GENETIC DIVERSITY OF KHAT (*Catha edulis*) IN ETHIOPIA REVEALED BY ISSR MARKERS

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Genetic Diversity of Khat as Revealed by ISSR Markers

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Abstract

Khat (*Catha edulis*) is a plant that is habitually chewed for its euphoric and stimulatory effects. In Ethiopia there are many khat types produced which are identified only by their place of growth. No studies were conducted on khat genetic diversity and this study is designed to fill this gap. This study aimed at evaluating genetic diversity of seven populations of khat obtained from different locations of Ethiopia. DNA was extracted using modified CTAB method of Wang *et al*. (1996). Fourty amplified fragments were obtained using five ISSR primers. Nei's genetic diversity ranged from 0.22 to 0.36 with mean of 0.32 and Shannon information index ranged from 0.31 to 0.52 with mean of 0.47. Within population diversity based on polymorphic bands ranged from 52.5% to 85% with mean of 77.14 %. The AMOVA indicated within population diversity of 79% and among population diversity of 20.9%. The highest diversity was obtained from Gurage, Gelemso and Wendo populations and the lowest was from Hirna. This study showed that there is a considerable genetic diversity among and within khat populations. This was indicated by the allele observed and clusters generated. PCoA, NJ and UPGMA showed comparable results with strong cluster in majority of individuals of the populations. The objective of the study was to identify the extent of genetic diversity exist among khat population.

Keywords: *Catha edulis*, Genetic diversity, ISSR, Khat

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Table of Contents **Page**

List of tables

Page

List of figures

Page

Acronyms and Abbreviations

AMOVA	Analysis of molecular variance
AFLP	Amplified Fragment Length Polymorphism
CTAB	Cetyltrimethyl Ammonium Bromide
I	Shannon information index
ISSR	Inter Simple Sequence Repeat
NBP	Number of polymorphic band
PCoA	Principal Coordinated Analysis
PPB	Percentage of polymorphic band
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction fragment length polymorphism
SSR	Simple Sequence Repeats
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

1. INTRODUCTION

Catha edulis Forek. (*Celastraceae*) commonly known as qat or khat, is an evergreen tree indigenous to East Africa, South Arabia and Yemen (Luqman and Danowski, 1960*).* The leaves and tender twgis are chewed in certain countries of East Africa and Arabian Peninsula, mainly Yemen (Dhaifalah and Santavy, 2004; Warfa *et al*., (2007) for their central nervous system stimulating properties (Luqman and Danowski, 1976). Khat chewing had become an integral part of these region's' culture and represents an element of ethnic identification (Rousseau, 2004). It has a deep-rooted social and cultural tradition (Kalix and Braenden, 1985) and is usually chewed to enhance working capacity (Elmi 1983; Kalix and Braenden 1985; Kenndy, 1987).

The habit of chewing khat has been common in many countries. Approximately 20million people chew khat on a daily basis worldwide while hundreds of millions depend on it as a main source of livelihood (Pennings and Van Amsterdam, 2008; Bogale and Yisma ,2016) . The earliest record with more factual bases showed the Arabic source Al-Biruni (973-1051 A.D.), indicated the ancient use of khat (Schopen, 1978). Historically, khat has been used for medicinal purposes (Kennedy *et al*., 1987) as well as an aphrodisiac (Margetts 1967; Krikorian, 1984), though it was also used for recreational purposes (Kennedy, 1987). It is most valued for its stimulant effects (Baasher, 1980). It is also used for avoiding sleepiness euphoriatic effects. Chewing is the most common mode of administration. It has been taken as a tea and occasionally smoked (Hodgkinson 1962; Kennedy, 1987).

In Ethiopia, processed leaves and roots are used to treat influenza, cough, gonorrhea, asthma and other chest problems. The roots are used to treat stomachache and an infusion made from them is taken to treat boils (Lemessa, 2001). Cathinone is believed to be the main active ingredient in fresh khat leaves (Hassan *et al*., 2007).

Local cultivars of khat were described based on geographical location, growth habit, and physical appearance, i.e. colour of the leaf, stem sizes, and potency of effect. It has been known for a long time that different kinds of khat have different degrees of pharmacological action. Yemeni farmers recognized four cultivars based on colours of shoots and twings as 'Abyadh' predominantly pale green in colour, ' Azraq' purplish, 'Aswad' crimson and 'Ahmar'an intermediate between 'Azraq' and 'Aswad' reddish. In Ethiopia two prominent cultivars have been described as dimma (red) and ahde (white) (Getahun and Krikorian, 1973; Krikorian, 1984 ; Brbier, 1883) classified khat into three types, namely 'Madness-ausing', 'intoxicating-like sprit' and 'insomnia –causing' based on their effect while Getahun and Krikorian, 1973) classified them into two major groups, namely "kudda" and "kudda kerti" mainly based on harvesting time. Forty kinds of khat based on geographical origin were recognized in Yemen by Ramadan *et al*. (1981).

Genotypic studies have not been conducted for this crop in Ethiopia where it has become a major income earner. Farmers plant different cultivars obtained from various sources resulting in mixed populations.

The level of genetic variability can be a good indicator of productivity especially when different forms of biotic or abiotic stresses confront the plant (De Vicente, M.C. and Fulton, T. (2003)). Further, large gene pool, that is, if the genetic composition of individuals in the population varies significantly, such group has a greater chance of surviving and flourishing than a population with narrow genetic variability.

The information on intra-specific gene variability is, therefore, important to policy makers and khat crop farmers in formulation and implementation of sound strategies, such as breeding or propagation programs that ensure preservation of genetic resources (Subudhi & Rosenow, 2002). Genetic diversity information is essential for the germplasm management and developing conservation approaches (Das & Lakshmikumaran, 1999; Govindaraj, 2015).

Molecular characterization is an important tool used in genetic diversity studies to detect variations that exist in DNA sequences or specific genes or modifying factors (Matheri, 2016). Various molecular markers have been used in differentiating morphologically similar individuals among cultivars of the same plant species (Mohammadi & Prasanna, 2003; Wanjala and Hanson, 2013). Molecular markers are a source of reliable information for genetic analysis and genotyping of different cultivars within a species (Sarkhosh *et al.,* 2006). Various molecular markers have been used in population genetics studies including simple sequence repeats (SSR) (Gupta *et al.,* 1996), restriction fragment length polymorphisms (RFLP) (Sambrook & Maniatis, 1989) and Sarkhosh & Ebadi, 2006). Random amplified polymorphic DNAs or (RAPDs) (Williams, *et al.,* 1990), amplified fragment length polymorphism (AFLP) (Vos & Zabeau, 1995), and inter simple sequence repeats (ISSR) (Matheri & Kamau, 2016 and Sarkhosh *et al.,*2006).

2. STATEMENT OF THE PROBLEM

Production and consumption of khat is very high in many countries. Ethiopia produces and exports khat to other countries. For many people in Ethiopia, khat is the sources of income and livelihood. There are many types of khat produced which are identified by their place of growth such as Bahirdar, Wendo, Hirna, etc. However, the genetic diversity within and among these khat population is not well known. To the best of our knowledge, there are no genetic diversity studies on this crop.

Analyzing the genetic diversity of this crop will be used in order to manage, conserve and improve in a way that it doesn't have negative health impact to people (for medicinal purpose). Therefore, the present study is aimed to assess the genetic diversity of this crop in Ethiopia.

2.1 OBJECTIVE OF THE STUDY

General Objective

This study was aimed to quantify the extent of genetic diversity exist in khat (*Catha edulis*) by using Inter Simple Sequence Repeat ISSR Markers

Specific Objectives

- To evaluate the genetic diversity and relation within and among different khat populations
- To generate genetic diversity information for its conservation, management and use.

3. LITRATURE REVIEW

3.1 Ethno botany of khat

Khat (*Catha edulis*) belongs to *Celastraceae* family which contains 60–70 genera and 850–900 species. It is an erect, evergreen, glabrous shrub or tree 2–25 m high with reddish stems, shiny green leaves and white flowers. The leaves are opposite on flowering shoots and alternate on older ones. Khat is an indigenous plant to Ethiopia and Yemen as the countries of origin. However, it is found in many other east African countries and southern Africa (Al-Meshal *et al.*1986; Gelaw & Haile-Amlak 2004). The first botanical description of khat was given by the Swedish botanist Peter Forsskal who in 1775 named it as *Catha edulis* (Connor& Makonnen, 2002).

Fig 1.Image of khat

3.2. Propagation of khat

Dechassa , (2001) reported Catha edulis is a plant that may be difficult to start from seed, so a common method is asexual propagation via cuttings. Green, herbaceous stem material of this species will not be able to produce roots and is not viable material, the stem must have some woody tissue. The most successful method is propagating the suckers that the plant will often produce, and suckers can be easily induced with BAP (Benzylaminopurine, a cytokinin plant growth regulator) applied to the base of the stem.

The propagation method of Khat is by cuttings or suckers having root (s). These planting materials are the only means of propagating, as seeds are not used for propagation due to poor germination rate of the seeds.

3.3. Distribution of khat in Ethiopia

The total area of land under *khat* cultivation in Ethiopia in the year 1997/98 was estimated at 78,570 hectare (Central Statistics Authority, 1997/98). Oromia, mainly East and West Hararghe zones, is the most important centre of *khat* production East Hararghe zone alone contributes 53.4% of the total production area in Ethiopia. Hararghe is considered to be the most important producer of quality *khat* in the world (Peter, 1952).

The expansion of chat plant in Ethiopia has been increasing radically. If we investigated the total area of chat plant some thirty years back was 3000ha,3500ha and 6997ha in 1954, 1957 and 1961 respectively(Amare and Krikorian ,1973).
According to CSA the total area of land under chat cultivation in the year 1998 was estimated at 78,570 ha in 2008 increased to 163,227haand then 204,648 ha in 2011 and in 2015 it reached 248,964 ha. Correspondingly the yield gained has increased.

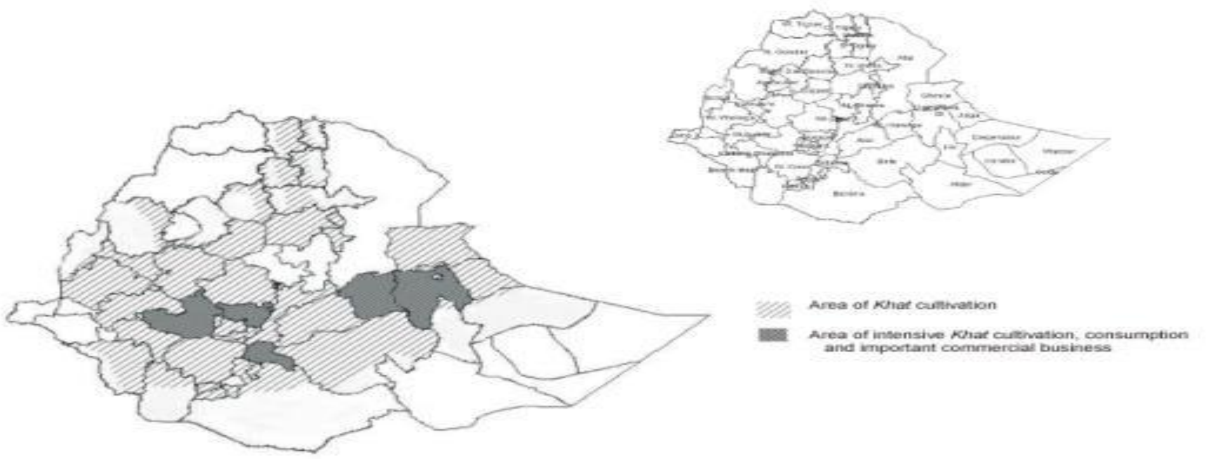


Fig 2. Map showing distribution (rough) of khat in Ethiopia

3.4. Medicinal uses of khat

Khat is traditionally used as remedy for cough and influenza (Feyissa *et al.,*2008), stomach troubles (Amare and Krikorian, 1973), chest problems (Al-Hebshi *et al*., 2010) and infusion of leaves to treat diseases (Michel *et al*., 2008). Some of the traditional medical uses have been corroborated by research. For example, petroleum ether and chloroform extracts of the leaves have shown antimicrobial activities (Elhag, *et al*., 1999); to lower serum glucose, triglyceride and cholesterol levels (Al-Habori & Al-Mamary, 2004), against leukemia and prostate cancer (Dimba *et al*., 2004). It is interesting to note that in vitro studies showed khat extracts have anti cariogenic properties, i.e., counter the development of tooth decay (Al-Hebshi & Ghandour, 2010). A recent study revealed khat leaves possess high antioxidant activity which is comparable to green tea (Vinokur *et al*., 2008).

3.5. Genetic variability

The existence of genetic diversity is a pre-condition in development of an effective plant breeding program. Allard (1960) and Falconer and Mackay (1996) defined genetic diversity as the occurrence of variability among the individuals due to difference in genetic composition. If the expression of traits for two individuals is measured in similar environment and a difference recorded, these variations would be caused from genetic control and named genetic variation (Falconer and Mackay, 1996). Genotypic variation is the component of variation caused by the genotypic differences among individuals within a population. Therefore, this type of variation among individuals is a core for plant breeders in crop species improvement and this can buffer against seasonal fluctuation (Sharma, 1998). Phenotypic variation is observable variation present in traits which include both genotypic and environmental components of variation and, as a result, its magnitude differs under different environmental conditions (Singh, 1977).

Hence, phenotypic variation is the result of genotypic variation and environmental deviation (Falconer and Mackay, 1996).

3.6. Marker Systems and their Applications in Genetic Diversity Study

Biological markers could have many different types but mainly classified into morphological, biochemical and molecular types. The morphological (classical or visible, agronomic traits) markers are phenotypic traits, while; biochemical markers are isozymes, and are based on protien. The DNA (molecular) markers which reveal sites of variation in DNA is marker with better resolution of diversity.

Each type of marker system has its own advantages and disadvantages. The type of marker is selected based on the species types, availability and cost benefit analysis (Winter and Kahl, 1995; Semagn *et al.*, 2006).

3.7. Morphological markers

Morphological markers are usually visually described phenotypic characters such as flower color, seed shape, growth habits or pigmentation (Winter and Kahl, 1995). Morphological markers are phenotypic markers with distinguishable trait that have evident to human eyes (Bagali *et al.*, 2010). Morphological marker systems are the earliest, simple and inexpensive genetic markers which lie on phenotypic appearance (Vos *et al.*, 1995). It is the oldest method and considered as the first step in description and classification of germplasm (Hedrick, 2005). Its estimations are more dependent on environment and are more subjective than other measurements (Li *et al.*, 2009).

3.8. Biochemical (Isozyme) markers

Isozyme markers are differences in enzymes that are detected by electrophoresis and specific staining, these are coomassie blue, silver and fluorescent staining (Winter and Kahl, 1995). Biochemical analysis is based on the separation of proteins into specific banding patterns. The technique is rapid, economical and co-dominant nature of allozyme data makes it useful for the characterization of genetic variation in plant species (Weising *et al.,* 2005). Although protein markers circumvent environmental effects, the numbers of detectable markers are limited and they are typically tissue and developmental stage-specific and have no power to discriminate genetic diversity between closely related varieties (Park *et al.,* 2009). For this reason, most researchers began to focus on the use of DNA marker systems for genetic and ecological analyses of plant populations.

3.9. Molecular markers

Molecular markers, also called DNA markers, are thought of as signs along the DNA trail that pinpoint the location of desirable genetic traits or indicate specific genetic differences. Characterizations of plant genetic resources have been greatly facilitated by using a number of molecular marker systems due to their abundance in the genome. Molecular markers are not subject to environmental influences; so assessment can be carried out at any time during plant development (DeVicente and Fulton, 2003). DNA-based molecular markers have several advantages over the conventional phenotypic markers since their presence is not dependent on the growth stage of the crop and can be found in all tissues (Mondini *et al*., 2009).

The common types of dominant DNA markers that are used for genetic diversity study are Random Amplified Length Polymorphism (RAPD), Inter Simple Sequence Repeat (ISSR), Amplified Fragment Length Polymorphism (AFLP), DNA amplification fingerprinting (DAF) and arbitrarily primed polymerase chain reaction (AP-PCR). Whereas the co-dominant markers includes RFLP, SSR, sequence characterized amplified region (SCAR), cleaved amplified polymorphic sequence (CAPS), expressed sequence tag (EST) and single nucleotide polymorphism (SNP) (De Vicente and Fulton, 2003).

3.10. Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) has much greater power and was originally developed for mapping human genes than anything previously available (Botstein *et al.*, 1980). RFLP analysis is based on the ability of restriction enzymes (also called restriction endonucleases) to cleave DNA in to pieces (digested) at specific target nucleotide sequences consisting usually of a four or six nucleotide pairs. Variations in the characteristic pattern of a RFLP digest can be caused by base pair deletions, mutation, inversions, translocations, transpositions and unequal crossing over which result in the loss or gain of a recognition site resulting in a fragment of different length and polymorphism (not specific to the recognition site) (Schlotterer & Tautz, 1992).

3.11. Random Amplified Polymorphic DNA (RAPD)

RAPD were the first PCR- based molecular markers to be employed in genetic variation analysis (Welsh and McClelland, 1990; Schierwater and Ender, 1993). It is a type of PCR reaction, but the segments of DNA that are amplified are random. This marker is first introduced by Williams *et al.* (1990). RAPD markers are useful DNA based method for initial assessment of genetic variation, especially the assessment of genetic diversity in plant species (Song, 2005).

3.12. Inter- Simple Sequence Repeat (ISSR)

ISSR are DNA fragments of about 100-3000 bp located between adjacent, oppositely oriented microsatellite regions (Joshi *et al.,* 2000; Bussell *et al.,* 2005). ISSR marker was first developed by Zietkiewicz *et al.* (1994). Primers based on microsatellites are utilized to amplify inter-SSR DNA sequences.

ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16–18 bp). About 10–60fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored as the presence or absence of fragments of particular size.

Since the ISSR technique can produce a much large number of fragments per assay unit, it is a more powerful fingerprint tool than SSR; RFLP and RAPD marker system (Marsh and Ayres, 2002).

ISSR markers are also more reliable and reproducible than RAPDs because of the higher annealing temperature contributed by longer primers (Zietkiewicz *et al.,* 1994; Domyati *et al.,*1996; Powell *et al.,* 1996; Fang and Roose, 1997; Kojima *et al.*, 1998; Moreno *et al.,* 1998;Denduangboripant *et al.,* 2010).

ISSR markers target divergence in regions containing dispersed repetitive DNA and can rapidly differentiate closely related individuals, it is especially useful in detecting clonal variation and fingerprinting of related individuals. It has great potential in the study of natural populations for addressing questions ranging from conservation biology to molecular ecology and systematic (Bornet and Branchard, 2001; Song, 2005; Mudibu *et al.,* 2011).

This technique is useful to endangered, rare and less commercially important species, which are generally little studied for their genetic structure (Song, 2005). So, ISSR markers have been widely used for detecting genetic diversity of rare and endangered plant species (Marsh and Ayres, 2002). Therefore, ISSR is mainly used in many respects such as the study of genetic diversity in many plant species (Brantestem *et al.,* 2004).

3.13. Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism is a DNA fingerprinting technique, which detects DNA restriction fragments by means of PCR amplification. AFLP involves the restriction of genomic DNA, followed by ligation of adaptors complementary to the restriction sites and selective PCR amplification of a subset of the adapted restriction fragments. AFLP gives high level of resolution to allow delineation of complex genetic structures, to differentiate individuals in a population gene flow experiments and also to register plant varieties (Misra *et al.,* 2010). AFLP was first developed by Vos *et al.* (1995).

3.14. Simple Sequence Repeats

Microsatellites are short nucleotide tandem repeats of a motif, usually one to six bases (Morgante and Olivieri, 1993; Bhat, 1999 and Kylin, 2010). Microsatellite repeats are sets repeated sequences found within eukaryotic genomes (Bell and Ecker, 1994; Gupta and Varshey, 2000; Ghislain *et al.,* 2004). This marker system was first developed by Akkaya *et al.* (1992). Microsatellite sequences are especially suited to distinguish closely related genotypes; because of their high degree of variability, they are favored in population studies and for the identification of closely related cultivars. If nucleotide sequences in the flanking regions of the microsatellite are known, specific primers (generally 20–25 bp) can be designed to amplify the microsatellite by PCR.

3.16. Diversity Studies in khat

According to Al-Thobhani *et al.* (2008) populations of *Catha edulis* from different geographical regions and growth habits in Yemen showed a clear genetic differentiation. These authors assessed the genetic relationships among 40 khat genotypes from diverse locations of Yemen using RAPD marker. They reported 71.7% were polymorphic and 21.7% were monomorphic.

They also reported four variants of *C. edulis* are not strongly differentiated from each other genetically. This could be the collection of small number 'Abyadh'(10), 'Aswad'(5), 'Azraq'(16) and 'Ahmar'(9). However, a small sample size can lead to significant bias in the population genetics (Ficher *et al.,* 2000). Sample size of 10-15 individuals is adequate for largely unbiased results (Isabel *et al.,* 1999). Low to moderate polymorphism was (11-40%) obtained among the genotypes which could be the result of propagation of khat by cutting and suckers, since seeds of *C. edulis* show poor germination rate (Lemessa, 2001).

Although somatic mutation could occur in populations where asexual propagation is common (Lemote *et al*. 2002) recent studies reveal that genetic diversity will decrease at a constant rate with an increase in the rateof asexual reproduction (Baloux *et al.* 2003; Bengtsson *et al.* 2003).
Al-Thobhani *et al.* (2008) reported that tree genotypes had lower similarity index as compared to the shrub types.

Ngari *et al. (*2018) evaluated genetic diversity of selected khat cultivars grown in Embu and Meru Counties of Mt Kenya region using 5 SSR markers. They reported average gene diversity among all the selected samples was 0.2883. The Polymorphism Information Content (PIC) value of each marker varied for all tested SSR loci.

The PIC values range from 0 to 1 with values that are greater than 0.5 being highly informative. The PIC values ranging from 0.25 to 0.5 show a reasonably informative marker while those with values less than 0.25 are slightly informative (Musyoki, 2015; Matheri *et al*.,2016 and González-Pérez *et al*., 2013) Based on this, markers CE 37 and CE 39 were reasonably informative. Marker CE 37 had PIC value of 0.2878, which was lower than 0.89 as observed by Moges *et al*.(2016) in 23 khat genotypes.

Ngari *et al. (*2018) reported genetic diversity within population is 93% and among population is 7%. Different types of mutations accumulate over time which serves as a source of polymorphism within a species.

Variations within a population can be determined through an assessment of gene diversity and allelic richness (Biron *et al*., 2002).

Ngari *et al. (*2018) reported populations from different geographical regions and growth types in Mt. Kenya region showed genetic differentiation.

4. MATERIALS AND METHOD

4.1. Plant Material

Young leaf khat (*Catha edulis*) samples were collected from different shops in Addis Ababa. A total of 70 individual plants representing 7 populations. These populations collected came from Gelemso, Matakesha, Wendo, Hirna, Gurage, Leyu and Bahirdar (table 1).

Table1. Populations of khat their geographic location

Region of pop	Pop Name	Pop Code
Oromia	Gelemso	GMS01
		GMS02
		GMS03
		GMS04
		GMS05
		GMS06
		GMS07
		GMS08
		GMS09
		GMS10
Oromia	Matakesha	MTK01
		MTK02
		MTK03
		MTK04
		MTK05
		MTK06
		MTK07
		MTK08
		MTK09
		MTK10
Southern	Wendo	WDO01
		WDO02
		WDO03
		WDO04
		WDO05
		WDO06
		WDO07

		WDO08
		WDO09
		WDO10
Southern	Gurage	GUR01
		GUR02
		GUR03
		GUR04
		GUR05
		GUR06
		GUR07
		GUR08
		GUR09
		GUR10
Amhara	Bahir dar	BDR01
		BDR02
		BDR03
		BDR04
		BDR05
		BDR06
		BDR07
		BDR08
		BDR09
		BDR10
HARERI	Hirna	HIR01
		HIR02
		HIR03
		HIR04
		HIR05
		HIR06
		HIR07
		HIR08
		HIR09
		HIR10
Oromia	Leyu	LEY01
		LEY02
		LEY03
		LEY04
		LEY05
		LEY06
		LEY07
		LEY08
		LEY09
		LEY10

4.2. DNA Isolation

About 0.5 g of young leaves were harvested from the tip of young shoots on the stem or branches of khat and dried in silica gel (Chase and Hills, 1991). Total genomic DNA was isolated from silica gel-dried leaves using the CTAB method by Wang *et al.* (1996). Fifty (50) mg of collected dried leaf samples from seven representative plant were bulked and filled in 2ml labeled Eppendorf-caps containing two autoclaved beads with 5mm diameter and grinded with Mixer Mill (Retsch GmbH, Germany) for three minutes at frequency of 30 Hz/sec. A total of 700 µl of CTAB solutions (10ml (1M Tris-HCl), 40ml (5M NaCl), 4ml (0.5M EDTA), 2g CTAB and 1g PVP) was added to each grinded sample to break open plant cells, solubilize the contents and disrupt the cell, and incubated at 65oC for 30 minutes in water bath and centrifuged at 16000 rpm for 5 minutes at 10ºC. After taking the supernatant, 700 µl cold chloroform was added and centrifuged at 16000 rpm for 5 minutes. This step was repeated for better cleanup of the genomic DNA. After taking the supernatant 50 µl,3M NaAc and mix gently. Cooled (4 ºC) Iso-propanol (2/3 of volume) was added to solution and allowed to freeze for 2 hrs at -20ºC and then centrifuged at 16000 rpm for 3 minutes to pour off supernatant. The DNA pellet was washed with 1µl of 70% ethanol, wash the pellet until it gets dissolved in ethanol.

The centrifuge at 16000 rpm for 3 minutes and this step was repeated. Aspirated and then dried at room temperature for 1to 2 hrs. Finally, the DNA pellet was suspended in 100 µl of TE (1x, p.a. grade), precipitated in salt solution (Ammonium acetate and Sodium acetate) and stored in refrigerator at -20oC.

4.3 DNA Quantification and Quality Test

DNA quantification and quality test was done by using gel electrophoresis and Nano-drop spectrophotometer methods.

An agarose gel (50 ml 1 x TBE, 0.5 g Agarose, and 1% ethidium bromide (10 mg/ml)) was prepared using casting tray. Stock solution of 10X Tris Borate EDTA (TBE) (108 g Tris base; 55 g Boric acid; 40 ml (0.5M) EDTA, pH 8.3 components per liter) was prepared and stored at room temperature. From the stock, working solution of 10X TBE prepared and then further diluted to 1x TBE and used to prepare the gel as well as to fill the electrophoresis tank (Bio-rad, Sub-cell®GT). Agarose gel with 1% (0.5 g) was prepared by boiling 1x TBE in 100 ml Erlenmeyer flask in micro oven for 2:30 minutes. After the agarose solution was cooled down at room temperature, 2 µl of ethidium bromide was added for better visualization of the gel. Then it was poured on to the gel tray with the inserted comb and the gel was left for 30 minutes. The comb was carefully removed and put the gel tray into the electrophoresis tank properly filled with electrophoresis buffer (1XTBE). The extracted DNA samples (4 µl), and 2 µl of 6x loading dye was loaded on the gel and electrophoresised at constant voltage of 80 v for 45 minutes. Gel picture was taken under UV light (Gel documentation system, Biosenc SC750) and digitally photographed. Finally, those with high band intensity and fewer smears were selected for PCR amplification out of the first two extraction fraction.

The quantity and qualities of extracted DNA were ao tested using Thermo Scientific Nano Drop spectrophotomer (NanoDrop 2000/2000c) by reading their absorbance at 260 nm wave length. Based on the reading obtained; the quantity and quality of first and second DNA extraction were evaluated, and the second extraction for the most of the samples was selected for PCR amplification based on the absorbance ratio at 260/280 reading.

4.4. PCR amplification and electrophoresis

Seven primers were screened on a total of seven individuals of different populations and five primers that produced clear, reproducible and polymorphic band pattern were selected for the study (table 2). PCR amplification was carried out in the total volume of 10µL containing 1XPCR buffer B (0.8mm Tris HCl,0.2m $(NH4)_2SO4$, 0.2% w/v Tween-20), 2.25 mm of $MgCl_2$,1U firepol Taq DNA Polymerase , 200µM of each of dNTPs, 0.5µM of primers and 0.5 µL of 20ng/µL total genomic DNA. Amplification will be performed by using Teche, Model TC41H2D thermal cycler.

Amplification was performed following temperature profiles: an initial denaturation step of 4 min at 94^{o}C, followed by 36 cycles of 30s 94^{o}C (denaturation), 30s at 40-47^{o}C (depending on specific annealing temperature of the primers) and then 1 min at 72^{o}C (extension). The last cycle was followed by a final extension step of 10 min at 72^{o}C. and, finally, the PCR was adjusted to store the product for 24 hr at 4 ^{o}C.

The PCR products were then electrophoresed on 0.5 % agarose in 0.5 mTAE buffer at constant voltage of 75 V for 2 h. The gel was then stained with ethidium bromide and visualized with Biorad gel documentation system. Band size was estimated using 100bp DNA ladder.

Table 2. Lists of primers, their sequence, repeat motif, Annealing temperature and Nucleotide

Primer	Repeat motif	Annealing temp.(ºC)	Nucleotide types
834	(AG)8YT	43 ºC	Di-nucleotide
835	(AG)8YC	45 ºC	Di-nucleotide
824	(CT)8G	43 ºC	Di-nucleotide
810	(GA)8T4	42 ºC	Di-nucleotide
889	DBD(AC)7	45 ºC	Di-nucleotide

4.5. Data Scoring and Analysis

The fragments generated from five primers with clearly distinguishable and reproducible bands were selected for scoring. Data scoring was performed as discrete statistics using a binary matrix “0” coded for absence, and “1” for presence of a band. Scored data were directly entered into computer for making analysis by using different software. Data generated were analyzed by using POPGENE version1.32 (Yeh *et al.,* 1999) to investigate and characterize genetic diversity in terms of number of polymorphic loci, percent polymorphism, means of Nei’s genetic diversity and Shannon index. NTSYS- pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek *et al.,* 1999) softwares were used to compute Jaccard’s similarity coefficient by using the following formula:

$$Sij=aa+b+c$$

Where; ‘a’ is the total number of bands shared between individuals i and j, ‘b’ is the total number of bands present in individual i but not in individual j and ‘c’ is the total number of bands present individual j but not in individual i.

NTSYS- pc version 2.02 (Rohlf, 2000) was used to generate cluster analysis based on the Un-Weighted Pair Group Method with Arithmetic mean (UPGMA)) for analyzing and comparing of individual genotypes and standardized genetic distance analysis (Nei, 1972). Neighbor joining (NJ) clustering method was computed by using Free Tree 0.9.1.50 Software (Pavlicek *et al.,* 1999) to compare individual genotypes and evaluate patterns of genetic diversity. Principal coordinated analysis (PCoA) was figured using PAST version 1.18 (Hammer *et al.,* 2001) and STATISTICA version 5.5 (Statsoft Inc, 2001) softwares based on Jaccard’s coefficient similarity for further examining of the patterns of variation among individual genotypes.

Areliquin version 3.01 (Excoffier *et al., 1992*) was used for analysis of molecular variance (AMOVA) to partition the total variance into among and within populations.

5. Results

5.1. ISSR band variation and level of polymorphism within 7 populations of khat

A total of 40 clear and scorable bands were amplified by five primers. An average of 8 bands per primer having ISSR fragment size ranged from 100 to1400 bp were obtained (Table 3). The number of polymorphic bands ranged from 2 to 9 within 34 total polymorphic bands and 6.8 average polymorphic band per primer. Percentage of polymorphic bands (PPB) ranged from 28.57 to100 (Table 3). The highest gene diversity (0.4450) and Shannon information (0.6363) were recorded by primer 835. On the other hand, lowest gene diversity (0.1326) and Shannon information index (0.1875) were recorded by primer 889 (Table 3). The mean gene diversity and Shannon information index for all primers were 0.3545 and 0.5051, respectively. Primer 834 and 835, dinucleotide repeat, produced nine polymorhphic bands, which is the highest number (Table 3).

Table3. ISSR primers with their scored band, polymorphism, and level of gene diversity

Primer	Sequence (5’-3’)	Anealing temperature (ºC)	Total band	NBP	PPB%	H ± SD	I ± SD
834	(AG)5Yt	43	9	9	100	0.442±0.049	0.634±0.052
835	(AG)8Yc	45	8	9	100	0.445±0.047	0.636±0.050
824	(Tc)8)Yc	43	9	8	88.89	0.423±0.227	0.594±0.225
889	DBD(Ac)7	45	7	2	28.57	0.132±0.227	0.187±0.320
810	(GA)8T4	42	7	6	85.71	0.319±0.181	0.473±0.245
Total			40	34			
Mean			8	6.8	80.63	0.352±0.133	0.505±0.178

Key:NPB number of polymorphic band, PPB percent of polymorphic band, hgene diversity, I shannon's information index

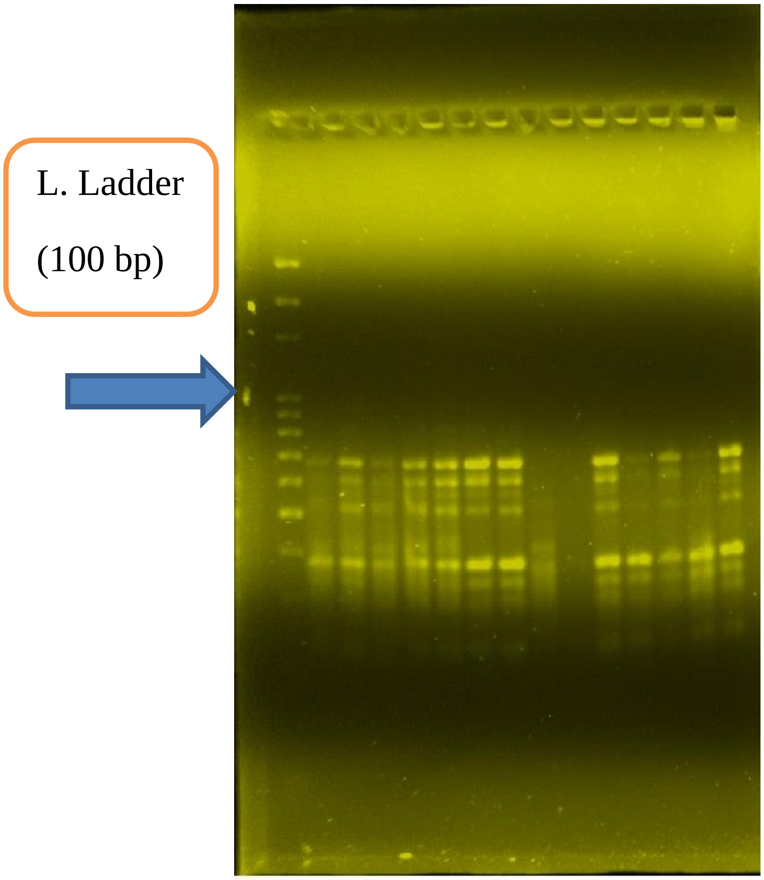


Fig.3. Generated bands using primer 824 on agarose gel. L Ladder (100bp)

5.2. Genetic diversity among khat populations

PPB ranged from 52.59% for Hirna to 85% for Gurage indicating Gurage having highest genetic diversity but Hirna having the lowest (Table 4). Gene diversity ranged from 0.22 for Hirna to 0.36 for Gurage . Shannon information index ranged from 0.31 for Hirna to 0.52 for Gurage. Gelemso, Wendo, Matakesha and Bahir Dar showed almost the same gene diversity (0.36) and (0.34) respectively, (Table 4).

The least percentage of polymorphic band (52.59%), gene diversity (0.22) and Shannon information index (0.31) were scored by Hirna population. Gurage and Gelemso populations showed the highest gene diversity (0.366 and 0.362), PPB (82.5 and 85%), and Shannon index 0.51 and 0.52), respectively (Table 4).

Table 4. Measure of genetic diversity in the 10 population of *Catha edulis*

Pop	NPB	PPB	h ± SD	I ± SD	Gst*	Nm*
Gelemso	33	82.5	0.362±0.181	0.519±0.252	-	-
Matakesha	33	82.5	0.348±0.191	0.500±0.642	-	-
Wendo	33	82.5	0.349±0.178	0.505±0.249	-	-
Herna	21	52.5	0.220±0.222	0.318±0.316	-	-
Gurage	33	85	0.366±0.169	0.527±0.235	-	-
Leyu	30	75	0.303±0.204	0.440±0.284	-	-
Bahir Dar	32	80	0.345±0.190	0.495±0.266	-	-
Mean	30.71	77.14	0.327±0.191	0.472±0.320	0.251	1.488

Key: Nm estimate of gene flow from Gst. Nm=0.5(1-Gst)/Gst, h Nei’s gene diversity, I Shannon index, NBP number of polymorphic bands, PPB percentage of polymorphic bands

5.3. Genetic difference within seven khat populations

The result shows that Jaccard similarity coefficient ranged from 0.71 to 0.89. Gurage, Gelemso and Wendo are close populations with similarity coefficient of 0.89, 0.87 and 0.84 respectively. Hirna is the most distantly related population with 0.74 similarity coefficient (Table 5).

Table 5.Jaccard pairwise similarity between populations

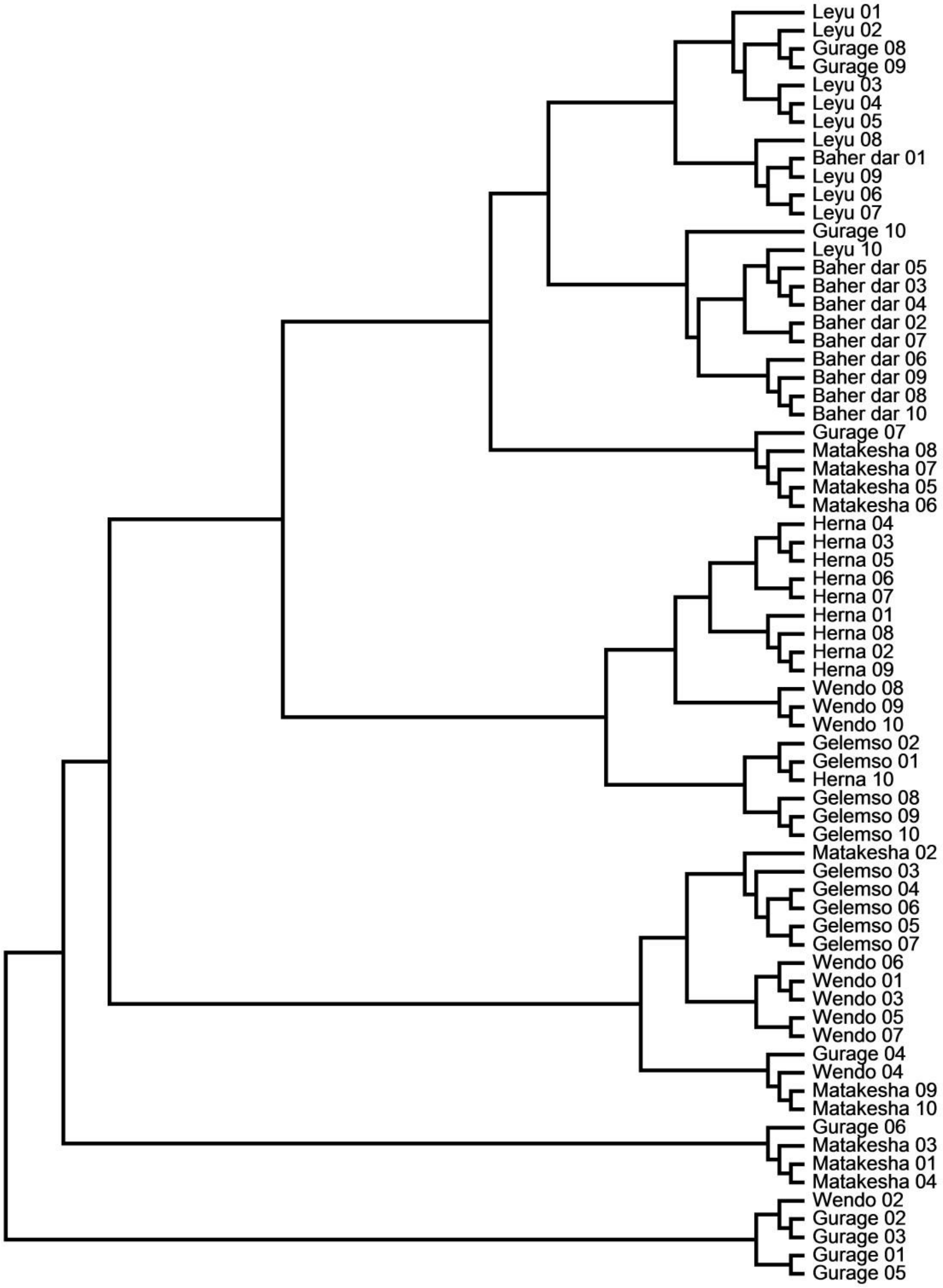
Pop ID	Gm	Mt	Wd	He	Gu	Le	Bd
Gm	****	0.8482	0.8728	0.7629	0.8392	0.8014	0.8424
Mt		****	0.8128	0.7191	0.8208	0.8192	0.8261
Wd			****	0.7402	0.8751	0.8437	0.8509
He				****	0.7446	0.7733	0.7415
Gu					****	0.8990	0.7983
Le						****	0.8078
Bd							****

Key:Gm, Gelemso, Mt, Matakesha, Wd, Wendo, He, Hirna, Gu, Gurage, Le, Leyu, Bd, Baher Dar

5.4. Clustering analysis of khat

UPGMA and Neighbor Joining tree construction methods was used to construct dendrogram for seven populations and 70 individuals based on 40 PCR bands amplified by five primers. The dendrogram derived from neighbor-joining analysis of the whole ISSR data with 70 khat populations showed three distinct clusters and two sub-clusters within each major cluster (Fig. 4).

The UPGMA-based dendrogram of 7 populations of *C. edulis* generated four distinct major clusters. The first top cluster contains populations of Gelemso; the second cluster contains Wendo, Baher Dar, Gurage ; the third cluster contains Hirna and Leyu. The final cluster contains Matakeha. The highest within-cluster was observed between Wendo and Bahir Darwhich are geographically distant from each other.


Leyu 01
Leyu 02
Gurage 08
Gurage 09
Leyu 03
Leyu 04
Leyu 05
Leyu 08
Baher dar 01
Leyu 09
Leyu 06
Leyu 07
Gurage 10
Leyu 10
Baher dar 05
Baher dar 03
Baher dar 04
Baher dar 02
Baher dar 07
Baher dar 06
Baher dar 09
Baher dar 08
Baher dar 10
Gurage 07
Matakesha 08
Matakesha 07
Matakesha 05
Matakesha 06
Herna 04
Herna 03
Herna 05
Herna 06
Herna 07
Herna 01
Herna 08
Herna 02
Herna 09
Wendo 08
Wendo 09
Wendo 10
Gelemso 02
Gelemso 01
Herna 10
Gelemso 08
Gelemso 09
Gelemso 10
Matakesha 02
Gelemso 03
Gelemso 04
Gelemso 06
Gelemso 05
Gelemso 07
Wendo 06
Wendo 01
Wendo 03
Wendo 05
Wendo 07
Gurage 04
Wendo 04
Matakesha 09
Matakesha 10
Gurage 06
Matakesha 03
Matakesha 01
Matakesha 04
Wendo 02
Gurage 02
Gurage 03
Gurage 01
Gurage 05

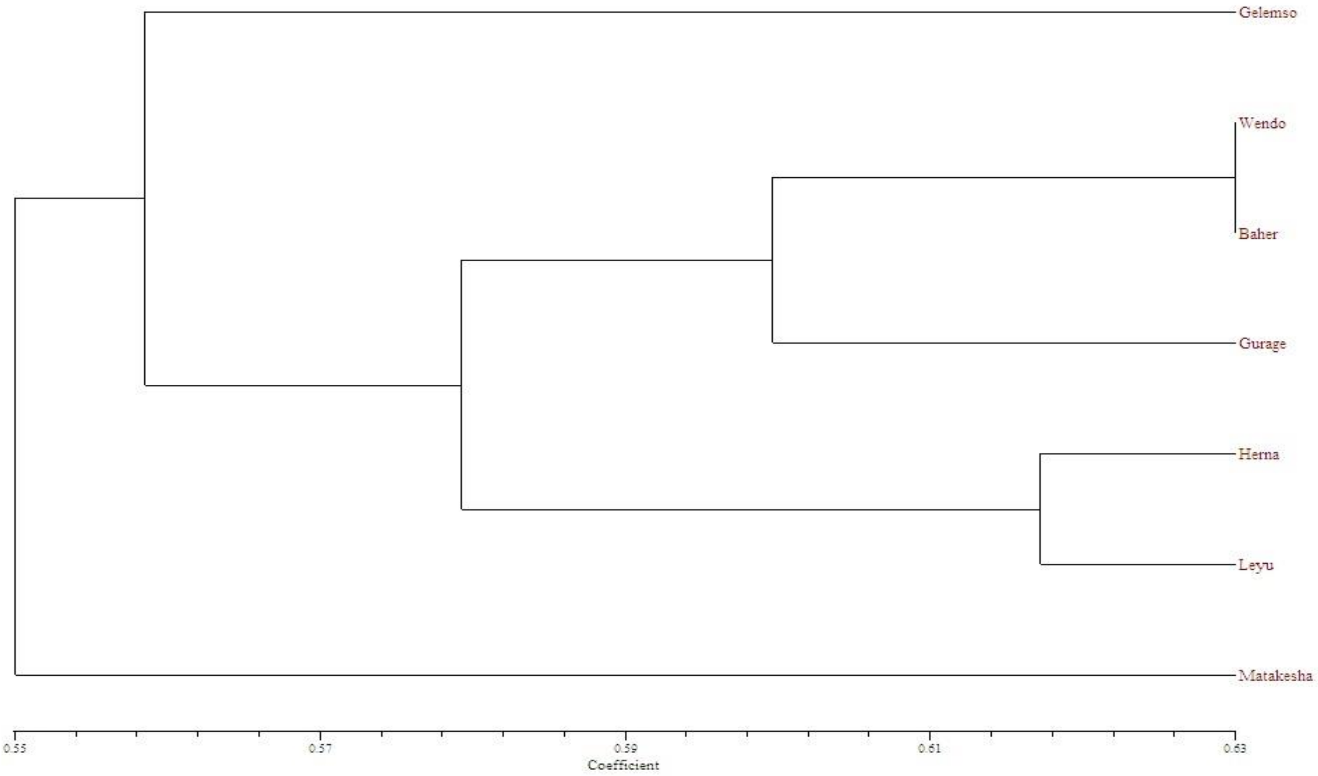


Fig. 5 UPGMA dendrogram for 7 populations of *C. edulis*

5.5. Results of AMOVA among seven khat populations

Analysis of molecular variance (AMOVA) indicated that a total of 79.13% within ($p<0.05$) and 20.86% among population with fixation index (Fst 0.208) shows 20.86 genetic differentiation among populations ($p<0.05$) (Table 5).

Table 6.AMOVA design and results among seven populations without grouping

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	6	133.400	1.61365 Va	20.93
Within populations	63	384.100	6.09683 Vb	79.07
Total	69	517.500	7.71048	
Fixation Index FST :		0.20928		

5.6. Principal coordinates analysis (PCOA) of khat

All the data obtained using the five ISSR primers were used in PCO analysis using Jaccard's coefficients of similarity. The first three coordinates of the PCO having Eigen values of 3.34, 2.44 and 1.92 with variance of 10.37%, 7.6% and 6.46%, respectively were used to show the grouping of individuals using two and three dimensional coordinates indicated in figures 3 and 4.

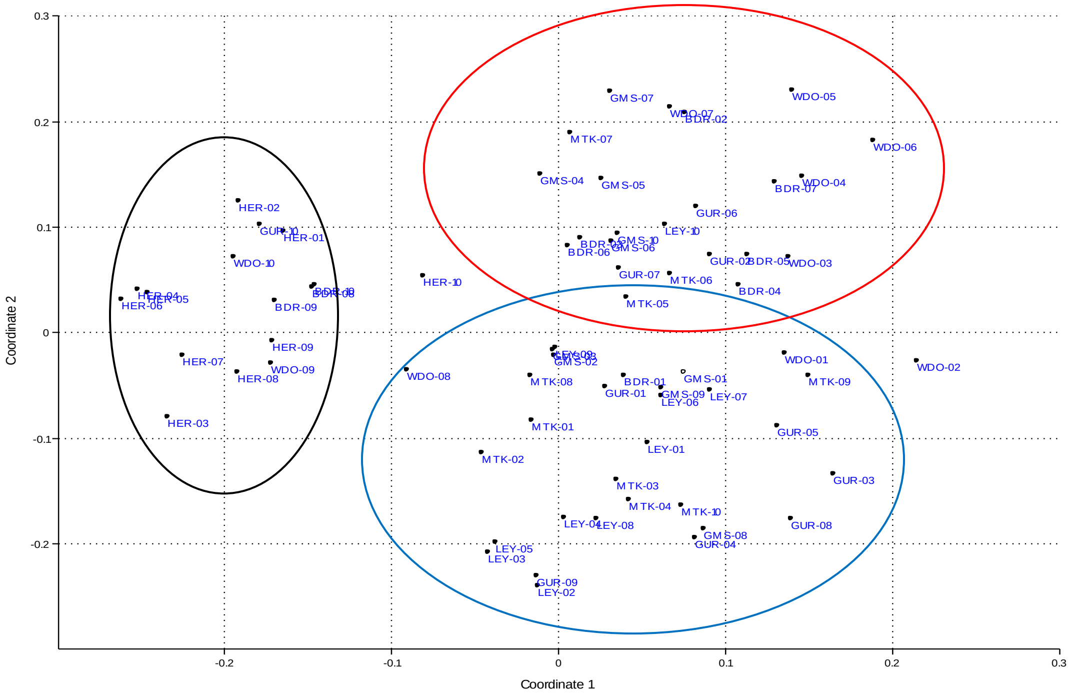


Fig. 6 Two-dimensional representation of principal coordinate analysis of genetic relationships among individuals of 7 *Catha edulis* populations using ISSR data

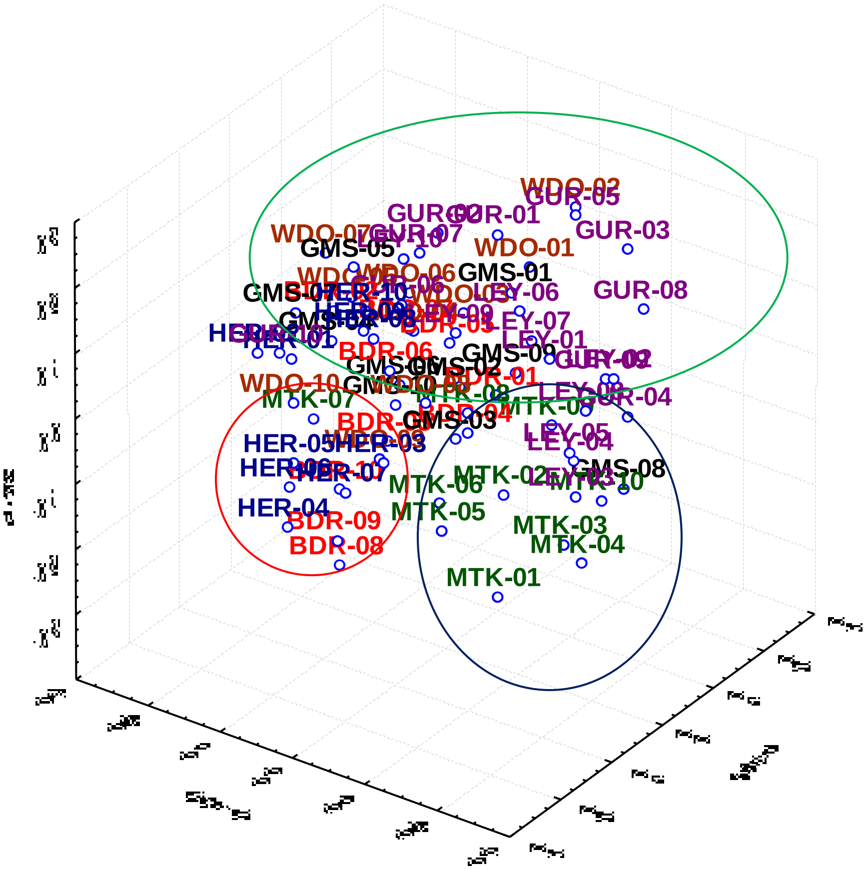


Fig. 7.Three-dimensional representation of principal coordinate analysis of genetic relationships among individuals of 7 *Catha edulis* populations using ISSR data

6. Discussion

The work presented here is the first to describe genetic variation among cultivated populations of *Catha edulis* in Ethiopia. The current study describes genetic diversity of *C. edulis* in Ethiopia. From best of our knowledge this is the first attempt *C. edulis* genetic diversity report using ISSR markers. Five ISSR markers generated Forty bands were out of which 34 of them polymorphic.

The mean gene diversity and Shannon information index for all five primers were 0.50 and 0.35, respectively. The result of gene diversity ranged from 0.22 to 0.36, close genetic diversity was reported in Yemn (Al-Thobhani&Sondur, 2008). It showed between 0.202 and 0.172 on a genotype Abyad (is it Ethiopian/Yemen Population...). The different gene diversity attained could be attributed to the use of different set of primers and khat cultivars under study.

Percentage of polymorphic bands, gene diversity (h), and Shannon information index (I) were highest for Gurage (85%, 0.366, 0.52), Gelemso (82.5%, 0.362, 0.51) and Wendo (82.5%, 0.34, 0.50), respectively. The lowest percentages of polymorphic bands, gene diversity and Shannon information index were shown by Hirna population (52.50%, 0.22, and 0.31) indicating that Hirna populations have uniform gene diversity (low genetic diversity). This low genetic diversity might be explained by the loss of wild relatives (National Research Council 1991) mainly anthropogenic destruction of natural habitats, existence of uniformity or monoculture possibly due to vegetative way of propagation prevailed in the area, homogeneity due to single or few seed sources during its introduction.

Wendo and Bahir Dar showed highest similarity but have longest geographical distance between them. However, Bahir Dar khat is recently introduction which is not more than thirty years. This might be due to Bahir Dar founding population taken from Wondo.

Molecular Variance (AMOVA) within and among 70 khat individuals genetic variations in the populations were compared. Percentage variations showed that there was genetic diversity among the seven khat populations. The results indicated that 79.1% of the total variation was within the populations, while 20.8% was among populations. It is reported a closely related result that observed 79.1% of total variation in Eritrean barley using SSR markers (Backeset *et al.*, 2009). It is reported similar huge differences in percentage variations between and among a group of khat genotypes studied using SSR markers (Curto*et al.*, 2013). And also related with result that observed 93 % of total variation in Kenya khat using SSR markers (Ngari *et al*., 2018) PCoA, NJ and UPGMA showed comparable results with strong cluster in majority of individuals of the populations. Populations that cluster in a one quadrant are closely related. Then populations that are located further to one another have less similarity than those that are closer. The parameters also have some intermixed individuals may be due to admixture that may be resulted from gene flow, through pollen or seed, migration of people from place to place, marketing of either seeds or vegetative parts. Hollingsworth and Ennos (2004) stated that grouping together of individuals from different populations in the same cluster of tree has been interpreted as evidence for gene flow between populations.

Mutations could be the origin of high genetic variation within the sample population (Epps& McCullough, 2005 and Alice *et al.,* 2016). On the other hand, sharing of the same ISSR profiles among the populations could account for the relatively low genetic variation among these khat populations. Also common ancestry of cultivars could contribute to low variability despite the fact that they are grown in different countries (Alice *et al.,* 2016).

7. Conclusion

Understanding of genetic diversity of crop facilitates efficient conservation and utilization of genetic resource. The result of the present study showed that the existence of genetic diversity in khat populations. Populations of Gurage, Gelemso and Wendo exhibited high genetic diversity compared to the other populations. Hirna population showed the lowest genetic diversity.

Analysis of molecular variance for the accessions studied showed that the highest proportion of genetic variation was attributed to within population than among populations.

Based on UPGMA data Gelemso clustered in one group, Matakesha clustered in one group, Leyu,Hirna and Gurage clustered in one group. Whereas Wendo and Bahir Darcrusted in one group they are geographically far from each other. Finally the study showed that the khat populations from Ethiopia did not cluster based on their geographical origin.

8. Recommendations

The study could help in conservation of genetically distinct *C. edulis* cultivars collected in different locations of Ethiopia. Due to financial limitation samples are collected in Addis Ababa, it is better further studies can be done on khat population by collecting samples from their place of growth. This would help to increase the accuracy of the result. Further studies on chemotypes and their correlations with morphological and molecular markers would help to understand the variation among khat. Understanding the spatial organization of genetic diversity within plant populations is of critical importance for the development of strategies designed to conserve genetic variation. More reliable and advanced marker systems such as genotyping by sequencing should be conducted on these khat cultivars to give more details on genetic diversity.

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