Genetic Diversity of Hot Pepper (*Capsicum annuum*) from Selected Areas of Ethiopia Using Inter Simple Sequence Repeats (ISSR) Marker

BY:

Alebachew Molla Nibret

A Thesis Submitted to Applied Biology Program in Partial Fulfillment of the Requirement of the degree of Master of Science in Biology (Biotechnology)

School of Applied Natural Science

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

June, 2018

Genetic Diversity of Hot Pepper (*Capsicum annuum*) from Selected Areas of Ethiopia Using Inter Simple Sequence Repeats (ISSR) Marker

BY:

Alebachew Molla Nibret

Advisor: Dr. Mulugeta Kebede

Co-Advisor: Dr. Kassahun Tesfaye

A Thesis Submitted to Applied Biology Program in Partial Fulfillment of the Requirement of the degree of

Master of Science in Biology (Biotechnology)

School of Applied Natural science

Office of Graduate Studies

Adama Science and Technology University

Adama, Ethiopia

June, 2018

APPROVAL SHEET

Adama Science and Technology University

School of Applied Natural Science

Applied Biology Program

This is to certify that the thesis prepared by Alebachew Molla Nibret entitled "**Genetic Diversity of Hot Pepper (*Capsicum annuum*) from Selected Areas of Ethiopia Using Inter Simple Sequence Repeats (ISSR) Marker"** submitted in partial fulfillment of the requirement of applied biology program for the award of master science in Biology (Biotechnology) complies with the regulation of the university.

Alebachew Molla Student Name	Signature	26/12/10 Date
Dr. Mulugeta Kebede Advisor	Signature	03-07-18 Date
Dr. Kassahun Tesfaye Co-Advisor	Signature	05-07-18 Date
Dr. Gelete Dugassa Internal examiner	Signature	03-07-18 Date
Dr. Wendaweke Abebe External examiner	Signature	05/07/18 Date
Department chairman/chairperson	Signature	05/07/18 Date
School Dean	Signature	01-07-18 Date
Postgraduate Dean	Signature	Date

DECLARATION

I hereby declare that this M.Sc. thesis is my original work and has not been presented for a degree in any other university, and all sources of material used for this thesis have been duly acknowledged.

Name: Alebachew Molla Nibret

Signature: -

This M.Sc. thesis has been submitted for examination with my approval as thesis advisor.

Name Dr- Mulugeta Kebede

Signature:-

Date of submission 05-07-18

ACKNOWLEDGEMENTS

I want to praise the almighty God for all my achievements. Next I would like to gratefully acknowledge my major Advisor Dr. Mulugeta Kebede for his strong commitment, enthusiasm and paternal approach during the research. I am also grateful to my co-Advisor Dr. Kassahun Tesfaye, for his follow up from the beginning to the end of this work and for his continuous technical advice during laboratory session and provision of expensive laboratory materials, enzymes and chemicals.

I extend my deep gratitude to Debre Zeyit Agriculture Research Center for their cooperation and providing me with all the necessary facilities for Greenhouse work of this study. Moreover, I am indebted to Adama Science and Technology University for giving me the opportunity to join the post graduate program and provision of financial support. Also, I would like to thank Institute of Biotechnology, Addis Ababa University, for accepting me to do my thesis experiment in Plant genetics research laboratory and provision of all facility during this study. I would like to thank Ethiopian Institute of Biodiversity Conservation for providing germplasms with full passport data of *Capsicum annuum* assembled from Ethiopia.

My heartfelt appreciation also goes to Dr. Fasil for his support during the Green house work of this study. Hawi Nigusse, Gizachew woldesembet and Birhanu Kumbi are acknowledged for their limitless technical assistance during the laboratory works.

Finally, I am grateful to extend my deepest and heartfelt gratitude to my parents Ato Molla Nibret and W/ro Birhan Gelanew, my brothers, sisters and close relatives, for their care, support, encouragement, experience sharing and for all what they did for me. Their all rounded and unconditional moral support enabled me to realize my educational goal.

Table of content

Contents **Page**

LIST OF TABLES

LIST OF TABLES IN THE APPENDIX

Tables in the appendix page

LIST OF FIGURES

LIST OF FIGURES IN THE APPENDIX

Appendix figure page

LIST OF ACRONYMS AND ABBREVIATIONS

CAPS	Cleaved Amplified Polymorphic Sequence
DGGE	Thermal Gradient Gel Electrophoresis
DNA	Deoxy Ribonucleic Acid
EEPA	Educational Evaluation And Policy Analysis
H	Gene Diversity
HPLC	High Performance Liquid Chromatography
I	Shannon Index Information
IAR	International Agricultural Research Center
IBC	Institute of Biodiversity and Conservation
IBPGR	International Board of Plant Genetic Resource
IPGRI	International Plant Genetic Resource Institute
ISSR	Inter Simple Sequence Repeat
M.A.S.L	Meter Above Sea Level
MARC	Melkassa Agricultural Research Center
NJ	Neighbor-joining
NPL	Number of Polymorphic Loci
NTSYS	Numerical Taxonomy and Multivariate Analysis System
PCR	Polymerase Chain Reaction
PP	Percent Polymorphism
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SD	Standard Deviation
SNNPR	South Nation, Nationality and Peoples Region
SSCP	Single Strand Conformational Polymorphism
SSR	Simple Sequence Repeat
STMS	Sequence Tagged Microsatellite
TGGE	Thermal Gradient Gel Electrophoresis
UBC	University of British Colombia
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

ABSTRACTS

Hot pepper (Capsicum annuum) is the most important vegetable crop belonging to the Solanaceae family. Despite its economic importance, little research has been undertaken on genetic characterization of hot pepper germplasm and cultivars. Genetic diversity of 53 hot pepper (Capsicum annuum) accessions was studied using Inter Simple Sequence Repeat (ISSR) markers at plant Genetics research Laboratory of Addis Ababa University, Ethiopia. The main objective of this study was to investigate the genetic diversity among the Ethiopian hot pepper germplasm collections using ISSR markers. Genomic DNA was extracted from 53 accessions of individuals using CTAB extraction method. A diluted genomic DNA was subjected to PCR amplification. Two di-nucleotide and two penta nucleotide repeat primers amplified a total of 29 clear and reproducible bands. All bands were 100% polymorphic. Both UPGMA dendrogram and neighbor joining (NJ) trees were constructed for the individual cultivars using Jaccard's similarity coefficient. The dendrogram clearly indicated two major clusters and three sub-clusters. The genetic diversity among C. annuum populations and cultivar groups considered in the present study indicated that Mirab Gojjam population exhibited a genetic diversity value of 0.31 and Shannon diversity index of 0.47, while the Gurage, Kembata, Bale and Semen Gondar population of C. annuum showed 0.26, 0.22, 0.12 and 0.11 for genetic diversity and 0.39, 0.33, 0.19 and 0.17 Shannon diversity index values respectively. Generally Mirab Gojjam population showed higher genetic diversity but Semen Gondar population showed least genetic diversity. Furthermore Analysis of molecular variance (AMOVA) demonstrated highly significant (p=0.0) genetic diversity within populations (55.3%) than among populations (44.7%). Generally on the basis of bands generated by all primers, ISSR revealed high levels of genetic diversity within and among population of Ethiopian C. annuum. Further study of ISSR using more geographic area coverage and primers is recommended to obtain confirmative results.

Key words: *C. annuum*, Genetic diversity, ISSR marker, Genomic DNA, Polymorphism

1. INTRODUCTION

1.1. Background Justification

Hot pepper (*Capsicum annuum*) is the most important vegetable crop belonging to the *Solanaceae* family and originated in the new world tropics and subtropics (Mexico, Central America and Andes of South America) over 2000 years ago (Rodriguez *et al.,* 2008). The genus *Capsicum* consists of 22 wild species and five domesticated species, which include C. *annum,* C. *frutescens*, C. *chinenses*, C. *baccatum*, and C. *pubescens* (Bosland and Votava, 2000). Species *Capsicum annum* is closely related with species *C. frutescens* and *C. chinenses* which are widely grown in the Americas and worldwide. *C. annum* has been domesticated in the highlands of Mexico, and includes most of the Mexican hot peppers, most of the hot peppers in Africa and Asia and the various cultivars of sweet peppers grown in temperate countries. *C. frutescens* and *C. chinenses* are cultivated in Africa and Asia as spice crop, as intact fruits or for their oleoresin content and the other two species namely *C. baccatum* and *C. pubescens* are predominantly confined to Latin America (Pickersgill, 1997). *C. annuum* was introduced to Ethiopia by the Portuguese in the 17^{th} century (Haile and Zewde, 1989).

Hot Pepper is grown in many countries of the world and its production for cooking and vegetable uses has been increased from time to time. In Ethiopia today, it is extensively produced and considered as a national spice. Even though the exact time of introduction of pepper not certainly known, it has been cultivated in Ethiopia for long period of time. In Ethiopia, pepper grows under warm and humid weather conditions and the best fruit is obtained in a temperature 21-27^0C during the day time and 15-20^0C at night (IAR, 1996). It is extensively grown in most parts of the country, with the major production areas concentrated at altitude of 1100 to 1800 m.a.s.l. (MOARD, 2009). Hot pepper covers 67.98% of all the area under vegetables in Ethiopia (CSA, 2011/2012).

Hot pepper is one of the major vegetable crops produced in Ethiopia and the country is one of a few developing countries that have been producing paprika and *C. oleoresins* for export market. Because of its wide use in Ethiopian diet, hot pepper is an important traditional crop mainly valued for its pungency and color. The average daily consumption of hot pepper by Ethiopian adult is estimated 15 g, which is higher than tomatoes and most other vegetables (MARC, 2004). According to Lin *et al*. (2013), uses of pepper are generally grouped into five broad market categories: (i) fresh market (green, red, multi-color whole fruits), (ii) fresh processing (sauce, paste, canning and pickling), (iii) dried spice (whole fruits and powder), (iv) industrial extracts (paprika, oleoresin, capsaicinoids and

carotenoids) and (v) ornamental (plants and fruits). According to the EEPA, (2003), in the major pepper producing regions of Ethiopia which are Amhara, Southern Nations and Nationality People's Regional State (SNNPR) and Oromia pepper generated an income of 122.80 million Birr for farmers in 2000/01. This value jumped to 509.44 million Birr for smallholder farmers in 2004/05. This indicates that hot pepper serves as one of the important sources of income to smallholder farmers and as exchange earning commodity in the country (Beyene and David, 2007).

Pepper is a dicotyledonous small shrub in suitable climatic conditions and more cultivated in the tropical South and Central America. Conventionally, morphological descriptors like plant height, flower color, fruit length and fruit shape and seed characteristics are routinely used to distinguish hot pepper genotypes (Sitthiwong *et al*., 2005). In the last century, morphological and biochemical based genetic profiling has been used to study genetic diversity within the genus *Capsicum.* However, these methods suffer from many discrepancies like, influence of environment on morphological trait. Inter simple sequence repeat markers are efficient than morphological or biochemical markers, since ISSR markers do not require previous knowledge of DNA sequence to design primers, the technique is simple and low cost (Sitthiwong *et al*., 2005).

Many research groups have examined genetic diversity in the *C. annuum*, using restriction fragment length polymorphism (RFLP) (Prince *et al*., 1995), RAPD technique for analyzing genetic diversity (Thul *et al*., 2011) genetic distance (Sanatombi *et al*., 2010), genetic relationship (Ince *et al*., 2010), phylogeny (Adetula *et al*., 2006) and amplified fragment length polymorphisms (AFLP) (Toquica *et al*., 2003) in region specific accessions and landraces (Makari *et al*., 2009) and national germplasm repositories. There is scanty information on the use of ISSR markers for examining genetic diversity of hot pepper varieties cultivated in Ethiopia.

1.2. Statement of the problem

In spite of its importance, the hot pepper production system for green and dry pod has stayed as low input and low output with a national average yield of 7.6 t/ha for green pod whereas it was 1.6 t/ha for the dry pod respectively (CSA, 2006). The decline of hot pepper production is also attributed to poor varieties, poor cultural practices, the prevalence of fungal (blights) and bacterial as well as viral diseases (Fekadu and Dandena, 2006).

The assessment of genetic diversity on the base of morphological traits is not very reliable, as it may be influenced by the environmental factors and the number of traits with known inheritance is small. Molecular markers have the distinct advantage of being independent of climatic variables and very numerous traits and one of the most convenient and popular methods to identify and study of intraspecific genetic polymorphism is inter simple sequence repeat (ISSR) PCR based technology (Grishin *et al*., 2011). The variability of detailed information about the production system, genetic status and genetic potential are the major limitations of Ethiopian hot pepper varieties. Evaluations of the genetic diversity are extremely fundamental to understand the level and distribution of diversity in the species and for the design of improvement and appropriate conservation strategies (Endashaw, 1985). Despite its economic importance, little research has been undertaken on genetic characterization of hot pepper germplasm and cultivars. Information about genetic diversity and germplasm characterization is important for any breeding program and conservation.

1.3. Objectives of the study

- **General objective**

  - The general objective of the study was to investigate the genetic diversity of some Ethiopian hot pepper collections using inter simple sequence repeat markers.

- **Specific objectives**

The specific objectives of this study were:

- To determine the level and pattern of distribution of variations within and among the overall collection of hot pepper germplasm.
- To identify populations and regions with higher diversity for genetic improvement and conservation.
- To compare inter-population variability within Ethiopian hot pepper population.

1.4. Significance of the study

The result of this study:

- Will provide information on the general genetic variability of *C. annum* accessions which may assist in the identification and selection of the genetic materials for conservation and improvement for different regions of Ethiopia.
- Can indicate the possible application of inter simple sequence repeat markers to genetic diversity of *C. annuum.*
- Can be used as a reference point for other researchers to do similar works.

2. LITERATURE REVIEW

2.1. Origin and Distribution

The origin of *Capsicum annuum* is extended from Mexico in the North to Bolivia in the South of Latin America, where it has been part of human diet since about 7500BC (Purseglove *et al.*, 1981). Spanish and Portuguese explorers spread pepper around the world. Pepper was introduced to Spain in 1493, England in 1548 and Central Europe in 1585. From Europe it went to Asia. Currently the crop is produced in various countries around the world including India, China, Pakistan, Indonesia, Sri Lanka, Thailand and Japan in Asia, and Nigeria, Uganda and Ethiopia in Africa, India and Indonesia were the largest producers and currently China is the main producer and exporter in the world (Yemane, 2017).

Capsicum annuum is produced in all the continents except Antarctica. In Antarctica there are stories about pepper being kept in flower pots to spice up their food. This is reflected in the large lands devoted to their production in such countries as India, Mexico, China, Korea, USA and Africa. In addition, interest in both sweet and pungent types of peppers is growing in many countries not traditionally associated with spicy cuisine; protected culture has developed in northern latitude countries such as Holland and Canada and also in Mediterranean countries such as Spain, and Israel, to supply the increased demand (Wien, 1997). In Ethiopia, pepper is cultivated in many parts of the country and it is an important source of cash earning for smallholder producers both in green and dry forms.

2.2. Taxonomy and Morphology

The genus *Capsicum* to which pepper belongs is a member of the *Solanaceae* family that consists of about 22 wild species and five domesticated species. *C. annuum* species can be divided in to several groups depending on their fruit characteristics ranging in pungency, color, shape, intended use, flavor, and size. Despite their vast trait differences, most commercially cultivated peppers in the world belong to the species *C. annuum*, which is the most common species cultivated in Ethiopia (Amare**,** 2013). *C. annuum* is commercially classified by the concentration of capsaicin ($C_{18}H_{27}NO_3$) which determines a variety of 'hotness". *C. annuum* species are diploid and most having 12 chromosome number (2n=24). But recent studies indicated the chromosome number for non-pungent (wild) species is (2n=26) (Tong and Bosland, 2003). They vary in size, shape, color, flavor and degree of hotness, from mild to very hot.

Capsicum annuum have a solitary (single) flower that starts at the axils of the first branching node with subsequent flowers forming at each additional node. Flower differentiation is not affected by day length, but the most important factor determining differentiation is air temperature, especially at night. The *Capsicum* flower is complete, bisexual, hypogenous and usually pentamerous (Bosland and Votava, 2000). Depending on the environmental conditions and variety, the period of receptivity of the stigma is 5-8 days, from several days before flower to fewer days afterwards, with maximum fertility on the day of flower period. The most actively growing organ of hot pepper plant after flowering is the fruit. The fruit is ordinarily seeded, but parthenocarpic forms do exist. The seed set affects development and subsequent growth of the fruit. On average there is a direct linear relationship between the number of seeds per fruit and final fruit size, until saturation at perhaps over 200 seeds per fruit (Marcel *et al*., 1997). Typically cultivated fruit reaches the mature green stage in 35-50 days after the flower is pollinated. The fruits are characterized as non-climacteric in ripening (Bosland and Votava, 2000).

2.3. Cultivation and importance of hot pepper

Hot peppers like most other plants, prefer well drained, moisture holding loam soil (sandy loam) containing some organic matter. A pH of 6.5-7.5 is suitable and the land should be level to 0.01- 0.03 % slope to allow adequate drainage and prevent root diseases. Adequate water supply is essential. Water stress can cause abscission of fruit and flowers, especially when it occurs during flowering and reduces yield through reduced pollination. The extreme case can result in increased risk of diseases. Poorer soil types and water stress are believed to produce lower yields (Haigh *et al.,* 1996). The potential areas in the country for *C. annuum* production is estimated to be about 59,991 hectares of land with the total production of 72,466 tone for dry pod and 4783 hectare of land with production of 44,273 tones for fresh pod (CSA, 2006).

According to Salter (1985), their production and consumption have steadily increased worldwide during the 20th century due to their roles as both vegetable and spices. Pepper is the world's second important vegetable ranking after tomatoes and it is the most produced type of spice flavoring and color to food while providing essential vitamins and minerals. The nutritional value of hot pepper merits special attention. It is a rich source of vitamin A and E. Both hot and sweet peppers contain five to six times as much vitamin C as an orange or a lemon and this fact makes pepper ideal to prevent flu colds more than any other vegetable crop (Bosland and Votava, 2000). *C .annuum* fruits are consumed as fresh, dried or processed, as table vegetables and as spices or condiments as it increases the acceptance of the colorless basic nutrient foods. Oleoresin and paprika are the two

important industrial extracts of hot pepper. In many households, pepper provides the only needed flavor to enhance intake of otherwise bland diets. The range of food products that contain pepper or its chemical constituent is broad, and it includes ethnic foods, meat, salad dressings, mayonnaise, dairy products, and candies, packed foods, slack foods, salsa, and hot sauces. Rubatzky and Yamaguchi (1997) pointed out in addition to their uses as food, uses for cosmetic production, condiment and medicine.

According to Bosland and Votava (2000), hot pepper is the most recommended tropical medication for arthritis. The pharmaceutical industry uses capsaicin as a counter-irritant balm (cream), for external application of sore muscles. Creams containing capsaicin have reduced pain associated with postoperative pain for mastectomy patients and for amputees suffering from phantom limb pain. Prolonged use of the cream has also been found to help reduce the itching of dialysis patients, the pain from shingles and cluster headaches. It is not only their nutritional quality and medicinal value that makes peppers an important food crops, but hot pepper also stimulate the flow of saliva and gastric juices that serve in digestion. It has been said that pepper raises body temperature, relieve cramp, stimulate digestion, improve the complexion, reverse inebriation, cure a hangover, soothe gout and increase passion. On the other hand among its many modern innovative uses, it has been tried to use it as a barnacle repellent. For example, anti-mugger aerosols with chilies pungency as the active ingredient have replaced mace and tear gas in more than a thousand police departments in the United States. The spray will cause attackers to gasp and twitch helplessly for 20 minutes (Bosland and Votava, 2000).

2.4. Plant Genetic Diversity

Genetic diversity is defined as the extent to which heritable material differs within a group of plants, while genetic differentiation is defined as the extent to which heritable material differs between groups of plant. It is the result of evolution, including domestication and plant breeding. The processes of natural evolution resulted in a buildup of genetic diversity in natural populations whereas domestication caused further differentiation of small parts of the diversity of wild species, which became adapted to human requirement and natural adoption. Genetic diversity can be assessed at four levels of organization: among species and among populations, within populations and within individuals. Subdividing the variation into its components may assist in genetic conservation and utilization, and establishment of *in situ* gene conservation (Endashaw, 1985).

Several research results showed that studying the extent and patterns of distribution of genetic variation of a crop species is essential for effective utilization of germplasm in plant breeding programs, devising appropriate sampling procedures for germplasm collection and conservation, obtaining core collections for efficient germplasm management and elucidating the taxonomy, evolution and origin of crop species. Hence, knowledge of genetic diversity and relatedness in germplasm is needed for the crop improvement programs, management and evaluation. Furthermore, knowledge about genetic diversity and population structure is a good baseline for formulating effective conservation plans and can often provide novel conservation- relevant site, where an effective conservation strategy for species can be made only after detailed population genetic information becomes available (Avise, 1994). Genetic variation can be measured by using different genetic markers.

2.5. Understanding diversity within germplasm collections

Understanding the diversity and specific traits of each accession allows selection of more appropriate genotype to much environmental and livestock production system and support increased use available genetic resources. Standardized observation strategies have been tested for morphological characterization of forages and descriptors have been proposed for forge species to cover passport and agronomic characterization data. However, more general descriptors are available for genetic marker technologies. Data from morphological, nutritional and genetic characterization can be combined to find similar or dissimilar accessions. Once similar groups identified individual dissimilar accessions could be selected and designated as a core collection, which is defined as a limited set of accessions that represent the genetic diversity in the whole collection and extremely useful to rapidly screen a species to potential use from few accessions in a cost effective manner (Pengelly, 2001).

3.6. Loss of Plant Genetic Diversity

The disappearance of the genetic resources is taking place at an alarming rate more particularly in the last two to three decades (Gole *et al.*, 2003). Such loses occur due to deforestations, habitat destructions, development activities, modern agriculture, intentional and accidental introduction of exotic plants and animals, the consequence of which have been aggravated in the tropical countries where the livelihood of the majority of the peoples are dependent on the existing genetic diversity (Gole *et al.*, 2003)

2.7. Conservation Methods for Plant Genetic Resource

Evaluations of the genetic diversity are extremely fundamental to understand the level and distribution of diversity within a species and for the design of appropriate conservation strategies. No single conservation approaches can be applied to conserve the full range of plant genetic resources of the target gene pool or species effectively. Although, there are various conservation methods, essentially two main conservation strategies can be applied for plant genetic resource conservation namely: *in-situ* and *ex-situ* conservation (Endashaw, 1985).

The *in-situ* conservation involves maintaining and conserving the viable genetic resources in their natural habitats where the dynamic process of evolution of the species is occurring and increase the genetic diversity of species to be conserved. It is the methods of choices for conserving germplasm of wild forest species and wild relatives and their cultivars in the natural environments where the target species are under natural evolutionary forces. Moreover it is the vital mechanism of storing the world's genetic diversity or gene pool of important crop plants and their wild relatives for future potential use or vice versa. This, in turn, is crucial to meet the world's future development needs in terms of improving productivity of crops (IPGRI, 2005).

Ex-situ conservation approach on the other hand is a mean of conservations biodiversity from threats of destructions, replacement or deterioration. *Ex-situ* conservation involves storage of seeds at low temperature and low moisture contents in the seed banks of the species, or as living in the seed gene banks or botanical gardens. Besides, pollen storage and DNA bank, tissue culture and cryopreservation also provides another means of indirect *ex-situ* conservation of plant germplasm. However, Karp *et al.* (1997) suggested the use of the combinations of both conservation methods for effective conservation of genetic resources.

2.8. Markers and their Application in Genetic Diversity

Biological markers could have many different types but mainly classified into morphological, biochemical and molecular marker. The morphological (classical or visible, agronomic traits) markers are phenotypic traits, while; biochemical markers are isozymes, and are based on gene product. The DNA (molecular) markers which reveal sites of variation in DNA are 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 (Semagn *et al.,* 2006).

2.8.1. Genetic diversity using morphological markers

Morphological markers are usually visually described phenotypic characters such as flower color, seed shape, growth habits or pigmentation. Morphological markers are phenotypic markers with distinguishable trait that have evident to human eyes (Bagali *et al.*, 2010). Morphological characterization does not require expensive technology, but large tracts of land are often required for these experiments, making it possibly more expensive than molecular markers assessment. These traits are often susceptible to phenotypic plasticity conversely; this allows assessment of diversity in the presence of environmental variation. Although phenotypic traits cannot be reliable measures of genetic differences because of the influence of the environment on gene expression, it is appropriate to mention salient traditional approaches employed for assessment of variability (Singh *et al.*, 2010).

Phenotypic variation is positively associated with genetic diversity, but it is also dependent on environmental factors as well as on the interaction between genotypes and environment. Morphological characters may be unstable due to environmental influences; therefore methods to assess and detect genetic diversity have extended from analysis of discrete morphological traits to biochemical and molecular traits (Muthusamy *et al.*, 2008).

2.8.2. Genetic Diversity Using Biochemical Markers

Biochemical markers are differences in enzymes that are detected by electrophoresis and specific staining. Biochemical analysis is based on the separation of proteins in to specific banding patterns. It is a fast method which requires only small amount of biological material; however only a limited number of enzymes are available and thus, the resolution of diversity is limited (Mondini *et al.*, 2009).

The number of polymorphic enzymatic systems available is limited and the enzymatic loci present only a small and not random part of the genome (the expressed part), therefore the observed variability may not be representative of the entire genome, although it allows large numbers of samples to be analyzed, comparisons of samples from different species, loci, and laboratories are problematic, since they are affected by extraction methodology, plant tissue, and plant storage (Mondini *et al.*, 2009).

2.8.3. Genetic Diversity using Molecular Markers

Molecular genetic markers are regions in the genome that are heritable as simple Mendelian traits, are easy to document (Schulman *et al*., 2004) and can be used to quickly detect genetic variation. Genetic variation results from differences in the DNA sequences which may have arisen due to mutations caused by insertion, deletion, duplication or inversion of DNA fragments. They may be 'functional' causing changes in metabolic or phenotypic traits or 'neutral' when not subjected to positive, negative or balancing selection (Marsjan and Oldenbroek, 2007).

Molecular or DNA markers are relatively recent but most strong tools for genetic diversity analysis. It overcome most of the shortcomings of morphological and biochemical markers. When compared with other markers systems, DNA based diversity estimates were shown to reflect the actual differences between genotypes, relatively simple to detect, abundant throughout the genome, completely independent of environmental conditions and can be detected at any stage of plant development (Barrett and Kidwell, 1998). DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers. Molecular markers are commonly used to characterize genetic diversity within or between populations or groups of individuals because they typically detect high levels of polymorphism. The DNA based marker systems are generally classified as hybridization-based (non-PCR) markers and PCR based markers (Joshi *et al*., 1999).

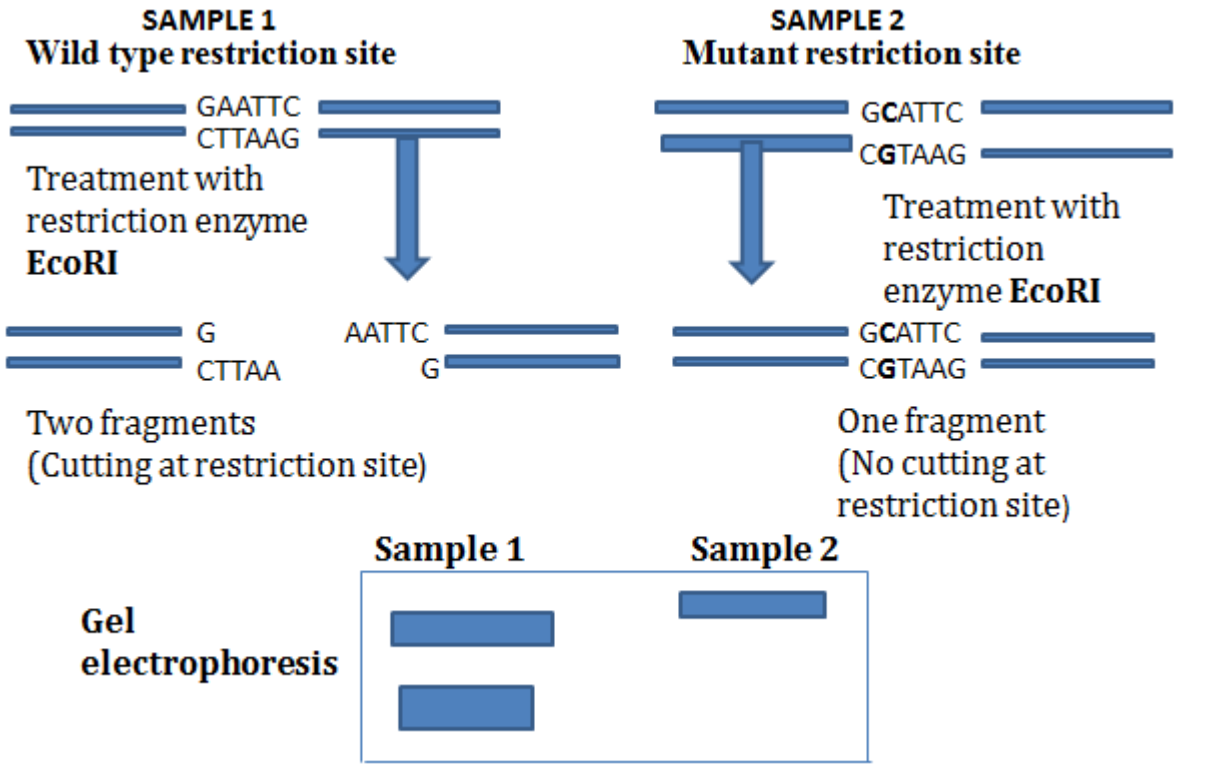
2.9. Non-PCR based Molecular markers

The two main non-PCR-based techniques are restriction fragment length polymorphism (RFLP) and microarray technology. RFLP are co-dominant markers and are more informative than dominant markers. However, this technique requires relatively large amounts of purified and high molecular weight DNA, is time consuming and laborious (Said Mohammed, 2012).

2.9.1. Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is DNA markers based on the premise that some DNA molecules in a population contain a particular restriction site, whereas others lack it because of mutation. This polymorphism is shown by placing highly purified nuclear DNA in contact with an enzyme recognizing and restricting a sequence of 4-8 nucleotides. Fractionating the fragments on a gel and doing a southern blot allows hybridization of a radio labeled or fluorescent probe (Figure 1). The probe will reveal a pattern of DNA fragments based on the number of restriction sites present or absent along its sequence or flanking sequence (Hartl, 2000).

The analysis involves digestion of complex genomic DNA into small DNA fragments using restriction enzyme such as restriction endo nucleases that recognize and cut specific short sequences of DNA followed by separation of these fragments by gel electrophoresis. They differentiate genotypes based on mutations in endo nuclease restriction sites across the genome in addition to genome rearrangements. However, the low level of DNA polymorphism exhibited by RFLP markers, time consumption, laborious, expensive and its requirement of radioactively labeled probes limited its application and for these reasons many researchers have turned to PCR based methods (Reiter, 1994).



:

Figure 1: Principle of restriction fragment length polymorphism (RFLP)

2.9.2. Microarray Technology

A microarray is a pattern of ssDNA probes which are immobilized on a surface (called a chip or a slide). The probe sequences are designed and placed on an array in a regular pattern of spots. The chip or slide is usually made of glass or nylon and is manufactured using technologies developed for silicon computer chips. Each microarray chip is arranged as a checkerboard of 105 or 106 spots or features, each spot containing millions of copies of a unique DNA probe. Like Southern & northern blots, microarrays use hybridization to detect a specific DNA or RNA in a sample. But whereas a Southern blot uses a single probe to search a complex DNA mixture, a DNA microarray uses a million different probes, fixed on a solid surface, to probe such a mixture. The exact sequence of the probes at each feature/location on the chip is known. Wherever some of the sample DNA hybridizes to the probe in a particular spot, the hybridization can be detected because the target DNA is labeled (and unbound target is washed away). Therefore one can determine which of the million different probe sequences are present in the target. Additionally, the amount of signal directly depends on the quantity of labeled target DNA. Thus microarrays can give a quantitative description of how much of a particular sequence is present in the target DNA. This is particularly useful for studying gene expression, one common application of microarray.

These novel functional molecular biology technologies are extraordinary tools to simultaneously monitor different mutations in the genome, monitoring all gene activities in one experiment, and analyze protein expression differences between diverse biological samples in a comparative way at different levels: genome, transcriptome or proteome. The basic role of functional biology is to identify new genes and gene functions, explore new regulatory networks involved in different cellular processes (Ágnes *et al*., 2015).

Diversity Arrays Technology is a microarray-based DNA marker technique for genome-wide discovery and genotyping of genetic variation. Diversity arrays technology allows simultaneous scoring of hundreds of restriction site based polymorphisms between genotypes and does not require DNA sequence information or site-specific oligonucleotides. There is no doubt that the DNA microarray is a powerful tool that has revolutionized the field of genetic diversity. A single hybridization can quickly yield vast quantities of data on the relatedness of one organism to another, at a single base-pair resolution. Microarrays offer a comprehensive and unbiased approach to analyzing diversity and permit observations that would be overlooked with established techniques where only small regions of a given genome are investigated (Claire and Elizabeth, 2005).

2.10. PCR based molecular markers

There are a variety of PCR based marker systems currently available for the genetic diversity assessment. The introduction of PCR based methods enables investigators to overcome the limitations of probe hybridization based methods and it constituted a new milestone in the field of DNA fingerprinting using molecular markers. With the development of the polymerase chain reaction (PCR), many PCR based molecular techniques have been, and still are being developed for plant genome analysis. The techniques could be categorized in to two main groups: arbitrary (or semi arbitrary) primed techniques and site targeted PCR techniques (Karp *et al*., 1997).

Techniques in the first category use primers which are designed arbitrarily or semi arbitrarily, i.e., without the knowledge of flanking sequence of the region to be amplified. These techniques includes Random Amplified Polymorphic DNA (RAPD) which was introduced in 1990 (Williams *et al*., 1990), Inter Simple Sequence Repeats (ISSR) (Zietkiewicz *et al*., 1994), Simple sequence repeat (SSR) and Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995). These are also called dominant molecular markers. These classes of dominantly expressed markers (RAPD, AFLP and ISSR) are multi-locus, and scattered throughout the entire genome.

Techniques in the second category depend on primers that target a single known site, such as a gene or known segments. These are alternative approaches to multi-locus profiling. Microsatellites (SSR), PCR-DNA sequencing, sequence-tagged microsatellite (STMs), thermal gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE), cleaved amplified polymorphic sequences (CAPS), single strand conformational polymorphisms (SSCP), are important examples of these categories. Since they give information on a single locus, they are particularly important when information is required on gene frequency or genealogical information for genetic diversity management and when information on heterozygosity is required.

PCR utilizes oligonucleotide primers as priming sites for polymerase from *Thermus aquaticus* to begin the *in vitro* amplification of DNA. Using nucleotides, magnesium, and repeated heating and cooling cycles to copy a small segment of the genomic DNA, one molecule of template DNA can be amplified into microgram quantities within hours. PCR itself is technically simple, because reagents are easily mixed and heating is done by a programmed machine. Primer information can be PCR based markers such as RAPD, ISSR, AFLP and SSR which are superior to RFLP in that they show high diversity even in autogamous crops with a narrow genetic base like soybean and wheat (Russel *et al*., 1997).

2.10.1. Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) is the first PCR-based molecular markers to be employed in genetic variation analysis (Schierwater and Ender, 1993). This marker is first introduced by Williams *et al.* (1990). This technique is based on the use of short, arbitrary primers in PCR reaction and can be used to produce relatively detailed and complex DNA profiles for detecting amplified fragment length polymorphisms between organisms. The RAPD primers are very sensitive to PCR conditions and this may lead to poor reproducibility (Figure 2). It is a type of PCR reaction, but the segments of DNA that are amplified are random. RAPD markers are useful DNA based method for initial assessment of genetic variation, especially the assessment of genetic diversity in plant species (Song, 2005). Because of its simplicity, RAPD technology is being extensively used in genetic variability analysis of various plant and animal species (Beiranvand *et al.*, 2011).

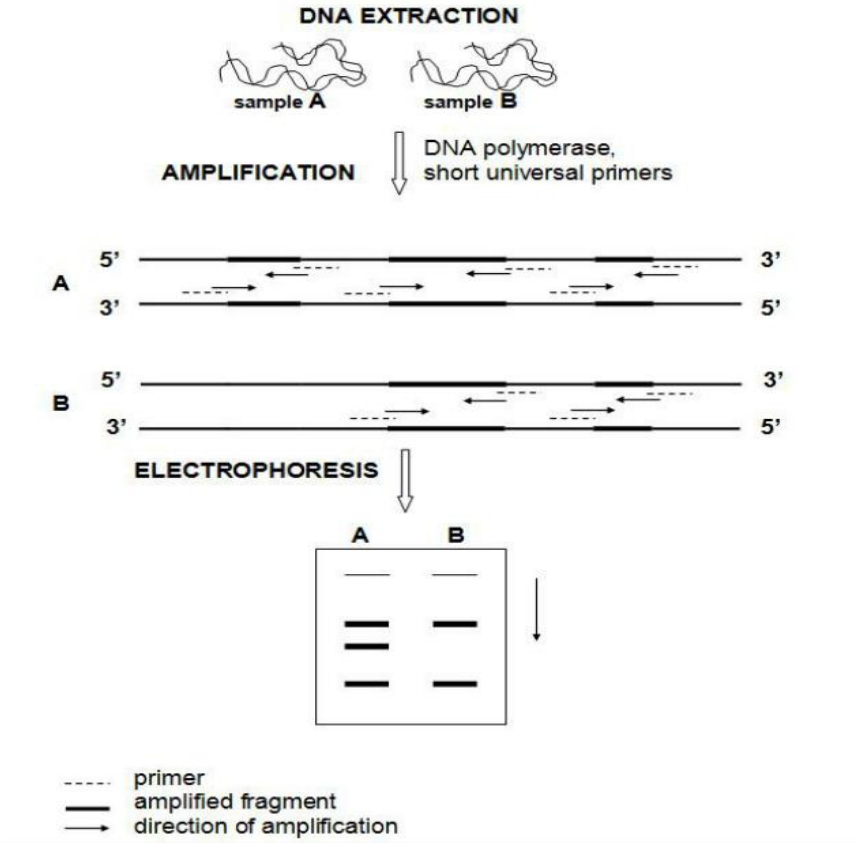


Figure 2: Principle of random amplified length polymorphism (RAPD)

2.10.2. Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length polymorphism is a PCR-based tool used in genetics research, DNA finger printing. AFLP was first developed by Vos *et al.* (1995). It uses restriction enzymes to cut genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. It is finger print of any DNA regardless of its sources and without any prior knowledge of DNA sequence, and used to distinguish closely related individuals in sub species level (Althoff *et al.*, 2007). It utilizes fragments of DNA amplified using directed primers from restriction digested genomic DNA (Figure 3). 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).

Amplified fragment length polymorphism technique is more useful in detecting genetic diversity of rare and endangered species. Utilizing this technique current diversity status was also demonstrated for a number of trees and shrub species (Barker *et al.*, 1999). The AFLP is useful in genetic of large number of polymorphic markers (Song, 2005).

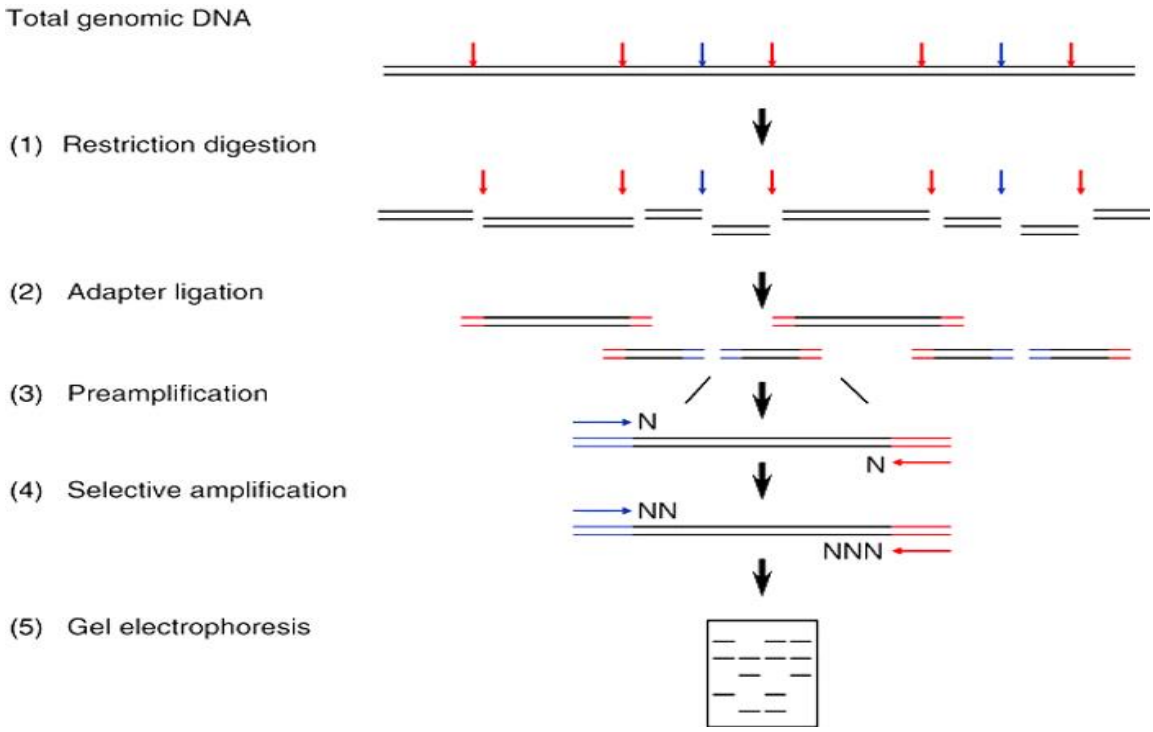


Figure 3: Principle of amplified fragment length polymorphism (AFLP**)**

2.10.3. Simple Sequence Repeat (SSR)

Simple Sequence Repeat (SSR) also known as microsatellites markers belong to a class of molecular markers whose primers are sequence dependent, SSRs are universal DNA motifs repeated randomly. Factors influencing the success of a particular motif include: frequency throughout genome, structural constraints of the DNA helix, and the size of the fragment to be amplified. Di-, tri-, or tetra nucleotide motifs are repeated perfectly at least three times (Figure 4). The variability in the number of repeat units is the basis of observed polymorphism. SSRs have become popular because they show easily distinguished co-dominance, are more easily reproduced than sequence independent markers, high rate of polymorphism, and distribution throughout eukaryotic genomes (Rakoczy- Trojanowska, and Bolibok, 2004).

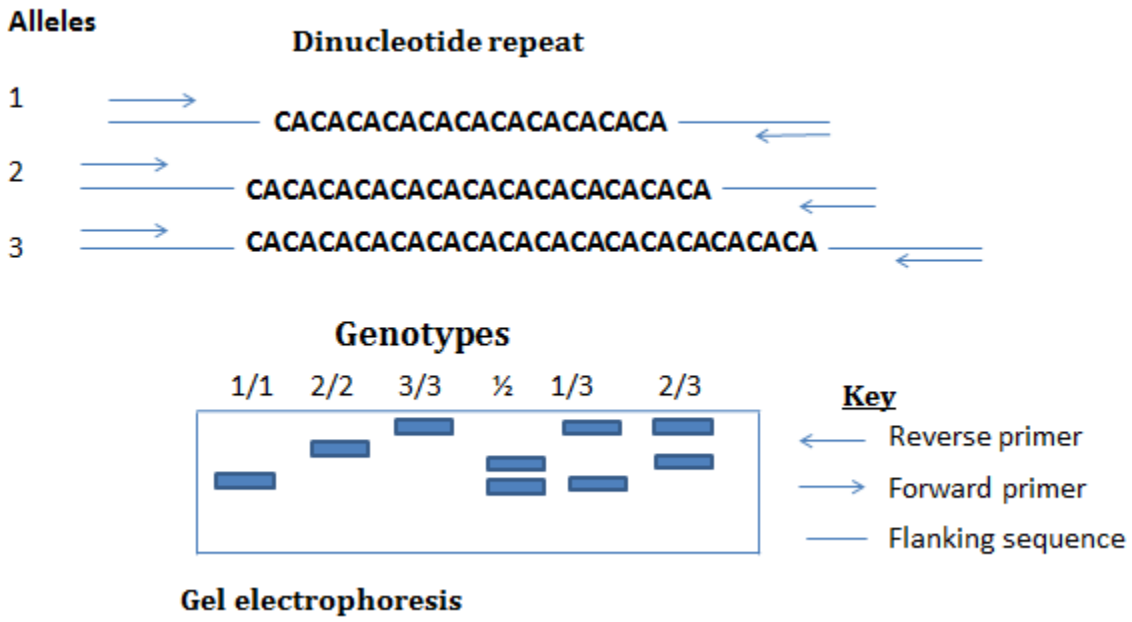


Figure 4: Principle of simple sequence repeat (SSR) marker

2.10.4. Inter simple sequence repeats (ISSRs) markers

The Inter Simple Sequence Repeat (ISSR) markers are regions of the nuclear DNA; located at specific sequences between two inverted simple sequence repeat regions when primers are annealed on two microsatellite sequences of DNA strands, the PCR reaction generates a band of a particular size for that locus. Each locus is scored for an individual in a binary way, as "1"or "0" (presence or absence of a product) respectively. Then, they allow detection of size polymorphism of the region lysing between microsatellites repeats (Zietkiewicz *et al*., 1994). Using longer primers (14-16 bases long) that allow more stringent annealing conditions during PCR amplification (Hillis *et al*., 1996).This is because Inter Simple Sequence Repeats involve the use of microsatellite sequences as primers in a polymerase chain reaction to generate multi-locus markers. Hence, ISSR markers are DNA sequence delimited by two inverted SSRs, that is, they are regions found between the simple sequence repeats (SSRs) composed of the same units which are amplified by a single PCR primer consisting of few units with or without anchored end. Each band corresponds to the DNA sequence delimited by the inverted microsatellites as ISSR amplify between SSR regions in the genome.

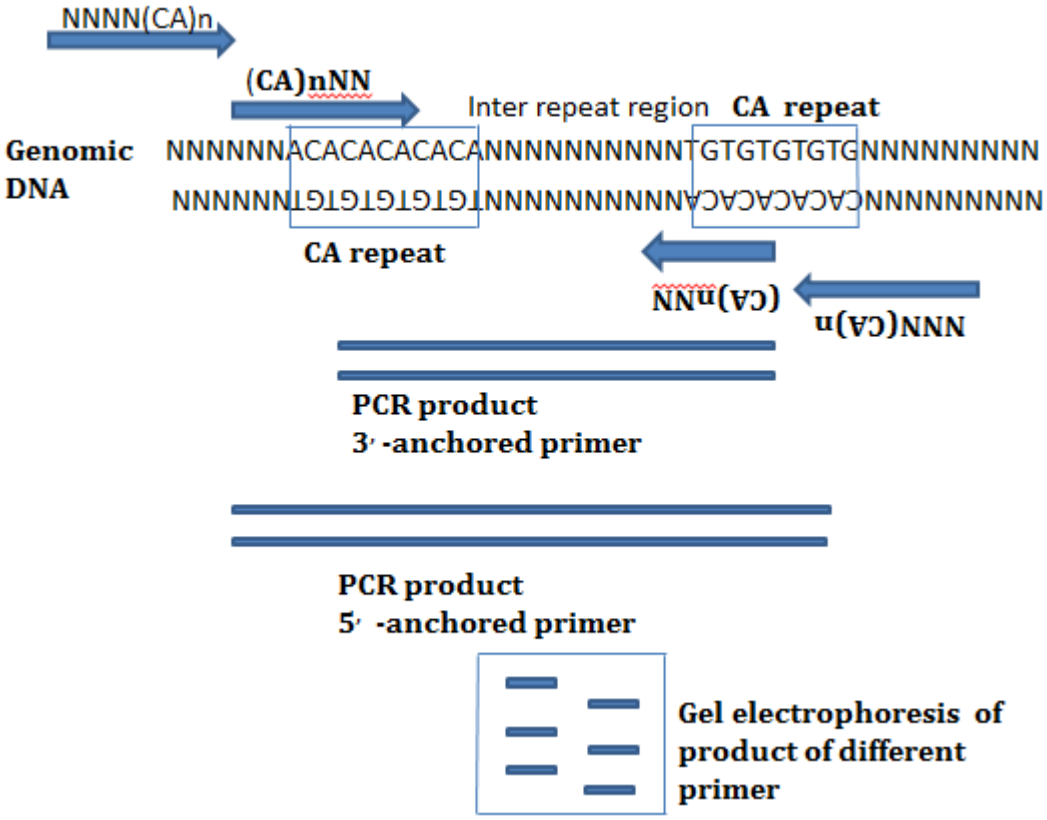


Figure 5: Principle of inter simple sequence repeat (ISSR) marker

Inter simple sequence repeat (ISSR) markers have been more specific than RAPD markers to study genetic diversity due to its longer SSR-based primers (16 -25bp) with higher primer annealing temperature (45 - 60) which enable for higher- stringency and band reproducibility. In addition to this, the major limitations such as low reproducibility of RAPD, high cost of AFLP, the need to know the flanking sequences to develop species specific primers for SSR polymorphism and large quantities of high quality genomic DNA for RFLP (Reddy *et al.*, 2002) are overcome by this markers (Zietkiewicz *et al*., 1994). Generally, technically ISSR markers are efficient, quick, reliable, very reproducible, highly discriminative and informative, independent of sequence information and require small quantity of sample DNA. These facts suggest that ISSR could be used extensively for assessing the molecular genetic variability within and among many living organisms (Kol and Lazebny, 2006).

The conservation and sustainable use of *C. annuum* population is mainly depends on proper characterization of its genetic resources. ISSR marker, in addition to its suitability to genetic diversity study in plants, is highly polymorphic, reproducible, and cost effective and requires no prior information of the sequence. In agronomicaly important crops such as *C. annuum*, this marker is used to study the patterns and level of diversity. Hence, the ISSR marker assay has been chosen to study the level of diversity and patterns of distribution of *C. annuum* genetic resources.

3. MATERIALS AND METHODS

3.1. Plant materials and sampling

Seeds of 47 *Capsicum annum* accessions were collected from Ethiopian Institute of Biodiversity (EIB), originated from different administrative regions of Ethiopia, and 6 newly released varieties (Mareko Fana, Melka Zala, Melka Awaze, Melka Dera and Melka Oli) used as a control were collected from Awash Melkasa Agricultural Research Center (AMARC). A total of 53 *C. annuum* were considered in this study (**Appendix I**). All the 53 *C. annuum* accessions seed samples were sown and grown in greenhouse in Horticulture research unit of Debre Zeyit Agricultural Research Center (DZARC). Fresh leaves from six week young leaves were collected using zip locked plastic bag containing silica gel to dry and preserve until genomic DNA extraction was executed in plant genetics research laboratory, Institute of Biotechnology, AAU.

3.2. Genomic DNA extraction

Genomic DNA was extracted following the modified CTAB method (Govers, 2005) and following methods described in Borsch *et al*. (2003). 700 µl (per sample) CTAB solution was poured in a 15ml-tube and 0.2 vol % mercaptoethanol was added and warmed in water bath up to 65°C. 50 mg of fresh leave was grinded using mixer miler with clean and sterilized bed for about 4 minutes. The powder was transferred into an eppendorf cap with warm CTAB solution immediately and dissolved and incubated 30 minutes at 65°C. After 30 minutes, centrifuged for 7 minutes at 13000 rpm and the supernatant (only clear liquid) was transferred in to new eppendorf –cap. 600 µl chloroform was added to the caps with supernatant and shake carefully a few times upside down and centrifuged for 7 min at 13000 rpm. The supernatant (only clear liquid) was transferred in to new eppendorf-caps. Cooled isopropanol (4°C) (**2/3** of the solution volume), was added in to caps containing supernatant and shakes carefully by inversing the eppendorf cap and freeze for more than 2 h at -20°C. After 2 h liquid was aspirated using yellow tips. 200 µl ethanol 70 % was added in to the pellet and the inner cap surface was rinsed by turning the cap and centrifuged for 15 min at 13000 rpm. Ethanol was aspirated using yellow tips and the pellet was dried at room temperature. The pellet was dissolved in 100 µl TE (1x, p.a. grade) and stored overnight at 4°C. Cooled 7.5 M NH4Ac-solution (4°C, half of the solution volume) was added and mixed. Cooled ethanol 100 % (double of the solution volume) was added, mixed and freeze for more than 2 h at -20°C. After 2 h the sample was centrifuge for 30 minutes at 13000 rpm, fluid was aspirated. 200 µl ethanol 70% was added and the inner cap surface was rinsed by turning the cap, centrifuged for 10 minutes at 13000 rpm and liquid was aspirated and

pellet was dried at room temperature. The pellet was dissolved in 100 µl TE (1x, p.a. grade) and stored at 4°C until gel electrophoresis test to check the quality of genomic DNA.

3.3. Genomic DNA gel electrophoresis test

One percent agarose gel (50 ml, 1xTBE and 0.5 g agarose powder) was prepared and 2 µl of each genomic DNA samples were mixed with 2 µl loading dye (1X bromophenol blue) was loaded on the gel and electrophoresed at constant voltage of 80 V for 30 minutes. Gel picture was taken under UV transilluminator by gel documentation system. From the two extractions following the protocol given by Borsch *et al*. (2003), those with high band intensity and fewer smears was selected for PCR (**Appendix III**).

3.4. Quantification and Purity Checking using Nano Drop

The yield of DNA isolated was measured using a Nano Drop ND-2000 UV (thermo fisher scientific) spectrophotometer with 1.0 µl sample volume. The concentration of total genomic DNA ranges from 115.6 ng/µl to 3525 ng/µl. The purity of DNA was also determined by estimating the ratio of absorbance at 260/230 and at 260/280. The ratio of diluted samples at 260/230 was lied between 1.75-2.53 which is near for pure DNA detection standard of 2-2.2. The ratios of diluted sample at 260/280 were lied between 1.86-2.47 which is approached to fit a pure DNA ratio approaches to 1.8.

3.5. Primer selection and optimization

A total of twelve (12) available ISSR primers obtained from the University of British Colombia (primer kit UBC 900) were used for the initial testing of polymorphism and reproducibility of PCR products (Table 1). DNA from one individual plant was selected from each population to screen the primers. Based on polymorphism and reproducibility, four primers (UBC-810, UBC-812, BC-880 and UBC-881) were selected for the analysis of the study.

Table 1: List of primers, primer sequence, annealing temperature, amplification pattern and repeat motives used for optimization and screening

No	Primers Code	Annealing temperature	Primer sequence (5'-3')	Amplification Quality	Repeat motifs
1	UBC-810	45^{o}C	GAGAGAGAGAGAGAGAT	Reproducible and Polymorphic	Dinucleotide
2	UBC-812	45^{o}C	GAGAGAGAGAGAGAGAA	Reproducible and Polymorphic	Dinucleotide
3	UBC-815	45^{o}C	CTCTCTCTCTCTCTCTG	No Banding	Dinucleotide
4	UBC-818	48^{o}C	CACACACACACACACAG	No Banding	Dinucleotide
5	UBC-834	45^{o}C	AGAGAGAGAGAGAGAGYT	No Banding	Dinucleotide
6	UBC-840	49^{o}C	GAGAGAGAGAGAGAGAYT	No Banding	Dinucleotide
7	UBC-848	48^{o}C	CACACACACACACACARG	No Banding	Dinucleotide
8	UBC-854	48^{o}C	TCTCTCTCTCTCTCTCRG	No Banding	Dinucleotide
9	UBC-873	45^{o}C	GACAGACAGACAGACA	No Banding	Tetra nucleotide
10	UBC-876	45^{o}C	GATAGATAGACAGACA	No Banding	Tetra nucleotide
11	UBC-880	45^{o}C	GGAGAGGAGAGGAGA	Reproducible and Polymorphic	Penta nucleotide
12	UBC-881	48^{o}C	GGGTGGGGTGGGGTG	Reproducible and Polymorphic	Penta nucleotide

Source: Primer kit 900 (UBC 900); *Single-letter abbreviations for mixed base positions: R = (A, G) Y = (C, T).*

3.6. PCR amplification and electrophoresis

The polymerase chain reaction was done using Biometra 2000 T3 master cycler. PCR amplification was carried out in a 25 µl total reaction mixture containing 1 µl template DNA, 19 µl ddH_20, 1 µl dNTP (1.25 mM), 2.5 µl PCR buffer (10 x Hibuffer with 17.5 mM $MgCl_2$), 0.7 µl $MgCl_2$ (25 mM), 0.4 µl primer (20 pmol/µl) and 0.4 µl *Taq* Polymerase (5 U/µl). The amplification program was arranged as, 4 minutes for pre heating and 15 second initial denaturation at 94°C, 1 minutes primer annealing at (45 or 48°C) based on primers used, 1.30 minutes extension at 72°C. The final extension was followed for 7 minutes at 72°C. The PCR products were also stored at 4°C until loaded on gel for electrophoresis.

The amplification products were checked first on test gel (0.5 gm agarose powder with 50 ml 1xTBE) for the presence of ISSR-PCR products. The amplified products were run on to ISSR gel using 0.8 gram agarose, with 50 ml 1xTBE using gel electrophoresis chamber. The ISSR gel was prepared using 50 ml TBE mixed with 0.8 gram agarose using 500 ml Erlenmeyer flask and then boiled in micro oven for 2 minutes. Two µl Ethidium Bromide (10 mg/ml) was added immediately and the gel was poured on casting system and allowed to solidify for more than 40 minutes. Seven micro litter ISSR amplification products and 2 µl loading dye (6X bromophenol blue) was mixed thoroughly and loaded on the gel. A 100 base-pair ladder was used to estimate the molecular size of the DNA fragments. The electrophoresis was run for 1:30 hours with at constant voltage of 80 V. Gel picture was taken under UV transilluminator by gel documentation system (applied bio system).

3.7. Data scoring and analysis

ISSR bands were scored visually and each fragment, amplified using ISSR primers was treated as a unit character, and scoured as '1' for presence, '0' for absence and '?' for ambiguous and missing data.. POPGENE version1.32 software was used to calculate genetic diversity for each population as number of polymorphic loci, percent polymorphism, gene diversity and Shannon diversity index.

Shannon‟s index of diversity is used to measure the total diversity (Hsp) for the species as well as the mean diversity per population (Hpop). The proportion of diversity within and between populations is calculated as $\frac{\text{Hpop}}{\text{Hsp}}$ and $\frac{1-\text{Hpop}}{\text{Hsp}}$, respectively. Analysis of molecular variance (AMOVA) was used to calculate variation among and within population using Areliquin version 3.01 (Excoffier *et al*., 2006). Free Tree 0.9.1.50 software was used to calculate Jaccard‟s similarity coefficient which is calculated as: $\text{Sij} = \frac{a}{a+B+C}$

Where, **a** is the total number of bands shared between individuals i and j, **B** is the total number of bands present in individual i but not in individual j and **C** is the total numbers of bands present in individual j but not in individual i.

NTSYS- pc version 2.02 Rohlf (2000) was used to generate the unweighted pair group method with arithmetic mean (UPGMA) Dendrogram to analyze and compare the individual genotypes. The neighbor joining (NJ) method was used to compare individual genotypes and evaluate patterns of genotype clustering using Free Tree 0.9.1.50 Software (Pavlicek *et al*., 1999).

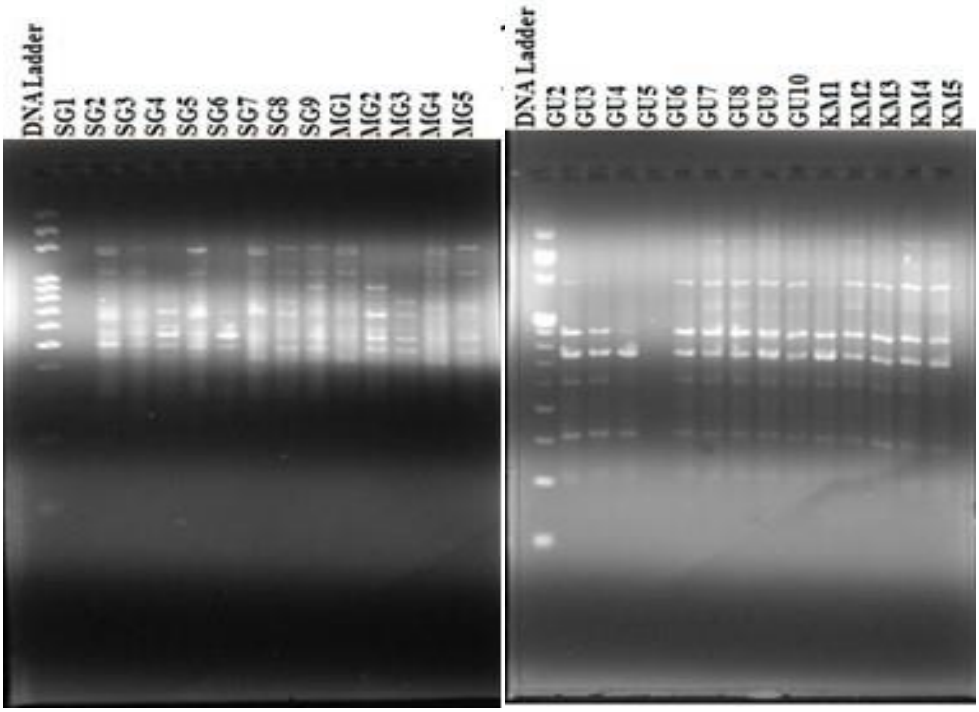
4. RESULTS AND DISCUSSION

4.1. ISSR primers and their amplification pattern

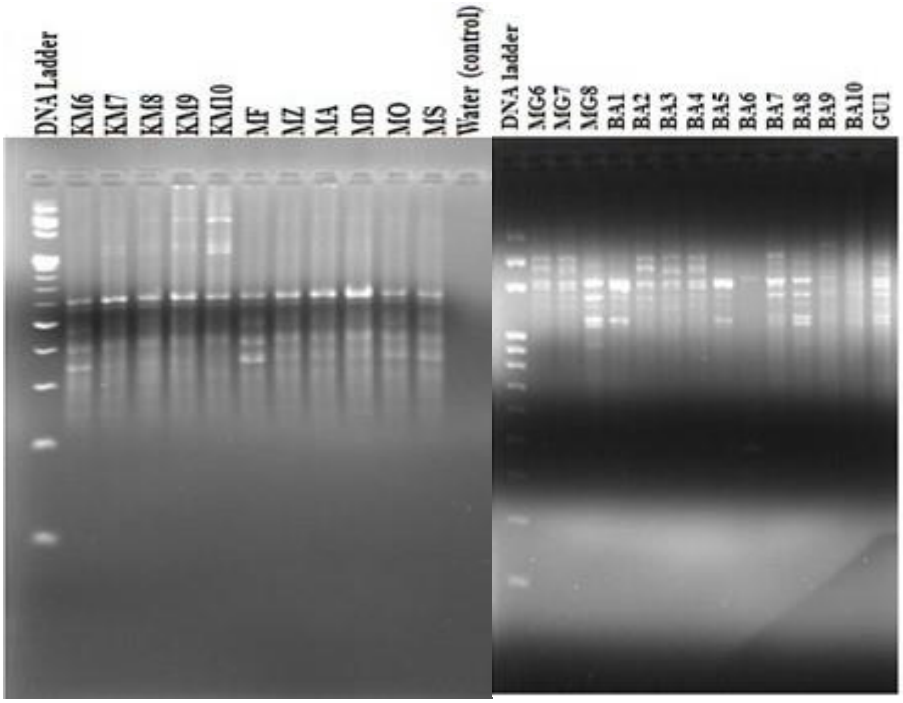
Out of the twelve primers tested initially, four of them gave relatively clear banding pattern and they were selected to distinguish hot pepper accessions and cultivars in this study (Table 2). But 8 primers did not amplify DNA of any hot pepper accessions and cultivars. These primers, which did not amplify DNA of all the genotypes, may not have found complementary sequences on the genomic DNA. The size of the fragments amplified by using four primers was in the range of 250 bp to 1000 bp. The highest number of bands (8) was recorded for primer UBC-810 and UBC-880 followed by primer UBC-812 and UBC-881 which generated 7 and 6 scorable bands respectively (Figure 6). Oumer Abdie (2015) has got the highest reproducible and scorable band for UBC-810 and UBC-880 primers with 10 and 9 fragments respectively on Inter simple sequence repeat (ISSR) analysis of Ethiopian white lupine (*Lupinus albus* L.). Kassahun Tesfaye (2006) has got the highest reproducible and scorable band for UBC-812 primers with 22 fragments on Genetic Diversity of wild *Coffea arabica* populations in Ethiopia.

Table 2: Selected ISSR primers with their amplification and banding pattern.

ISSR primers	**repeat motif**	**Number of Scored bands**
UBC-810	(GA)8T	8
UBC-812	(GA)8A	7
UBC-880	(GGAGA)3	8
UBC-881	(GGGTG)3	6
	Total	**29**



UBC - 810 UBC – 812



UBC - 880 UBC - 881

Figure 6: ISSR fingerprint generated from accessions and cultivars of *C. annuum* by primers UBC-810, UBC 812, UBC-880 and UBC-881.

4.2 Genetic Diversity as Revealed by Percent Polymorphism, Shannon Weaver and Gene Diversity Values

Out of the total 29 loci scored, all 29 loci were observed to be polymorphic which means all primers are 100% polymorphic in each individuals of *C. annuum* from Ethiopia (Table 3). In all individuals the number of polymorphic loci ranged from 6 for primer UBC-881 to eight for primer UBC-810 and UBC-880. Primer UBC-812 showed highest gene diversity and Shannon diversity index values (0.4 and 0.58, respectively) and primer UBC-810 was the least (0.28 and 0.45, gene diversity and Shannon diversity index values, respectively).

Table 3: Number of polymorphic loci (NPL), percent polymorphism (PP), gene diversity (H) and Shannon Index information (I) of all 53 *C. annuum* populations and cultivars based on each primer used.

			with individual primers	
primer code	**NPL**	**PP (%)**	**H±SD**	**I±SD**
UBC-810	8	100	0.28±0.09	0.45±0.11
UBC-812	7	100	0.40±0.07	0.58±0.07
UBC-880	8	100	0.39±0.09	0.58±0.10
UBC-881	6	100	0.33±0.10	0.50±0.12
Average	**7.25**	**100**	**0.35±0.08**	**0.53±0.1**

Among the *C. annuum* populations and cultivars evaluated using ISSR markers with all primers Mirab Gojjam *C. annuum* population exhibited a genetic diversity value of 0.31 and Shannon diversity index of 0.47, while the Gurage, Kembata, Bale and Semen Gondar population of *C. annuum* showed 0.26, 0.22, 0.12 and 0.11 for genetic diversity and 0.39, 0.33, 0.19 and 0.17 Shannon diversity index values respectively. Cultivar groups of *C. annuum* showed 0.07 genetic diversity value and 0.11 Shannon diversity index values. The average genetic diversity and Shannon diversity index values for all five populations and one cultivar group was 0.18 and 0.27 respectively (Table 4).

Table 4: Number of polymorphic loci (NPL), percent polymorphism (PP), gene diversity (H) and Shanon Index information (I) of five *C. annuum* populations and one cultivar groups based on all 4 primers used**.**

		with all primers		
Population	**NPL**	**PP (%)**	**H±SD**	**I±SD**
Semen Gondar	9	31.03	0.11±0.18	0.17±0.27
Mirab Gojjam	25	86.21	0.31±0.16	0.47±0.22
Bale	12	41.4	0.12±0.17	0.19±0.25
Gurage	22	75.86	0.26±0.19	0.39±0.27
Kembata	17	58.62	0.22±0.21	0.33±0.29
Cultivar group	6	20.7	0.07±0.15	0.11±0.23
Average	**15.2**	**52.3**	**0.18±0.17**	**0.27±0.25**

Thus, Mirab Gojjam *C. annuum* population was observed to show higher percent polymorphism and Shannon's diversity index than other populations and cultivars with an average genetic diversity of 0.31. This may be due to possible gene flow between individuals through effectors (human, insects, and birds) and can be further supported with presence of long roads from Addis Ababa to Gondar and other roads from Wollega and Debre Tabore to Mirab Gojjam, due to those roads there could be introduction of varieties of *C. annuum.*

Given the proliferation of molecular markers, a comparison between the markers seems highly inevitable on the basis of study objectives and the nature of the markers. From these desired qualities of molecular markers, automation (PCR-based), polymorphisms and reproducibility are the highly demanded features of the molecular techniques to be used in the intraregional diversity analysis. ISSR markers are thus one of the molecular markers that have these characteristics to study variability in different crops (Zietkiewicz *et al*., 1994). Moreover, this marker observed to be very useful in detecting genetic diversity and population structure of Coffee (Tesfaye, 2006), Tef (Assefa, 2003), and rice (Gezahagne, 2007) collected from all over Ethiopia

4.3. Analysis of Molecular Variance (AMOVA)

Analysis of molecular variance was carried out on the overall ISSR data scores of hot pepper populations and cultivars. It was done by computation of the distance between "haplotypes", each individual`s data pattern as one "haplotypes" and computing variance components for each level (Excoffier *et al*., 2006). Partition of genetic diversity by analysis of molecular variance revealed that higher percentage of variation was attributed to within population (55.3%) and the rest 44.7 percent variation was attributed to among populations (Figure 5). This could be due to pollinating agents and seed intermixing during harvesting.

AMOVA result of Kassahun Tesfaye (2006) in coffee, Oumer Abdie (2011) in white lupine, Edossa *et al.*, (2010) in lentil, and many other earlier studies support the higher genetic diversity within populations than among population. Similar study by Oumer Abdie (2011) on genetic diversity of Ethiopian white lupine using four ISSR primers and reported 74.62% of genetic diversity within populations and 25.38% among the populations. Morphological and molecular diversity study of Ethiopian lentil using four ISSR primers by Edossa also found 56.28% diversity within populations than among populations (43.72%).

Table 5: Analysis of Molecular Variance (AMOVA) of *C. annuum* in Ethiopia based on their population as Semen Gondar, Mirab Gojjam, Bale, Gurage and Kembata**.**

Source of variation	Sum of squares	Variance components	Percentage variation	Fixation Index	P
Among populations	8.93	0.18	44.72	0.44	0.00
Within populations	10.22	0.22	55.27		0.00
Total	**19.16**	**0.41**	**100**		

Since the crop is mostly self-pollinated species, higher genetic diversity was expected among populations than within populations. However, AMOVA result indicated larger genetic diversity within the population than among population. This could be due to high seed exchange among different regions and markets which could lead to intermix of populations between regions. Unlike other landraces of cultivated plants, *C. annuum* in Ethiopia is not restricted to a given area rather it is wildly exchanged among local community and markets. This shows that there is very high gene flow between populations.

4.4. Clustering Analysis

Unweighted pair group method with arithmetic mean (UPGMA) and Neighbor Joining (NJ) tree construction methods was used to construct dendrogram for five populations and one cultivar group and 53 individuals based on 29 PCR bands amplified by two di-nucleotides (UBC-810 and UBC-812), and two penta nucleotides (UBC-880 and UBC-881).

UPGMA analysis based on five populations and one cultivar of *C. annuum* revealed two major clusters. The first cluster contains semen Gondar, Gurage, Kembata and cultivar group, while the second cluster contains Bale and Mirab Gojjam populations (Figure 7). Each five populations and one cultivar group of *C. annuum* showed moderate grouping based on their collection areas. Similar study by Oumer Abdie (2011) on genetic diversity of Ethiopian white lupine using four ISSR primers and reported populations collected from Amhara region were observed to form moderate grouping based on their place of origin.

However UPGMA with individual accessions *C. annuum* showed intermixing of individuals to different groups, except Kembata population and Cultivar groups, where they forms their own groups without any intermixing with each other and with other populations (Figure 8). Some of the individual accessions collected from the same region tend to spread all over the tree without forming their own grouping, because of they could be genetically distant from their population. There is high genetic similarity with in Kembata populations and cultivar groups. The wider distribution of *C. annuum* accession all over the tree shows low genetic similarity within populations.

The dendrogram derived from neighbor-joining analysis of the whole ISSR data with 53 individual showed two major clusters and three sub-clusters and one outlier (MG 4), this could be genetically distant from other individuals (Figure 9). Similar study by Hana Alemu (2014) on genetic diversity of *chloris gayana* kunth populations using inter simple sequence repeat markers Based on NJ analysis, accessions of *Chloris gayana* revealed one major cluster and one outlier groups.

Like that of the UPGMA analysis of the individual accession the dendrogram derived from neighbor-joining analysis of the whole ISSR data were not showing a clear grouping (Figure 8). Few accessions from Kembata and cultivar group were tended to form their own cluster while some of the accessions distributed all over the tree i.e. individuals of all populations were inter-mixed. Generally, the dendrogram analysis using *C. annuum* individual plant form inter-mixed cluster between

populations since high levels of genetic variation is detected in almost all populations investigated. Genetic distance is a measure of the allelic substitutions per locus that have occurred during the separate evolution of two populations or species. Smaller genetic distances indicate a close genetic relationship whereas large genetic distances indicate a more distant genetic relationship. Crosses between distantly related individuals are expected to give better offspring than those between closely related genotypes.

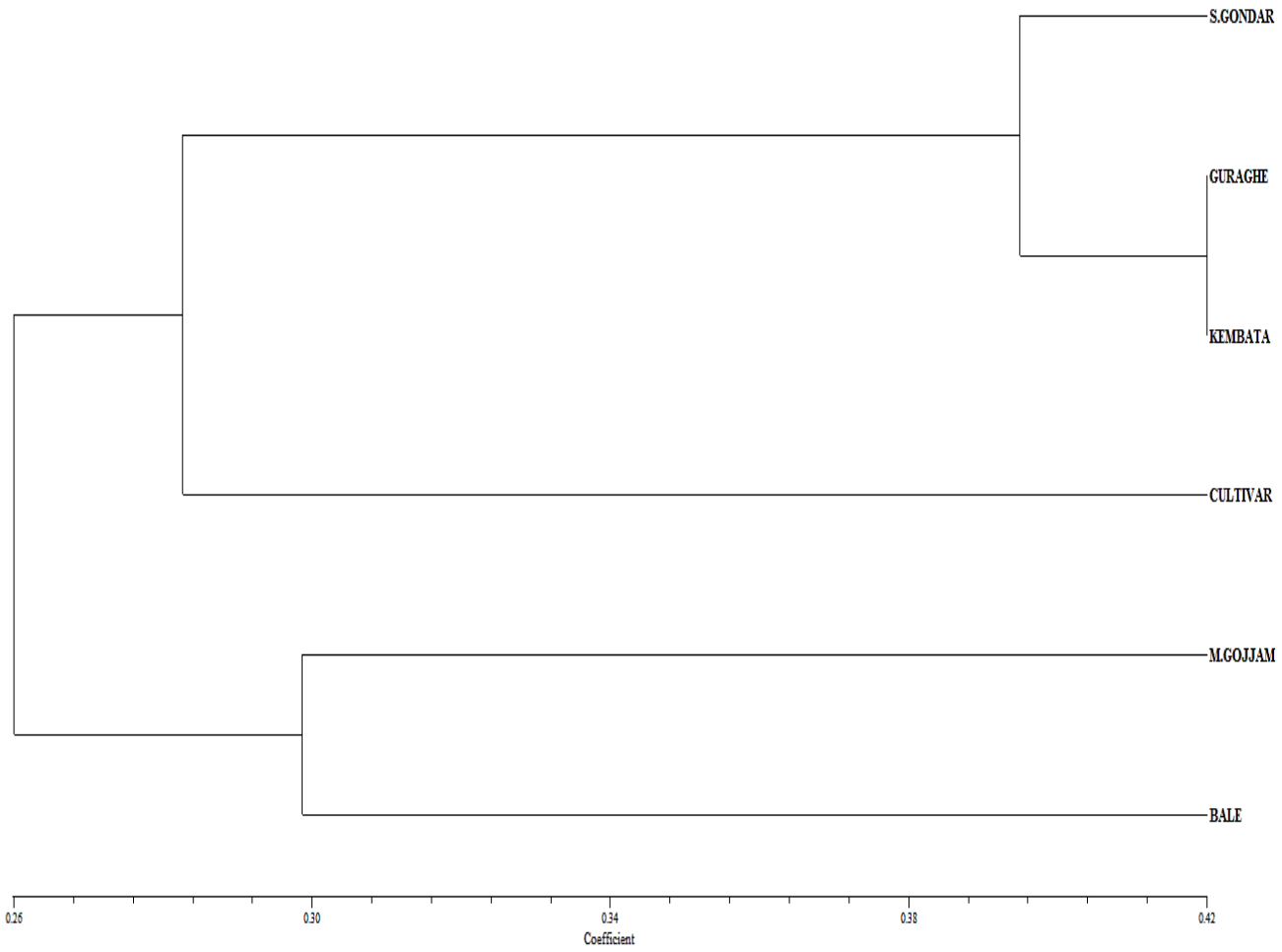


Figure 7: UPGMA based dendrogram for 5 *C. annuum populations* and one cultivar groups using 4 ISSR (2 di and 2 penta nucleotide) primers

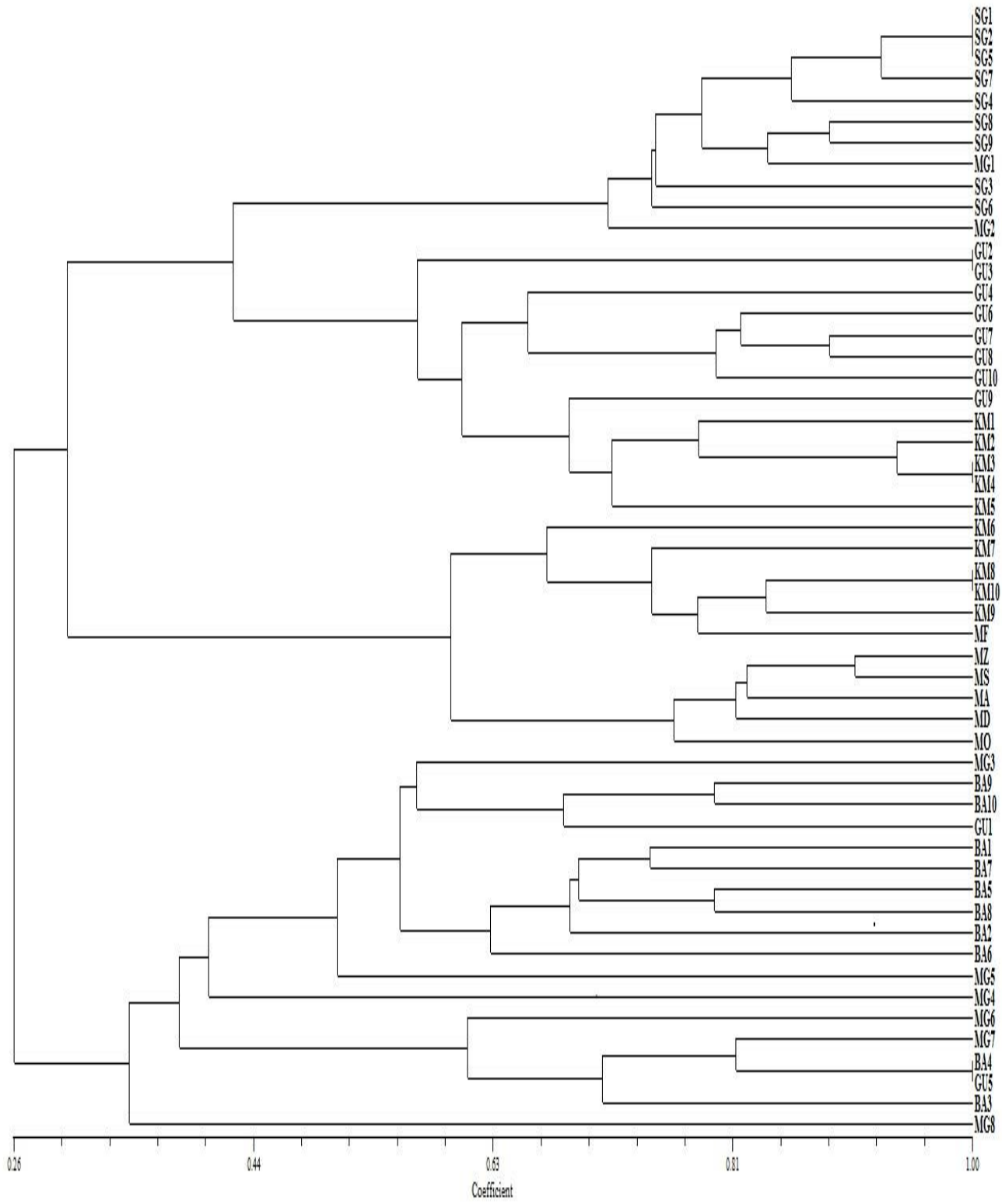


Figure 8: UPGMA based dendrogram for 53 *C. annum individuals* using 4 ISSR (2 di, and 2 Penta nucleotides) primers. Key: SG = Semen Gondar, MG = Mirab Gojjam, BA= Bale, GU = Gurage, KM=Kembata, MF=Mareko Fana , MD=Melka Dera, MO= Melka Oli, MZ= Melka Zala, MA= Melka Awaze and MS = Melka Shote

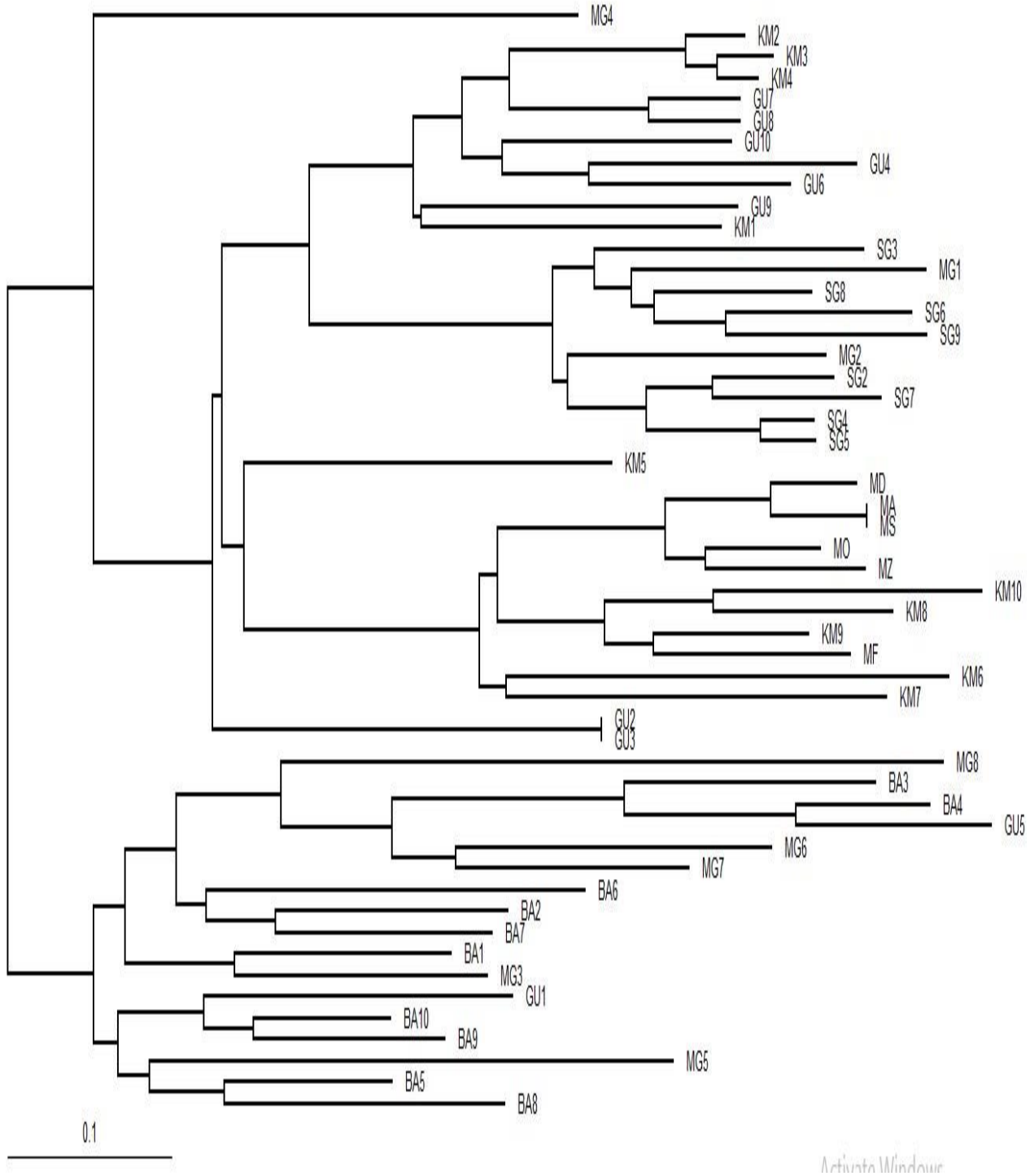


Figure 9: Neighbor-joining analysis of 53 individuals based on 29 PCR bands amplified using two dinucleotide (UBC-810 and UBC-812) and two penta nucleotide (UBC-880 and UBC-881) primers.

The neighbor joining algorithm is based on Jaccard's coefficient. *Key*: SG = Semen Gondar, MG = Mirab Gojjam, BA= Bale, GU = Gurage, KM=Kembata, MF=Mareko Fana , MD=Melka Dera, MO= Melka Oli, MZ= Melka Zala, MA= Melka Awaze and MS = Melka Shote.

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

In this study, ISSR markers were observed to be an appropriate molecular marker for generating the detailed intraspecific genetic diversity data to evaluate the extent and distribution of genetic diversity within and among *C. annuum* accessions. Out of the total 29 scorable bands produced with the total of four primers (two dinucleotide and two penta nucleotide), 29 bands were polymorphic.

Among five populations and one cultivars of *C. annuum* considered in the present study, Mirab Gojjam population shows higher gene diversity (0.31) than Gurage (0.26), Kembata (0.22), Bale (0.12), Semen Gondar (0.11) and cultivar group (0.07). Generally among all *C. annuum* populations Mirab Gojjam (0.31) population is highly diversified and Semen Gondar (0.11) population is least diversified, whereas cultivar group (0.07) is very least diversified than all the five populations of *C. annuum*.

Partitioning of genetic diversity by analysis of molecular variance using grouped populations revealed that out of the total genetic diversity, most of the ISSR diversity was distributed between individual plants within the populations (55.3%), with the remaining diversity being distributed among populations (44.7%).

Dendrogram of the present study by using UPGMA of jaccard's coefficient of similarity showed Kembata and Gurage populations of *C. annuum* were closely related. Therefore, prior knowledge of the genetic distance between genotypes or accessions is important in designing breeding program.

5.2. Recommendations

Although the ISSR analysis indicated the existence of diversity among the 53 *C. annuum* individuals, the total genetic diversity is narrower. This low genetic diversity among the Ethiopian *C. annuum* population indicated that the populations are closely related. Therefore, the National Breeding Program should take careful action to broaden the genetic base of the *C. annuum* in the country to reduce its vulnerability to diseases and insect pest outbreak. Hence, the Ethiopian *C. annuum* Breeding Program should revise its breeding approach in a way that assists in broadening the available narrower genetic diversity of the population. This could be achieved through gene transfer from wild relatives to cultivated species, implementing crossing programs, enhancing variability through mutation breeding, utilize biotechnological tools in identifying, characterizing and transfer of novel genes from distant relatives of the species.

Generally the patterns of genetic diversity obtained in this study suggested that:

- This study is not exhaustive in terms of geographic area coverage and amount of primers. Hence, the study should be repeated with more geographic area coverage and primers and markers systems.
- Germplasms from Mirab Gojjam and Gurage population should be targeted for conservation and yield improvement work by breeders.
- *Capsicum annuum* populations from Bale and Semen Gondar were observed to show low diversity and hence, special attention should be given to conserve these populations.
- Rates of self- pollination and cross-pollination require in-depth investigation to account for the speculated gene flow.

6. REFERENCES

Adetula, O. (2006). Genetic diversity of *Capsicum* using random amplified polymorphic DNAs. *Afr. J. Biotechnol.* **5:** 120-122.

Ágnes, Z., Klára, K., Nóra, F. and László, G. (2015). Microarray technology. Institute of Genetics, *Biological Research Center, Hungarian Academy of Sciences*. **59**(1):51-67.

Althoff, D., Gitzendanner, M. and Segraves, K. (2007). The utility of amplified fragment length polymorphisms in phylogenetics. A comparison of homology within and between genomes. *Syst. Boil.* **56:** 477- 484.

Amare Tesfaw, (2013). Benefit-Cost Analysis of Growing Pepper: A Trial At West Gojjam, Near The Source Of Blue Nile. *International Journal of Agriculture and Crop Sciences.* **6** (17): 1203-1214.

Assefa Kebebw, (2003). Phenotypic and molecular diversity in the Ethiopian cereal, Tef (Eragrostis tef (Zucc.) Trotter). Doctoral Dissertation, Department of crop Science, SLU. Acta Universitatis Agriculturae Sueciae. Agraria vol.**426**.

Avise, J. (1994). Natural History and Evolution of Molecular Marker, Champ and Hall, USA.

Bagali, P., Antonyheroldprabhu, P., Ragharenda, K., Hittalmani, S. and Vadivelu, J. S. (2010). Application of molecular markers in tissue culture. *J. Mol. Biol. Biotechnol.* **18:** 85-87.

Barker, J., Matthes, M., Arnold, G., Edwards, K., Ahman, I., Larsson, S. and Karp, A. (1999). Characterization of genetic diversity in potential biomass willows (Salix spp.) by RAPD and AFLP analysis.*Genome*, **42:** 173-18

Barrett, B. and Kidwell, K. (1998). AFLP-based genetic diversity assessment among wheat cultivars from the Pacific North West. *Crop Sci*. **38**: 1261–1271.

Beiranvand, N., Hallajian, M. and Majdabadi, A. (2011). Phenotypic and molecular analysis of M7 generation of soybean mutant lines through random amplified polymorphic DNA (RAPD). Marker and some morphological traits. *A.J.A.R.* **6:** 1179-1785.

Beyene, T. and David, P. (2007). Ensuring Small Scale Producers in Ethiopia to Achieve Sustainable and Fair Access to Pepper Market. *Uganda Journal of Agriculture*, **3**(2): 113-119.

Borsch, T., Hilu, K., Quandt, D., Wilde, V., Neinhuis, C. and Barthlott, W. (2003). Noncoding plastid trnT-trnF sequences reveal a well resolved phylogeny of basalangiosperms. *J. Evol. Biol*. **16**: 558-576.

Bosland, P. and Votava, E. (2000). Pepper: Vegetable and Spice *Capsicums*. CABI Publishing, New York.

Claire, K. and Elizabeth, A. (2005). Elucidating genetic diversity with oligonucleotide arrays. Chromosome Research **13**: 225–235.

CSA (Central Statistical Agency Agricultural sample survey) (2012). The federal democratic republic of Ethiopia (September-December, 2011/2012).

CSA (Central Statistical Authority of Ethiopia) (2006). Agricultural sample survey. 2005/2006(1998 E.C). Report on area and production of crops. *Statistical bulletin,* Addis Ababa, Ethiopia, **1**: 361.

EEPA (2003). Spice Potential and Market Study. Product Development and Market Research Directorate, Addis Ababa.

Edossa Fikiru, Kassahun Tesfaye and Endashaw Bekele, 2010. A comparative study of morphological and molecular diversity in Ethiopian lentil (Lens culinaris Medikus) landraces. *African Journal of Plant Science.* **4:** 242-254.

Endashaw Bekele (1985). The biology of cereal landrace population. I. Problems of gene conservation, plant breeding selection schemes and sample size requirements. *Hereditas,* **103**: 119-134.

Excoffier, L., Laval, G. and Schneider, S. (2006). Arlequin Version.3.01: An integrated software package for population genetics data analysis. *Evol.Bioinform. Online,* **1**: 47-50.

Fedlu Hassen, Endashaw Bekele, Workneh Ayalew and Tadelle Dessie, (2007). Genetic variability of five indigenous Ethiopian cattle breeds using RAPD markers. *Afr. J.Biotechnol*. **6:** 2274-2279.

Fekadu Moges. and Dandena, G. (2006). Status of Vegetable Crops in Ethiopia.*Ugandan Journal of Agriculture*, **12(**2): 26-30.

Gezahegn Girma, 2007. Relationship between wild rice species of Ethiopia with cultivated rice based on ISSR marker. M.Sc Thesis, Faculty of School of Graduate Studies, Biology department Addis Ababa University.

Govers, K. (2005). Standard Operation Procedure. A standardised procedure for th preparation of (stock) solutions. Specially written for the Nees Institute. Nees-Institut für Biodiversität der pflanzen. Rheinische Friedrich-Wihelms-Universität Bonn, Germany.

Grishin, S., Zayakin, V. and Nam, I. (2011). Comparison of the effectiveness of RAPD- and ISSR-analysis methods in identification of genetic polymorphism of *Lupinusangustifolius*L. cultivars.Proceedings of the scientific-practical conference, Bryansk, "Center for Business Education", pp. 70-72.

Haigh, A., Alee, M. and Sumardi, K. (1996). Water stress reduces yield in chilli by reducing the success of pollination.

Haile, Yohanise. and Zewdie, Lake. (1989). Hot pepper and tomato production research in Ethiopia. In:Tomato and Pepper Production In the Tropics: Proceedings of the International Symposium On Integrated Management Practices. Shanhua, Taiwan. Pp: 93-94.

Hartl, D. (2000). A Primer of Population Genetics, Third Edition.Sinaur Assoc. Inc., Sunderland, MA.20-45.

Hillis, D. M., Moritz, C. and Mable, B .K. 1996. *Molecular Systematics*. 2nd ed. Sunderland, MA: Sinauer Associates, Inc.

IAR (Inistitute of Agricultural Research), (1996). Progress report Addis Ababa. I-San Lin. R. 1994. Pharmacological properties and medical use of pepper (P.nigrum). In: Caralambous G. 1994. (Ed), Spices, herbs and edible fungi.Elsevier Science B.V. pp. 469- 479.

Ince, A., Karaca, M. and Onus, A. (2010). Genetic relationships within and between *Capsicum* species. Biochem. Genet. **48**: 48:83.

IPGRI, (2005). Forage Grass Descriptors. International Border for plant genetic resource and crop evolution **46**: 63-71.

Joshi, S., Prabharkar, K., Ranjek, R. and Vidya, S. (1999). Molecular markers in Plant Genetic Resources Conservation: A Guide to the Technologies. IPGRI Technical Bulletin No. 2.International Plant Genetic Resources Institute, Rome, Italy.

Karp, A., Kresovich, S., Bhat, K., Ayad, W. and Hodgkin, T. (1997). Molecular markers In Plant Genetic Resources Conservation: A Guide to the Technologies. IPGRI Technical Bulletin No. 2.International Plant Genetic Resources Institute, Rome, Italy.

Lin, S., Chou Y., Ching, S., Andreas, W., Ebert, S., Ravza, M., Albert, R. and Paul, A. (2013). Pepper (*Capsicum annum*) Germplasm Dissemination by AVRDC – The World Vegetable Center: an Overview and Introspection. Chronicahorticulturae, **53** (3): 345-375.

Makari, H., Patil, H., Abhilash, M. and Kumar, H. (2009). Genetic diversity in commercial varieties of chilli as revealed by RAPD method. *Indian J. Sci. Technol.* **2**(4): 456-578.

MARC (Melkasa Agricultural Research Center) (2004). Progress Report Addis Ababa Ethiopia.

Marcelis, I. and Baan Hofman-Eijer, (1997). Effect of seed number on competition and dominance among fruits in *Capsicum annum* L. *Annals of botany*, **79**: 687-693.

Mesfin Bogale, Wingfield, B., Wingfield, M. and Steenkamp, E. (2006). Characterization of *Fusarium oxysporum*isolates from Ethiopia using AFLP, SSR and DNA sequence analyses. *Fungal diversity* **23:** 51-66.

Misra, A., Shasany, A., Shukla, A., Darokar, M. and Singh, S. (2010). AFLP markers for identification of swertia species (Gentianaceae).*Genet. Mol.Res*. **9:** 1535-1544.

MOARD (Ministry of agricultural and Rural development) (2009). Variety Register. Issue No. 9. June 2006. Addis Ababa, Ethiopia.

Mondini, L., Noorani, A. and Pagnotta, M. (2009). Assessing plant genetic diversity by molecular tools.*Diversity* **1:** 19-35.

Muthusamy, S., Kanagarajan, S. and Ponnusamy, S. (2008). Efficiency of RAPD and ISSR marker system in accessing genetic variation of rice bean (*Vigna umbellate*) landraces. *Electron.J. Biotechnol*. **11:** 1-10.

Patel A. S., Sasidharan N., Ashish G. V. and Vinay K. (2011). Genetic relation in *Capsicum annuum* L. cultivars through microsatellite markers: SSR and ISSR. Electr. *J. Plant Breed,* **2**: 67 -76.

Pengelly, B. and Mass, B. (2001). Lablab purpureus (L.) sweet diversity, potential use and determination of a core collection of this multipurpose tropical legume. *Genetic resource and crop Evolution.* **48**: 261-272.

Prince, J., Lackney, V., Angeles, C., Blauth, J. and Kyle, M. (1995). A survey of DNA polymorphism within the genus *Capsicum* and the fingerprinting of pepper cultivars. Genome, **38**: 224-231.

Purseglove, J., Brown, E., Green, C. and Robbins, S. (1981). Spices. Longman Inc. New York. **1**: 365p.

Reddy, M., Sarla, N. and Siddiq, E. (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica. **128**: 9- 17.

Rodríguez, Y, Depestre, T. and Gómez, O, (2008). Efficiency of selection in pepper lines (*Capsicum annum*) from four subpopulations in characters of productive interest.*Ciencia e Investigació nAgraria,* **35**(1): 29-40.

Rubatzky, V. and Yamaguchi, M. (1997). World vegetables: principles, production and nutritive values, 2nd edition, Chapman and Hall International, Thomson Publishing, New York, pp. 843.

Russell, J., Fuller, J., Macaulay, M. and Hatz, B. (1997). Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. *Theor. Appl. Genet.* **95:** 714-722.

Said, Mohamed. and Kassahun, Tesfaye. (2012). Genetic Diversity Study of *Lepidium sativum* Populations from Ethiopia using Morphological Characters and ISSR Markers, M.Sc. *Thesis*, Addis Ababa University, Addis Ababa Ethiopia, **14**(17): 1461-1470.

Salter, P. (1985). Crop establishment, recent research and trends in commercial practice. *Scientia Horticultruae,* **36**: 32-47.

Sanatombi, K., Sen-Mandi, S. and Sharma, G. (2010). DNA profiling of *Capsicum* landraces of Manipur.Sci. Hortic. **124**: 405-408.

Schierwater, B. and Ender, A. (1993). Different thermo stable DNA polymerase may apply to different RAPD products. *Nucleic Acids Res.* **21:** 4647-4648.

Schulman, A., Flavell, A. and Ellis, T. (2004). The application of LTR retrotransposons as molecular markers in plants. *Methods Mol. Biol.* **260**: 145-173.

Semagn, Kasa., Bjornstad, A. and Ndjiodjop, M. (2006).An Over View of Molecular Markers Methods for Plants.Norwegian University of Life Sciences, Departmentof Plant and Environmental Science, Norway, **5**: 2540-2568.

Singh, P., Singh, S., Mishra, S. and Bhatia, S. (2010). Molecular Characterization of Genetic Diversity in *Jatrophacurcas L.* Molecular biology and tree physiology laboratory, Institute of Forest Productivity, India, **4**:1-8.

Sitthiwong, K., Matsui, T., Sukprakarn, S., Okuda, N. and Kosugi, Y. (2005). Classification of pepper (*Capsicum annuum* L.) accessions by RAPD analysis. Biotechnology, **4**(4): 305-309.

Kasahun, Tesfaye, (2006). Genetic diversity of wild *Cofea Arabica* populations in Ethiopia as a contribution to conservation and use planning. Ecology and Development series. Doctoral Thesis. University of Bonn, Germany. No. 44.

Thul, S., Darokar M., Shasany A. and Khanuja, S. (2011). Molecular profiling for genetic variability in *Capsicum* species based on ISSR and RAPD markers. *Molecular biotechnology*, **51:** 137-147.

Thul, S., Darokar, M., Shaseny, A. and Khanuja, S. (2012). Molecular profiling of genetic variability in *Capsicum* species based on ISSR and RAPD markers. *Mol. Biotechnol*. **10**: 2033-9446.

Tong, N. and Bosland, p. (2003). Observations on interspecific compatibility and meiotic chromosome behaviour of *Capsicum buforum* and *Capsicum lanceolatum. Genetic Resource and Crop Evaluation.* **50**: 193-199.

Toquica, S., Rodriquez, F., Martinez, E., Duque, M. and Tohme, J. (2003). Molecular characterization by AFLPs of *Capsicum* germplasm from the Amazon department in Columbia, characterization by AFLPs of *Capsicum*. *Genet. Res. Crop Evol.* **50**: 639-647.

Vos, P., Hogers, R., Bleeker, M. and Reijans, M. (1995). AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res*. **23:** 4407-4414.

Wien, H. (1997). The physiology of Vegetable Crops.CAB International Oxon, UK. Pp: 259-293.

Williams, J., Kubelik, A., Livak, K., Rafalski. J. and Tingey, S. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **18**: 6531-6535.

Yemane Kasa, (2017). Evaluation of Hot Pepper Varieties (*capsicum annum*) for Growth, Dry pod Yield and Quality at M/Lehke District, Tigray, Ethiopia, **5(**3): 2321-9939.

Zietkiewicz, E., Rafalski, A. and Labuda, D. (1994). Genome fingerprinting by simple sequence repeats (SSR) anchored PCR amplification. *Genomics*. **20**: 176–183.

7. APPENDICES

Appendix I. Hot pepper (*Capsicum annum*) accessions used in the study

Table 1: Hot pepper accessions, geographical coordinates, altitude and location used in the study

No	Acc. No	Acc. code	Region	Zone	Latitude (N)	Longtude (E)	Altitude (m.a.s.l)
1	19968	SG1	Amhara	Semen Gondar	12-30-57	37-06-05	1818.00
2	19969	S2G	Amhara	Semen Gondar	12-28-07	37-04-25	1971.00
3	19970	SG3	Amhara	Semen Gondar	11-12-05	37-05-44	1834.00
4	19971	SG4	Amhara	Semen Gondar	12-10-47	37-01-54	1806.00
5	19972	SG5	Amhara	Semen Gondar	12-16-46	37-06-00	1847.00
6	19973	SG6	Amhara	Semen Gondar	11-57-48	36-59-03	1864.00
7	19974	SG7	Amhara	Semen Gondar	12-2-08	36-55-28	2056.00
8	19978	SG8	Amhara	Semen Gondar	12-21-11	37-09-57	1842.00
9	19979	SG9	Amhara	Semen Gondar	12-26-05	37-46-02	1888.00
10	9083	MG1	Amhara	Mirab Gojjam	11-25-29	37-05-00	1962.00
11	9085	MG2	Amhara	Mirab Gojjam	11-29-07	37-06-01	1899.00
12	9099	MG3	Amhara	Mirab Gojjam	11-30-35	36-56-43	1964.00
13	9102	MG4	Amhara	Mirab Gojjam	11-29-46	36-53-45	2010.00
14	9103	MG5	Amhara	Mirab Gojjam	11-29-33	36-52-3	2050.00
15	9104	MG6	Amhara	Mirab Gojjam	10-38-21	37-05-10	1974.00
16	9106	MG7	Amhara	Mirab Gojjam	10-26-06	36-11-11	2045.00
17	9094	MG8	Amhara	Mirab Gojjam	11-31-25	36-56-43	1956.00
18	20840	BA1	Oromia	Bale	06-56-30	40-37-10	1570.00
19	20841	BA2	Oromia	Bale	7-6-57	40-19-09	2366.00
20	20844	BA3	Oromia	Bale	7-6-33	40-38-24	1970.00
21	20846	BA4	Oromia	Bale	7-9-44	40-39-00	2004.00
22	20848	BA5	Oromia	Bale	7-9-50	40-38-40	2011.00
23	20850	BA6	Oromia	Bale	7-19-24	40-48-08	1789.00
24	20853	BA7	Oromia	Bale	7-17-31	39-49-13	2437.00
25	229878	BA8	Oromia	Bale	06-61-00	39-04-00	2060.00
26	19049	BA9	Oromia	Bale	7-22-33	40-28-16	2144.00
27	19051	BA10	Oromia	Bale	7-24-32	40-14-56	2394.00

28	223633	GU1	SNNP	Gurage	8-9-00	38-45-00	1810.00
29	223634	GU2	SNNP	Gurage	8-10-00	38-44-00	1850.00
30	223635	GU3	SNNP	Gurage	06-50-00	39-05-00	1850.00
31	223638	GU4	SNNP	Gurage	7-10-00	38-45-00	1950.00
32	223642	GU5	SNNP	Gurage	8-8-00	38-26-00	2060.00
33	223644	GU6	SNNP	Gurage	8-9-00	38-36-00	2050.00
34	223645	GU7	SNNP	Gurage	8-7-00	38-30-00	1945.00
35	223646	GU8	SNNP	Gurage	8-10-00	38-45-03	2089.00
36	223647	GU9	SNNP	Gurage	8-7-00	38-42-03	2089.00
37	223640	GU10	SNNP	Gurage	8-8-00	38-45-03	2089.00
38	212912	KM1	SNNP	Kembata	38-03-00	7-21-00	1850.00
39	223648	KM2	SNNP	Kembata	7-24-00	38-06-00	1820.00
40	223649	KM3	SNNP	Kembata	7-24-00	38-06-00	1820.00
41	223650	KM4	SNNP	Kembata	7-24-00	38-06-00	1820.00
42	223652	KM5	SNNP	Kembata	7-24-00	38-06-00	1820.00
43	223653	KM6	SNNP	Kembata	7-24-200	38-06-00	1860.00
44	223654	KM7	SNNP	Kembata	7-25-200	38-06-00	1860.00
45	223655	KM8	SNNP	Kembata	7-25-00	38-06-00	1860.00
46	223656	KM9	SNNP	Kembata	7-25-00	38-06-00	1860.00
47	223657	KM10	SNNP	Kembata	7-26-200	38-07-00	1890.00

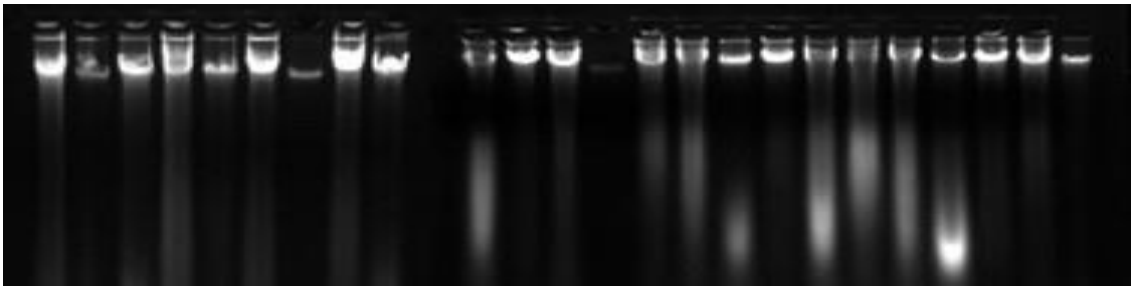
Table 2: Hot pepper cultivars and their code and source used in the study

No	Sample name	Sample code	Sample source
1	Mareko Fana	MF	MARC
2	Melka Zala	MZ	MARC
3	Melka Awaze	MA	MARC
4	Melka Dera	MD	MARC
5	Melka Oli	MO	MARC
6	Melka Shote	MS	MARC

Appendix II: Gel test of genomic DNA extraction

The following figure shows the genomic DNA extraction which was run on 1% TAE agarose gel to determine the quality of DNA

A.



B.

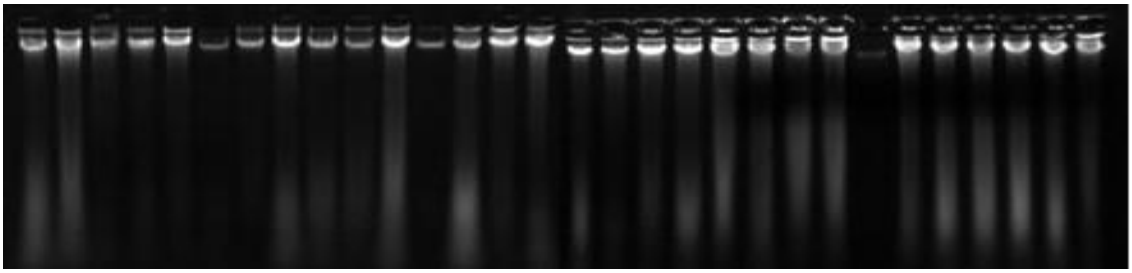
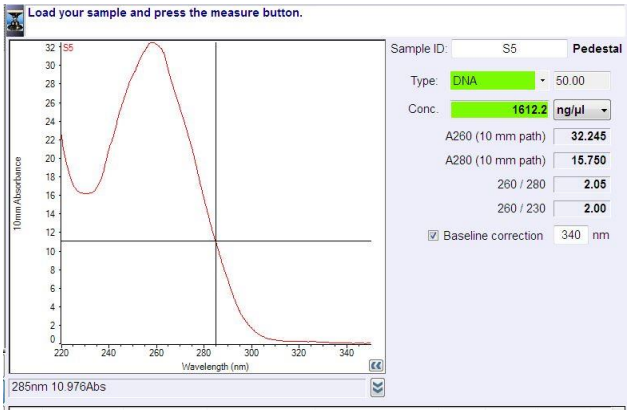


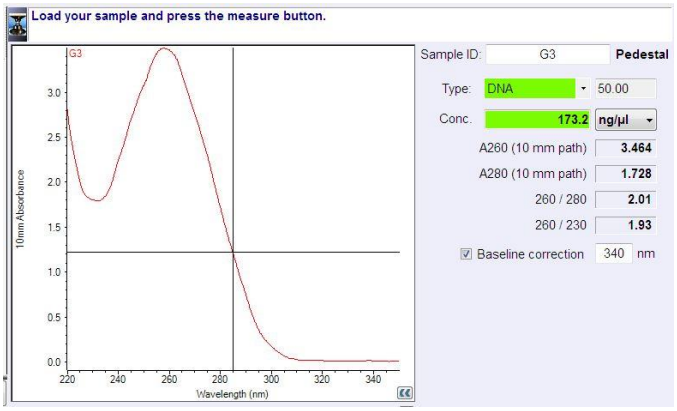
Figure 1: Agarose gel test of genomic DNA extraction.

Appendix III: Quantification and qualification of Genomic DNA using Nano Drop

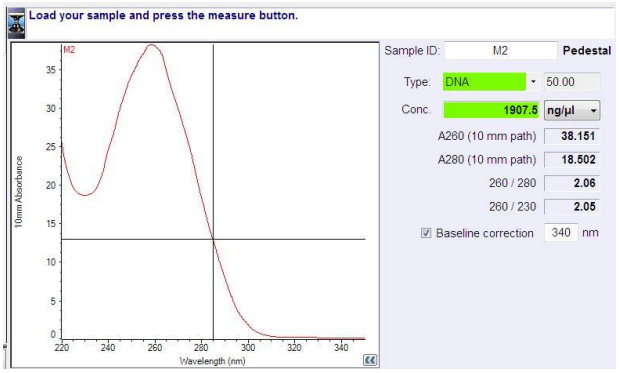
The following graph shows the absorbance patterns of some genomic DNA selected from each populations of *C. annuum* considered in this study.



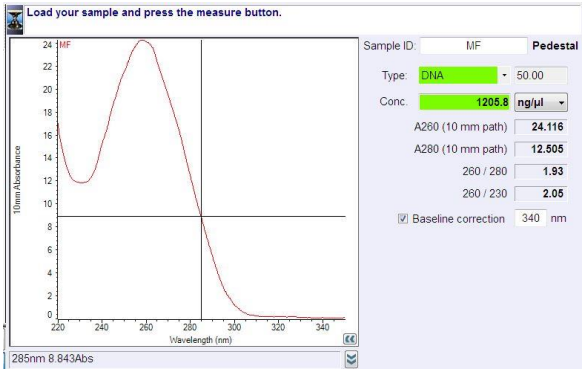
A.



B.



C.



D.

Figure 2: pictures taken from Nano drop qualification and quantification.

Appendix IV: Representative pictures to give further illustration of all practical works beginning from sample collection to the end of laboratory session

Figure 3: Pictures which taken during research work from sample collection to laboratory session (A= *C. annuum* growing, B= sample collection, C= collected sample in laboratory, D= leaf sample grinding by mixer miller, E= centrifugation during DNA extraction F= aspiration, G= quantification and qualification using Nano Drop, H= PCR master mix preparation and I= master mix with DNA interring to PCR machine)