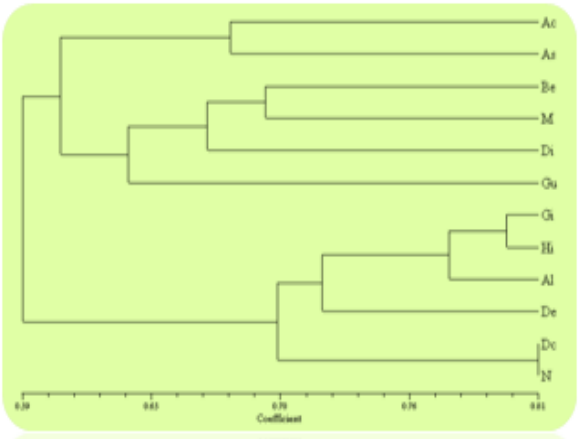
ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES
INSTITUTE OF BIOTECHNOLOGY

Genetic Diversity Study of Anchote (*Coccinia abyssinica* (Lam.) Cogn.) Using Inter Simple Sequence Repeat (ISSR) Markers



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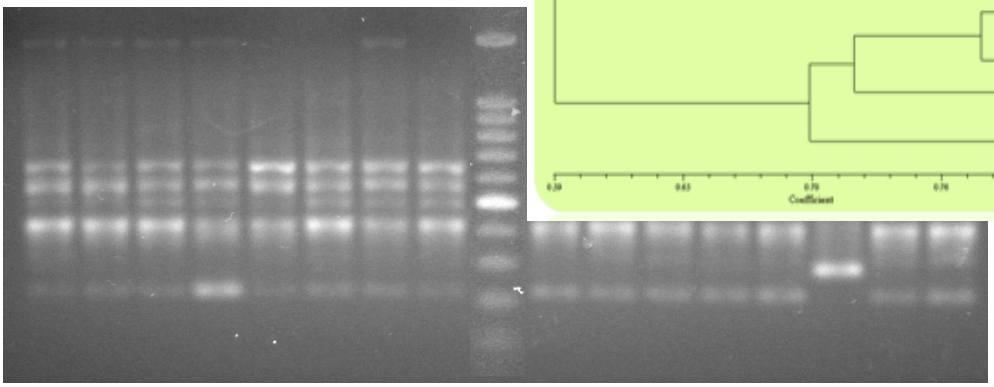

A Thesis Submitted to the School of Graduate Studies, Addis Ababa University, in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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This is to certify that the thesis prepared by Abreham Bekele Alemu, entitled: Genetic diversity study of anchote *(Coccinia abyssinica)* using ISSR markers and submitted in fulfillments of requirements for the degree of Master of Science in Biotechnology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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ABSTRACT

Anchote (*Coccinia abyssinica*) is perennial trailing vine, underutilized but very important endemic plant with high calcium content grown for its edible tuberous roots in Ethiopia. In spite of its importance as food security crop, there is no information on molecular genetic diversity of this crop. Therefore, the objective of this study was to assess genetic diversity within and among 12 populations of anchote using ISSR markers. A total of 87 scorable bands were generated using nine ISSR primers among which 74 were polymorphic. Within population diversity based on polymorphic bands ranged from 13.8% to 43.53% with mean of 33.05%, Nei's genetic diversity of 0.04 to 0.156 with mean of 0.118, Shannon information index of 0.07 to 0.23 with mean of 0.175 and AMOVA within population 51.4%. With all diversity parameters, the highest diversity was obtained from Gimbi, Bedele and Ale, whilst the lowest was from Manna. AMOVA showed 48.56% among populations variability and significantly lower than that of within population variation. Population differentiation with F_{ST} was 48.6%, impling high differentiation among population. From Jaccard's pairwise similarity coefficient, Decha and Nedjo were most related populations exhibiting 0.76 similarity and Manna and Nedjo were the most distantly related populations with similarity of 0.52. The penta nucleotide primer 880 (GGAGA)3 showed unique band in some individuals that appeared to be associated with morphological traits. Illubabor and Gimbi populations exhibited highest genetic diversity so that the populations should be considered as the primary sites in designing conservation strategy and improvement of this crop.

Key words: Anchote, Endemic, Genetic diversity, ISSR, Underutilized

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List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
BARC	Bako Agricultural Research Center
CAPS	Cleaved Amplified Polymorphic Sequence
CTAB	Cetyltrimethyl Ammonium Bromide
dNTPs	deoxy Nucleotide Tri-Phosphate
DZARC	Debre-Zeit Agricultural Research Center
EDTA	Ethylene Diamine Tetra Acetic acid
GD	Genetic Distance
GS	Genetic Similarity
HARC	Holleta Agricultural Research Center
IBC	Institute of Biodiversity Conservation
ISSR	Inter Simple Sequence Repeats
IPGRI	International Plant Genetic Resource Institute
MAS	Marker Assisted Selection
NPB	Number of Polymorphic Band
PCoA	Principal Coordinate Analysis
PGRC	Plant Genetic Resource Center
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SDS	Sodium Dodecyl Sulphate
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeats
TAE	Tris-Acetate EDTA buffer
TE	Tris-EDTA buffer
UPGMA	Unweighted Pair Group Method with Arithmetic mean

1.0. INTRODUCTION

Anchote (*Coccinia abyssinica* (Lam.) Cogn.) is perennial trailing vine that belongs to family *Cucurbitaceae* and genus *Coccinia*. Genus *Coccinia* contains about 30 species in the palaeotropics of which 8 species (five named and three unnamed) reported to occur in Ethiopia (Jeffrey, 1995). However, according to Holstein and Renner (2011) genus *Coccinia* contains 27 species almost all confined to sub-Saharan Africa where it is diversified into numerous habitat types. *Coccinia grandis* is the only species that escaped from sub-Saharan Africa and spread to the highlands of the Arabian Peninsula and tropical Asia, and now becomes an invasive weed on the Pacific Islands (Holstein and Renner, 2011). Although there was no information about chromosome numbers and ploidy level of *C. abyssinica, C.grandis* of this genus is a diploid plant with 24 chromosomes (2n=24). About 8 species *Coccinia* reported to occur in Ethiopia, only *C. abyssinica* is cultivated for human consumption (Abera Hora, 1995 and Endashaw Bekele, 2007). This plant is reported to be an endemic species that is found both in cultivated and wild form in Ethiopia (PGRC, 1996; IBC, 2008; Mkamanga, 2009).

Anchote is a tuberous plant with shoots having a simple tendril and grows at altitudes ranging from 1,300-2,800. According to anchote germplasm collection records of Institute of Biodiversity Conservation (IBC) of Ethiopia, majority of anchote populations were collected from Oromia regional state mostly around Wollega, a place with old history of cultivation which is supposed to be the center of diversity. According to

collection records of IBC, it is also found in Jimma, Illubabor, Gojjam, Benishangul-Gumuz and other western and south western parts of the country.

Coccinia abyssinica can be propagated both vegetatively and from seeds. Vegetative propagation is achieved by planting either the whole tuber or by slicing it into two or more pieces, each piece having rootlets and an external covering (Abera Hora, 1995; Endashaw Bekele, 2007). Although young shoots of anchote are eaten as a cooked vegetable; its cultivation is mainly for tuberous fleshy rootstock (Imbumi, 2004) adopted only from farmers own practice and past experience. The juice from fruits and other parts are also used as medicine. According to Desta Fekadu (2011), leaves of anchote are rich in protein, calcium, phosphorus, zinc and other dry matter compared to commonly consumable tubers that contains relatively less protein and dry matter when compared to leaf and fruits. Likewise, immature fruits that are rarely consumed are rich in calcium, potassium, phosphorus, magnesium and dry matter. From the above mentioned facts, one may generalize that all parts of anchote are consumable if the community is well aware of the nutritional composition and the ways of processing those parts.

Anchote is valuable food source in the Western and South Western region of Ethiopia. Juice prepared from roots of anchote has *saponin* as an active substance and is used to treat gonorrhoea, tuberculosis and tumor cancer by the local community. On top of this, local people have been using anchote for treatment of bone fracture, displaced joint, on ceremonial days, special guests and animal fattening (Abera Hora, 1995; Endashaw Bekele, 2007; Dandena Gelmesa, 2010).

The fact that indigenous vegetables have rescued thousands of hungry Ethiopians during famine period, it has bridged a gap during the periods of grain shortage, which is crucial for food security. However, little is known about their agronomy, nutritive value, germplasm management and improvement of their yield especially by the natives. According to Dandena Gelmesa (2010), there is an urgent need to identify the most effective commercialization, marketing and policy frameworks to promote their use and increase economic values.

It is expected that the genetic diversity study of the traditional crops and their wild relatives enables the research community to develop improved varieties. To this effect characterization and evaluation of conserved germplasm carried out to define core collections is of paramount importance in facilitating the utilization of genetic materials in Ethiopia. IBC country report (2007) emphasized that morphological and molecular data on the germplasm will support in depth evaluation of genetic materials for utilization. Interestingly, to strictly conserve elite genotypes, information on genetic diversity within and among plant populations has a tremendous significance for marker assisted selection, preservation and improvement of crop for sustainable agriculture.

Although people often use morphological markers to identify and name cultivars of anchote and other crops; morphological markers are constrained due to small number of observable phenotypic traits, environmental influence and long time it takes. Research towards investigation of genetic diversity of anchote has not yet been conducted for conservation and sustainable utilization of this underutilized native crop using any DNA

based methods. Therefore, this work is the first of its kind in establishing baseline information on anchote genetic diversity for its conservation.

Besides morphological and biochemical markers, various molecular markers are used to investigate the genetic diversity in crops. The most commonly employed ones are PCR-based assays such as Random Amplified Polymorphic DNA (Williams *et al*., 1990), Simple Sequence Repeats (Akkaya *et al*., 1992), Amplified Fragment Length Polymorphism (Vos *et al*., 1995) and Inter Simple Sequence Repeats (Zietkiewicz *et al*., 1994). Among these, Inter Simple Sequence Repeat (ISSR) is preferable as it is simple, fast, cost effective, discriminative, reproducible, informative, and require small quantity of sample DNA (Zietkiewicz *et al*., 1994; Bornet and Branchard, 2001; Bornet *et al.*, 2004; Bhagyawant and Srivastava, 2008; Emel, 2010). ISSR-based study involves the PCR amplification of regions between inversely oriented microsatellites. It does not need sequence information and prior genetic studies (Zietkiewicz *et al*., 1994; Jabbarzadeh *et al.*, 2010). Moreover, ISSR markers are considered to be more reproducible, reliable and generate more polymorphic markers than RAPD (Bornet and Branchard, 2001; Chowdhury *et al*., 2002). As was shown in Chowdhury *et al.* (2002), the ISSR method produces more complex marker patterns than the RAPD assay which is advantageous when differentiating closely related cultivars. Furthermore, ISSR analysis is less time consuming and less expensive as compared to SSR procedure.

Various papers on genetic diversity analysis of different types of plant pointed out that ISSR is potential marker system in discriminating within and among population without prior DNA information especially for crops that are very difficult to classify

morphologically. Some of this studies are: analysis of genetic diversity in the endangered tropical tree species *Hagenia abyssinica* using ISSR markers by Tileye Feyissa *et al*. (2007), ISSR variation study in forest coffee trees (*Coffea arabica* L.) populations from Ethiopia by Esayas Aga *et al*. (2005), ISSR and analysis of wild and cultivated rice species from Ethiopia by Gezahegn Girma *et al*. (2010). Therefore, this study aims at using Inter Simple Sequence Repeat (ISSR) molecular marker based technique to study genetic diversity of anchote populations representing different areas of the country and provide baseline information for future conservation and improvement of the crops.

2.0. LITERATURE REVIEW

2.1. Taxonomy, botanical description and distribution of anchote

2.1.1. Taxonomy

Kingdom: *Plantae*

Phylum: *Magnoliophyta*

Class: *Magnoliopsida*

Order: *Violales*

Family: *Cucurbitaceae*

Genus: *Coccinia*

Species: *Coccinia abyssinica*

According to its taxonomic classification, *Coccinia abyssinica* belongs to the family *Cucurbitaceae* that have about 90 genera further classified into 700 species and a genus *Coccinia*. Jeffrey (1980) classified the family *Cucurbitaceae* into two subfamilies: *Zanonioideae* and *Cucurbitoideae*, with several tribes; *Schizopeponeae, Joliffiea, Trichosantheae, Benincaseae, Cucurbiteae, Cyclanthereae,* and *Sicyoeae*. According to this classification, the genus *Coccinia* belongs to sub-tribe *Benincasinae* of the tribe *Benincaseae*. Although *Coccinia* contains about 30 species in Jeffrey (1995), Holstein and Renner (2011) reported that genus *Coccinia* contains 27 species almost all of which are confined to sub-Saharan Africa where it is diversified into various habitat types. Out of them eight species of *Coccinia* are found in Ethiopia (Jeffrey, 1995).

2.1.2. Botanical descriptions

The term anchote originated from the Oromo language as a vernacular name for the crop *Coccinia abyssinica* which refers to the edible tuber of the cultivated races of the crop on the one hand (Abera Hora, 1995) and entire plant on the other. It is also known by its different vernacular names at different places such as '*ushushe*' in Walaita, '*wushish*' in Tigragna *'shushe'* in Dawero and *'ajjo'* in Kaffa (Abera Hora, 1995). For this thesis a most popular name 'anchote' adopted by various research communities such as Ethiopian Institute of Agricultural Research, Ministry of Agriculture and Institute of Biodiversity Conservation was entirely used. In Ethiopia, currently it is cultivated mainly for its tuberous fleshy rootstock, though its young shoots are often eaten as a cooked vegetable and other parts are also found to be nutritive (Tilahun Gamta, 1989; Imbum, 2004, Desta Fekadu, 2011).

Anchote is a perennial plant with trailing annual shoots that climb up a support by means of simple tendrils having spherical to cone-shaped tuber. The tubers of cultivated variety are usually of economic concern whilst their fruits are not edible due to lack of necessary information about nutrition and anti-nutrition factors. Contrary to the cultivated ones, tubers of wild anchote are not edible although the fruits are edible (Amare Getahun, 1973) and have been used as animal feed in several localities.

The flowers of anchote are either male (staminate) or female (pistillate) with the male flowers in racemes while the female flowers are borne singly. The flowering period is from July to November (Abera Hora, 1995). Flowers produce abundant nectar which is

collected by honey bees along with some pollen. The fact that anchote assumes both cross- and self- pollinated crop, there is a need for further studies so as to determine pollination type of this plant. However, Desta Fekadu (2011) indicated anchote as self-pollinating plant with epigeal germination type.

2.1.3. Distribution of anchote

Ethiopia is one of the richest genetic resource centers in the world in terms of crop diversity with an estimated flora between 6,500 and 7,000 species, of which 10 – 12 percent is considered to be endemic. Anchote (*Coccinia abyssinica*), enset (*Ensete venricosum),* yams (*Dioscorea spp*.), and Oromo dinich (*Plectranthus edulis*) are the most threatened and underutilized but important native tuber crops of the country (IBC, 2008; 2009).

Anchote is an endemic, underutilized but important root and tuberous crops found both in cultivated and wild form in Ethiopia (PGRC, 1996; IBC, 2008; Mkamanga, 2009). However, its cultivation is sporadic and found widely in western, southeastern (western Sidamo, Gamu Gofa, and parts of Shewa), central and eastern provinces (previous Shewa and Hararge Administrative Regions, respectively) and northern parts (Amare Getahun, 1973; Abera Hora, 1995). Populations collected by the Institute of Biodiversity Conservation (IBC) of Ethiopia confirmed that there are also samples of anchote around Gojjam and other areas where it was not originally cultivated. However, there is no or limited information on whether people around Gojjam use anchote as food or not. This shows its wide ecological adaptation on one hand and its popularity on the other. In

Ethiopia, it is cultivated at elevations varying from 1,300 m to 2,800 m.a.s.l having average rainfall of 762 mm to 1,016 mm.

According to Amare Getahun (1973), anchote is a drought tolerant potential dry land crop that may adapt to wide ecological zones. It has an ability to thrive best on poor soil, moisture stress conditions and help in reducing soil erosion due to their rooting system and biomass coverage. In addition, the crop is known for its ability to tolerate termites probably, due to their moisture contents (Aschalew Sisay *et al.*, 2009). However, the seeds of 15 populations propagated in pots at the Addis Ababa University, College of Natural Science glasshouse showed that it was the only crop that was severely attacked and dried by fungi Powdery mildew compared to other crops such as sorghum (*S. bicolor*), Oromo Dinich (*Plectranthus edulis*), noug (*Guizota abyssinica*), and sweet potato *(Ipomoea batatus)* that were neighborly grown in the same glasshouse (personal observation). This may need further research to identify and characterize this specific pathogen and introduce suitable management strategy.

2.2. Propagation system and productivity

Although anchote is propagated both vegetatively and by seeds, propagation by seed is an easy technique and commonly employed. Vegetative propagation is performed by planting either the whole tuber or by slicing it into two or more pieces, each piece having rootlets and covers. This is usually done to establish mother plants, called *guboo,* that serve as a seed source for further plantings. Stem cuttings are also effective for propagating anchote especially when plantlets are found densely in the field.

Seed propagation involves taking out of seeds from fully matured red-ripe fruits which are harvested before they start rotting. Such fruits are processed from the fleshy juicy part and dried in sun and stored until sowing season. Women are the ones that exclusively work from fruit harvesting to seed extraction and storage. As practiced, the women harvest, prepares, process and store anchote seeds and/or either purchase or request seeds from their friends and make them available for sowing well ahead of the beginning of the rainy season. It is after obtaining the seeds that the women insist on their male partner to plough the land (Abera Hora, 1995; Endashaw Bekele, 2007).

In terms of its productivity, the total yield of anchote is 15-18 tones/hectare, which is in the range of the total yield of sweet potato and potato (Abera Hora, 1995; Habtamu Fufa, 1997). Similarly, Girma Abera and Hailu Gudeta (2007) reported that the average yield of anchote in western Oromia zones on several hectares of land was 10-15 t/ha. Likewise, Wayessa Garedew *et al.* (2008) also affirmed that productivity of anchote was from 10 t/ha to 20 t/ha from west to east Wollega zones. The yield can also be increased noticeably if appropriate management strategies and improved verities are released.

In comparison with other root and tuber crops, anchote is relatively of short life cycle which gives high yield in a relatively shorter period of time usually about 4 months from sowing to maturity (Girma Abera and Hailu Gudeta, 2007). This is important in the area of food shortage since it can easily substitute other foods in short time (Table 1).

Table 1. Maturity time of anchote as compared to other tuber crops

Common name	Scientific name	Planting to harvesting duration(in month)
Cassava	*Manihot esculenta*	10-14
Yam	*Dioscorea esculenta*	6-9
Taro	*Colocasia macrorrhiza*	7-9
Sweet potato	*Ipomoea batatus*	4.5- 8
Anchote	*Coccinia abyssinica*	4.5

Source: Abera Hora, 1995

2.3. Uses of anchote

2.3.1. Anchote as food and feed

Almost all parts of anchote (tubers, leaves and the immature fruits) are consumable by human as well as animals. However, anchote is mainly cultivated for its storage root (Fig. 1). Anchote, when compared to other root and tuber crops, contributes a significant amount of protein and calcium to the diet of the rural community. It is thus highly important in fighting protein malnutrition problems and contributes a lot to the diet of rural families.

Habtamu Fufa and Kelbessa Urga (1997), found out that anchote has a good nutrient composition with a good supplement of vitamins and minerals compared to other tuber crops (Table 2). According to their findings in100 gram of edible portion, peeling of anchote reduces protein, carbohydrate, and vitamins while relatively increases ash, fat,

fiber, calcium, iron and phosphorus content. Similarly, Habtamu Fekadu (2011) found out that boiling of anchote tuber after peeling reduces crude protein, total ash, crude fat, utilizable carbohydrate, gross energy, calcium, magnesium, zinc, and anti-nutritional factors such as phytate, oxalate, tannin and cyanide. However, boiling before peeling decreases crude fiber, iron and phosphorous content compared to raw and boiling after peeling.

Fig. 1. Different parts of anchote that contain valuable nutrients. Shoots/leaves of anchote (A), fruits on tendrils (B), uncoated anchote seeds (E), edible tubers of anchote (D, E and F). D and E refer to top and frontal view of unboiled anchote while F refer to boilied ones. *Source: Figs. A, B, D, E photo by author; fig C from Yosef Yambo, 2012 and Fig. F from Habtamu Fekadu, 2011.*

Table 2. Nutrient composition of anchote and other tubers in 100 gram of edible portion

Sample	protein	Carbohydrate	Ca	Fe	P	Vit-A	Vit-C	Vit-B2
	g	g	mg	mg	mg	µg	mg	Mg
Potato	1.3	18	13	1.1	51	0	21	0.03
Sweet potato	1.6	28	33	2	38	75	37	0.05
Taro	1.8	23	51	1.2	88	11	8	0.03
Yam	1.9	27	52	0.8	61	10	6	0.02
Cassava	1.2	35	68	1.9	42	30	31	0.05
Whole Anchote	3.9	25	327	4.6	104	53	8	0.08
Peeled Anchote	3.00	22	344	5.5	123	45	10	0.06

Source: Habtamu Fufa and Kelbessa Urga (1997)

As compared to other tuber crops such as potato, sweet potato, taro, yam, and cassava, anchote has got higher protein content, calcium, iron, and phosphorous. So it is believed to play a vital role in supplying the calcium requirements of infants and children especially in places that have scarcity of milk production. This is indeed useful in the utilization of the crops in food security programs by attracting the sight of investors and industrialists to produce different food items from anchote.

In addition, anchote flour has been used to prepare *soup* when boiled with bone-marrow of animals. Such soup is particularly served to patients with broken or fractured bones or sick people. A stew locally called *"anchote ittoo"* is prepared on ceremonial occasions solely from sliced anchote with butter.

Furthermore, culturally a variety of dish that has been served from anchote are *'kitifo'*, *'lankata'* (finely grounded tuber), '*wot'* (local stew from anchote), *soup*, and '*murmura*' (boiled tuber cut in pieces), *porridge* and other more culinary dishes. Foods prepared from anchote are more commonly consumed in areas like Wollega, Jimma, Illubabor and West Shewa mostly during wedding, birthdays, *meskel (finding of the true cross)*, *atete'* and other religious ceremonies.

2.3.2. Medicinal uses of anchote

Apart from its nutrition anchote has many social, cultural, economic and medicinal values. Anchote has got high protein and calcium content which enables consumers to defend some deficiency diseases. Traditionally, it is believed that anchote heals broken or fractured bones, helps sick people to recuperate and makes lactating mothers healthier and stronger (Abera Hora, 1995; Endashaw Bekele, 2007; Dandena Galmessa, 2010). Its higher calcium content makes the crop preferable to children, youth, elders, lactating and pregnant mothers. The report of Dawit Abebe and Estifanos Hagos (1991) cited in Endashaw Bekele (2007) showed that juice prepared from roots of anchote has *saponin* as an active substance and is used to treat gonorrhea, tuberculosis and cancer. Therefore anchote can interestingly be used as new, or alternative or substitutive input for modern pharmaceuticals. As a result, this could pave a way for developing modern new medicinal products from anchote and hence there is an urgent need immediate research in this area.

2.4. Anchote research and crop improvement effort

The fact that women play greater role in domestication, cultivation, selection and storage of the best genotypes of anchote for the next growing season than men do (Abera Hora, 1995), anchote farming is traditionally considered as human's own business. Nevertheless, researches being conducted in the country are somewhat in a very fragmented manner and hence no such improved variety of anchote exists so far. However, little effort in selection and morphological characterization of the crop has already been started by the Debrezeit Agricultural Research Center (DZARC) and Bako Agricultural Research Center (BARC), which should be strengthened and augmented by molecular characterization methods.

Furthermore, strengthening the research towards minimization of some anti-nutritional factors (if any) by modern biotechnological approaches (genetic engineering) should be considered so as to improve various bottleneck problems associated with anchote cultivation. The recently optimized cost-effective micro propagation protocol from seeds of anchote (Yosef Yambo, 2012 unpublished) was very important for targeting with genetic transformation activities for better variety development. Desta Fekadu (2011) also stated in his thesis that, despite all parts of anchote used for human consumption, not all of them are equally rich in nutrient composition for all populations he studied. So improvement of this crop through traditional breeding or genetic transformation is very important to generate marketable products.

2.5. Constraints of anchote production

Even though some fragmented research was conducted at few Universities by graduate students and few research centers (BARC and DZARC), the crop faced limited research coverage and lower attention both at a national and regional level. Amongst others, low seed production resulted in absence of seed on the market because of pest attack, very small scale cultivation coverage, inconsistent supply for consumers, absence of government support for the improvement and development of the crop, lack of improved varieties, poor cultural practices, limited information about the disease and insect pests that affects the production and productivity and damage by animals are the major bottlenecks of anchote production. Furthermore, poor harvesting mechanism is as well part of the challenges in enhancing its productivity since the tuber go deep into the soil up to 30cm (Abera Hora, 1995). The seeds grown in glasshouse of Addis Ababa University also confirmed that these crops are very sensitive to powdery mildew that resulted in total death of shoots.

2.6. Genetic diversity and markers for genetic diversity study

Genetic diversity refers to any variation in the nucleotides, genes, chromosomes or whole genomes of the plant or others. Assessment of genetic diversity is important in plant breeding if there is an improvement by selection. Genetic diversity is commonly measured by genetic distance (GD) or genetic similarity (GS = 1 - GD), both of which imply that there are either differences or similarities at the genetic level (Lamkey and Lee, 1993; Kurane *et al*., 2009).

Due to the rapid developments in the field of molecular genetics, varieties of different techniques have emerged to analyze genetic variation. These genetic markers may differ with respect to important features, such as genomic abundance, level of polymorphism, locus specificity, reproducibility, technical requirements and financial investment. No marker is superior to all others for a wide range of applications. The most appropriate genetic marker depends on the specific application, the presumed level of polymorphism, the presence of sufficient technical facilities and knowhow, time constraints and financial limitations. The three major classes of markers are morphological markers, biochemical markers and molecular markers (Kurane *et al*., 2009; Kumar *et al.,* 2009,). For assessment of genetic diversity, molecular markers have been generally superior to morphological, and biochemical data (Lamkey and Lee, 1993).

2.6.1. Morphological markers

Morphological markers generally correspond to the qualitative and qualitative traits tha can be scored visually. Several undesirable factors limited its application as appropriate marker system. Amongst them is high dependency on environmental factors, poly genetic and epistatic control, and long time it needs for observation (Spoone *et al,* 2005).

2.6.2. Biochemical markers

These markers are the products of the various alleles of one or several genes. Isozymes, the different molecular forms of the same enzyme that catalyze the same reaction, have been used for over 60 years for various research purposes. Biochemical markers are superior to morphological markers in that they are mostly independent of environmental factors. The main weakness of isozymes is their relatively low abundance and low level of polymorphism (Kassa Semagn *et al.*, 2006; Kumar *et al.*, 2009)

2.6.3. Molecular markers

Molecular markers are identifiable DNA sequences, found at specific locations of the genome, transmitted by the standard laws of inheritance from one generation to the next. Most of them are not protein coding genes and hence usually have no any biological effect, instead can be thought of as constant landmarks in the genome (Kassa Semagn *et al.*, 2006). These markers are numerous in number and can be found in nuclear, mitochondrial and chloroplast DNA. Their uses are based on the naturally occurring DNA polymorphism by deletion, insertion and substitution. Unlike protein based markers, DNA markers segregate as single genes and not affected by the environment.

A large number of molecular marker systems are now available which can directly evaluate the genome, ensure genome wide coverage and are neutral to the environmental factors and developmental stages and are not influenced by other genes and factors. They may differ in a variety of ways such as technical requirements; the amount of time, money and labor needed; the number of genetic markers that can be detected throughout

the genome; and the amount of genetic variation found at each marker in a given population.

Molecular markers are generally classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers. Hybridization-based markers are usually restriction fragment length polymorphism (RFLP) while polymerase chain reaction (PCR)-based markers include random amplified polymorphic DNA (RAPD), Inter simple sequence repeats (ISSR), simple sequence repeats (SSR), amplified fragment length polymorphism (AFLP), single nucleotide polymorphism (SNP), cleaved amplified polymorphic sequences (CAPS). In the former, DNA profiles are visualized by hybridizing the restriction enzyme-digested DNA, to a labeled probe, which is a DNA fragment of known origin or sequence. PCR based markers involve *in vitro* amplification of particular DNA sequences or loci, with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. The amplified fragments are separated electrophoretically and banding patterns are detected by different methods such as staining and autoradiography. PCR is extremely sensitive and operates at a very high speed. Its application for diverse purposes has opened up a multitude of new possibilities in the field of molecular biology (Kumar *et al.,* 2009).

Properties desirable for ideal molecular markers include: High polymorphism, co dominant inheritance, frequent occurrence in genome, selective neutral behaviors, easily scorable and access, easy and fast assay, high reproducibility, and easy exchange of data between laboratories. However, there is no specific molecular marker, which would meet

all the above criteria. Although the need for markers and their number is increasing from time to time annotated review of RFLP, RAPD, SSR, AFLP, SNP, CAPS, ISSR are given as follows.

2.6.3. 1. Restriction Fragment Length Polymorphism (RFLP)

RFLP were the first DNA based marker used for human genome mapping (Botstein *et al*., 1980) and later adopted for plant genomes (Helentjaris *et al*., 1986; Weber and Helentjaris, 1989) to distinguish plant species, genotypes and, in some cases, individual plants. RFLPs are markers detected by treating DNA with restriction enzymes. They have their origin in the DNA rearrangements that occur due to evolutionary processes, point mutations within the restriction enzyme recognition site sequences, insertions or deletions within the fragments, and unequal crossing over. Differences in the lengths of DNA fragments will then form different banding pattern on membrane after southern blotting from size fractionating gel electrophoresis. Fragments of interest are identified by hybridization with radioactive labeled probe.

RFLPs are codominant markers, reliable in linkage analysis and breeding and can easily determine if a linked trait is present in a homozygous or heterozygous state in individual. However the utility of RFLPs has been hampered due to the requirement of large quantities (1–10 μg) of purified high molecular weight DNA (Roy *et al.*, 1992; Young *et al.*, 1992), expensive and hazardous radioactive isotope, time-consuming and labor intensive assay and only one out of several markers may be polymorphic. To circumvent these drawbacks simpler marker systems have subsequently been developed latter.

2.6.3.2. Random Amplified Polymorphic DNA (RAPD)

RAPDs are DNA fragments amplified using single short random primers, generally 10 bases long individual primers operate in both forward and reverse directions, thus amplifying between inverted repeats of the binding sequence (Williams *et al*. 1990). Amplified fragments are generally separated by agarose gel electrophoresis and polymorphism is detected as the presence or absence of products. Polymorphisms arise primarily due to base variation at putative primer annealing sites, although length differences are also possible.

RAPD possesses several advantages; it does not need sequence information, each arbitrary primer can reveal several polymorphic loci, and loci are distributed through the genome and technically simple. RAPD is, however, less popular due to problems such as poor reproducibility, faint or fuzzy products, difficulty in scoring bands, and dominant in nature (Williams *et al.*, 1990; Rieseberg, 1996; Jones *et al.*, 1997). Despite these drawbacks, RAPDs have been used for many purposes, ranging from studies at the individual level to studies involving closely related species.

2.6.3.3. Amplified Fragment Length Polymorphism (AFLP)

AFLP first describbed by Vos *et al.* (1995), are DNA fragments, normally between 80 and 500 base pairs in length that are obtained by digesting DNA using restriction enzymes, then ligating oligonucleotide adaptors to digested products and finally amplifying these sequences by PCR. It combines the power of RFLP with the flexibility

of PCR-based technology and then selective amplification of restriction fragments giving rise to large numbers of useful markers (Lynch and Walsh, 1998).The PCR primers used are semi-specific, consisting of an adaptor sequence, a restriction enzyme specific sequence, and a 3' end of one to five other nucleotides. The higher the number of other nucleotides in the 3' end, the lower the number of bands obtained in PCR.

AFLP banding profiles are the result of variation in restriction sites and in intervening regions. The AFLP technique generates products from many sites in the genome, perhaps revealing 50 to 100 fragments in an individual reaction. Each AFLP reaction can reveal a large number of polymorphic loci randomly distributed throughout the genome (Powell *et al.*, 1996; and Russell *et al.*, 1997). Nonetheless, AFLPs loci distribution may not be totally randomly distributed around the genome as clustering in certain genomic regions, such as centromers, has been reported for some crops. The technique is considerably reproducible, which makes comparison between different studies and different laboratories possible, no sequence data for primer construction are required. The main disadvantages are the need for purified high molecular weight DNA, technically demanding and relatively expensive, the dominance of alleles, and the possible non-homology of co-migrating fragments belonging to different loci. Products are separated on polyacrylamide gels and visualized using radioactive, fluorescent, silver staining or other methods. Bands are then scored as presence or absence in individuals. When it comes to data analysis, AFLPs are generally assumed to have their origin in nuclear DNA. However, they may more rarely originate from organelle DNA (Muchug *et al.*, 2008 and Kumar *et al.*, 2009).

AFLPs can be applied in studies involving genetic identity, parentage and identification of clones and cultivars, and phylogenetic studies of closely related species. Their high genomic abundance and generally random distribution throughout the genome make AFLPs a widely valued technology for gene mapping studies (Vos *et al.,* 1995)

2.6.3.4. Microsatellites or Simple Sequence Repeat (SSR)

Simple sequence repeats are sections of DNA, consisting of tandemly repeating mono-, di-, tri-, tetra- penta, or hexa-nucleotide (2–6 bp repeats) units that are arranged throughout the genomes of most eukaryotic species (Akkaya *et al*., 1992, Powell *et al.* 1996). SSR polymorphism is based on variation in those short tandem repeats at a site. These repeat regions have been found to be hypervariable, possibly due to DNA polymerase slippage or mispairing at repeats during the normal replication process (Smith and Devey, 1994). Generally, polymorphism is studied in nuclear DNA, although variation in organellar DNA is sometimes also assessed. Length polymorphisms are generally visualized by running products on polyacrylamide gels. Radioactive, fluorescent, silver staining or other techniques are used for detection.

SSR markers are primarily codominant with exception of null alleles at some loci, can detect large variation within population, reproducible across mapping populations, show high levels of allelic variation at individual loci, and use lower quality DNA. Nevertheless as with any other technologies, it isn't without limitations. These include skill and high cost of developing species-specific primer which is unaffordable for most

developing countries as well as variation at a single locus only which is not desirable unlike that of dominant markers that sometimes reveal diversity at many loci simultaneously (Galli *et al.*, 2005; Amos *et al*., 2007). In addition, sometimes formation of null alleles due to change in sequence of primer binding sites, and problem in comparing data produced by different laboratories are also among the major drawbacks (Farooq *et al.*, 2002; Muchug *et al.*, 2008).

2.6.3.5. Cleaved Amplified Polymorphic Sequences (CAPS)

Cleaved amplified polymorphic sequences are combination of the PCR and RFLP (Konieczny & Ausubel, 1993). The technique involves amplification of a target DNA through PCR using specific 20–25 bp primers, followed by digestion of the PCR products with a restriction enzyme. Subsequently, length polymorphisms resulting from variation in the occurrence of restriction sites are identified by gel electrophoresis of the digested products.

Advantages of CAPS include the involvement of PCR requiring only low quantities of template DNA (50–100 ng), the co-dominance of alleles and the high reproducibility. Compared to RFLPs, CAPS analysis does not include the laborious and technically demanding steps of Southern blot hybridization and radioactive detection procedures. However, CAPS show only low levels of polymorphism that is more difficult to find because of the limited size of the amplified fragments (300–1800 bp), only assesses variation at one locus, and needs sequence information for synthesis of the primers.

2.6.3.6. Single Nucleotide Polymorphisms (SNP)

This approach can be considered as one of newest and highly automated genotyping techniques that can detect changes in single nucleotide sequences (Farooq *et al.,* 2002). Single nucleotide polymorphisms (SNPs) and insertions and deletions (indels) are highly abundant and distributed throughout the genome in various species including plants**.** The abundance of these polymorphisms in plant genomes makes the SNP marker system an attractive tool for mapping, marker-assisted breeding and map-based cloning. Hence, in contrast to all previous methods, allele discrimination cannot be based on size differences on a gel (Richard *et al*, 1995; Sunyaev *et al*, 1999).

2.6.3.7. Inter Simple Sequence Repeats (ISSR)

Inter-simple sequence repeat is a general term for a genome region between microsatellite loci. The technique using these microsatellites as primer annealing sites was first reported by Zietkiewicz *et al* (1994). It involves PCR amplification of DNA segments present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly inter simple sequence repeats of different sizes. The microsatellite repeats used as primers for ISSRs can be di-nucleotide, tri-nucleotide, tetra nucleotide or penta-nucleotide. The primers used can be either unanchored or more usually anchored at 3` or 5` end with 1 to 4 degenerate bases extended into the flanking sequences. The amplified products are usually 200–2000 bp long and amenable to detection by both agarose and polyacrylamide gel electrophoresis.

ISSRs exhibit the specificity of microsatellite markers, but need no sequence information for primer synthesis enjoying the advantage of random markers as with RAPDs and AFLPs. The technique is simple, quick, reveal quite high numbers of polymorphic loci that are distributed through the genome, less technically demanding than AFLPs, more reproducible than RAPDs, high stability, and the use of radioactivity is not essential (Wolfe, 2005; Muchug *et al.*, 2008; Kurane *et al.*, 2009).

It is used for applications such as diversity analysis, fingerprinting and genome mapping, parentage, phylogenetic studies, gene tagging, clone and strain identification, and taxonomic studies of closely related species. As no prior sequence knowledge is required, they are more rapidly applied than SSR markers, and they are more reliable and robust than RAPD markers. In most plant species tested, ISSR markers can suitably be applied to most situations, and have been shown to be particularly useful in genetic fingerprinting and diversity analysis (Zietkiewicz *et al*., 1994; Godwin *et al*., 1997; Bornet *et al*., 2004; Jabbarzadeh, 2010). The major limitations of ISSR marker are in most cases dominant in nature, possibility of no homology of similar sized fragments in some cases and some reproducibility problems.

ISSR products are separated by gel electrophoresis and generally scored as the presence or absence of bands. Though they are generally assumed to have their origin in nuclear DNA, they may rarely originate from organelle DNA. Nonetheless, in plants it is unlikely that ISSR markers result from amplification of plastid DNA because the microsatellites found in this genome are predominantly mononucleotide repeats (Powell *et al.*, 1995).

For this study, ISSR markers were chosen because the technique is simple, fast, cost effective, highly discriminative, reproducible, informative, reliable, and require small quantity of sample DNA (Zietkiewicz *et al*., 1994; Bornet and Branchard, 2001; Bornet *et al.*, 2004; Bhagyawant and Srivastava, 2008; Emel, 2010). Besides, they also do not need any prior primer sequence information and are non-radioactive.

2.7. Rationale of the study

Although anchote is a good source of protein, carbohydrate, high calcium level and iron (Habtamu Fufa and Kelbessa Urga, 1997, Habtamu Fekadu, 2011, Desta Fekadu, 2011), there is limited amount of research on this important tuber crop. It is well known that indigenous vegetables have rescued thousands of hungry Ethiopians during famine period. However, very little information is available about anchote agronomy, nutrition, and medicinal value, while nothing is known about genetic diversity using molecular techniques, yield improvement via conventional and modern biotechnology, and well designed management of its germplasm.

The genetic diversity study of the traditional crops and their wild relatives enables the research community to develop improved varieties, put forward conservation strategy and sustainable utilization of genetic resources, and enrich endogenous knowledge to facilitate the utilization of genetic materials. Likewise, information on genetic diversity within and among plant populations has a tremendous significance for Marker Assisted Selection (MAS) and Quantitative Trait Loci (QTL) mapping. Though people often use morphological marker for differentiating and naming anchote and other crops, its use as a

marker is often limited due to small number of phenotypic marker available and the fact that they are influenced by environmental conditions. Research towards investigation of genetic diversity of anchote using molecular marker is not yet conducted for conservation and sustainable utilization of this underutilized native tuber crop. Therefore, this work is first of its kind to characterize anchote genetic diversity for its conservation.

3.0. OBJECTIVE OF THE STUDY

3.1. General objective:

The general objective of this study is to investigate the within and among populations genetic diversity of anchote (*Coccinia abyssinica*) landraces collected from different regions of Ethiopia using ISSR marker and to generate information for possible conservation strategies.

3.2. Specific objectives:

- To analyze genetic diversity within and among populations;
- To analyze genetic identity among individuals of anchote;
- To evaluate genetic distance in relation to geographical location;
- To generate baseline information for future possible conservation strategy and recommend best conservation sites;
- To investigate some unique markers related to morphological traits.

4.0. MATERIALS AND METHODS

4.1. Plant material

Seeds of 12 populations of anchote were obtained from Debre Zeit Agricultural Research Center (DZARC). These seeds were originally collected from Oromia, Amhara, Southern Nation and Nationalities People (SNNP) and Benishangul-Gumuz regional states (Fig. 2) where it is distributed and cultivated.

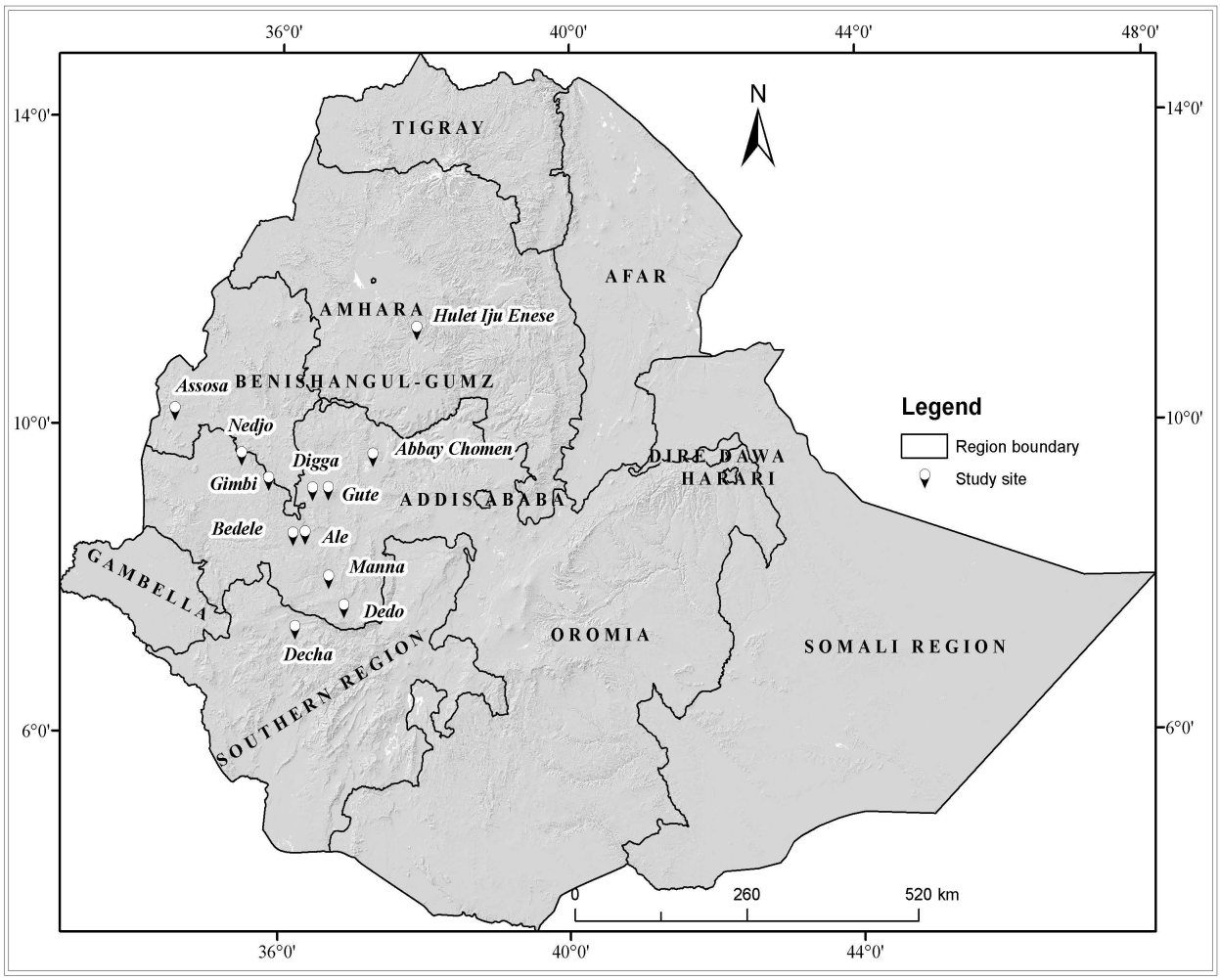

Fig. 2. Map of Ethiopia showing study populations collection sites. *Source: author*

Seeds of each anchote populations were sown in a glasshouse at Addis Ababa University, College of Natural Sciences and DNA were extracted from young leaves. The detailed data on the area from which seeds were originally collected for *ex situ* preservation was indicated in table 3.

Table 3. Lists of populations of *C. abyssinica* used for genetic diversity analysis

Collection (district)	Letterc ode	Region	Zone	Latitude	Longitude	Altitude (m.a.s.l)
Abbay Chomen	Ac	Oromia	Horro Guduru Wollega	9°28’29”	37°18’50”	2175
Assosa	As	Ben.Gum	Assosa	10°03’14”	34°33’18”	1400
Gimbi	Gi	Oromia	West Wollega	9°09’17”	35°51’03”	1581
Nedjo	N	Oromia	West Wollega	9°28’34”	35°28’37”	1834
Dedo	De	Oromia	Jimma	7°29’53”	36°53’29”	1764
Ale	Al	Oromia	Illubabora	8°26’03”	36°11’30”	1585
Bedele	Be	Oromia	Illubabor	8°27’11”	36°21’25”	1587
Manna	M	Oromia	Jimma	7°52’39”	36°41’00”	2031
Hulet Iju Enese	Hi	Amhara	East Gojam	11°08’02”	37°52’54”	2149
Digga	Di	Oromia	East Wollega	9°01’27”	36°27’12”	2131
Decha	Dc	SNNP	Keficho Shekicho	7°12’58”	36°13’31”	1952
Gute	Gu	Oromia	East Wollega	9°1’55”	36°40’17”	1893
Total	**12**					

4.2. Sampling method and sample size

Populations were selected depending on geographical distance and number of populations so far collected by Institute of Biodiversity Conservation of Ethiopia from specific collection sites and made to represent large area of the country. The more the populations from one collection site the higher chance of having more than one population for this work. For this study, 12 populations each with eight individuals were used.

4.3. DNA extraction

DNA was extracted according to modified CTAB method (Hu *et al*., 2009) with minor modification after cleaning the leaves several times with tap water in glass house and then taking plantlets overnight to laboratory. Young leaf tissue was homogenized in liquid nitrogen to fine powder using pre-chilled mortar and pestle. About 0.3 g of finely powdered leaf was weighed and transferred to a 2 ml eppendorf tube. To that 750µl extraction buffer (0.1M tris (Trisma base), 50 mM EDTA and 500 mM NaCl) and 100 µl (10%) were added. After gentle shaking of the eppendorf tubes up and down, it was incubated for 40 - 60 minutes at 65^{o}C in water bath followed by addition of 250 µl potassium Acetate (KAc) and mixed gently; the tubes were put on ice for at least 30 minutes; Centrifuged at 13000 rpm for 17 minutes; equal amount of supernatant (about 510 µl) was transferred to two new 1.5 µl eppendorf tubes; 1 volume of cold (-20 °C) isopropanol was then added and centrifuged at 13000 rpm for 13 minutes and the supernatant was discarded. The pellet was dissolved in 125 µl TE and after the pellet was dissolved entirely (if not using water bath at 65 ^{o}C to facilitate dissolution), 125 µl CTAB

was added and incubated at 65 ^{o}C for 15 minutes in water bath. One volume of chloroform was added and centrifuged at 13000 rpm for 17 minutes; the supernatant was collected in new eppendorf tubes (same samples was pooled in one tube) and chloroform extraction was repeated; it was then precipitated with 1 volume of cold isopropanol and centrifuged at 13000 rpm for 18 minutes at room temperature; the supernatant was discarded; the pellet was diluted in 100 µl of TE; 2.5 µl RNAse (1mg/ml) was added and incubated at 37 ^{o}C for 30 minutes and finally stored at -20 until concentration measurement and quality checking.

4.4. Concentration and quality analysis of DNA

DNA concentration and quality was checked using the nanodrop spectrophotometer at Molecular Biotechnology Laboratory of Holetta Agricultural Research Center (HARC) (Appendix 1). Intactness of DNA was confirmed by 0.8% agarose taking some representative samples of low, medium and high concentration. For PCR, since the extracted DNA was highly concentrated, each DNA sample was then diluted to 20 ng/µl after optimization of 10, 15, 20, 25 ng/µl.

4.5. Screening and optimization of ISSR primers

Twenty three potential ISSR primers purchased from Sigma-Aldrich were screened with different PCR condition and reaction components. Two individuals were selected from two representative populations to screen the primers for polymorphism and reproducibility first at 49 ^{o}C and second at 52 ^{o}C annealing temperature (Ta). Band

intensity and reproducibility of both conditions were compared. Finally, nine primers were selected. Six of selected primers have di-nucleotide repeat and the remaining three have tri, tetra and penta nucleotide repeat. All di-nucleotide repeat have anchored 3'sequence and the remaining has unanchored 3' sequence. The primers screened to amplify 96 individuals of anchote are listed in table 4 while the nine chosen primers after screenng are listed in table 5.

Table 4. Total primers used for initial screening and PCR optimization.

Primers code*	Sequence (5'-3')	Repeat motifs	Amplified products	Reproducibility*	comment
..	(AGC)4 Y	tri-	absent	-	Rejected
CR-2	$(CA)_8$ AG	di-	absent		Rejected
809	$(AG)_8$ G	di-	Present(good)	not reproducible	rejected
810	$(GA)_8$ T	di-	absent	-	Rejected
812	$(GA)_8$ A	di-	absent	-	Rejected
818	$(CA)_8$ G	di-	Present (poor)	IP	Rejected
822	$(TC)_8$A	di-	absent	-	Rejected
824	$(TC)_8$ G	di-	Present (good)	reproducible	Accepted
825	$(AC)_8$ T	di-	Present (good)	reproducible	Accepted
827	$(AC)_8$ G	di-	Present (good)	reproducible	Accepted
834	$(AG)_8$ YT	di-	Present (good)	reproducible	Accepted
835	$(AG)_8$YC	di-	absent	-	Rejected
836	$(AG)_8$ YA	di-	Present(good)	not reproducible	rejected
841	GAAG $(GA)_6$ YC	di-	Present (good)	reproducible	Accepted
844	$(CT)_8$ RC	di-	Present (poor)	IP	Rejected
857	$(AC)_8$ YG	di-	Present (poor)	IP	Rejected

860	$(TG)_8$ RA	di-	Present (good)	reproducible	Accepted
866	$(CTC)_6$	tri-	Present (good)	reproducible	Accepted
872	$(GATA)_4$	tetra-	Present (poor)	IP	Rejected
873	$(GACA)_4$	tetra-	Present (good)	reproducible	Accepted
878	$(GGAT)_4$	tetra-	absent	-	Rejected
880	$(GGAGA)_3$	penta-	Present (good)	reproducible	Accepted
881	$(GGGTG)_3$	penta-	Present (poor)	not reproducible	Rejected
	Total = 23			**Nine selected**	

IP = reproducibility test was not performed because of initial poor amplification and monomorphism
.. = primer without code
- = reproducibility test was not performed because of initial no amplification at all

Table 5. Primers optimized for total amplification of all DNA samples

Primers code	Sequence (5'-3')	Repeat motifs	Tm	GC %	Ta
824	$(TC)_8$ G	di-	49.1	52.9	48.0
825	$(AC)_8$ T	di-	49.3	47.0	49.0
827	$(AC)_8$ G	di-		52.9	48.0
834	$(AG)_8$ YT	di-	49.8	44.4	50.0
841	GAAG $(GA)_6$ YC	di-	49.0	50.0	51.0
860	$(TG)_8$ RA	di-	49.3	44.4	49.0
866	$(CTC)_6$	tri-	60.5	66.6	53.0
873	$(GACA)_4$	tetra-	45.1	50.0	50.0
880	$(GGAGA)_3$	penta-	49.1	60.0	50.0

Tm = melting temperature, Ta = annealing temperature, Y = (A, T) R = (G, A

4.6. PCR amplification and electrophoresis

PCR reaction containing 23 µl of master mix and 2µl of 20 ng/µl working DNA were optimized with different concentrations of $MgCl_2$, primers and dNTPS. The optimized

master mix contained 1x PCR buffer A of Himedia, India and 1x PCR buffer B (0.8 M Tris-HCl, 0.2 M $(NH4)_2SO4$, 0.2% w/v Tween-20) of Solis Biodyne, Estonia; 2.25mM of $MgCl_2$; 200 µM of each of dNTPs (Himedia, India); and 0.4 µm of primers (Sigma-Aldrich, German) and 1U taq DNA polymerase (Solis biodyne or Himedia). Out of the 23 primers 9 primers were selected having good band intensity and reproducible results with PCR condition operated at 94^oc initial denaturation for 4 minutes followed by 40 cycles of 94^oC denaturation for 25 seconds, 48-53 ^oC for 1 minute annealing (depending on primer GC content and Tm), 72 ^oC for one and half minute extension and final extension at 72^oC for 6 minutes for Himedia taq DNA polymerase. Initial denaturation, was little increased to 95^oC for Solis biodyne firepol DNA polymerase since the enzyme is provided as an inactive form. PCR products were stored at 4 ^0C until loading on gel for electrophoresis.

For electrophoresis of ISSR products, 1.7 % agarose was dissolved in 1x TAE buffer and melted by microwave for about 3 minutes by gentle shacking with interval of one minutes. Then 5 µl of 10 mg/ml ethidium bromide was added to 200 ml melted agarose after it cooled to about 55^oC. The agarose was casted and 10 µl of PCR products were mixed with 2 µl of 6X loading dye on parafilm, loaded to respective wells and electrophoresed at constant voltage of 75V for three and half to four hours depending on molecular size of amplified product during optimization and stained in staining solution containing 50 µl of ethidium bromide in 500 ml double distilled water for 45 minutes. It was then destained in equal amount of double distilled water for 20 minutes. Molecular weights were estimated using a 100 bp DNA ladder.

4.7. Band scoring and data analysis

Bands were counted manually after capturing of gel image with gel documentation system and scored as binary data; '1' for band present, '0' for band absent and '?' for missing data. Only unambiguously amplified ISSR bands were scored while faint bands were excluded.

Various software's were used for analysis of binary data matrix. Percentage of polymorphic bands (PPB), Nei's (1973) gene diversity (*h*), Nei's (1978) unbiased genetic distances (*D*) separating populations which is an accurate estimate of the number of gene differences per band when populations are small , Shannon–Weaver diversity index (*I*), Gene differentiation between populations that is estimated from the coefficient of gene differentiation (*Gst*) and gene flow (*Nm*) which is evaluated from *Gst* according to McDermott and McDonald (1993), where $Nm = 0.5(1 - Gst)/Gst$ were estimated using POPGENE Version 1.32 (Yeh *et al*., 1999) under the assumption of Hardy-Weinberg equilibrium. The two comparable estimators: Nei's gene diversity (*h*) and Shannon's information indices (*I*) were used to calculate genetic diversity for each population.

Jaccard's similarity coefficient (J) was calculated from NTSYS- pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek *et al.,* 1999) and in turn used to calculate similarity between pairs of populations with the formula:

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

Where, **'a'** is the total number of bands shared between individual i and j,

'b' is the total number of bands presents in individual i but not in individual j

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

An analysis of molecular variation (AMOVA) was used to estimate genetic variance within and among each population using Areliquin version 3.01 (Excoffier *et al.,* 2006).

To examine the genetic relationship among individuals, pairwise similarity indices were estimated using the Jaccard coefficient of similarity (Jaccard, 1908) from NTSYS- pc version 2.02 (Rohlf, 2000) that is used for Unweighed Paired Group Method with Arithmetic mean (UPGMA) tree construction. Likewise, Free Tree 0.9.1.50 (Pavlicek *et al.,* 1999) was used to construct Neighbor Joining (NJ) trees (Saitou and Nei, 1987). Unlike that of UPGMA that assumes equal rates of evolution (molecular clock assumption), NJ assumes variations in the rate of change and often preferred than UPGMA. In addition to NJ and UPGMA tree, two dimensional (2D) and three dimensional (3D) Principal Coordinate Analysis (PCoA) were used to reveal patterns of variation among individual samples based on Jaccard's similarity coefficient using PAST software version 1.18 (Hammer *et al.,* 2001). The first three axes were used to plot the three dimensional PCoA with STATISTICA version 6.0 software (Hammer *et al.,* 2001; Statistica soft, Inc., 2001).

5.0. RESULTS

5.1. ISSR band Variation and level of polymorphism

A total of 87 clear and scorable bands were amplified by nine primers with an average of 9.67 bands per primer having molecular weight ranging from 200-1900 bp. The number of polymorphic bands ranged from 5 – 15 attributing to 74 total polymorphic bands and 8.2 average polymorphic bands per primer. Percentage polymorphic bands (PPB) were from 62.5 (primer 825) to 100 % (Primers 824, 860, and 880). Primer 825 resulted in least polymorphic bands (5) with 62.5 % in overall individual comparison while primer 824, 860, and 880 generated 100% with 6, 8, and 15 polymorphic bands, respectively (Table 6). All primers resulted in 85.06% polymorphism at species level.

Contrary to PPB measurement (100% for three different primers in this study), the highest Nei's gene diversity (0.41) and Shannon information index (0.59) were exhibited by primer 824 followed by 860 (0.36 and 0.53, Nei and Shannon information index respectively). Similar to PPB, Primer 825 showed least Nei (0.20) and Shannon information index of 0.21. The mean Nei's and Shannon information index for all primers were 0.285 and 0.426 respectively (Table 6).

Table 6. ISSR primers with their scored band polymorphism, and level of gene diversity

primer	Sequence 5'-3'	Scored band			Diversity		
		Band size (bp)	Total bands	NPB	PPB (%)	h+SD	I+SD
824	$(TC)_8$ G	350-900	6	6	100.00	0.412+0.148	0.592+0.178
825	$(AC)_8$ T	350-1400	8	5	62.50	0.205+ 0.217	0.309+0.303
827	$(AC)_8$ G	300-1380	9	8	88.89	0.356+0.166	0.520+0.225
834	$(AG)_8$ YT	200-1250	12	9	75.00	0.239+0.205	0.361+0.283
841	GAAG $(GA)_6$ YC	230-950	11	9	81.82	0.281+0.198	0.420+0.268
860	$(TG)_8$ RA	300-1000	8	8	100.00	0.360+ 0.153	0.532+0.189
866	$(CTC)_6$	500-1400	6	5	83.33	0.349+0.201	0.505+0.274
873	$(GACA)_4$	445-1200	12	9	75.00	0.220+ 0.166	0.346+0.242
880	$(GGAGA)_3$	210-1900	15	15	100.00	0.261+0.186	0.399+0.251
All primers		**200-1900**	**87**	**74**	**85.06**	**0.285+0.187**	**0.426+0.255**

h = Nei's (1973) gene diversity, I = Shannon's Information index

NPB = Number of Polymorphic Band,

PPB = Percentage of Polymorphic Band

Both highest polymorphic band and rare unique bands were obtained from primer 880 (penta- nucleotide repeat). A largest (1900 bp) unique single band was amplified from one individual of Gimbi population (Gi-7). Similarly, another band (1500 bp) with unique pattern was observed in 12 individuals. Amongst them, Decha population collected from Keficho Shekicho around Bonga has got this unique band in five individuals and Hulet

Iju Enase in two individual. However, only single individual found to contain this band from populations of Nedjo (N), Digga (Di), Ale (Al), Gimbi (Gi) and Abbay Chomen (Ac). Banding profiles of some populations using primer 824 is shown on Fig. 3, and using primers 880 and 873 are shown on Fig. 4 A and B.

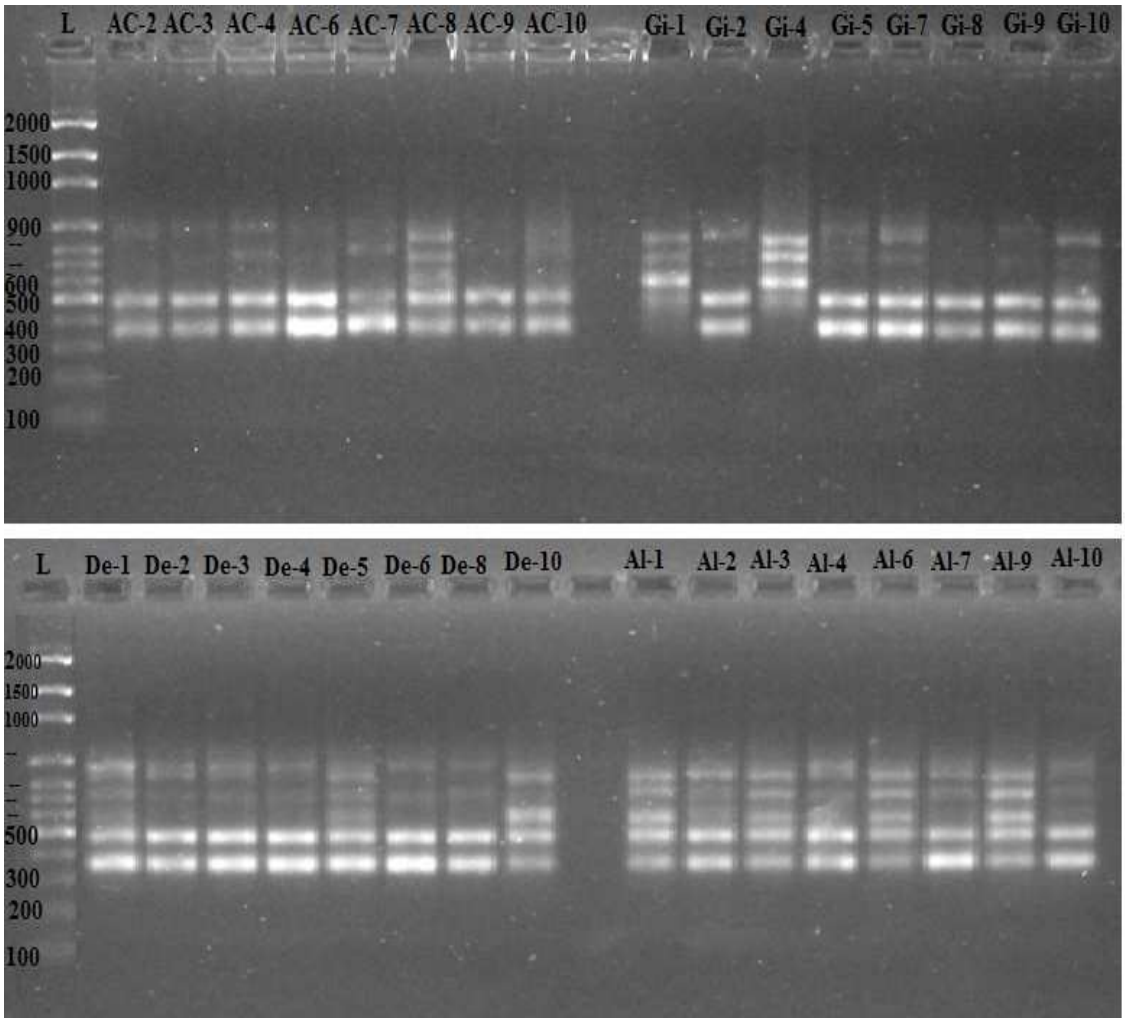

Fig. 3. ISSR profiles of anchote individuals generated by primer 824 using agarose gels. L = Ladder (100 bp); C = Control; Ac, Gi, De, Al = refers to populations of Abbay Chomen, Gimbi, Dedo and Ale respectively and the number system refers to individual code in each population.

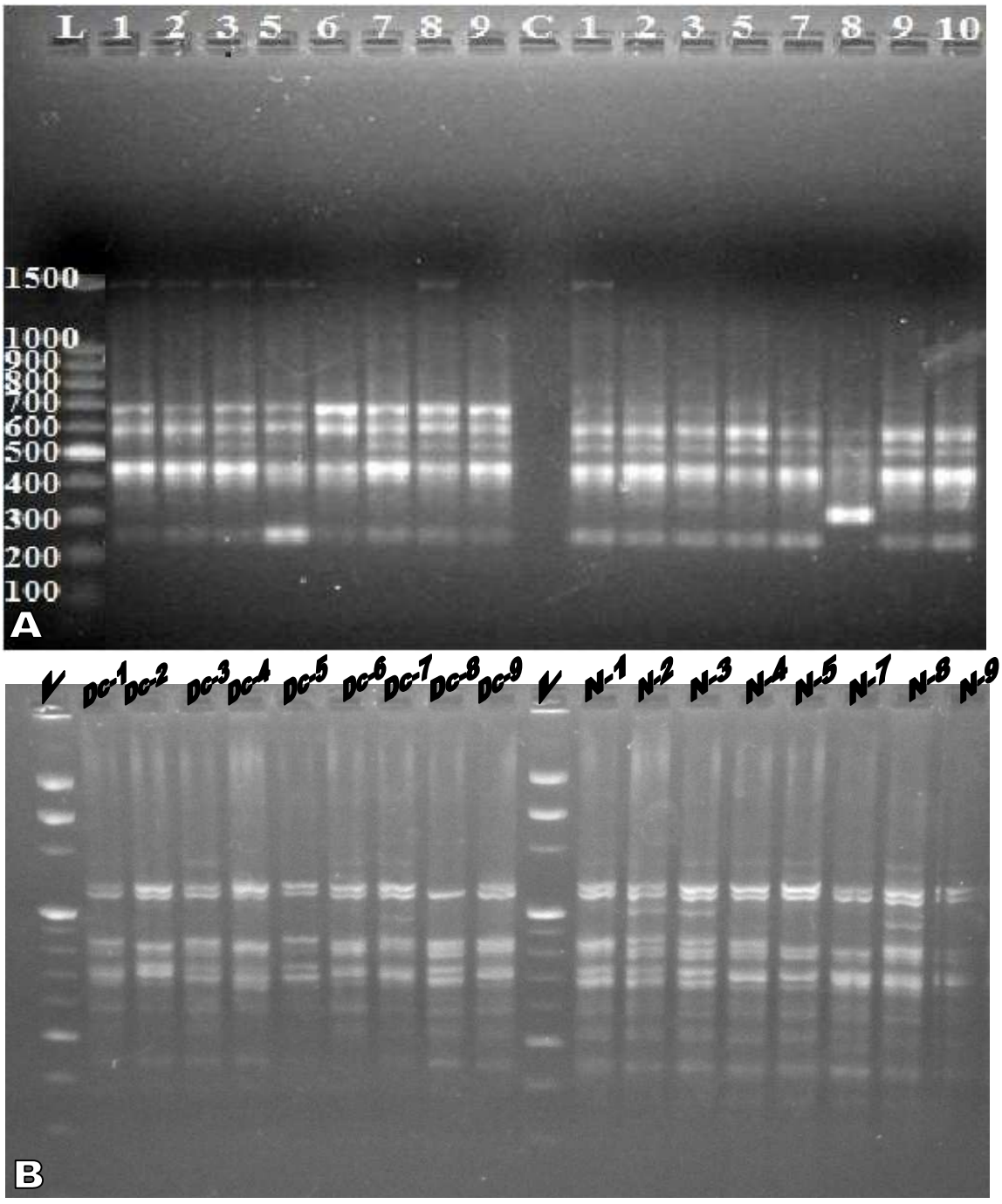

Fig: 4. ISSR profiles generated by primer 880 (A) and primer 873 (B). Primers are penta and tetra- nucleotide repeat respectively. L = Ladder (100 bp); NL = Not Loaded; C = contol; Dc, and N refers populations of Decha and Nedjo respectively; the number system refers to individual code in each populations. Both fig. was for Decha and Nedjo.

5.2. Within population genetic diversity

Within populations, percentage polymorphic band ranged from 13.79 % for Manna to 43.68% for Gimbi followed very closely by Bedele (42.53%) with a mean of 33.05 %, indicating Gimbi as having highest genetic diversity, whilst Manna with lowest genetic diversity. Gene diversity ranged from 0.047 for Manna to 0.156 for Bedele with mean of 0.118, and Shannon information index ranged from 0.07 for Manna to 0.23 for Bedele with mean of 0.175. Though Gimbi has highest percentage of polymorphic band, it has little lower gene diversity and Shannon index as compared to Bedele. Similarly, Gimbi has bit higher percentage of polymorphic band compared to Ale (43.68 and 39.08) respectively, but their gene diversity was not significantly different (0.146 and 0.143 respectively). Populations of Digga, Decha, Gute, Nedjo, and Assosa have almost equal gene diversity (0.11). The least percentage of polymorphic band, gene diversity and diversity index were found within population of Manna (13.79, 0.047 and 0.07, respectively) that formed their own separate groups with principal coordinate and with both dendrogram based on UPGMA and NJ suggesting that this population was the most homogenous.

Populations collected from Illubabor (Ale and Bedele) have got highest gene diversity (0.146 and 0.156), PPB (39.1 and 42.53), and Shannon index (0.21 and 0.23) compared to other collection sites. Though both Gimbi and Nedjo were collected from very closer sites (both from West Wollega), Gimbi has relatively higher gene diversity (Table 7).

Table 7. Analysis of gene diversity, population differentiation and gene flow estimate in each and overall populations

Pop.	Code	NPB	PPB	h+SD	I+SD	Gst*	Nm*
Abbay Chomen	Ac	32	36.78	0.107 + 0.164	0.167+ 0.242	-	-
Assosa	As	30	34.48	0.117 + 0.184	0.175+ 0.265	-	-
Gimbi	Gi	38	43.68	0.143 + 0.187	0.217+ 0.271	-	-
Nedjo	N	26	29.89	0.117 + 0.194	0.171+ 0.276	-	-
Dedo	De	33	37.93	0.133 + 0.190	0.198+ 0.274	-	-
Ale	Al	34	39.08	0.146 + 0.205	0.214+ 0.290	-	-
Bedele	Be	37	42.53	0.156 + 0.202	0.231+ 0.289	-	-
Manna	M	12	13.79	0.046 + 0.131	0.069+ 0.189	-	-
Hulet Iju Enese	Hi	25	28.74	0.106 + 0.182	0.157+ 0.262	-	-
Digga	Di	28	32.18	0.113 + 0.186	0.167+ 0.265	-	-
Decha	Dc	23	26.44	0.112 + 0.194	0.161+ 0.275	-	-
Gute	Gu	27	31.03	0.117 + 0.191	0.172+ 0.273	-	-
Mean		**28.75**	**33.05**	**0.118+0.184**	**0.175+0.264**	**0.546**	**0.415**

** Nm = estimate of gene flow from Gst. Nm = 0.5(1 - Gst)/Gst, h = Nei's (1973) gene diversity, I = Shannon's Information index, NPB = Number of Polymorphic Band, PPB = Percentage of Polymorphic Band.*

5.3. Analysis of Molecular Variance (AMOVA)

Analysis of molecular variance was performed without grouping the regions of anchote cultivation and indicated that bit higher percentage of total variation was accounted from

within population variation (51.44 %). Among population variations attributed about 48.56 % of total variation (Table 8). Fixation index (F_{ST}) indicated about 48.6 % genetic differentiation among populations with significant components of molecular variations (*P* =0.05).

Table 8. Analysis of Molecular Variance among the 12 populations without grouping

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation Index (F_{ST})	P-value
Among populations	11	493.760	4.95485 Va	48.56	0.486	0.05
Within populations	84	440.875	5.24851 Vb	51.44		
Total	95	934.635	10.20336			

5.4. Among population divergence

The overall mean percentage of polymorphism, gene diversity, diversity index, gene flow (Nm) and genetic differentiation from AMOVA (F_{ST}) for all study populations with nine ISSR primers of current study are 85%, 0.285, 0.426, 0.415, and 0.486 respectively (Table 6, 7 and 8). Nei's (1978) unbiased measures of genetic distance (D) pair wise comparison of all populations showed that Manna and Nedjo are very distantly related populations having genetic distance of 0.38 compared to the overall study populations. However, populations of Dedo (Jimma) and Ale (Illubabor) with genetic distance of 0.067 and Nedjo and Decha with genetic distance of 0.079 showed low genetic distance (Table 9).

Table 9. Nei's (1978) Unbiased Measures of Genetic distance between populations

Pop	Ac	Gi	De	Al	As	Be	M	Di	Gu	Hi	Dc	N
Ac	***											
Gi	0.113	***										
De	0.102	0.138	***									
Al	0.090	0.087	**0.067**	***								
As	0.095	0.156	0.160	0.126	***							
Be	0.111	0.117	0.135	0.100	0.080	***						
M	0.175	0.215	0.247	0.206	0.147	0.140	***					
Di	0.270	0.249	0.292	0.203	0.238	0.209	0.172	***				
Gu	0.235	0.222	0.234	0.181	0.286	0.224	0.182	0.185	***			
Hi	0.200	0.157	0.175	0.137	0.226	0.178	0.264	0.226	0.149	***		
Dc	0.273	0.245	0.216	0.164	0.234	0.234	0.361	0.246	0.250	0.113	***	
N	0.317	0.264	0.253	0.209	0.284	0.279	**0.382**	0.273	0.242	0.106	**0.079**	***

Jaccard similarity coefficient of pairwise comparison (Table 10) of the 12 populations showed that Decha and Nedjo as well as Ale and Dedo are the most closest with similarity coefficient of 0.76 and 0.74 respectively. However, Manna and Nedjo are the most distantly related populations with 0.52 similarity coefficient. Jaccard coefficient of pairwise similarity was found to be consistent with Nei unbiased measures of genetic distance in revealing similarity or diversity even if the values are different.

Table 10. Jaccard pairwise similarity between populations above the diagonal

Pop	Ac	Gi	De	Al	As	Be	M	Di	Gu	Hi	Dc	N
Ac	***	0.688	0.696	0.700	0.676	0.679	0.642	0.583	0.619	0.645	0.576	0.567
Gi		***	0.701	0.727	0.630	0.640	0.609	0.588	0.629	0.701	0.608	0.591
De			***	**0.742**	0.641	0.672	0.600	0.577	0.629	0.699	0.608	0.606
Al				***	0.646	0.670	0.641	0.611	0.652	0.715	0.644	0.623
As					***	0.65 5	0.636	0.561	0.562	0.615	0.584	0.556
Be						***	0.673	0.636	0.610	0.652	0.588	0.574
M							***	0.670	0.686	0.614	0.546	**0.516**
Di								***	0.673	0.644	0.638	0.614
Gu									***	0.698	0.614	0.597
Hi										***	0.724	0.723
Dc											***	**0.762**
N												***

The mean G_{ST} obtained from Nei's genetic diversity analysis (0.54), and the fixation index (F_{ST}) from AMOVA (0.486) indicated that 54% and 48.6% of the total genetic variability was distributed in overall populations respectively. Both estimators resulted in closer value showing high degree of population differentiation. The overall number of migrants (*Nm*) estimated from nine ISSR primers were 0.41, showing an evidence of gene flow in this crop.

5.5. Principal Coordinate Analysis (PCoA)

Pair wise comparisons of all 96 individuals using Jaccard's similarity coefficients exhibited a range of 0.42 (for the most distantly related N-1 from Nedjo and M-6 from

Manna having bootstrap support of 100) to 1.0 for identical individuals of Manna (M-5 and M-9) followed by M-4 and M-5/6 having similarity coefficient of 0.98 with bootstrapping support of 62 and 69 respectively (Appendix 4).

PCoA grouping was performed utilizing the first three coordinates of the PCoA having Eigen values of 6.62, 4.96 and 3.04, accounted for 12.11 %, 9.07 % and 5.56 % variation respectively (Figs. 5 and 6) using both two dimensional and three dimensional PCo.

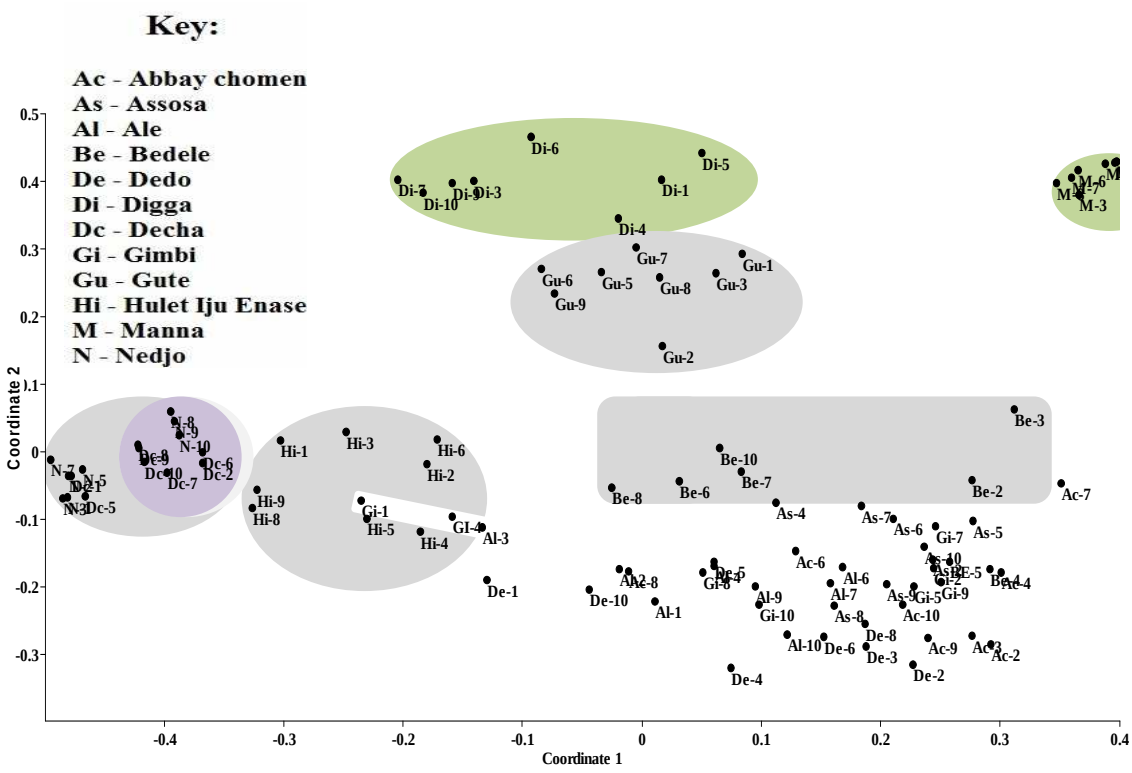

Fig. 5. Two dimensional representation and relationships of 96 individuals of 12 *C.abyssinica* populations on principal coordinate analysis

On first principal coordinate, populations having relatively major variation both within and among them aggregated together. The Decha and the Nedjo populations that showed close similarity between them upon clustering with both NJ and UPGMA, formed closer

pattern on first coordinate towards negative side. The Hi (Hulet Iju Enase of Gojjam collection) was also aggregated dispersly towards negative Eigen values of first coordinate following Nedjo and Decha. Populations of Assosa (As), Abbay Chomen (Ac), Bedele (Be), Ale (Al), Gimbi (Gi) and Dedo (De) were dispersly clustered on first coordinate towards positive Eigen values. Meanwhile, some individuals of these populations found intermixed with other populations.

On the second principal coordinate, populations having lesser variation formed some sort of clustering. Population of Manna that showed the least within diversity (the most similar) formed very closest pattern on positive Eigen values of second coordinate. Similarly, the Gute and Digga populations (both from East Wollega) dispersily found on this coordinate by indicating relatively higher variation within themselves compared to the Manna population.

The three dimensional PCoA also revealed similar result with 2D analysis that individuals of Manna population formed very close and clear grouping in space. The Decha and the Nedjo, populations showed close similarity on 2D analysis, here formed their own group on 3D PCoA. Likewise, individuals of Digga and Gute formed their own grouping relative to others. Similar to the observation from 2D PCo, some individuals of of Assosa (As), Abbay Chomen (Ac), Bedele (Be), Ale (Al), Gimbi (Gi) and Dedo(De) populations were intermixed with other populations.

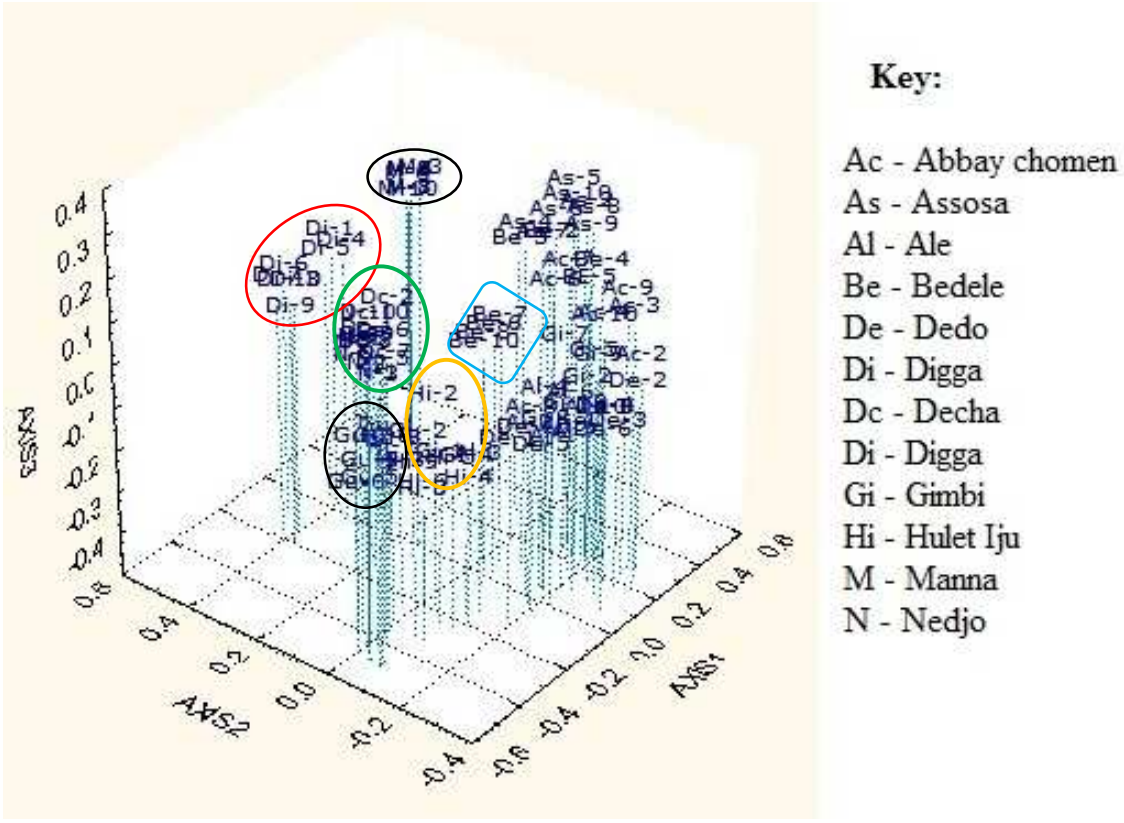

Fig. 6. Three dimensional representation and relationships of 96 individuals of 12 *C. abyssinica* populations on principal coordinate analysis.

5.6. Cluster analysis

UPGMA (Sneath and Sokal, 1973) based on 96 individuals of anchote (Fig. 7) formed three main clusters (I, II and III) at cutoff value of 0.64. In the first cluster (cluster I), all individuals from Digga (Di), Gute (Gu), and Manna formed their own separate groups. The second cluster (cluster II) comprised individuals from Nedjo (N), Decha (Dc) and Hulet Iju (Hi). Whereas one individual from Dedo (De-1) seems to be an outlier of the three and two individuals of Gimbi (Gi) and one of Ale (Al) generated another internal separate cluster. Although majority of the individuals from the remaining populations

(Ac, As, Be, De, and Gi) formed pattern of clustering (cluster III), some individuals of these populations found to be escaped from their own group.

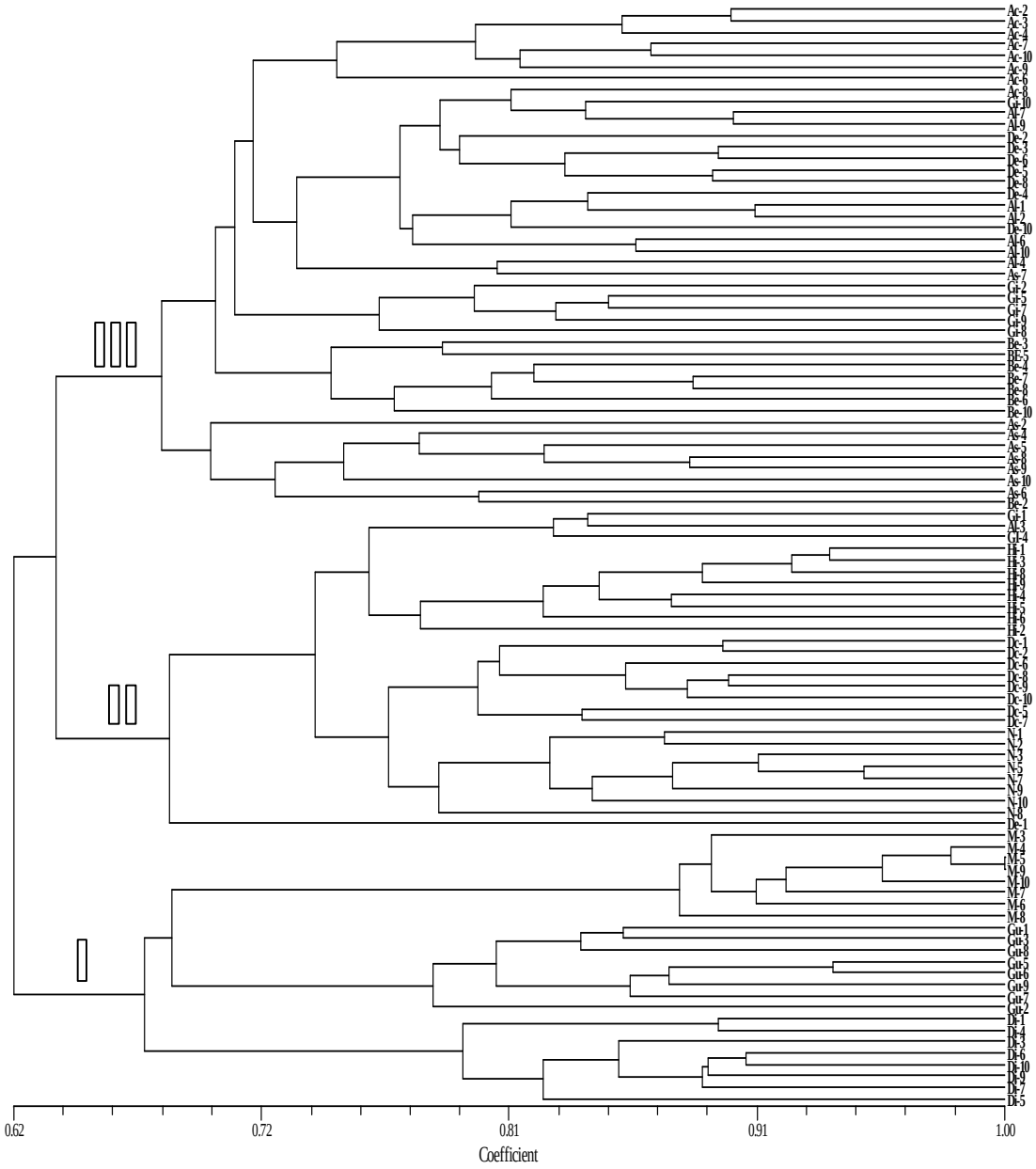

Fig. 7. Dendrogram obtained with UPGMA based on Jaccard's similarity coefficients for 96 individuals of *Coccinia abyssinica.*

Clustering based on NJ also resulted in two main clusters (A and B) without considering one outlier of Gimbi (Gi-10) and with no any cutoff value (fig. 8). The second cluster (B) branched into two sub-clusters (B-1 and B-2), while the first cluster is only individuals of Gimbi (Gi). In the second cluster sub cluster one (B-1), three individuals of Ale (Al) formed separate very small cluster. Under sub cluster B-2, majority of individuals of the populations formed clear separate within cluster groups (Di, M, Gu,Hi, Dc, N, Be, AC,) while some individuals were escaped from the remaining populations. Interestingly, observations from both UPGMA and NJ were nearly similar both indicating intermixing of some individuals of certain populations.

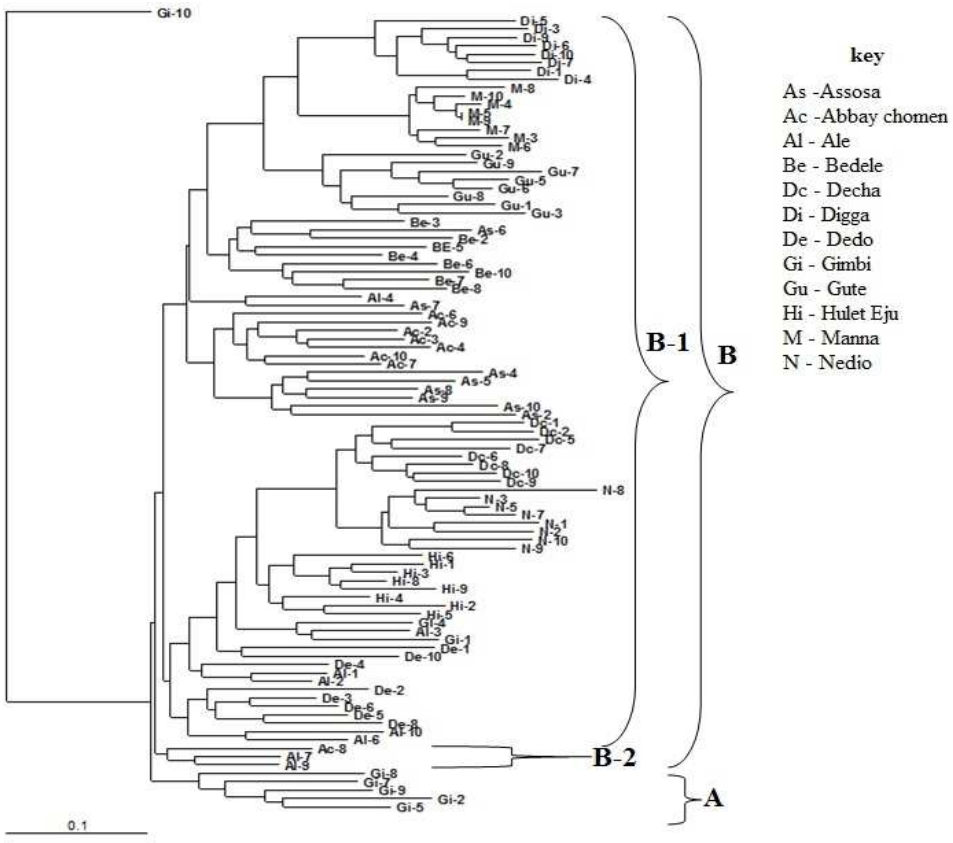

Fig. 8. NJ based analysis of 96 individuals of *C. abyssinica* using nine ISSR primers after pairwise comparisons using Jaccard's similarity coefficient.

UPGMA based dendrogram for 12 populations generated three distinct major cluster at cutoff value of 0. 64 (fig. 9). The first bottom cluster contains populations of Decha (Dc), Nedjo (N), Dedo (De), Ale (Al), Hulet Iju (Hi) and Gimbi (Gi). N and Gi are both from west wollega, Hi from East Gojjam, Ale from Illubabor, Dedo from Jimma and Decha from Keficho Shekicho. Among these, De and Dc as well as Gimbi and Nedjo are geographically found in close vicinity. The top cluster contained populations of Abbay Chomen (Ac) and Assosa (As). In the middle of dendrogram, populations of Bedele, Manna, Digga and Gute were clustered together; Digga and Gute were both from East Wollega, while Manna was from Jimma and Bedele from Ilubabor so that they are geographically close to each other.

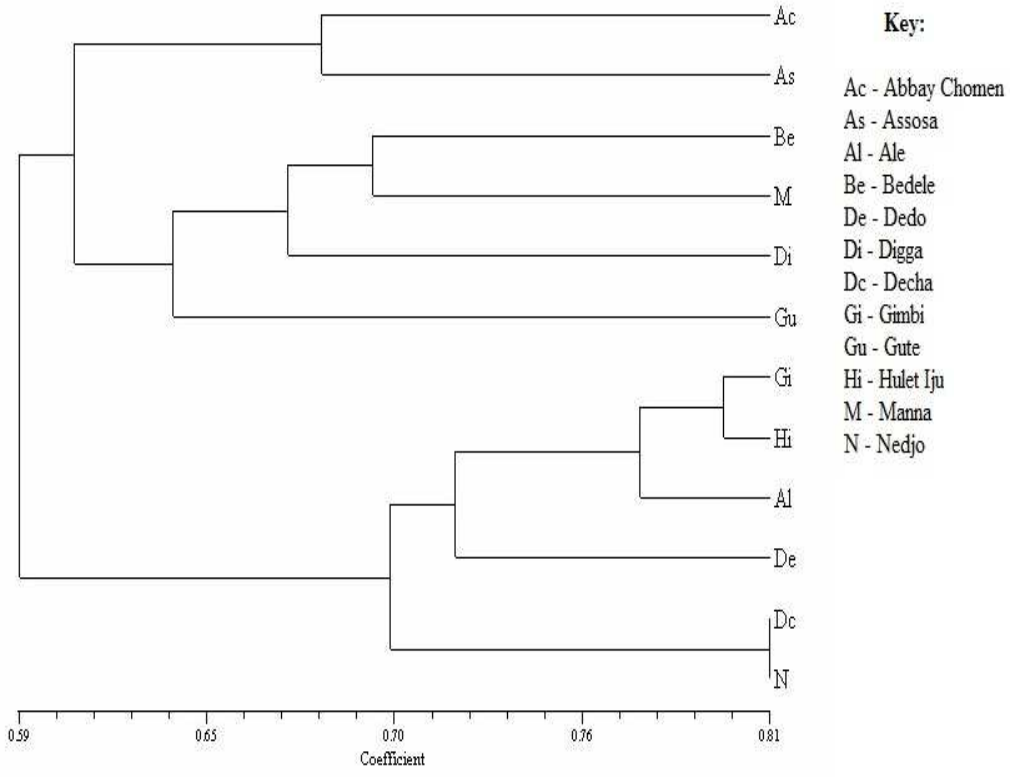

Fig: 9. UPGMA based dendrogram for 12 populations of *C. abyssinica.*

6.0. DISCUSSION

6.1. ISSR polymorphism and implication for diversity study in anchote

Nybom (2004) concluded that estimates of genetic variation derived by the dominantly inherited markers RAPD, AFLP and ISSR are similar and may even comparable to codominant marker types. However, ISSR tend to produce somewhat higher estimates of within-population variation which is also in agreement with our investigation. In our present study, we utilized ISSR marker system and investigated genetic variability and relationships within and among 12 population of *C. abyssinica* making this report the first attempt to study genetic variability within and among anchote populations using molecular marker.

In this study, nine microsatellite primers (six di-, one tri-, one tetra- and one penta-nucleotide repeats) were used for investigating and estimating genetic diversity of anchote. Penta nucleotide primer 880 (GGAGA)3 generated high number of polymorphic bands (15) as well as two bands with unique patterns. Single band approximately at 1900 bp was found from Gimbi populations and another band (1500bp) was found in 12 individuals out of 96. Out of individuals with second unique pattern, about 42 % (5 individuals) were the Dc (Decha population collected from Keficho Shekicho around Bonga) whilst, 16 % were from Hulet iju enase (East Gojjam). Nedjo (N), Digga (Di), Ale (Al), Gimbi (Gi) and Abbay Chomen (Ac) have got each 8%. Desta Fekadu (2011) in his study of phenotypic and nutritional characterization of 36 anchote populations of Ethiopia found out Decha population with large diameter of fruit (51cm), lowest seed

number per locule (11), highest protein in its leaf (41%) and fruit (36.4%). Very recently, Daba Mengesha *et al*. (2012) found out Decha populations with lowest vine length and leaf number. Likewise, we also confirmed that individuals of the Decha population and some others having this band seem shorter with thick leaf in first 30 days after germination. Therefore, it may be evident that this gene may be involved in above mentioned qualitative and quantitative traits that might be agronomically important. Similarly, Bhagyawant and Srivastava (2008) found unique patterns in their study of genotyping of chickpea using the same primer (GGAGA)3 and correlated it with temperature tolerant variety. Hence, a unique band from our current investigation could probably be attributed to these agronomic traits in anchote crop. From this information, one can say ISSR could also be used to reveal agronomically important genes that might be candidate gene for crop improvement via modern biotechnology.

The mean Nei's, Shannon information index and PPB for all primers were 0.285, 0.426 and 85.06% respectively, indicating that there is an immense genetic diversity at species level. Similarly, polymorphism of 90·62 %, 83%, 85.1%, and 92.4% were found in other tubers such as *Tacca chantrieri*, yam, and sweet potato (Zhang *et al*. 2006; Zhou *et al*. 2008; and Moulin *et al*. 2012). Genetic diversity of the *C. abyssinica* was higher than species with endemic distribution (*PPB* = 40.5%, *H* = 0.10) (Hamrick and Godt, 1996). Various studies showed low level of genetic diversity in endangered and endemic species (Li and Xia, 2005; Xiao et al., 2006), many others resulted opposite findings (Da-Wei , et al., 2004; Ni et al., 2006; Luan et al., 2006; Li and Jin, 2007; Wang et al., 2012).

6.2. Within and among population diversity

Genetic diversity estimators: percent polymorphic band, gene diversity (h), and Shannon's diversity (I) were highest for Gimbi (43.68%, 0.143, 0.22), Bedele (42.5%, 0.15, 0.23), and Ale (39.08%, 0.146, and 0.214 respectively) suggesting that probably these places are centers of genetic diversity of anchote. In fact, Ale and Bedele were collections of Illubabor while Gimbi collection was from west Wollega; the three populations being collected from an area closer to one another and found in little disturbed forest areas of the country. Thus, probably this high diversity may have been arised from both random migration of people towards these areas from several directions and sharing of seeds from various cultivation sites supposed to have dissimilar genetic makeup. Populations of Digga, Decha, Gute, Nedjo, and Assosa have almost equal gene diversity (0.11). The least percentage of polymorphic band, gene diversity and diversity index found in intra-population of Manna indicates individuals of Manna had high similarity within themselves. This lack of genetic diversity might be explained by the existence of low external gene flow to the area, vegetative way of propagation in the area, inherent narrow internal diversity existed and homogeneity in nature or collection from very specific and uniform site.

The overall mean percentage of polymorphism, gene diversity, diversity index and gene flow and genetic differentiation from AMOVA (F_{ST}) for all study populations with nine ISSR primers of current study were 85%, 0.285, 0.426, 0.415, and 0.486, respectively. The Nei genetic diversity (0.28) is relatively closer to what was recently found in other tuber crops by Wangsomnuk *et al*. (2011) in their effort to study genetic structure and

relatedness of Jerusalem artichoke (0.32) germplasm. In light of this, our current study revealed Manna and Nedjo as well as Manna and Decha as very distantly related populations with genetic distance of 0.38 and 0.36 respectively. Manna and Nedjo populations are geographically very far apart, while Manna and Decha are closer to one another. From this respect, agro-ecological zone or geographical distance were not correlated with similarity of genetic diversity.

The result from Jaccard's similarity showed that Decha and Nedjo were the closest with similarity coefficient of 0.81 from UPGMA. However, Manna and Nedjo were observed to be the most distantly related populations with 0.52 (from Jaccard's pairwise similarity). Accordingly, in this study, genetic distance was not entirely correlated with geographic distance. This result is congruent with the studies of Moulin *et al*. (2012) with ISSR and Veasey *et al*. (2008) with SSR who did not find correlations between geographic distances and genetic differences among sweet potato populations. Similarly, Almaz Negash *et al*. (2002), using ALFP and Genet Birmeta *et al.* (2004), using RAPD both on enset didn't find any correlation between genetic diversity and agro-ecological zone in their studies. As Desta Fekadu (2011) also indicated in his morphological study, majority of populations of anchote were clustered irrespective of agro-ecological zone but with their quantitative traits. Hence, this could be due to the widespread practice of exchanging seeds among the neighboring farmers at the common open market in the region and between relatives and migrants from one location to another.

6.3. Genetic differentiation and population structure

AMOVA indicated that relatively more than half of the total variation (51.44 %) was accounted from within population variation, while among population variations attributed to 48.56 % of the total variation. Genet Birmeta (2004) obtained far higher variation (88 %) within populations of enset. Unlike that of Desta Fekadu (2011) result assuming anchote as self-pollinator crops; however, our result from gene flow, population differentiation, within and among population variation and tree topology showed that it seems both self and cross pollinator. However, this aspect still needs better informative codominant markers systems. Genetic differentiation among populations using fixation index (F_{ST} = 0.486) also further confirmed anchote as both inbreeder and outbreeder.

According to compiled data of Nybom (2004) with RAPD, indicating comparable estimate with ISSR, genetic diversity is strongly associated with life form, geographic range, breeding system, seed dispersal mechanism, and successional status. The estimate of population differentiation of *C. abyssinica* using F_{ST} and G_{ST} were closer (0.48, and 0.54). However the value of F_{ST} is recommended in various literatures (Laurentin, 2009) especially for dominant markers. In Nybom (2004) the value of 0.48 is most similar with short-lived perennial (0.41), regional geographical range (0.42), mixed breeding system (0.40), and mid successional taxa (39). Therefore, it may be evident that, *C. abyssinica* is typically of short lived perennial; follows mixed breeding system and mid-successional species.

Clustering based on PCoA, NJ and UPGMA showed comparable results with strong cluster in majority of individuals. The parameters also revealed some intermixed individuals from one population to another and vice versa. This type of admixture may be resulted from migration of people from one place to another; taking the seeds and adapting to new environment of their final destination and/ or other market exchange of seeds. High similarity of Decha and Nedjo irrespective of geographical distant may also be mainly because of migration of people from one location to the other and also because of old market and merchant movement from southern to northern Ethiopia (pers. com., Tesfaye Tafesse, 2009).

From the three distinct major clusters of UPGMA based dendrogram, the first bottom cluster contains populations of Decha, Nedjo, Dedo, Ale, Hulet Iju, and Gimbi. Nedjo and Gimbi are both from west Wollega, Hulet Iju from East Gojjam, Ale from Ilubabor, Dedo from Jimma and Decha from Keficho Shekicho. Among these, Dedo and Decha as well as Gimbi and Nedjo are geographically found in closer proximity. The highest within cluster similarity was, however, found between Decha and Nedjo followed by Gimbi and Hulet Iju Enase. Interestingly, this might be because of ancient market of Ethiopia from southern Ethiopia to the northern and human movement from Gojjam, Gondar and Wollo to the majority of provinces of Wollega during 1970 to 2000 who usually take seeds together with them to their destination. The migration is often because of multitude of reasons including environmental degradation, vulnerability, food insecurity, natural disaster, land scarcity and other push factors they faced, settled there for many years and latter displaced because of inter-ethnic conflicts (Tesfaye Tafesse,

2009). In particular, this inter-ethnic conflict has resulted in both returning to their original home land, and bypassing to other provinces towards southern Ethiopia. This fact is indeed believed to have contributed to the distribution of seeds of anchote to aforementioned areas since local people have been using anchote as popular cultural medicine. So that similarity observed between Decha (coffee growing area) and Nedjo might be because of bypassing migrants while similarity between Hulet Iju Enase (East Gojjam) and Gimbi (West Wollega) was probably due to seed transfer upon their homecoming.

On top of strong similarity between Nedjo and Decha; the five unique bands found in majority of Decha, but only one in Nedjo may indicate propagation system on one hand and loss of alleles on the other hand. Farmers around Decha might have used vegetative propagation method which resulted in the overall increase of this band. The reduction in the number of this unique band in Nedjo on the other hand may imply the dramatic reduction of genetic diversity from the area may be because of activities of migrants and local peoples seed exchange and intermixing with seeds of other sites.

The top cluster contained populations of Abbay Chomen and Assosa indicating that anchote seeds might have been taken to Assosa from Abbay Chomen by Oromo settlers. According to Desta Fekadu (2011) tubers and leaves of anchote populations of Abbay Chomen and Assosa contained highest copper and zinc content. In the middle of dendrogram, populations of Bedele, Manna, Digga and Gute all found in closer geographic area were clustered together. Digga and Gute were both from East Wollega,

while Manna was from Jimma and Bedele was from Illubabor. Sharing, borrowing and purchasing of seeds during sowing seasons by local people residing in this closer geographic proximity may have mainly attributed to the observed similarity among these populations.

6.4. Implications for the conservation

Knowledge on genetic diversity of crop facilitates the efficient conservation and utilization of genetic resources. To our knowledge, no genetic information study using any of the currently used molecular markers has so far been conducted on anchote. The result of the present study showed the existence of immense genetic diversity in some collection sites while evidence of allelic loss in others (Nedjo for example). Accordingly, better conservation is extremely important to protect the existing genetic diversity of anchote. Though there are undocumented evidences that Wollega was both the center of origin and cultivation for anchote, the majority of population from the area (except Gimbi) indicated low genetic diversity. In contrary to our assumption, populations of Illubabor exhibited higher genetic diversity compared to populations collected from Wollega. Hence, there is an urgent need to collect and conserve the existing populations starting with the most diverse places so as to minimize the loss of genetic diversity.

Continuous monitoring of the germplasm variability, including studies of allelic loss, effective population size, and the assessment of adaptive genetic variation, is useful in the design of effective conservation strategies. Institute of Biodiversity Conservation of

Ethiopia shall make use of this measure given that the major objective of a gene bank is preserving genetic variability and maintaining the genetic identity of the accessions. .

6.5. Implications for breeding

The identification of crossing parents with high divergent genetic variability has been a goal of many breeding programs that aim to explore the heterosis. Thus, germplasm characterization is necessary to provide information on gene pool for future use and to prevent the loss of these resources. In our current study, the unique pattern revealed from certain individuals might control number of seeds (lower seed), protein content (higher), fruit size (larger) and vine length (shortest) that needs further research by increasing both number of populations and primers. These unique band patterns could be informative for parental selections and cross breeding programs in anchote breeding for its improvement. Moreover, the results obtained from the present investigation showed that the ISSR primers could be informative markers which can be used to examine and correlate banding patterns with the agronomic characteristics. For instance, getting necessary information from the unique band produced by the (GGAGA)3 primer in the majority of Decha populations may serve an as candidate gene that may be used for future crop improvement program.

7.0. CONCLUSIONS

- Results from all diversity parameters showed that Ale and Bedele populations from Illubabor and Gimbi population from Wollega exhibited high genetic diversity indicating that conservation strategies must primarily focus on these areas.
- One of the two populations collected from Jimma (Manna population) had lowest genetic diversity. However, individuals of Manna are very similar within themselves and distantly related to others and need conservation attention.
- AMOVA of the present study showed that intra-population diversity was little higher than inter-population diversity. From this parameter
- Clustering analysis with PCoA, NJ and UPGMA suggested that there were some intermixed individuals from one to other populations and no entire strong association made between geographic and genetic distance.
- The overall gene flow information (Nm) and population differentiation value from Gst and F_{ST} supports mixed breeding system of anchote.
- Finally, ISSR markers found to be helpful in revealing unique pattern that could be useful for Marker Assisted Selection (MAS), and future characterization of populations in gene bank.

8.0. RECOMMENDATIONS

1. *C. abyssinica* is multi-purpose crop and every part of it is nutritionally feasible having important nutritional components. However, unlike that of other tubers, this plant is underutilized and little study has been carried out on it. Study on reproductive biology, karyotyping, medicinal importance and other economic aspects should receive due emphasis.
2. Pollination type or pattern of mating of the plant is not well documented. It is therefore important to deal with and at the same time devise a rational approach for breeding programs and germplasm managements.
3. This study is the first attempt to reveal existence of genetic diversity in *C. abyssinica* at molecular level using ISSR markers. However, a study (using other markers or combination of markers) such as the codominant markers like SSR are so indispensible for comparative analysis for better inference and estimate of gene flow.
4. Characterization of more populations is also very important and an in depth study should be made to indicate level of diversity among populations and agro-ecological zones.
5. The currently obtained unique marker need further study to validate if the results from qualitative and quantitative trait (lowest seed number, high protein content, largest fruit size and shortest vine length) are linked to this unique band.
6. Fungus that attacks shoot parts of *C. abyssinica* has to be studied in detail and management strategy should be forwarded.

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10. APPENDICES

Appendix 1. Concentration and quality of DNA of all individuals

Well	Conc.	Units	A260	A280	260/280	Well	Conc.	Units	A260	A280	260/280
Ac-1	866	ng/ul	17.32	8.708	1.99	Gw-6	257.5	ng/ul	5.149	2.578	2
Ac-2	51.38	ng/ul	1.028	0.553	1.86	GW-7	341.7	ng/ul	6.834	3.461	1.97
Ac-3	210.4	ng/ul	4.207	2.126	1.98	Gw-8	172.4	ng/ul	3.448	1.729	1.99
Ac-4	180	ng/ul	3.601	1.821	1.98	GW-9	1175	ng/ul	23.5	11.53	2.04
Ac-5	3.724	ng/ul	0.074	0.035	2.1	GW-10	29.33	ng/ul	0.587	0.302	1.94
Ac-6	28.77	ng/ul	0.575	0.287	2.01	JA-1	81.62	ng/ul	1.632	0.897	1.82
Ac-7	8.085	ng/ul	0.162	0.097	1.66	JA-2	1646	ng/ul	32.92	16.51	1.99
Ac-8	4.646	ng/ul	0.093	0.051	1.81	JA-3	292.6	ng/ul	5.852	2.943	1.99
Ac-9	843.2	ng/ul	16.86	8.313	2.03	JA-4	900.7	ng/ul	18.01	9.057	1.99
Ac-10	5.54	ng/ul	0.111	0.06	1.85	JA-5	257.6	ng/ul	5.151	2.603	1.98
SS-1	48.43	ng/ul	0.969	0.545	1.78	JA-6	782.3	ng/ul	15.65	8.259	1.89
SS-2	2.998	ng/ul	0.06	0.034	1.79	JA-7	1170	ng/ul	23.4	11.63	2.01
SS-3	1056	ng/ul	21.12	10.7	1.97	JA-8	1698	ng/ul	33.96	16.82	2.02
SS-4	13.71	ng/ul	0.274	0.186	1.47	JA-9	683.6	ng/ul	13.67	6.929	1.97
SS-5	114.5	ng/ul	2.29	1.218	1.88	JA-10	72.81	ng/ul	1.456	0.746	1.95
SS-6	6.128	ng/ul	0.123	0.069	1.77	De-1	442	ng/ul	8.84	4.461	1.98
SS-7	9.51	ng/ul	0.19	0.071	2.67	De-2	153.8	ng/ul	3.077	1.559	1.97
SS-8	230.1	ng/ul	4.603	2.317	1.99	De-3	424	ng/ul	8.479	4.277	1.98
SS-9	168.2	ng/ul	3.365	1.706	1.97	De-4	38.27	ng/ul	0.765	0.392	1.95
SS-10	276.6	ng/ul	5.533	2.751	2.01	De-5	11.25	ng/ul	0.225	0.125	1.8
Gi-1	87.5	ngul	1.75	0.877	2	De-6	7.92	ng/ul	0.158	0.094	1.69
Gi-2	51.45	ng/ul	1.029	0.516	1.99	De-7	4.665	ng/ul	0.093	0.043	2.16
GI-3	1009	ng/ul	20.17	9.882	2.04	De-8	10.36	ng/ul	0.207	0.097	2.15
GI-4	45.97	n/ul	0.919	0.47	1.96	De-9	9.089	ng/ul	0.182	0.113	1.6

Gi-5	40.47	ng/ul	0.809	0.39	2.07	De-10	816	ng/ul	16.32	8.143	2
Gi-6	128.2	ng/ul	2.565	1.287	1.99	Al-1	2940	ng/ul	58.8	29.82	1.97
Gi-7	30.3	ng/ul	0.606	0.311	1.95	Al-2	641.6	ng/ul	12.83	6.574	1.95
Gi-8	22.07	ng/ul	0.441	0.22	2.01	Al-3	996.8	ng/ul	19.94	9.931	2.01
Gi-9	16.39	ng/ul	0.328	0.165	1.99	Al-4	48.99	ng/ul	0.98	0.496	1.98
Gi-10	30.08	ng/ul	0.602	0.302	1.99	Al-5	8.947	ng/ul	0.179	0.085	2.1
GW-1	25.41	ng/ul	0.508	0.275	1.85	Al-6	156.8	ng/ul	3.136	1.561	2.01
GW-2	18.78	ng/ul	0.376	0.195	1.93	Al-7	125.3	ng/ul	2.507	1.265	1.98
Gw-3	106.6	ng/ul	2.131	1.092	1.95	Al-8	202.4	ng/ul	4.047	2.001	2.02
Gw-4	402.7	ng/ul	8.053	3.997	2.01	Al-9	87.5	ng/ul	1.75	0.877	2
Gw-5	272	ng/ul	5.44	2.725	2	Al-10	51.45	ng/ul	1.029	0.516	1.99
As-1	12.18	ng/ul	0.244	0.16	1.53	Gu-4	743.5	ng/ul	14.87	7.396	2.01
As-2	8.161	ng/ul	0.163	0.077	2.13	Gu-5	389.7	ng/ul	7.794	3.894	2
As-3	14.99	ng/ul	0.3	0.112	2.68	Gu-6	351.2	ng/ul	7.023	3.486	2.01
As-4	128.2	ng/ul	2.565	1.287	1.99	Gu-7	424	ng/ul	8.479	4.277	1.98
As-5	30.3	ng/ul	0.606	0.311	1.95	Gu-8	438.3	ng/ul	8.766	4.391	2
As-6	22.07	ng/ul	0.441	0.22	2.01	Gu-9	433.3	ng/ul	8.666	4.323	2
As-7	16.39	ng/ul	0.328	0.165	1.99	Gu-10	273.6	ng/ul	5.473	2.712	2.02
As-8	30.08	ng/ul	0.602	0.302	1.99	Hi-1	3589	ng/ul	71.79	38.18	1.88
As-9	25.41	ng/ul	0.508	0.275	1.85	Hi-2	2571	ng/ul	51.41	25.67	2
As-10	18.78	n/ul	0.376	0.195	1.93	Hi-3	2763	ng/ul	55.25	28.17	1.96
Be-1	112.2	ng/ul	2.244	1.12	2	Hi-4	3571	ng/ul	71.43	37.64	1.9
Be-2	381.9	ng/ul	7.638	3.822	2	Hi-5	2874	ng/ul	57.48	29.66	1.94
Be-3	229.9	ng/ul	4.599	2.286	2.01	Hi-6	2257	ng/ul	45.14	22.7	1.99
Be-4	658.8	ng/ul	13.18	6.711	1.96	Hi-7	1798	ng/ul	35.96	17.73	2.03
BE-5	497.5	ng/ul	9.951	5.061	1.97	Hi-8	2653	ng/ul	53.06	27.06	1.96
Be-6	1270	ngul	25.4	12.49	2.03	Hi-9	3980	ng/ul	79.61	42.4	1.88
Be-7	103.4	ng/ul	2.067	1.046	1.98	Hi-10	2821	ng/ul	56.41	28.54	1.98

Be-8	89	ng/ul	1.78	0.935	1.9	Dc-1	1057	ng/ul	21.14	10.55	2
Be-9	2019	ng/ul	40.39	20.18	2	Dc-2	2175	ng/ul	43.51	21.58	2.02
Be-10	84.88	ng/ul	1.698	0.846	2.01	Dc-3	2032	ng/ul	40.65	20.35	2
M-1	14.6	ng/ul	0.292	0.14	2.08	Dc-4	1687	ng/ul	33.74	16.62	2.03
M-2	100.6	ng/ul	2.012	0.948	2.12	Dc-5	2721	ng/ul	54.43	27.53	1.98
M-3	125.9	ng/ul	2.518	1.254	2.01	Dc-6	1828	ng/ul	36.55	18.09	2.02
M-4	1895	ng/ul	37.91	19.06	1.99	Dc-7	5829	ng/ul	116.6	65.85	1.77
M-5	1175	ng/ul	23.5	11.53	2.04	Dc-8	2171	ng/ul	43.42	22.02	1.97
M-6	29.33	ng/ul	0.587	0.302	1.94	Dc-9	2959	ng/ul	59.18	30.17	1.96
M-7	400.2	ng/ul	8.004	4.01	2	Dc-10	1301	ng/ul	26.03	12.84	2.03
M-8	296.8	ng/ul	5.937	2.951	2.01	N-1	1016	ng/ul	20.33	10.33	1.97
M-9	26.2	ng/ul	0.524	0.264	1.98	N-2	1119	ng/ul	22.38	11.23	1.99
M-10	1148	ng/ul	22.95	11.48	2	N-3	748.4	ng/ul	14.97	7.916	1.89
Di-1	172.3	ng/ul	3.446	1.727	2	N-4	1025	ng/ul	20.49	10.15	2.02
Di-2	53.43	ng/ul	1.069	0.527	2.03	N-5	892.3	ng/ul	17.85	9.173	1.95
Di-3	38.27	ng/ul	0.765	0.394	1.95	N-6	967.8	ng/ul	19.36	9.613	2.01
Di-4	112.8	ng/ul	2.255	1.144	1.97	N-7	679	ng/ul	13.58	7.11	1.91
Di-5	81.62	ng/ul	1.632	0.897	1.82	N-8	1820	ng/ul	36.41	18.29	1.99
Di-6	1646	ng/ul	32.92	16.51	1.99	N-9	2647	ng/ul	52.95	27.03	1.96
Di-7	292.6	ng/ul	5.852	2.943	1.99	N-10	705.4	ng/ul	14.11	7.223	1.95
Di-8	900.7	ng/ul	18.01	9.057	1.99						
Di-9	116.7	ng/ul	2.334	1.167	2						
Di-10	86.84	ng/ul	1.737	0.883	1.97						
Gu-1	303.6	ng/ul	6.072	3.011	2.02						
Gu-2	137.8	ng/ul	2.756	1.381	2						
Gu-3	242.3	ng/ul	4.846	2.486	1.95						

Ac= Abay Chomen

SS= Sibu sire

Gi = Gimbi

GW = Guto wayu

As = Assosa

Be = Bedele

M = Mana

Di = Diga N = Nadjo

Gu = Guto

JA = jimma Argo

De = Dedo

Hi = Hulet Iju enase

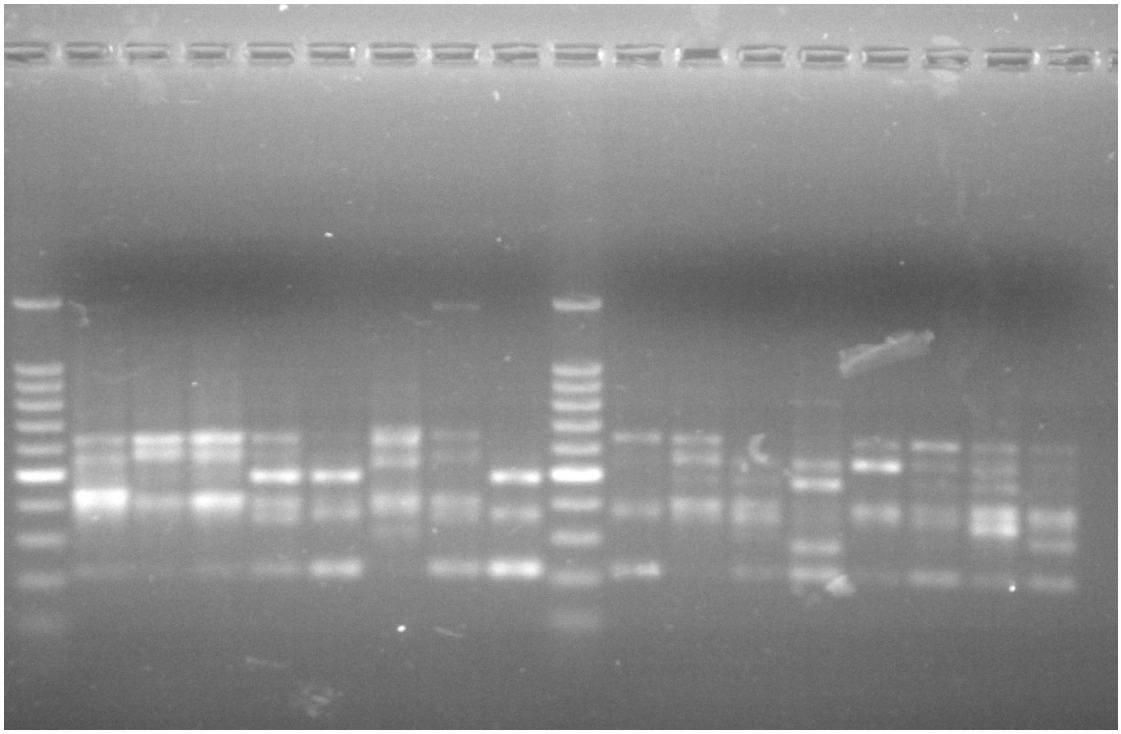
Dc = Decha

Al = Ale

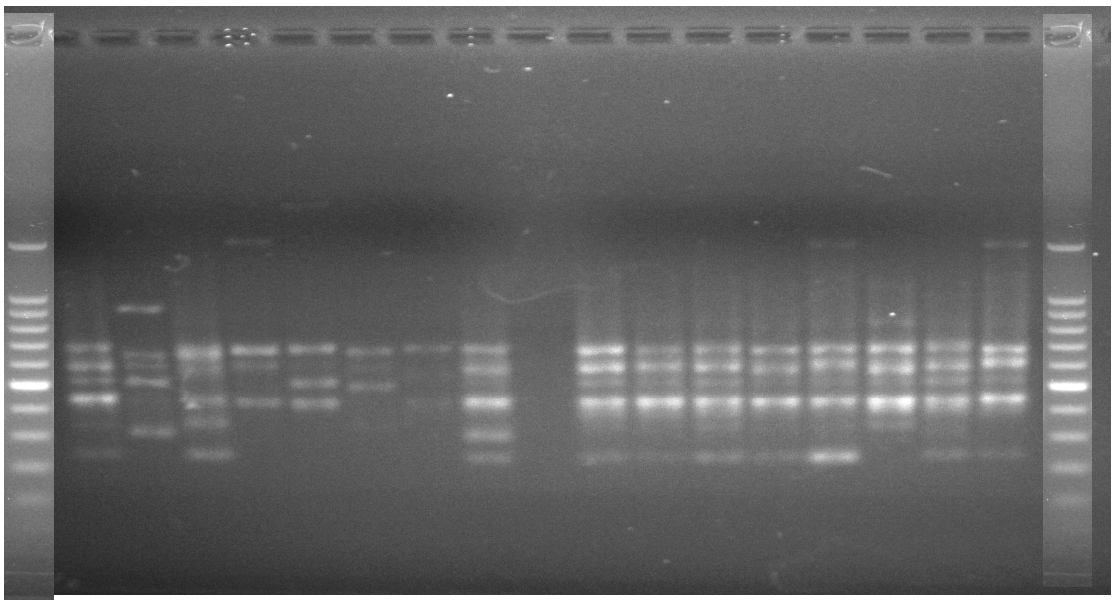
Appendix 2. Numbering of individuals found in selected populations

Appendix.3. banding patterns revealed from some study population with different primers

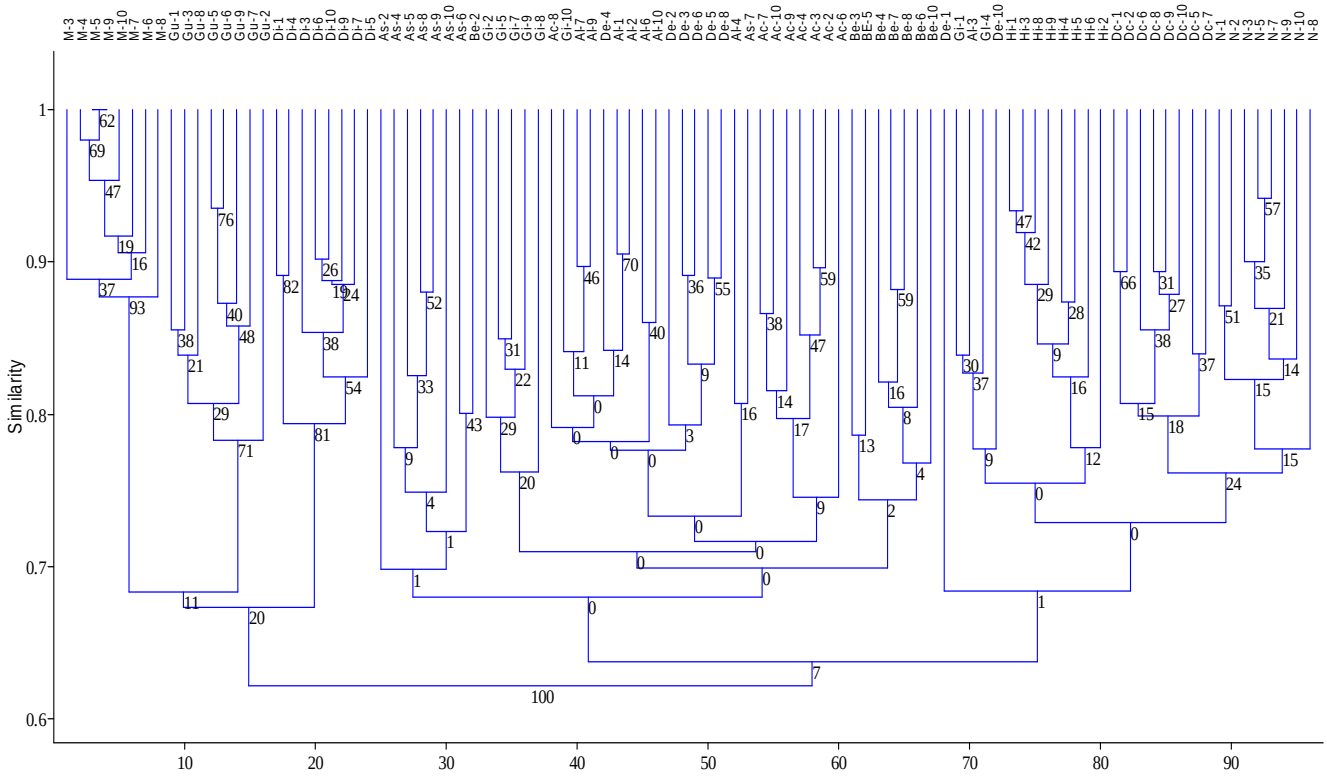
1).Banding pattern of Ale and Assosa with primer 880(left to right)



2) Badding pattern of Gimbi and Hulet iju enase from primer 880



Appendix 4: Bootstrapped dendrogram obtained from Jaccard pair wise similarity



Declaration

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other university. All source of the material used for this thesis have been duly acknowledged.

Name: Abreham Bekele Alemu

Signature _____

Date of submission_____

I hereby certify that this thesis has been done under the guidance of me and I read and evaluated the thesis entitled as *Study of genetic diversity of anchote (Coccina abyssinia) using ISSR markers***.** I recommend that it can be submitted as fulfilling thesis requirement.

Advisor: Dr. Tileye Feyissa

Signature_____

Date _____