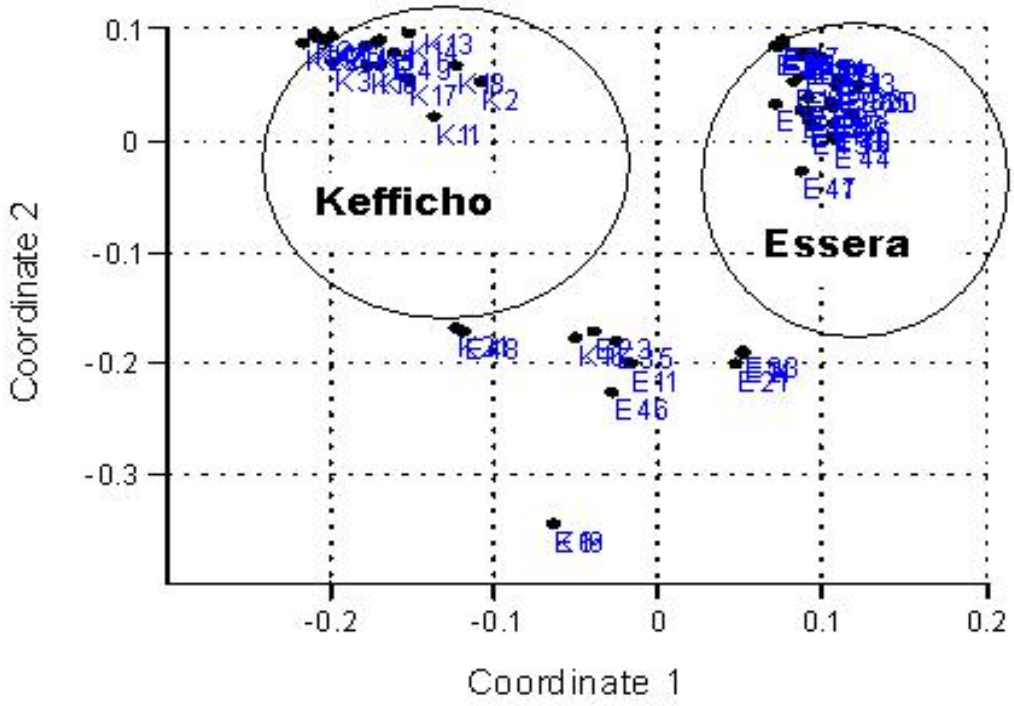


Figure 7: - Two dimensional representations of two enset populations based on Jaccard's similarity coefficients. The symbols K and E indicate the region from where individuals were collected, Kefficho and Essera respectively

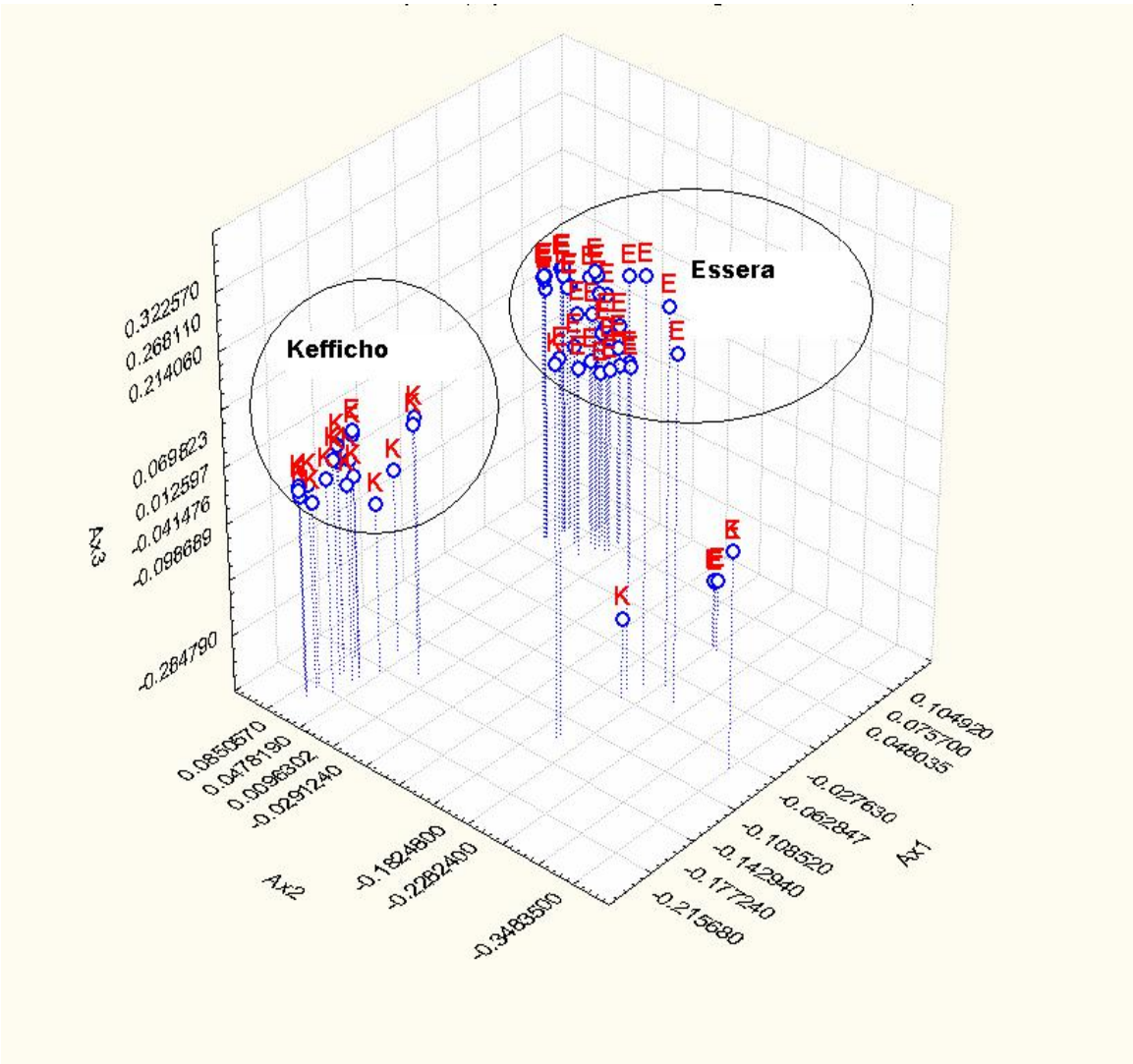


Figure 8: - Three-dimensional representation of a principal coordinate analysis of the genetic relationship among 71 individuals of cultivated *E.ventricosum*, inferred from a similarity matrix using the Jaccards index. The symbols K and E indicate the region from where individuals were collected, Kefficho and Essera respectively.

6. Discussion

In this study, the extent of genetic diversity in cultivated enset obtained from two sites in the southwestern part of Ethiopia was determined using ISSR marker. The PCR- based technique, ISSR has been found immensely useful in detecting genetic diversity of coffee (Tesfaye *et.al.,* submitted), Tef (Assefa, 2002) lentil (Fikru, *et al.,* 2007), rice (Girma, 2006, Unpublished) collected from different parts of Ethiopia. ISSR markers are also important to study intra specific variation in plant species, as they are effective in determining very low levels of genetic variation (Zietkiewics *et al.,* 1994).

In a study on white lupin it has been demonstrated that among 10 primers used any two were sufficient to distinguish all the 37 accessions studied (Gilbert *et al.,* 1999). Therefore the two primers selected for this study will be sufficient to distinguish the selected 71 clones of enset. The two ISSR primers generated a total of 26 scorable bands. The number of polymorphic bands generated by primers 843 and 826 was 12 and 14, respectively. In the previous enset DNA polymorphism studies, the number of scorable bands produced ranged from 9-13 using RAPD (Birmeta *et al.,* 2004) and a total of 180 AFLP fragments were scored (Negash *et al.,* 2002).

In this study ISSR analysis was a powerful tool in assessing the genetic diversity of *E.ventricosum.* Furthermore, the data suggest the existence of polymorphism within and between populations of *E. ventricosum.* This result is in agreement with other results obtained by AFLP (Negash *et al.,* 2002) who indicated that there is a considerable diversity in the crop, despite the reported loss of several important clones from farmers' fields. (Birmeta *et al.,* 2002) also indicated that the diversity and number of clones in enset cultivation regions could be as high as the number of vernacular naming used by local farmers. Nybom and Bartish (2000) as cited by Birmeta *et al.* (2002) indicated that even though each cultivated enset clone is propagated vegetatively, the genetic diversity among the clones was found to be relatively high compared to that of most other out

breeding crops. The high genetic variation observed in this study may be due to the efficient utilization of the crop by the peoples based on growing of each clones of enset crop for its specific utility to meet the use value of each to the peoples.

In this study, the genetic diversity parameters: genetic diversity (GD), percent polymorphic loci and Shannon's diversity index indicated that the genetic diversity in the enset populations of southwestern Ethiopia is indeed high. Shannon diversity index result showed that populations of enset from Kefficho are more diverse (0.67) than populations from Essera area (0.59). Similarly genetic diversity (GD) result indicated enset populations from kefficho have high genetic diversity than from Essera.

There was a negative correlation between estimates of Shannon index and analysis of molecular variance (AMOVA) which indicated the variation among populations of enset is greater than the within variation. The difference in estimation of variation with AMOVA and Shannon diversity index may be due to the fact that AMOVA is more reliable to co dominant markers than dominant markers like ISSR. Therefore for this study it is better to take Shannon diversity index as a true measure of the within and the between variation of the studied population.

Shannon diversity index indicated that the within population variation (73.5%) of enset from the two site is higher than the variation among the two population (26.5%). The analysis made by Popgne software also showed that the within population variation Hs= 0.269 (67.02% of the total 0.4018) is greater than the between population diversity Dst= 0.133 (32.97% of the total 0.4018). Similar to the present result, in previous RAPD study (Birmeta *et al.,* 2002), the within genetic variation (86%) was found to be greater than the between variation (14%). In an AFLP analysis of 146 cultivated enset clones from 5 regions of Ethiopia (70-200 km apart), Negash *et al.* (2002) found 4.8% between population variation which is less than the within population variation (95.2%) using AMOVA. The

mating system of a species has implications for the patterns of intraspecific genetic diversity. The higher within genetic diversity in cultivated enset could be due to the vegetative propagation mode of reproduction and other factors such as protection of enset clones due to cultural reasons and due to the differential values obtained from each clones of the crop.

The genetic structure of plant populations reflects the interactions of various factors, including the long-term evolutionary history of the species (shifts in distribution, habitat fragmentation, and population isolation), genetic drift, mating system, gene flow and selection (Schaal *et al.,* 1998 as cited by Fikiru, 2006). In the present study supporting the above result (the Shannon's diversity index), Nei's diversity parameters Gst (Genetic differentiation value) = 0.329 indicated also slightly high level of genetic differentiation among enset populations. This value is greater than the differentiation among the 5 wild enset populations (Gst= 0.12) obtained by RAPD analysis (Birmeta *et al.,* 2004). Generally, the between population variation estimate of the present study is greater than the among population variation obtained by both RAPD (Birmeta *et al.,* 2002) and AFLP (Negash *et al.,* 2002) analysis. This could be attributed to high mutation of annealing site of ISSR primers thus reflecting difference in sensitivity of different molecular markers.

A cluster analysis (UPGMA and Neighbor joining) was used to construct a dendrogram in order to see relationships among all individuals. Both dendrograms indicate a clear separation between the enset clones from Essera and Kefficho. The separation and formation of a cluster by the two populations may suggest that either of the two populations may have been isolated from each other for a longer period in time. Similarly PCO analysis based on two and three coordinates also show similar result except some observation in which a sample from Essera intermixed with a sample from Kefficho. Some enset clones from Essera clustered together with enset clones from Kefficho. Looking at the geographic location of Kefficho and Essera would explain this fact. Essera is

found in Dawro zone which shares a boundary in the west with Kefficho. Therefore, limited enset material transfer between people living in these two areas is a possibility. The Neighbor joining tree also shows that few clones of enset from both groups have longer branch than the other clones. This is due to the fact that these groups are highly differentiated from the other groups of enset clones.

The values obtained from Nm in this study show the approximate number of individuals migrating from one population to the other, in a typical island model. As Nm is indicative of the number of migrants, it suggests that the average number of migrants per generation (Nm) between the enset populations of Ethiopia included in the present study is 1.02. According to Slatkin, as cited by Fekadu and Ledin (1997) Nm values can be grouped into three categories: high if Nm>= 1, intermediate if Nm ranges from 0.25 to 0.99 and low if Nm ranges from 0.000-0.249. In this study, the relatively high to medium level of gene flow detected may be due to the fact that the presence of limited exchange of materials between peoples of the two region and hence limited gene flow will not be restricted between clones from the two regions.

7. Conclusions and implications for conservation and improvement of clone of enset in Ethiopia

There are only two reports where the genetic diversity of clonal enset was analyzed using molecular marker: Birmeta et al. (2002) based on RAPD and Negash et al. (2002) based on AFLP markers. Lack of empirical knowledge about the genetic diversity of a crop hampers the efficient conservation and utilization of its genetic resource. The present study dealt with analyzing enset clonal diversity in Kefficho and Essera using ISSR marker.

In conclusion, the present study using ISSR analysis, all the diversity parameters confirm that there is high diversity in cultivated *E. Ventricosum* populations of South Western Ethiopia and that ISSR markers can be successfully applied for the assessment of genetic diversity of this species. Diversity parameters also indicated that clones of enset from Kefficho region are highly diverse than clones of enset from Essera region. Therefore, intrapopulation improvement programs should target selection of individual plants with desirable traits from Kefficho populations. This is because the probability of getting individual plants with good traits from such diverse population is very high as compared to those that are less diverse. However, this study is not complete in terms of sample size and area coverage; hence a more extensive genetic diversity investigation including areas not covered yet should be done and allow its effective conservation.

Furthermore, all the clustering analysis (UPGMA, NJ and PCOs) and Nm value in this study have clearly shown that there is a limited gene flow among Ethiopian cultivated enset populations. Estimates of gene flow indicate that there could be a possibility of sampling plants with the same genetic constitution from different administrative regions. Therefore, representing a population by as many collections as possible would be the best approach.

8. Recommendations and research needs

- The distribution of cultivated enset in Ethiopia appears to be expanding, especially after periods of famine, when people in other regions learn about the benefits of this crop and attempt to incorporate it into their own agricultural system (Birmeta *et al.,* 2004). Enset cultivation is constrained by bacterial wilt and other diseases. Therefore, research and extension activities should be carried out that address those constraints limiting production to provide protection of enset against hazards, as well as a focus on other factors that could lead to increased output. These factors interact in determining the food security and nutritional status leading to the improved health and welfare of the nation's people.

- Further research on analysis of genetic diversity of cultivated enset population including divergent production areas has to be done.

- Agro-botanical investigations including collection, classification and selection of clones with superior characteristics, including bacterial wilt resistance, to standardize materials for further research.

- Promoting public awareness about the importance and values of conservation and sustainable utilization of enset crop genetic diversity and strengthening the conservation capacity of institutes both for *in-situ* and *ex-situ.*

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