GENETIC DIVERSITY OF ETHIOPIAN *DEKOKO* (*Pisum sativum* L. var. *abyssinicum*) LANDRACES USING INTER-SIMPLE SEQUENCE REPEAT (ISSR) MARKERS

M.SC. THESIS

KIROS TEKLE

MARCH 2017
HARAMAYA UNIVERSITY, HARAMAYA

Genetic Diversity of Ethiopian Dekoko (*Pisum sativum* L. var. *abyssinicum*) Land Races Using Inter-Simple Sequence Repeat (ISSR) Markers

A Thesis Submitted to the Department of Biology,

Postgraduate Program Directorate

HARAMAYA UNIVERSITY

In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

Kiros Tekle

March 2017

Haramaya University, Haramaya

HARAMAYA UNIVERSITY
POSTGRADUATE PROGRAM DIRETORATE

As Thesis Research Advisors, we hereby certify that we have read and evaluated this Thesis prepared, under our guidance, by Kiros Tekle, entitled **"Genetic Diversity study of Ethiopian *Dekoko* (*Pisum sativum* L. var. *abyssinicum*) Landraces using Inter-Simple Sequence Repeat (ISSR) Markers".** We recommend that it be submitted as fulfilling the thesis requirement

Teklehaimanot Haileselassie (PhD)	________________	____________________
Major Advisor	Signature	Date
Yohannes Petros (PhD)	________________	____________________
Co-Advisor	Signature	Date
Kassahun Tesfaye (PhD)	________________	____________________
Co Advisor	Signature	Date

As members of the Examining Board of the Final MSc Thesis Open Defense Examination, we certify that we have read, evaluated the thesis prepared by Kiros Tekle and examined the candidate. We recommend that the thesis be accepted as fulfilling the Thesis requirement for the degree of Master of Science in Biotechnology.

____________________	____________________	____________________
Chairperson	Signature	Date
____________________	____________________	____________________
Internal Examiner	Signature	Date
____________________	____________________	____________________
External Examiner	Signature	Date

Final approval and acceptance of the thesis is contingent upon the submission of its final copy to the council of graduate of studies (CGS) through the candidate's department or school graduate committee (DGC or SGC).

DEDICATION

This thesis is dedicated to my father, TEKLE HAFTU, and my mother, KEBEDU TEKLU, who committed their lives with hard - work and strong prayers for the betterment and success of my life.

STATEMENT OF THE AUTHOR

First, I declare that this thesis is my legitimate work and that all sources of materials used for this thesis have been appropriately acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MSc. Degree at Haramaya University and is deposited at the University Library to be made available to borrowers under the rules of the Library. I soberly declare that this thesis is not submitted to any other institution anywhere for an award of any academic Degree, Diploma, or Certificate.

Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of the source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the Department of Biology or the Director for Postgraduate Program Directorate when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

Name of the Author: Kiros Tekle Signature: _______________

Place: Haramaya University, Haramaya.

Date of Submission: March 31/ 2017

BIOGRAPHICAL SKETCH OF THE AUTHOR

The author was born on December 17, 1988 in Hintalo Wejerat Woreda, South Eastern Zone of Tigray National Regional State from his father Tekle Haftu and his mother Kebedu Teklu. He attended his primary and junior secondary educations at Adi-Keyh Junior Secondary School from 1996-2002. He then joined Adi-Gudom Senior Secondary School in 2003 for grades 9 and 10 and completed in 2004. From 2005-2006, he attended his preparatory school education at Weldu Nigusse Secondary and Preparatory School.

In 2007, he joined Haramaya University and received his Bachelor of Science (B.Sc.) Degree in Applied Biology in July 2010. After his graduation, he was given the opportunity to serve as a Senior Technical Assistant in the Department of Biology, Haramaya University. He was then promoted to the rank of Graduate Assistant II and Assistant Lecturer in 2013& 2014, respectively. In October 2014, he joined the Postgraduate Program of Haramaya University to study M.Sc Biotechnology.

ACKNOWLEDGEMENTS

I wish to express my deep gratitude and appreciation to my Advisors, Dr. Teklehaimanot Haileselassie, Dr. Yohannes Petros and Dr. Kassahun Tesfaye who devoted their precious time and energy in the critical evaluation of the research work and for their encouragement, excellent comments, guidance and careful supervision beginning from the research proposal to the write-up of the thesis. The successful accomplishment of this research work wouldn't have been possible without their unreserved guidance and assistance. Thus, I am very much indebted to them for their tireless efforts that enabled me to successfully complete my studies.

Special thanks also goes to Dr. Kassahun Tesfaye for allowing me to conduct the laboratory activities in his research laboratory by providing the required resources such as laboratory chemicals for DNA Extraction, primers for PCR amplification as well as his follow up during the laboratory work.

A sincere thanks is also extended to the Haramaya University for the study leave and grant which enabled me to pursue my graduate studies. In addition, I would like to thank the Research Office of Haramaya University for providing me with additional funds for the purchase of chemicals for the research work.

My gratitude is extended to Mr. Haftom Hagos, Head of Agriculture and Rural Development Office, Endamekoni Woreda, Tigray, for his unreserved support during data collection.

I would also like to acknowledge Addis Ababa University and the technical assistance by the members of the Plant Genetics Laboratory of the Department of Microbial, Cellular and Molecular Biology and Institute of Biotechnology during the laboratory work. Due thanks is also extended to all the *P. sativum* L. var. *abyssinicum* seed owners for their willingness to provide me with the seed used for this study.

ABBREVIATIONS AND ACRONYMS

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
CSFL	Cool Season Food Legume
CTAB	Cetyl Trimethyl Ammonium Bromide
EBI	Ethiopian Biodiversity Institute
EDTA	Ethylenediaminetetraacetic acid
ISSR	Inter Simple Sequence Repeat
NJ	Neighbor Joining
NPL	Number of Polymorphic Loci
NTSYS-pc	Numerical Taxonomy and Multivariate Analysis System
O.D	Optical Density
PCO	Principal Coordinate
PCR	Polymerase Chain Reaction
PPL	Percent polymorphic Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SSR	Simple Sequence Repeat
TBE	Tris-borate-EDTA
TE	Tris-EDTA
UBC	University of British Colombia
UPGMA	Unweighted Pair Group Method with Arithmetic average

TABLE OF CONTENTS

STATEMENT OF THE AUTHOR **IV**

BIOGRAPHICAL SKETCH OF THE AUTHOR **V**

ACKNOWLEDGEMENTS **VI**

ABBREVIATIONS AND ACRONYMS **VII**

LIST OF TABLES **X**

LIST OF FIGURES **XI**

LIST OF TABLES IN THE APPENDIX **XII**

ABSTRACT **XIII**

1. INTRODUCTION **1**

2. LITERATURE REVIEW **4**

2.1. Origin and Taxonomy *P. sativum L. var. abyssinicum* **4**

2.2. Economic Importance of *P. sativum L. var. abyssinicum* **5**

2.3. Production Potential of *P. sativum L. var. abyssinicum* **6**

2.4. Genetic Diversity and Population Genetics **7**

2.4.1. Population genetics 7

2.4.2. Genetic diversity 9

2.5. Genetic Markers and Genetic Diversity Analysis **11**

2.5.1. Morphological markers 12

2.5.2. Biochemical markers 13

2.5.3. Molecular markers 14

2.5.3.1. Non-PCR based molecular markers 15

2.5.3.2. PCR based methods 16

3. MATERIALS AND METHODS **21**

3.1. Plant Material Collection **21**

3.2. Germination Condition **22**

3.3. Extraction of Genomic DNA **22**

TABLE OF CONTENTS (continued)

3.4. Purification of DNA 23

3.5. DNA Quantity and Quality Determination 23

3.6. Genomic DNA Amplification 24

3.6.1. Selection of primers 24

3.6.2. PCR amplification 25

3.6.3. PCR product gel electrophoresis 26

3.7. Data Analysis 26

3.7.1. Gel band scoring and recording 26

3.7.2. Genetic diversity 26

3.7.3. Genetic structure analysis 27

3.7.4. Clustering analysis 27

4. RESULTS AND DISCUSSION 28

4.1. ISSR Primers and their Banding Patterns 28

4.2. Genetic Diversity Study of *P. sativum L. var. abyssinicum* 29

4.3. Genetic Structure and Classification of Genetic Diversity 32

4.4. Cluster Analysis of *P. sativum L. var. abyssinicum* Genotypes 34

4.4.1. Unweighted pair group method with arithmetic average (UPGMA) 34

4.4.2. Principal coordinates (PCO) analysis 39

5. SUMMARY, CONCLUSSIONS AND RECOMMENDATIONS 41

5.1. Summary 41

5.2. Conclusions 42

5.3. Recommendations 43

6. REFERENCES 44

APPENDICES 53

LIST OF TABLES

Table Page

1. Number of populations of *P. sativum L. var. abyssinicum* used in this study 22
2. List of primers used for primer screening 25
3. Number of Scorable Bands, Number and percentage of polymorphic loci, gene Diversity and Shannon information index of each primer for the 80 accessions 29
4. The Number of polymorphic loci, Percent polymorphic loci, gene diversity and Shannon's information Index, using data generated from all the six primers. 30
5. Analysis of Molecular Variance among and within the seven populations. 33
6. Pair wise Jaccard's similarity coefficient between pairs of the seven populations 34
7. List of accessions which are 100% similar in UPGMA clustering analysis 36

LIST OF FIGURES

Figure	Page
1. ISSR-PCR: A schematic representation of a single primer (AG) 8.	20
2. Geographic distribution of seven populations used in this study.	21
3. Banding pattern of ISSR primer 881.	32
4. Population based UPGMA dendrogram for the seven populations.	35
5. Individuals based UPGMA dendrogram for the seven populations.	38
7. Two dimensional principal coordinate analyses of the seven populations.	39
8. Three dimensional principal coordinate analyses of the seven populations.	40

LIST OF TABLES IN THE APPENDIX

Appendix Table **Page**

1. List of the 83 *P. sativum L. var. abyssinicum* accessions used in this study 53

2.The ND measurement of the quality and quantity of DNA from the 83 accessions 59

Genetic Diversity of Ethiopian Dekoko (*Pisum sativum* L. var. *abyssinicum*) Land Races Using Inter - Simple Sequence Repeat (ISSR) Markers

ABSTRACT

P. sativum L. var. abyssinicum *is a marginalized, economically important and endemic, cool - season legume - crop in Ethiopia. However, no genetic diversity study has been employed using molecular markers on this crop species sofar.* Therefore, this study is aimed to investigate genetic diversity and population structure of P. sativum L.var abyssinicum collected from Southern Tigray and Northern Amhara regions of Ethiopia. *Six ISSR (Inter simple sequence repeat) primers were used to study the genetic diversity of 83 accessions of* P. sativum L. var. abyssinicum *collected from southern Tigray and northern Amhara regions of Ethiopia. Out of the 91 well defined bands generated, 55(64.44%) revealed polymorphic and higher percentage of polymorphic bands (24.18%) were observed in the EM, EA and OF populations. The seven populations revealed a significant level of genetic diversity at the species level. Jaccard's pairwise similarity coefficients ranged from 0.58 to 1.00 indicating the presence of moderate level of genetic relationship at molecular level among the studied accessions. Analyses of Molecular Variance (AMOVA) also revealed genetic diversity among and within populations, 46% and 54% respectively and showed highly significant genetic differences ($p<0.001$). Cluster analysis clearly discriminated the accessions into two major clusters. Result of principal coordinate analysis was in agreement with cluster analysis. Both the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis and Principal Coordinates Analysis (PCO) supported the grouping of all seven populations into six groups. The high genetic diversity among populations implies that the conservation efforts should aim to preserve all the extant populations of this endangered species. In addition, the accessions collected from EBI can also be targeted for further evaluation of their unique character for breeding purposes.*

Keywords: P. sativum L. var. abyssinicum, Genetic diversity, Genetic structure, ISSR markers, Polymorphism

1. INTRODUCTION

Fabaceae is the third largest family of flowering plants, with over 750 genera and 20,000 species worldwide (Upadhyaya *et al.,* 2011). The genus *Pisum* is the largest cool - season food legumes (CSFL) crop in this family. *P. sativum* is one of the oldest and widely grown food crops in this genus. It is a largely self-pollinated diploid (2n=14) annual crop. Kosterin and Bogdanova (2015) reported that *P. sativum, P. fulvum* and *P. abyssinicum* are the three major sub-species in this genus. *P. sativum* var. *sativum* is widespread across the Middle East and has affinity with the wild *P. elatius* while *P. sativum* var. *abyssinicum* is restricted to highland regions of Ethiopia and Southern Yemen (Vershinin *et al.,* 2003).

The primary centers of its origin and diversity are the countries of Western Asia (Iran, Afghanistan, Pakistan, and Turkmenistan) and the entire Mediterranean (Greece, Italy, Spain, and Morocco) (Smykal *et al.,* 2011) and Ethiopia is now considered as a secondary center of pea diversity and origin. *P. sativum L. var. abyssinicum*, locally known as *Dekoko* (*minute seeded*) in Tigrigna and Yagere ater (pea of my country) or Tinishu ater (the smallest pea) in Amharic is grown at a few localities in the southern zone of Tigray and North Wollo zone of Amhara regions (Yemane and Skejelvag, 2003) and south-eastern parts of Ethiopia (Arsi) (Messiean *et al.,* 2006*).*

Ethiopia is one of the largest producers of field pea (*P. sativum*) in Africa (Messiean *et al.,* 2006). In Ethiopia CSLC are consumed daily and serve in various ways, mainly as sauces and snacks (Yemane and Skejelvag, 2003). But recently, the production of local landraces of field peas including *P. sativum L. var. abyssinicum* has been decreased by diseases like powdery mildew and downy mildew as well as by lodging (Mulusew *et al.,* 2010) and pea weevil (Abel *et al.,* 2015). Studies conducted so far on *P. sativum* var. *abyssinicum* include: effect of salinity on germination and early seedling growth (Berhanu and Berhane, 2014), effect of moisture on physical properties (Tamrat, 2014), physicochemical properties (Yemane and Skejelvag, 2003), characterization and preliminary evaluation of accessions using quantitative traits (Haddis and Tsegay, 2013), evaluation of seed yield and seed yield components in red–yellow (*P. fulvum*) and *P. sativum L. var. abyssinicum* (Mikic *et al.,* 2012). Jing *et al. (*2010); Smyka*l et al*. (2011) and Kwon *et al.* (2012) also conducted a genetic diversity study in *P. sativum L. at a Genus*

level including few accessions of P. sativum L. var. abyssinicum (only five). All these studies attempted to measure the genetic similarity of *P. sativum L. var. abyssinicum* with other *Pisum* varieties and they reported that, it as distinct variety that were clearly separated from all *Pisum* sub-species during clustering but show some similarity with the wild type *P. elatius*.

Knowledge of genetic diversity patterns play an indispensable role in understanding the evolutionary history of a species, assessment of future risks of erosion of diversity and provides a means for designing effective conservation strategies (Neel and Ellstrand, 2003). Assessment of genetic variation among populations of endangered species is also very essential for prioritization of sites and management choices for future conservation programs. Petit *et al.* (1998) reported that greatly diverse or differentiated populations could be targeted for conservation, while genetically poor populations might be targeted for management plans to restore diversity. In Ethiopia limited Breeding efforts have resulted in the release of a few of *P. sativum L. cultivars* (Messiean *et al*., 2006). But farmers in the *P. sativum L. var. abyssinicum* growing areas still use their own farm-saved seeds of local cultivars since there are no commercial improved *P. sativum L. var. abyssinicum* variaties. Traditional unimproved adapted landraces might be an important genetic resource for plant breeders and gene banks because of their considerable genotypic variation and their adaptation to environmental conditions in many years of cultivation (Messiean *et al*., 2006). Identifying the existing genetic diversity also provides plant breeders with options to develop productive crops that are resistant to virulent pests and diseases, adapted to changing environments and effective management and use of genetic resources (Wangari *et al*., 2013).

Assessment of Genetic diversity can be based on morphological, biochemical and molecular markers. But PCR-based DNA marker systems like random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs) and inter - simple sequence repeats (ISSRs) are efficiently used to assess genetic diversity. ISSR markers have proved to be good tools in the assessment of genetic variation both within and among plant populations due to its highly polymorphic and dominant nature; low developmental and running cost; it does not require prior sequence information and it is highly reproducible (Vijayan, 2005). The technique has also been used successfully in diversity studies in *P. sativum* (Taran *et al*., 2005; Baranger *et al.,* 2004; Lazaro and Aguinagalde, 2006) and other

plant species (Zhou *et al.,* 2008; and Liu *et al.,* 2013). Eventhough *P. sativum L. var. abyssinicum* is economically very important crop in Ethiopia; no breeding attempts were made for the development of disease and drought resistant varieties with superior yield potential. To the best of our knowledge, conservation and management strategies for this variety are also not yet reported. Moreover, no molecular assays have previously been utilized to analyze genetic diversity within and among populations of *P. sativum L. var. abyssinicum* landraces of Ethiopia.

General Objective:

This study is aimed to investigate genetic diversity and population structure of *P.* abyssinicum from southern Tigray and northern Amhara regions of Ethiopia by using inter - simple sequence repeat (ISSR) Markers.

Specific Objectives:

- To estimate the genetic diversity of *P. sativum L. var. abyssinicum* populations collected from Southern Tigray and north Amhara regional states of Ethiopia.
- To reveal genetic structure within and among populations of *P. sativum L. var. abyssinicum* and
- To identify populations of *P. sativum L. var. abyssinicum* with high genetic diversity for future improvement and conservation programs in the country.

2. LITERATURE REVIEW

2.1. Origin and Taxonomy *P. sativum L. var. abyssinicum*

Among the world's oldest domesticated crops, field pea (*P. sativum* L.*)* is one of the most widely grown food crop. There are several taxonomic levels and nomenclatures given to the species in the genus *Pisum.* Some of them classified as *P. sativum*, *P. abyssinicum*, *P. elatius and P. fulvum* species (Jing *et al*., 2010) while other researchers limit the scope of the genus to three, *P. sativum* with two sub-species (subsp. *sativum* and subsp. *elatius*) *P. abyssinicum*, and *P. fulvum* (Maxted and Ambrose*,* 2000). Others scholars are also recognize only two species, *P. sativum and P. fulvum* (Dyachenkoa *et al.,* 2014). It is also classified as *P. fulvum* found in Jordan, Syria, Lebanon and Israel; the cultivated species *P. abyssinicum* grows in Yemen and Ethiopia, possibly independently domesticated, *P. sativum* and a large aggregate of both wild *P. sativum subsp. elatius* and cultivated forms of *P. sativum subsp. sativum* (Smykal *et al.,* 2011*;* Upadhyaya *et al.,* 2011).

Another study also reported that the genus *Pisum* has two species, *P. sativum* with five sub-species (*P. sativum subsp. Sativum, P. sativum subsp. Elatius, P. sativum subsp. abyssinicum, P. sativum ssp. asiaticum,* and *P. sativum subsp. transcaucasicum*) and *P. fulvum*. Under subsp. s*ativum* there are two domesticated varieties (var. *sativum and* var. *arvense*) and under subsp. e*latius* there are three wild varieties (var. *elatius*, var. pumilio and var. *humile*) (Zong *et al*., 2009). Kosterin and Bogdanova (2015) also reported that *P. Sativum, P. fulvum* and *P. abyssinicum* are the three major species in this genus. Despite the controversies on the taxonomic status of the species in this genus, Conicella and Errico (2014) reported chromosomal evidence that considers *P. sativum L. var. abyssinicum* together *with pumilio* and *elatius* to be an ecotype or a botanical variety of *P. sativum* through conducting a Karyotype Variations study in *P. sativum L. var. abyssinicum*. Jing *et al.* (2010) and Vershinin *et al.,* (2003) also suggested that *P. sativum L. var. abyssinicum* may have been derived from a cross between *P. fulvum and P. elatius*. Kosterin and Bogdanova (2008) also reported that *P. sativum L. var. abyssinicum* and red–yellow pea (*P. fulvum*) as two close neighboring branches within the pea genus. Zong *et al.* (2009), in a comparative analysis of Chinese and global wide pea collections also found that *P. sativum L. var. abyssinicum* is a unique Ethiopian subspecies that

differed from other subspecies, and its domestication was independent from *P. sativum* subsp. s*ativum*

Due to the early cultivation of pea, over 9000 years ago (McPhee, 2003), it is difficult to identify the precise location of center of its diversity. However, based on its genetic diversity, four centers of origin were proposed by Vavilov (1926), namely Central Asia, the Near East, Abyssinia (Ethiopia) and the Mediterranean. Accordingly, Ethiopia and mountainous areas of the Near East and Central Asia were the primary centers of origin for pea and the Mediterranean was the secondary center. But, Smykal *et al.* (2011) has reported that the primary centers are the countries of Western Asia (Iran, Afghanistan, Pakistan, and Turkmenistan) and the entire Mediterranean (Greece, Italy, Spain, and Morocco) and Ethiopia is now considered a secondary center of pea diversity and origin. *P. sativum L. var. abyssinicum* is one of the important cultivated *Pisum* varieties in Ethiopia and Yemen (Smykal *et al.,* 2011*;* Messiean *et al.,* 2006). It is a food legume plant which is grown at a few localities in the southern zone of Tigray and to a much lesser extent in Wag-Himra and North Wollo zones (Yemane and Skejelvag, 2003). Messiean *et al.* (2006) also reported that *P. sativum L. var. abyssinicum* is cultivated in the south-eastern parts of Ethiopia in Arsi.

2.2. Economic Importance of *P. sativum L. var. abyssinicum*

The seeds of field pea serve as a protein-rich food for humans and livestock in the world. On a worldwide basis, legumes contribute about one-third of humankind's direct protein intake. Dry pea seeds have high levels of the essential amino acid lysine and tryptophan, which appeared in low amounts in cereal grains and it can supplement the low amount of protein present in food and feed processed from cereal grains (Rodino *et al*., 2009). It is also serving as an important source of fodder and forage for animals and edible and industrial oil for humans (Smykal *et al.,* 2012). Other most important attributes of legumes are their capacity for symbiotic nitrogen fixation, underscoring their importance as a source of nitrogen in both natural and agricultural ecosystems and serve as a break crop which further minimizes the need for external inputs. Recent studies have also highlighted the potential health benefits of pulses in human diet including a reduced risk of type II diabetes and cardiovascular disease (Rizkalla *et al*., 2002).

Ahmad *et al.* (2015) also stated that, crop genetic diversity studies of marker-trait association of lipid content for biodiesel industry is an agenda in Canada and field pea is found to be one of the promising crops for such alternative low cost and sustainable oilseed. Yemane and Skejelvag (2003) also reported that fat content of *P. sativum L. var. abyssinicum* in the cotyledon flour is 18.9 g/kg dry matter, which is almost close to the upper value indicated for peas. However, pea also contains proteases, tannins, lectins, etc. which may reduce livestock feed gain when their concentration is high in the diet (Rodino et al., 2009). *P. sativum L. var. abyssinicum*, the indigenous food legume in Ethiopia is also highly appreciated for its higher crud protein, fat content, sugar content and amino acid content like lysine and glutamine but less mineral content compared to other legumes like *Faba bean (Vicia faba L.)* used for the same purpose (Yemane and Skejelvag, 2003). *P. sativum L. var. abyssinicum* is also highly appreciated for its taste, as a result has high demand by local people and consumers and it obtains premium price in local markets. Main dishes include '*shiro wot'* (split pea seeds ground and made into stew) and *'kik wot'* (split pea seeds boiled and made into stew) (Haddis and Dargie, 2013).

2.3. Production Potential of *P. sativum L. var. abyssinicum*

Efficient global food production depends on the careful use and conservation of agricultural biodiversity and genetic resources. Crops and their wild relatives comprise the genetic resources for breeding new crop varieties in response to environmental and demographic changes. Grain legumes including pea, are commonly produced in rotation with cereal grains to break cereal disease cycles, allow weed control through herbicide application and enhances soil fertility by forming a symbiotic relationship with the soil bacterium. Canada, USA, Europe and Australia are the major producers of peas and China and India take majority of the production in Asia (FAOSTAT, 2015). Ethiopia is one of the biggest producers of CSFL crops such as beans and peas in Africa. Field pea (*P. sativum*) is the fourth most important legume crop in Ethiopia after faba bean, haricot bean and chick pea in terms of both area and total amount of production (Haddis and Dargie, 2013). Field pea production in Ethiopia has shown steady increase during the last decade and the annual consumption per person of pea seeds is estimated to be 6–7 kg (Messiean *et al*., 2006). But the increase in production is not due to increase in yield/hectare rather due to an increase in land cover by the crop (FAOSTAT, 2015).

The crop is popular among farmers due to its high return value even when grown in degraded soil, and it requires less management input than cereals to give good yield. In recent times, the production of local landraces has been hindered by diseases like powdery mildew and downy mildew as well as by lodging (Mulusew *et al*., 2010) but the major problem in field pea production is the pea weevil (Abel *et al*., 2015b). The economic loss due to this pest is getting worse and it is forcing farmers to give up field pea production in certain parts of the country. Since most farmers are unable to afford appropriate pesticides, they rely on fumigation as post-harvest containment alternative. With regard to *P. sativum L. var. abyssinicum*, its nutritional value, test and agronomic benefits have contributed more to its sustained production and use in cropping system.

2.4. Genetic Diversity and Population Genetics

2.4.1. Population genetics

Population genetics is a field of biology that studies the genetic composition of biological populations, and the changes in genetic composition that result from the operation of various factors, including natural selection (Samir, 2016). Its main purpose is to understand the ways in which the forces mutation, natural selection, random genetic drift, and population structure interaction to produce and maintain the complex patterns of genetic variation that are observed among individuals within a species (Çiftci and Okumu, 2002). Determining and measuring population differentiation important to quantify the nonrandom distribution of genetic variation in several local population by computing the difference in the genetic composition of the subpopulations from the expectation of random mating in the total population. It is used to compute the variance of allele frequencies between subpopulations, to calculate differences in the effective number of alleles between subpopulations, to estimate the difference in genetic distance between genes in subpopulations (e.g. the analysis of molecular variance, AMOVA, to determine the genetic diversity within and between subpopulations (Excoffier, 1992), to compare the discrepancy in heterozygosity or homozygosity between subpopulations and the total population, to assess the deviation population, to determine the co-ancestry coefficient (correlation or relatedness) for alleles within a subpopulation relative to the total population and to quantify the difference in private alleles between subpopulations (Ma *et al*., 2015).

There are many important factors in genetic variability, like selection, migration and genetic drift are also affected by human activities. One very important population genetics parameter is gene flow (*Nem),* i.e. the number of individuals immigrated to and interbred in a population per generation. Restriction on gene flow may lead to genetic subdivision and justify that subpopulations may treated as separate stocks (Chakraborty and Leimar, 1987). It is difficult to monitor gene flow directly so it has to be inferred indirectly from the spatial distributions of genetic markers by statistical approaches (Nei, 1973). i.e. Nm =0.5(1 - Gst)/Gst.

Another the most widely applied statistic for measuring population genetic differentiation is Nei's that analyzes allele frequency variation among subpopulations in terms of heterozygosity or gene diversity as defined by Nei (1973). The differentiation relative to the total population, named by Nei the coefficient of gene differentiation is given by $G_{ST} = (H_T - H_S) / H_T$ where H_S and H_T are the expected heterozygosity within subpopulations and for the total population, respectively (Nei, 1975). Sewall Wright, also found the theoretical population genetics, developed the So-called *F*-statistics (also known as fixation indices) in the 1950s to measure population differentiation (Wright, 1951). Fixation refers to the fixation of a single allele at a locus to form a homozygote.

$$F_{ST} = \frac{\sigma_p^2}{\overline{p}(1-\overline{p})}$$

σ^2_p is the variance of the frequency of allele *A* of subpopulations and $\overline{p}$ is the average frequency of allele *A* in the total population for biallelic systems (Wright 1965).Population heterozygosity (*H*), the mean number of alleles per locus and the percentage of polymorphic loci (*PLL*) are also important measures genetic differentiation among populations of (Nei, 1973).

Two popular sets of tree methods tend to give consistently different results and, in some cases, even gross differences in topology. Typical representatives of the first set are maximum likelihood (ML) or the numerically convenient short cut called the Unweighted Pair Group method using Arithmetic Averages (UPGMA) and the second set is represented by Neighbor Joining (NJ) methods (Saitou and Nei, 1987). To demonstrate levels of genetic relatedness (similarity) or genetic distance (*D*) between pairs of populations within group of populations, a

large number of different algorithms are available. In essence the algorithms measure the similarity of allelic frequencies over all loci among populations. The commonly used measures reviewed and demonstrated by Nei (1978). A genetic similarity value allows an examination of which pairs of populations are the most related and which are the least related.

2.4.2. Genetic diversity

Genetic diversity is defined by the total number of genetic characteristics in the genetic makeup of a species (Begbulem and Ita, 2016).Genetic diversity is a study undertaken to classify an individual or population compared to other individuals or populations. This is a relative measure, as the distance between any pair of entries is greater or lesser depending on all pairwise comparisons that can be made in the study. As it adapts to a wide range of climates and altitudes, accessions of Pisum have been collected, characterized and maintained in several major gene banks worldwide. These includes: Vavilov Institute , Russia; John Innes Center (JIC), UK; Nordic Gene Bank (NGB), Sweden; International Center of Agricultural Research (ICARDA), Lebanon; United States Department of agriculture (USDA), USA; and Ethiopian Biodiversity Institute (EBI), Ethiopia (Smykal et al., 2011). Some of these collections have been characterized with morphological and molecular markers but still P. sativum lags behind in terms of characterization of its gene pool as compared to cereals (Abel et al., 2015).

The largest collection of *P. sativum* germplasm in Africa is located at the Ethiopian Biodiversity Institute, Addis Ababa, Ethiopia, with over 1600 accessions (Messiean *et al*., 2006) but very few accessions of *P. sativum L. var. abyssinicum* (only ten) are found in EBI. Moreover, the institute doesn't recognized *P. sativum L. var. abyssinicum* as a distinct sub-species or variety and recorded as *P. sativum L. var. sativum* in the gene bank. Since no commercial cultivar was developed, landraces have been kept as original crops by a few dedicated farmers, cultivated on a small scale farming and handed down from generation to generation. This could help agricultural research centers and gene banks (Ethiopian Institute of Biodiversity Conservation) to collect and preserve relatively large number of *P. sativum L. var. abyssinicum* landraces. Nevertheless, there are limited efforts to characterize these field pea germplasm collections with morphological and molecular markers (Mulusew *et al*., 2010). Of these few works, a collection of 148 Ethiopian *P. sativum* genotypes has been assessed by Gemechu *et al*. (2005) for 12

agronomically important traits and significant differences have been detected in all, except for number of seeds per pod. Another study has also attempted to assess the genetic diversity of Ethiopian field pea (*P. sativum)* accessions using newly developed EST-SSR markers and observed significant differences (Abel *et al*., 2015). However, this study has only considered a few number of accessions and does not fully represent the existing diversity and a number of original pea landraces have not been included (Kumari *et al*., 2013).

The genetic diversity of field pea genetic resources have also been studied globally with DNA markers (Baranger *et al*., 2004; Zong *et al*., 2009; Jing *et al*., 2010). Some of These studies showed that pea cultivars have a small pool of genetic diversity (Jing *et al*., 2010) and the European feed peas, have a very narrow genetic basis (Baranger *et al*., 2004). But landraces and wild relatives are rich in genetic variation with valuable adaptive traits for certain areas. As far as the *P. sativum L. var. abyssinicum* landraces are concerned, nothing is known about the within and among population genetic diversity. Assessment of genetic variability between varieties is of interest not only for the conservation of genetic resources but also to know the source of genes for a particular trait within the available germplasm for breeding purposes. Genetic variability is considered as an important factor which is essential prerequisite for crop improvement program for obtaining high yielding progenies (Tiwari & Lavanya, 2012).

To overcome this problem, Global Crop Diversity Trust (GCDT)) supports the development of global crops (including some legumes) and regional strategies for ex situ conservation and utilization of crop diversity in different parts of the world (Upadhyaya *et al*., 2011). These strategies were designed for initiating the development of regional strategies by conducting surveys on genetic resources conservation and use via involvement of regional expert consultations. Conservation of plant genetic resources is essential to meet the current and future needs of crop improvement programs. The management of genetic resources includes (i) regenerating and conserving already collected genetic resources, (ii) enriching the genetic resources through collections of new germplasm and creation of new genetic variability, (iii) characterizing, evaluating, documenting and assessing the pattern of genetic diversity to identify gaps in the collection, (iv) assessing the impact of plant genetic resources in crops breeding and (v) promotion and awareness raising (Upadhyaya *et al*., 2011).

The use of plant genetic resources (PGR) in crop improvement is one of the most sustainable ways to conserve valuable genetic resources for the future while simultaneously increasing agricultural production and food security. Key to successful crop improvement is a continued supply of genetic variability in breeding programs, including new or improved variability for target traits. Cultivation of newly developed high performing pea cultivars is quit beneficial for pea growers. This indicates that sufficient genetic diversity increases productivity. However, limited parental material and intensive breeding in the development of pea varieties has resulted in low genetic diversity. In addition, self-pollination and increasing homozygosity of pea varieties may also have contributed to a loss of genetic diversity (Cieslarova *et al*., 2011). But cross pollination and geographical distribution could lead to increase in genetic diversity (Zhang *et al*., 2009).). Lack of sufficient genetic variability for economically important characters hinders breeding efforts and decreases the progress of crop improvement *(*Georgieva *et al.,* 2016). Beranger *et al*. (2004) and Taran *et al*. (2005) reported that a scarce amount of *Pisum* genetic diversity is available for breeding programs in Canada. In such breeding programs, estimates of genetic relations among parental line are essential in determining which material should be utilized in crosses to maximize genetic gain. Knowledge of genetic diversity among parental lines provides allelic variation for the assembly of novel gene combinations and favorable phenotypes.

Several approaches have been adopted in various studies to assess genetic diversity in both cultivated and wild pea germplasm but molecular markers can be effectively used to assess genetic diversity of pea (Simioniuc *et al*., 2002; Smykal *et al*., 2008a; Taran *et al*., 2005; Zong *et al*., 2009). The commonly used polymerase chain reaction (PCR)-based DNA marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs) and inter simple sequence repeat (ISSRs).

2.5. Genetic Markers and Genetic Diversity Analysis

The assessment of genetic diversity within and among populations is routinely performed at the molecular level using various laboratory-based techniques such as DNA analysis, which measures levels of variation directly. Genetic diversity may be also gauged using

morphological, and biochemical characterization and evaluation (Ana *et al*., 2009; Mondini *et al*., 2009). However, molecular markers have advantages over other kinds of markers, where they show genetic differences on a more detailed level without interferences from environmental factors (Smykal *et al.*, 2008b).

Some of the more important applications of genetic markers include: (1) Describing mating systems, levels of inbreeding, and temporal and spatial patterns of genetic variation within stands (2) Describing geographic patterns of genetic variation (3) Inferring taxonomic and phylogenetic relationships among species (4) Evaluating the impacts of domestication practices, including forest management and tree improvement, on genetic diversity (5) Fingerprinting and germplasm identification in breeding and propagation of populations (6) Constructing genetic linkage maps and (7) Marker assisted breeding (Duran *et al*., 2009).

2.5.1. Morphological markers

Identification and analysis of genetic diversity within and among populations were traditionally based on morphological characteristics such as leaf area, plant height/width, seed number, seed size, flowering time, germination time, etc. In addition this kind of analysis also requires a long period especially for ligneous plant and easily be affected by developmental stage, environmental condition, cultivation condition and even sampling error (Wang *et al*., 2011). Morphological markers reflect variation of expressed regions of genome. Plant species recognition has long been based on morphological characters. Plant morphology is highly polymorphic and phenotypic characters may, in principle, allow plant species classification. Especially, if the traits are highly heritable, morphological markers are one of the choices for diversity studies because the inheritance of the marker can be monitored visually (Wang *et al*., 2011). However, different individuals of the same species may present a variation in their morphology either naturally or in connection with local adaptations. Alternatively some species of the same genus can be morphologically very similar and may be grouped into the same species (Duminil and Michele, 2009).

Morphological features also have a number of limitations including being restricted to relatively few traits, display a low degree of polymorphism, are often environmentally variable in their manifestation, can depend on the expression of several unlinked genes (Schulman, 2007).

Another weakness of morphological markers for the discrimination of species is based on their accessibility. Indeed it is often difficult to have access to the vegetative part of adult woody individuals, especially in tropical forest ecosystems (Duminil *et al.,* 2006). This difficulty increases when the morphological characters of interest are reproductive traits that are absent during most of the year.

2.5.2. Biochemical markers

Allozymes are allelic variants of enzymes encoded by structural genes. Enzymes are proteins consisting of amino acids, some of which are electrically charged. As a result, enzymes have a net electric charge, depending on the stretch of amino acids comprising the protein. When a mutation in the DNA results in an amino acid being replaced, the net electric charge of the protein may be modified, and the overall shape (conformation) of the molecule can change. Because changes in electric charge and conformation can affect the migration rate of proteins in an electric field, allelic variation can be detected by gel electrophoresis and subsequent enzyme-specific stains that contain substrate for the enzyme, cofactors and an oxidized salt (e.g. nitro-blue tetrazolium). Usually two, or sometimes even more loci can be distinguished for an enzyme and these are termed isoloci. Therefore, allozyme variation is often also referred to as isozyme variation (Kephart, 1990).

The strength of allozyme markers is that it does not require DNA extraction, no need of sequence information, primers or probes, quick and easy to use, co-dominancy of markers, high reproducibility (Spooner et al., 2005). Disadvantages of isozyme include: (i) there are only few isozyme systems per species (no more than 30) with correspondingly few markers; (ii) the number of polymorphic enzymatic systems available is limited and the enzymatic loci represent only a small and not random part of the genome (the expressed part) - therefore, the observed variability may be not representative of the entire genome; (iii) although these markers allow 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 stage (Mondini et al., 2009). For this reason, most researchers began to focus on the use of molecular markers systems for genetic and ecological analyses of plant populations.

2.5.3. Molecular markers

Molecular markers work by highlighting differences (polymorphisms) within a nucleic acid sequence between different individuals. These differences include insertions, deletions, translocations, duplications and point mutations. They do not, however, encompass the activity of specific genes. In addition to being relatively not influenced by environmental factors, an ideal molecular marker should possesses the following features: (1) be polymorphic and evenly distributed throughout the genome; (2) provide adequate resolution of genetic differences; (3) generate multiple, independent and reliable markers; (4) be simple, quick and inexpensive; (5) need small amounts of tissue and DNA samples; (6) link to distinct phenotypes; and, (7) require no prior information about the genome of an organism. Nevertheless, no molecular marker presents all the listed advantages (Joshi *et al* 2000; Kundan *et al*., 2014). The different techniques employed are based either on restriction-hybridization of nucleic acids or techniques based on Polymerase Chain Reaction (PCR), or both.

In addition, the different techniques can assess either multi-locus or single-locus markers. Multi -locus markers allow simultaneous analyses of several genomic loci, which are based on the amplification of casual chromosomic traits through oligonucleotide primers with arbitrary sequences. These types of markers are also defined as dominant since it is possible to observe the presence or the absence of a band for any locus, but it is not possible to distinguish between heterozygote (a/-) conditions and homozygote for the same allele (a/a). By contrast, single -locus markers employ probes or primers specific to genomic loci, and are able to hybridize or amplify chromosome traits with well-known sequences. They are defined as co-dominant since they allow discrimination between homozygote and heterozygote loci. Therefore, molecular markers which detects variation at DNA level, overcomes the limitations of morphological and biochemical markers (Mondini *et al*., 2009).

DNA based marker techniques such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Inter Simple Sequence Repeats (ISSR) and Amplified Fragment Length Polymorphism (AFLP) are now in common use for ecological, evolutionary, taxonomical, phylogenic and genetic studies of plants (Mondini *et al*., 2009). The different methods of

molecular assessment differ from each other with respect to important features such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and cost. Moreover; a good marker for fingerprinting studies can be cheap to run, or gives a lot of information per run; very repeatable between assays; experience very low error rate and easy, unambiguous to score; and contain many alleles (high information content).

2.5.3.1. Non-PCR based molecular markers

Genetic marker systems based on DNA-DNA hybridization was developed in the 1970s (Southern, 1975). Eukaryotic genomes are very large and there was no simple way to observe genetic polymorphisms of individual genes or sequences. The property of complementary base pairing allowed for methods to be developed whereby small pieces of DNA could be used as probes to reveal polymorphisms in just the sequences homologous to the probe. The genetic system derived using this approach is called Restriction Fragment Length Polymorphism (RFLP).

Restriction Fragment Length Polymorphism

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). This technique quickly proved their utility in virtually all species. Variations in the characteristic pattern of a RFLP digest can be caused by base pair deletions, mutations, inversions, translocations and transpositions which result in the loss or gain of a recognition site resulting in a fragment of different length and polymorphism. Only a single base pair difference in the recognition site can cause the restriction enzyme not to cut. If the base pair mutation is present in one chromosome but not the other, both fragment bands can be present on the gel, and the sample is said to be heterozygous for the marker (Beckmann and Soller, 1986). Only co-dominant markers exhibit this behavior which is highly desirable. Dominant markers exhibit a present/absent behavior which can limit data available for analysis. RFLP markers are relatively highly polymorphic, codominant inherited, highly replicable and allow the simultaneously screening of numerous samples. However, RFLPs are highly expensive, time

consuming, labor intensive, a larger amount of DNA required and reveals limited polymorphism especially in closely related lines (Mondini *et al*., 2009).

2.5.3.2. PCR based methods

Advances in Molecular Biology led to the development of polymerase chain reaction (PCR) technology. Before PCR, the analysis of a specific DNA fragment generally required cloning of the fragment and amplification in a plasmid or comparable vector. The polymerase chain reaction enables the production of a large amount of a specific DNA sequence without cloning, starting with just a few molecules of the target sequence. The advantage of PCR-based marker methods over DNA-DNA hybridization marker methods is (1) A small amount of DNA is required,(2) Elimination of radioisotopes in most techniques,(3) The ability to amplify DNA sequences from preserved tissues,(4) Accessibility of methodology for small labs in terms of equipment, facilities, and cost; (5) No prior sequence knowledge is required for many applications, such as AFLP and ISSR, and (6) High polymorphism that enables to generate many genetic markers within a short time (Semagn *et al*., 2006).The polymerase chain reaction has three basic steps: (1) Denaturing of the double stranded DNA template; (2) Annealing of a pair of primers to the region to be amplified; and (3) Amplification using a heat-resistant DNA polymerase called Taq polymerase.

a. Random Amplified Polymorphic DNA

Randomly Amplified Polymorphic DNA (RAPD) was the first PCR based molecular marker technique developed and it is by far the simplest (Williams *et al.,* 1990). Short PCR primers (approximately 10 bases) are randomly and arbitrarily selected to amplify random DNA segments throughout the genome. The resulting amplification product is generated at the region flanking a part of the 10 bp priming sites in the appropriate orientation. RAPD products are usually 0.5-5 kb and visualized on agarose gels stained with ethidium bromide.

RAPD markers are easily developed and because they are based on PCR amplification followed by agarose gel electrophoresis, they are quickly and readily detected. RAPD technique was used extensively in studying genetic diversity between plant species. For example, it was used to study application of RAPD for molecular characterization of 11 plant species of medicinal value

from an arid environment in Saudi Arabia (Arif *et al*., 2010). As a result, RAPD's may permit the wider application of molecular maps in plant science. Most RAPD markers are dominant and therefore, heterozygous individuals cannot be distinguished from homozygotes. This contrasts with RFLP markers which are co-dominant and therefore, distinguish among the heterozygotes and homozygotes (Mulivi, 1999). The advantages of RAPDs include being quick and simple and inexpensive and the facts that multiple loci from a single primer are possible and a small amount of DNA is required (Arif *et al*., 2010,). However, the results from RAPDs may not be reproduced in different laboratories and it can only detect the dominant traits of interest (Collard *et al*., 2005) due to the short primer length and low annealing temperature.

b. Amplified Fragment Length Polymorphisms

Amplified Fragment Length Polymorphisms (AFLP) based genomic DNA fingerprinting is a technique used to detect DNA polymorphism. AFLP is a polymerase chain reaction (PCR) based technique, (Mohan *et al*., 1997) and it has been reliably used for determining genetic diversity and phylogenetic relationships between closely related genotypes. AFLP analysis combines both the reliability of restriction fragment length polymorphism (RFLP) and the convenience of PCR-based fingerprinting methods. AFLP markers are generally dominant and do not require prior knowledge of the genomic composition. AFLPs are highly reproducible and this enables rapid generation and high frequency of identifiable AFLPs, making it an attractive technique for identifying polymorphisms and for determining linkages by analyzing individuals from a segregating population (Mohan *et al*., 1997).

In higher plants AFLP was used in variety of applications which includes examining genetic relationships between species (Ipek *et al*., 2009), investigating genetic structure of gene pool and assessment of genetic diversity among populations (Zhang *et al*., 2009). Moreover, AFLP does not require knowledge of the genomic sequence to design primers with specific selective bases. Dominant markers such as RFLP as well as RAPD are very limited in their ability to precisely determine parentage. They can readily be used to establish that two individuals are not the same, but the statement, that two individuals are identical is usually only approximate and no formal statistics can be attached to this assertion. There are several advantage of this technique : a) It is highly reliable and reproducible b) It does not require any DNA sequence information c) It is information-rich due to its ability to analyze a large number of polymorphic loci

simultaneously d) Co-migrating AFLP amplification products are mostly homologous and locus specific with exceptions in polyploidy species (Kundan *et al*., 2014). Moreover, The AFLP combination of two different sources of variation leads to highly reproducible, efficient and reliable result that makes it an extremely useful tool for molecular variability studies in plant breeding and conservation program (Sirelkhatem and Gaali, 2009). Disadvantages of this technique are that allele are not easily recognized, has medium reproducibility, labor intensive and has high operational and development costs (Kundan *et al*., 2014).

c. Microsatellite Markers

Microsatellites or Simple Sequence Repeats (SSR) are sets of repeated sequences found Within eukaryotic genomes. These consist of sequences of repetitions, comprising basic short motifs generally between 2 and 6 base-pairs long. Polymorphisms associated with a specific locus are due to the variation in length of the microsatellite, which in turn depends on the number of repetitions of the basic motif. 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. Because the technique is PCR-based, only small quantities of template DNA (10–100 ng per reaction) are required. Due to the use of long PCR primers, the reproducibility of microsatellites is high and analyses do not require high quality DNA. Of the various molecular markers available, microsatellites, also known as simple sequence repeats (SSR), are highly popular genetic markers due to their accuracy, reliability, co-dominancy, reproducibility and high polymorphism (Becher *et al*., 2000; Smykal *et al*., 2008a). For these reasons, microsatellites have been widely used for genome fingerprinting, gene tagging, genome mapping, and marker-assisted selection for various crops including pea (Burstin *et al*., 2001). However, the development of microsatellites requires extensive knowledge of DNA sequences.

d. Inter - Simple Sequence Repeats

Inter simple sequence repeat (ISSR) technique is a PCR based method, which involves amplification of DNA segment present at an amplifiable distance between two identical

microsatellite repeat regions oriented in opposite direction (Zietkiewicz *et al*., 1994; Kundan *et al*., 2014). The technique uses microsatellites, usually 16–25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter- SSR sequences of different sizes. The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetra nucleotide or penta-nucleotide (Semagn *et al*., 2006). It does not require genome sequence information; it leads to multilocus, highly polymorphic patterns and produces dominant markers (Li *et al*., 2009). ISSR PCR is a fast, inexpensive genotyping technique based on variation in the regions between microsatellites.

As suggested by Reddy *et al.*(2002), ISSR marker has a wide range of uses, including the characterization of genetic relatedness among populations, genetic fingerprinting, gene tagging and marker assisted selection, phylogenetic analysis, gene mapping determining SSR motive frequency and studies on natural population/ speciation. He also determined its source of variation is the template DNA, nature of primer and the detection method used. The first ISSR studies were published in 1994 (Zietkiewicz et al., 1994). Inter simple sequence repeat marker technologies are promising for analysis of the molecular genetic variability in plants due to their simplicity, reliability, reproducibility, and ability to detect high level of polymorphism (Zietkiewicz *et al.,* 1994), estimate genetic diversity among closely related populations (Abbot 2001; Deshpande *et al*. 2001), low running cost and does not require prior information of the genome for its development (Kebour *et al.,* 2012 ;Vijayan *et al*., 2006 and Godwin *et al.* 1997). ISSRs have been successfully used to estimate the extent of genetic diversity at inter- and intra-specific level in a wide range of crop species which include common bean (Sadeghi and Cheghamirza, 2012*;* yam, Zhou *et al.,* 2008; field pea, Taran *et al., 2005*). Moreover, since the primers are SSR motifs, the band generation potential of ISSR markers depends on the frequency and distribution of microsatellites in different species (Depeiger *et al.,* 1995), so that the Potential for integrating ISSR–PCR into plant improvement program is enormous and their applications in different crop species are also sufficiently reviewed (Reddy *et al*. 2002).

Like RAPDs, ISSRs markers are quick and easy to handle, but they seem to have the reproducibility of SSR markers because of the longer length of their primers (Kebour *et al*., 2012). Amplification in this technique leads to multi-locus and highly polymorphous patterns and involves longer (16-25 nucleotides) primers encoding microsatellite elements that amplify

DNA segments of Intra-micro-satellites repeats. ISSR markers also overcome most of the limitations of the common PCR- based molecular markers (Zietkiewicz *et al*., 1994) like low reproducibility of RAPD; high cost of AFLP and the need to know the flanking sequences to develop species specific primers for SSR polymorphism (Reddy *et al*., 2002).

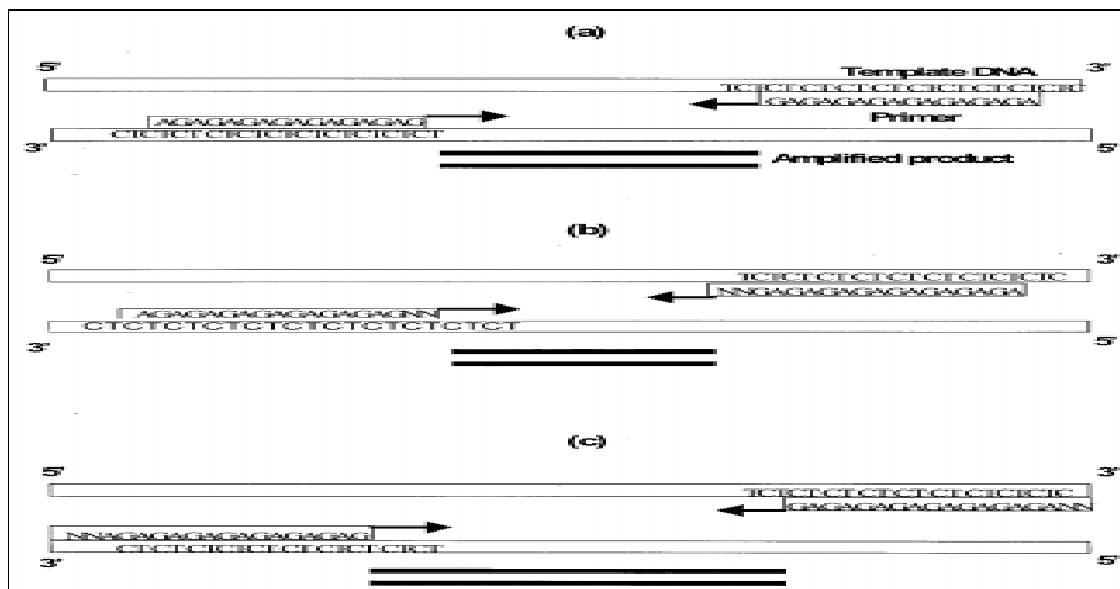

Figure 1. ISSR-PCR: A schematic representation of a single primer (AG) 8.

***Source*: Reddy *et al*. (2002)**

(a), 3'-anchored (b) and 5'-anchored (c) targeting a (TC)n repeat used to amplify inter simple sequence repeat region flanked by two inversely oriented (TC)n sequences. (a) Unanchored (AG)n primer can anneal anywhere in the (TC)n repeat region on the template DNA leading to slippage and ultimately smear formation (b) (AG)n primer anchored with 2 nucleotides (NN) at the 3' end anneals at specific regions on the template DNA and produces clear bands (c) (AG)n primer anchored with 2 nucleotides (NN) at the 5' end anneals at specific regions and amplifies part of the repeat region also leading to larger bands.

3. MATERIALS AND METHODS

3.1. Plant Material Collection

A total of 80 accessions were collected from different areas of southern Zone of Tigray and North Wollo Zone of Amhara regional states based on differences in geographical origin of landrace and three accessions were also obtained from the Ethiopian Biodiversity Institute (EBI) for comparison in this study. The germplasm was collected from woredas, namely, Ofla, Endamekoni, Emba Alaje, Raya Azebo and Hintalo-Wejerat in Southern Zone of Tigray, Raya Kobo and Guba-lafto in North Wello Zone of Amhara regional states (figure 2). The germplasms were collected having prier information about the production potential of kebelles and farmers found from the agricultural offices of each woredas and the germplasms were collected from homes. Number of populations of *P. sativum L. var. abyssinicum* used in this study is listed in table 1.

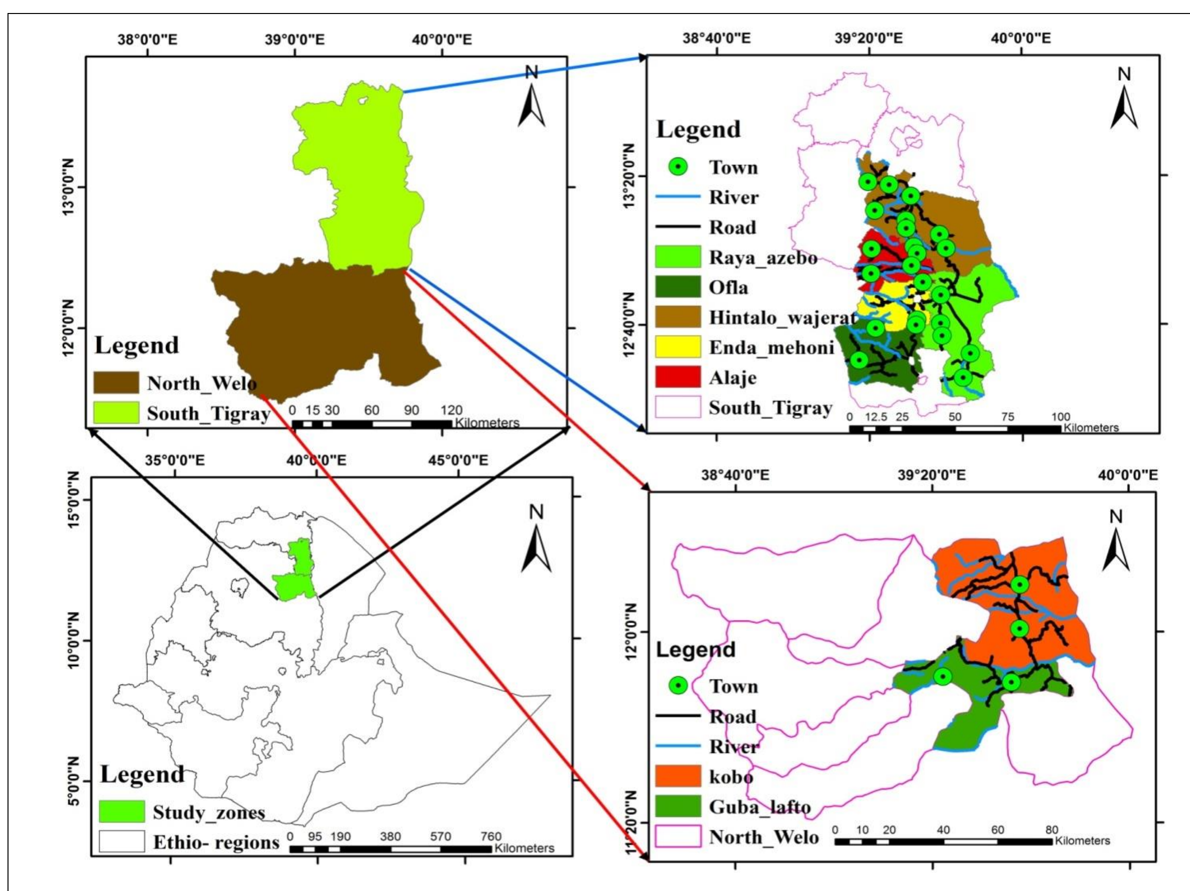

Figure 2. Geographic distribution of the seven populations used in this study.

Table 1. Number of populations of *P. sativum L. var. abyssinicum* used in the study by Woreda

No.	Population	Region	No. acc
1.	Hintalo Wejerat (HW)	Tigray	16
2.	Enda-Mekoni (EM)	Tigray	16
3.	Emba Alaje (EA)	Tigray	22
4.	Ofla (OF)	Tigray	8
5.	Raya Kobo (RK)	Amhara	8
6.	Raya Azebo (RA)	Tigray	5
7.	Guba Lafto (GL)	Amhara	5
8.	EBI (32774, 237507 and 237509	Tigray & Amhara	3
	Total		83

3.2. Germination Condition

The collected germplasms were germinated in a green house in Haramaya University for about three weeks. Young leaves from five randomly chosen plants per accession were dried in silica gel. The leaves were cut into small pieces with a sterile blade. Approximately 50mg of the dried leaf samples were bulked in 2ml Eppendorf tube with two beads and ground with grinding machine (MM 400). The ISSR marker assay and DNA extraction was conducted at the Plant Genetics Laboratory at the department of Microbial, Cellular and Molecular Biology, Institute of Biotechnology, Addis Ababa University.

3.3. Extraction of Genomic DNA

Total genomic DNA was extracted by using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Borsch *et al.,* 2003) with minor modifications. Seven hundred (700) µl of warm CTAB solution sublimated with 0.2 vol % Mercapto-ethanol was Added to the powdered sample to dissolve the powder and the sample was incubated for 30 minutes at 65°C. The sample was centrifuged for 5 minutes at 15000 rpm. The supernatant was transferred to a new Eppendorf-cap with cut blue pipette tips (***1st extraction***) and 700 µl new CTAB solution was added to the tissue pellet and stirred slightly with a new1000 µl pipette tip, and the solution was incubated at 65°C for another 30 min. The sample was centrifuged for 5 minutes at 15000 rpm the and

supernatant was transferred to another new Eppendorf-cap with cut blue pipette tips (2nd extraction). Six hundred (600) µl Chloroform solutions were immediately added to the cap with supernatant (***2nd extraction***) and were shaken carefully for a few times upside down. The samples were shaken thoroughly by turning inversing the Eppendorf caps for approximately 5 minutes and centrifuged for 5 min at 15000 rpm. The supernatant was carefully transferred in a new Eppendorf-cap using cut blue pipette tips. The chloroform extraction was repeated to make sure that all impurities are removed. Then approximately 2/3 of the solution volume of cool Isopropanol (4°C) was added and shaken carefully by inversing the Eppendorf cap. At this step the DNA was becoming visible as white threads for all samples. The DNA sample was then frozen for more than 2h at -20°C and Centrifuged for 10 min at 15000 rpm. Finally the liquid part was aspirated using yellow tips (without touching the pellet).

3.4. Purification of DNA

For purification of the extracted DNA, 200 µl 70 % Ethanol was added to the pellet and the inner cap surface was rinsed by turning the cap. The sample was then centrifuged in a cooled centrifuge for 10 min at 15000 rpm. The Ethanol was aspirated using yellow tips and the DNA-pellet was dried at room temperature for 15 min to remove the liquid drops. The Pellet was dissolved in 100 µl TE and stored at 4°C for more than 2 hrs. Half of the solution volume of cooled 7.5 M NH4Ac-solution (4°C,) was added and the solution was mixed carefully. Then, double of the solution volume of cool Ethanol 100 % was added and mixed carefully. The solution was then allowed to Freeze for more than 2 h at -20°C and centrifuged for 30 min at 15000 rpm. The liquid was aspirated carefully and 200 µl of 70% Ethanol was added and the inner cap surface was rinsed by turning the cap. The solution was then centrifuged for 10 min. at 15000 rpm. The liquid was aspirated and the DNA pellet was dried at room temperature and dissolved in 100 µl TE. The salting and cleaning step was repeated with 3 M NaAC-solution (4°C). Finally the DNA pellet was dissolved in 100 µl TE and stored at -20°C for PCR amplification.

3.5. DNA Quantity and Quality Determination

The quality of genomic DNA was tested using agarose. One percent (1%) Solution of agarose was prepared by boiling 0.5 g agarose in 50 ml of 1x TBE buffer in a microwave oven until the

solution is completely clear without any visible suspension. After allowing the solution to cool for a couple of minutes, ethidium bromide was added and thoroughly mixed by swirling. The solution was poured slowly on the gel tray that was well prepared with the comb fixed properly. Genomic DNA from all samples (5 µl each), along with 2 µl of loading dye, was loaded on walls of the gel and electrophoresed at a constant current of 100V for 30-40 minutes. Gel picture was taken under UV trans-illuminator by Gel Doc system (Biosens SC750) with digital canon camera.

The quantity and quality of the Genomic DNA was also determined using spectrophotometer (Nano Drop) by measurement of optical density (O.D). For this, 1 µl DNA sample (diluted with 1X TE buffer) was taken and the optical density was measured at 260 nm against a 1X TE buffer in a Bio-Spectrophotometer. The DNA concentration was then calculated based on Optical density (O.D). An O.D value was also taken at 280 nm (corresponding to protein), 230 nm (corresponding to RNA) and 320nm (contamination). Total DNA purity was tested by a ratio of O.D values at 260:280 and it was up to the standard that is between 1.8 and 2.00. Then working solution for PCR amplification was made in 1 to 5 dilutions for each sample (i.e. 1 µl of genomic DNA in to 4 µl of ddH_2O).

3.6. Genomic DNA Amplification

3.6.1. Selection of primers

A total of 20 Primers available at Addis Ababa University, Department of Microbial, Cellular and Molecular Biology were screened for polymorphism, reproducibility and clarity of bands for the present study. To screen the primers, 1 to 5 diluted DNA samples from 14 accessions selected from different geographical locations (2 from each population) was used. Out of total 20 primers tried in the PCR amplification, only 6 UBC primers, UBC826, UBC834, UBC835, UBC857, UBC873 & UBC881 showed relatively high polymorphism, reproducible, relatively clear bands and unambiguous amplification while the rest of the primers did not give amplification as they produced very faint or fuzzy lanes in the different reactions tried (one to three) (Table 2). The four di-nucleotide ISSR primers UBC826 $(AC)_8C$, UBC834$(AG)_8YT$, UBC835 $(AG)_8YC$, and UBC857 $(AC)_{8YC}$, one tetra-nucleotide ISSR primer UBC873 with

$(GACA)_4$ and one penta-nucleotide ISSR primer UBC881 with $(GGGTG)_3$ were used to study genetic diversity within and among populations of *P. sativum L. var. abyssinicum.*

Table 2. List of primers used for primer screening

No.	Primer	Sequence	Ta	Repeat motif	Amplification pattern
1.	UBC811	(GA)8C	47	Di-nucleotide	poor band
2.	UBC812	(GA)8A	45	Di-nucleotide	Poor band
3.	UBC813	(CT)8T	45	Di-nucleotide	Not polymorphic
4.	UBC826	(AC)8C	47	Di-nucleotide	Reproducible, Polymorphic
5.	UBC834	(AG)8YT	48	Di-nucleotide	Reproducible, Polymorphic
6.	UBC835	(AG)8YC	50	Di-nucleotide	Reproducible, Polymorphic
7.	UBC836	(AG)8YA	47	Di-nucleotide	Poor band
8.	UBC844	(CT)8RC	48	Di-nucleotide	Not polymorphic
9.	UBC841	(GA)8YC	48	Di-nucleotide	Not polymorphic
10.	UBC854	(TC)8RG	48	Di-nucleotide	Not polymorphic
11.	UBC857	(AC)8YG	48	Di-nucleotide	Reproducible, Polymorphic
12.	UBC845	(CT)8RG	48	Di-nucleotide	Not polymorphic
13.	UBC824	(TC)8C	47	Di-nucleotide	Poor band
14.	UBC860	(TG)8RA	45	Di-nucleotide	Poor band
15.	UBC866	(CTC)6	55	Tri- nucleotide	Poor band
16.	UBC873	(GACA)4	43	Tetra-nucleotide	Reproducible, Polymorphic
17.	UBC848	(CA)8RG	48	Di-nucleotide	No band
18.	UBC878	(GGAT)4	43	Tetra-nucleotide	No band
19.	UBC880	(GGAGA)3	43	Penta-nucleotide	Not polymorphic
20.	UBC881	(GGGTG)3	49	Penta-nucleotide	Reproducible, Polymorphic

UBC= University of British Colombia

3.6.2. PCR amplification

PCR amplifications were conducted in a total volume of 26 µl comprising 15.8 µl ddH2O, 2 µl of template DNA (1in 5 dilution), 0.3 µl primer and 0.3 µl of Taq polymerase, 4 µl 25 mM MgCl2, 3 µl of B reaction buffer, 0.6 µl of 25 mM dNTP. Negative control in which the template

DNA was replaced by double distilled water was also included in each round of reactions to check for absence/presence of contamination. Amplifications were performed on a Biometra 2003 T3 Thermo - cycler with the following profile: 94 °C initial denaturation for 4 min, followed by 40 cycles of 15 s at 94 °C, annealing at 43- 50 °C (based on the primer used) for 1minute, extension at 72 °C for 1 min 30s, final extension at 72 °C for 7 minutes and finally stored at 4°C until loaded for gel electrophoresis.

3.6.3. PCR product gel electrophoresis

PCR products were tested on 1% agarose gel stained with ethidium bromide (EtBr) in Tris Borate EDTA (TBE) buffer. Amplification products were finally separated by electrophoresis in 1.67 % agarose gels. Ten micro litter (10 µl) PCR product of each sample along with 2 µl of loading dye (6x concentrated) was loaded on to the agarose gel. Fifteen wells Comb was used for each ISSR gel slab. The first lane was loaded with 2 µl of 100 bp ladder with 2 µl of loading dye as size standard and the last lane was the control (without DNA template). The ISSR electrophoreses were done for about 3 hours at constant current of 100 V. Then the banding patterns were visualized under UV light and photographed using canon camera in the Gel Doc system (Biosens SC750) and documented for band scoring and analysis.

3.7. Data Analysis

3.7.1. Gel band scoring and recording

An ISSR marker was treated as dominant marker and each locus was considered as a bi-allelic locus with one amplifiable and one null allele. Scoring was performed manually for each primer based on presence (1) and absence (0) or ambiguous (?), and each band was recorded as a locus. Amplified bands were recorded, followed by generation of a "0" and "1" data matrix on GeneAlex versions 6.5, genetic analysis in excel.

3.7.2. Genetic diversity

Based on generated 0 and 1 matrix different softwares were used for analysis. POPGENE version 1.32 Software (Yeh *et al.,* 2000) was used to calculate genetic diversity for each

population as number of polymorphic loci, percent polymorphism, gene diversity (h) and Shannon–Weaver diversity index (I).

The genetic diversity was further investigated using Nei's gene diversity statistics, including the total genetic diversity (H_T), genetic diversity within populations (H_S), and the relative magnitude of genetic differentiation among populations ($GST = (H_T - H_S)/H_T$). An estimate of gene flow among populations (*Nm*) was computed by the formula of $Nm = (1 - G_{ST})/G_{ST}$

3.7.3. Genetic structure analysis

Analysis of molecular variance (AMOVA) was used to calculate variation among and within population using the program GeneAlex version 6.5 (Peakal and Smouse, 2012).

3.7.4. Clustering analysis

The unweighted pair group method with arithmetic average (UPGMA) (*S*neath and Sokal, 1973) was used in order to determine the genetic relationship among accessions and generates phenogram using NTSYS- pc version 2.02 (Rohlf, 2000).

The neighbor joining (NJ) method (Saitou and Nei, 1987; Studier and Keppler, 1988) was used to compare individual accessions and evaluate patterns of accession clustering using Free Tree 0.9.1.50 Software (Pavlicek *et al.,* 1999) softwares were used to calculate Jaccard's similarity coefficient which is calculated with the formula:-

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

Where,

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

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

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

The patterns of variation among individual samples in each population was further examine using two dimensional principal coordinate analysis (2D PCO), performed by the program GeneAlex version 6.5 (Peakal and Smouse, 2012). The three dimensional principal coordinate analysis (3D PCO) was also done to further confirm the clustering pattern in the 3Dplot with STATISTICA version 6.0 software (Hammer *et al.,* 2001).

4. RESULTS AND DISCUSSION

4.1. ISSR Primers and their Banding Patterns

Using the 6 UBC primers, a total of 91 scorable bands were found. The number of scorable bands for individual primer ranged from 10 to 20 while the average number of bands per primer was 15.2. The highest number of bands was generated with primer 834, while the lowest number was amplified with 835 and 873. The fragment size amplified with these primers was in the range of 200 to 3000 base pair. Details regarding ISSR primers and their sequences, Ta (annealing temperature), repeat motive, amplification patterns and NSB (number of scorable bands) are shown in Table 3.

In this study, ISSR markers were used to assess the level and pattern of genetic diversity among *P. sativum L. var. abyssinicum* accessions collected. The 6 primers led to amplification of 91 scorable bands and detected 5 to 11 polymorphic loci with average 9.2 in 83 accessions of *P. sativum L. var. abyssinicum* ranging from about 200 bp (primer 881) to 3000 bp (primer 834) (Table 3). Among the polymorphic loci, maximum percentage of polymorphic loci (68.75 %) was generated by 881 followed by 857 which accounted for 64.71%. Primers 826 and 873 also shared 61.11 and 60.00% polymorphism respectively. However, primers 834 and 835 generated the least percentage of polymorphic loci which revealed only 55.00% and 50.00% respectively. Furthermore, penta-nucleotide primer 881 also generated high number of percent polymorphism within population as compared to the di-, tetra- and penta-nucleotide, showing that the penta-nucleotides interestingly contributed more to the polymorphism. The highest gene diversity (0.236) and Shannon’s information index (0.352) were obtained from primer 857, while primer 834 showed the least gene diversity of 0.141 and Shannon’s information index of 0.225 (Table 3).

Table 3. Number of Scorable Bands, Number and percentage of polymorphic loci, gene Diversity and Shannon information index of each primer for the 80 accessions

No.	Primer	NSB	NPL	PPL	H ± SD	I±SD
1	UBC826	18	11	61.11%	0.142 ± 0.167	0.227 ± 0.249
2	UBC834	20	11	55.00 %	0.141 ± 0.167	0.225 ± 0.245
3	UBC835	10	5	50.00%	0.183 ± 0.228	0.266 ± 0.324
4	UBC857	17	11	64.71 %	0.236 ± 0.202	0.352 ± 0.289
5	UBC873	10	6	60.00 %	0.194 ± 0.206	0.293± 0.295
6	UBC881	16	11	68.75%	0.163 ± 0.180	0.256 ± 0.253
Overall		91	55	60.44 %	0.176 ± 0.185	0.258 ± 0.267

UBC = University of British Colombia, NSB = Number of Scorable Bands, NPL = Number of polymorphic Loci; PPL = Percent of polymorphic Loci; H = gene Diversity; I = Shannon's information index and SD= standard deviation

4.2. Genetic Diversity Study of *P. sativum L. var. abyssinicum*

Based on aggregated data from all the six primers (Table 4), the highest number of polymorphic loci (22) were detected from populations, Enda-Mekoni, Emba Alaje and Ofla while the lowest (0 & 7) number of polymorphic loci (NPL) was found from Guba Lafto and Raya Azebo respectively. The highest percent polymorphism (24.18) was also obtained from Enda-Mekhoni, Emba Alaje and Ofla populations. The lowest number of polymorphic loci and (0.00) least percent polymorphism (0.00%) was detected in accessions collected from Guba Lafto.

Out of the 91 scorable bands generated 55 (60.44%) bands were found to be polymorphic with 45 (39.56%) monomorphic bands. This result is lower than the result obtained by Choundhury *et al.,* (2006) which was 74.8% in 24 Indian released varieties of *P. sativum* L. by employing 60 RAPD primers. Kapila *et al*. (2012) also reported 139 (72.3%) polymorphic bands out of the total 192 RAPD amplicons and whereas 73 (89.0%) polymorphism was observed out of the total 82 ISSR amplicons in case of 31 genotypes of field and *garden pea*. In contrast Taran *et al.* (2005) reported relatively lower level of polymorphism (51%, 44% & 43%) using SSR, RAPD & ISSR primers respectively in 65 varieties and 21 wild accessions including five accessions of *P. sativum L. var. abyssinicum.* Simioniuc *et al*. (2002) also found 55.7% & 48.7% polymorphic fragments using RAPDs and AFLPs of 21 pea cultivars. These differences could be attributed to the genotypes evaluated and the number of primers used in the study.

Moreover, the choice of appropriate primer motives in ISSR fingerprint is critical to detect high polymorphism and reveal relationship within and among populations. Since the ISSR primers are SSR motifs, the abundance and distribution of SSRs in the genomes of *P. sativum L. var. abyssinicum* could be another factor that determined the levels of polymorphism.

Table 4. The Number of polymorphic loci, Percent polymorphic loci, gene diversity and Shannon‘s information Index, using data generated from all the six primers.

Pop.	**HW**	**EM**	**EA**	**OF**	**RK**	**RA**	**GL**	**Mean**	**Total**
Na	1.205	1.256	1.247	1.256	1.209	1.077	1.00	1.179	1.604
Ne	1.110	1.168	1.151	1.170	1.130	1.056	1.00	1.112	1.273
NPL	18.00	22.00	22.00	22.00	19.00	7.00	0.00	15.71	55.00
PPL	19.78	24.18	24.18	24.18	20.88	7.69	0.00	17.27	60.44
H	0.067	0.094	0.087	0.096	0.073	0.031	0.00	0.064	0.167
I	0.103	0.139	0.129	0.141	0.109	0.045	0.00	0.095	0.258
H_T									0.119
H_S									0.062
G_{st}									0.482
N_m									0.538

Na= Observed number of alleles, Ne = Effective number of alleles, NPL= Number of polymorphic, PPL= Percent polymorphic loci, H=Nei's gene diversity, I= Shannon's information index, H_T *= Total genetic diversity)),* H_S *= Intermodulation differentiation,* G_{ST} *= Interpopulation differentiation, and* N_m *= Estimated gene flow.*

The mean observed number of alleles (Na) ranged from 1.00 to 1.256 with an average of 1.79, while the mean effective number of alleles (Ne) varied from 1.00 to 1.170 with an average of 1.112. Nei's genetic diversity (H) varied from 0.00 to 0.096, with an average of 0.064, and Shannon's information indices (I) ranged from 0.00 to 0.139, with an average of 0.095. At species level, H and I were 0.167 and 0.258, respectively and Na and Ne values equaled to 1.604 and 1.273 respectively.

The estimated genetic diversity ranging from 0.00 to 0.096 (Table 4) among *P. abyssinicum* accessions based on ISSR markers was lower than that reported by Taran *et al.* (2005) whose values of which were ranged from 0.00 to 0.66 in 21 *P. sativum*. Varieties including five *P.*

sativum L. var. abyssinicum accessions. Kumari *et al*. (2013) also found values ranging from 0.05 to 0.82 based on SSR markers in 28 field pea genotypes including varieties released in India and Zong *et al*. (2009) reported values ranging from 0.64 to 0.93 in a comparative analysis of genetic diversity structure within *Pisum* genus based on microsatellite markers. Among the seven populations investigated, population Ofla exhibited relatively higher level of variability while population Guba Lafto possessed the lowest value of variability. The difference may not be related to the sample size. For example, population Emba Alaje with a larger sample size possesses a low genetic diversity (H) value, while population Ofla with the smallest sample size among the seven populations contains a high genetic diversity (H) value. The results demonstrated that the uneven distribution of genetic diversity is substantial and apparently related to the habitat. But the lowest genetic diversity (0.00) in Guba Lafto population indicates that, the accessions might be duplicates. The reason why the sample size in some populations in this study is small is due to the scarcity of seed sample during data collection. This is because many farmers have lost *P. sativum L. var. abyssinicum* crop fields due to recurrent drought and diseases in the past few years as well as constraints such as low productivity (personal communication). Therefore, the endangered status of this species currently reflects a small number of extant populations in the hands of few farmers.

The natural populations of *P. sativum L. var. abyssinicum* often occur in adjacent regions with almost similar topography including mountains, plateaus and valleys in the north Wollo and south Tigray highlands. The similarity in altitude and overall topography tends to decrease genetic diversity. The largely self-pollinating nature of *P. sativum L. var. abyssinicum* that has a great contribution for the relatively low genetic diversity in this study. The low genetic diversity in this study could also partly be caused by spatially similar ecological environment that lead to decrease of selection pressures on mutations. Farmers also purposefully preserve their own farm seeds for their next production season rather than obtaining it from the market. This results to a lower genetic exchange among population. The demand for high productivity also results in crop uniformity because of the use of limited, selection for high yielding varieties leads to the loss of many local varieties and brings about genetic erosion and finally loses of genetic diversity.

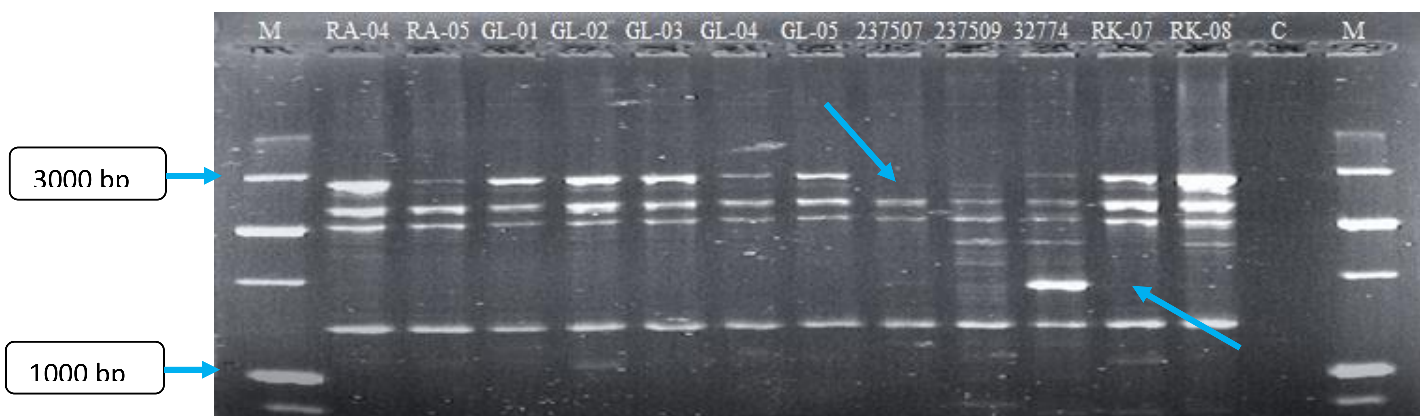

Figure 3. Banding pattern of ISSR primer 881.

M represents a 100 bp DNA ladder as a standard molecular marker; while C represents control (a PCR reaction mix without template DNA).

4.3. Genetic Structure and Classification of Genetic Diversity

The population genetic structure of a species reflects the interactions among different processes during a long evolutionary history of the species including shifts in distribution, habitat fragmentation and population isolation, mutation, genetic drift, gene flow, and selection. In addition pollination mechanism and mating system of a species also play an important role in determining population genetic structure (Sun *et al*., 1998). In the present study analysis of molecular variance (AMOVA) using the overall 91-ISSR bands generated by the six primers in seven *P. sativum L. var. abyssinicum* populations revealed that higher percent of variation (54 %) is attributed to the within population variation while 46 % is due to among population variation the FST value of 0.456 (Table 5). Hamrick et al., 1989 states that, if interpopulation gene flow is as high, gene movement within populations should reduce variation among population subdivisions. The AMOVA results revealed highly significant genetic differences with a p value of ($p<0.001$) between the seven populations of *P. sativum L. var. abyssinicum.* Relatively high genetic variation within populations indicated that low genetic similarities among the individual plants sampled from a single population.

The analysis of the population genetic structure revealed a moderate level of gene flow among the seven populations of *P. sativum L. var. abyssinicum* investigated. The total genetic diversity (H_T) of the species and genetic diversity within populations (H_S) were 0.119 and 0.062, respectively (Table 4). The proportion of genetic variation contributed by the coefficient of

genetic diversity among populations (G_{ST}) was 0.482, thus leaving 51.8% of the total genetic variation harbored within the populations. It was also consistent with the results of AMOVA, which detected highest genetic variation within population (54%), whereas the variance among populations was only 46 %. This was further confirmed by the moderate of gene flow (Nm, the number of migrating individuals among populations per generation) estimated to be 0.538.

The relatively higher within population genetic variation *in P. sativum L. var. abyssinicum* in this study might be due to a limited amount of genetic exchange or gene flow, which actually has a more homogenizing effect on the genetic variation among populations. Although a small distance, suitable geographical location, people with similar culture and many common markets in the *P. sativum L. var. abyssinicum* growing areas play a big role in seed exchange among population, farmers do not use seeds from unknown sources and from market for agricultural purposes. They only purchase those seeds for home consumption.

Understanding the extent and patterns of genetic diversity in *P. sativum L. var. abyssinicum* has an implication for its conservation and exploitation. It is important to understand that efforts should be made to effectively manage and conserve populations of this species. The AMOVA results show that each of the seven studied populations currently maintain a considerable among population genetic diversity. As a result, Conservation measures should therefore focus on establishing large numbers seeds to preserve the existing genetic diversity as much as possible.

Table 5. Analysis of Molecular Variance among and within the seven populations.

Source of variation	**d.f**	**S.S**	**M.S**	**EST.VAR**	**P.V**	**F_{ST}**	**P-value**
Among population	6	399.27	66.55	5.508	46%	0.456	P<0.001
Within populations	73	480.23	6.58	6.578	54%		P<0.001
Over all	79	879.50		12. 086	100%		

d.f = Degree of freedom, S.S = Sum of squares, MS = Mean square, V.C = Estimated Variation, PV = Percent of variation and FST = Fixation indices

4.4. Cluster Analysis of *P. sativum L. var. abyssinicum* Genotypes

4.4.1. Unweighted pair group method with arithmetic average (UPGMA)

The Percent pairwise similarity coefficient among *P. sativum L. var. abyssinicum* populations was calculated using Jaccard's similarity coefficient (Table 6). The degree of genetic relatedness among populations varied considerably from 0.697 (between RK and HW) to 0.943 (Between RA and RK). GL-RA (0.935), RK-OF (0.924), GL-RK (0.913) were among the highest pairwise similarity values, whereas RA-HW (0.716), OF-HW (0.738), GL-HW (0.744) were the lowest pairwise similarity values. The highest similarity coefficient (0.943) is obtained for adjacent populations (RA and RK) and the lowest similarity (0.697) is also obtained from distant populations (RK and HW). This result may be attributed to the level of gene flow due to their geographic distance and their habitat.

Table 6. Pair wise Jaccard's similarity coefficient between pairs of the seven populations

Populations	HW	EM	EA	OF	RK	RA	GL
HW	1						
EM	0.824	1					
EA	0.800	0.879	1				
OF	0.738	0.782	0.806	1			
RK	0.697	0.766	0.790	0.833	1		
RA	0.716	0.825	0.850	0.877	0.943	1	
GL	0.744	0.823	0.924	0.850	0.913	0.935	1

HW= Hintalo Wejerat, EM= Endamekoni, EA= Emba Alaje, OF= Ofla, RK= Raya Kobo, RA=Raya Azebo, GL= Guba Lafto

Cluster analysis of UPGMA and NJ analysis was carried out to construct dendrogram for the seven populations and 83 individuals based on 91 PCR bands amplified by six ISSR primers. UPGMA analysis of *P. sativum L. var. abyssinicum* populations revealed two major clusters (I and II) using Jaccard's similarity coefficient at 78 % similarity (Figure 4). Major cluster-I consisted of two sub-clusters (sub-cluster-I and sub- cluster-II) at 82 % similarity. Sub-cluster-I consisted of accessions collected from Hintalo Wejerat. Sub-cluster-II consisted accessions collected from Endamekoni. Major cluster-II also consisted of two sub-clusters (sub-cluster-I

and sub- cluster-II) at 84.5 % similarity. Sub cluster I consisted accessions of Emba Alaje, Raya Kobo, Guba Lafto and Raya Azebo populations while Sub cluster-II consisted of Ofla population.

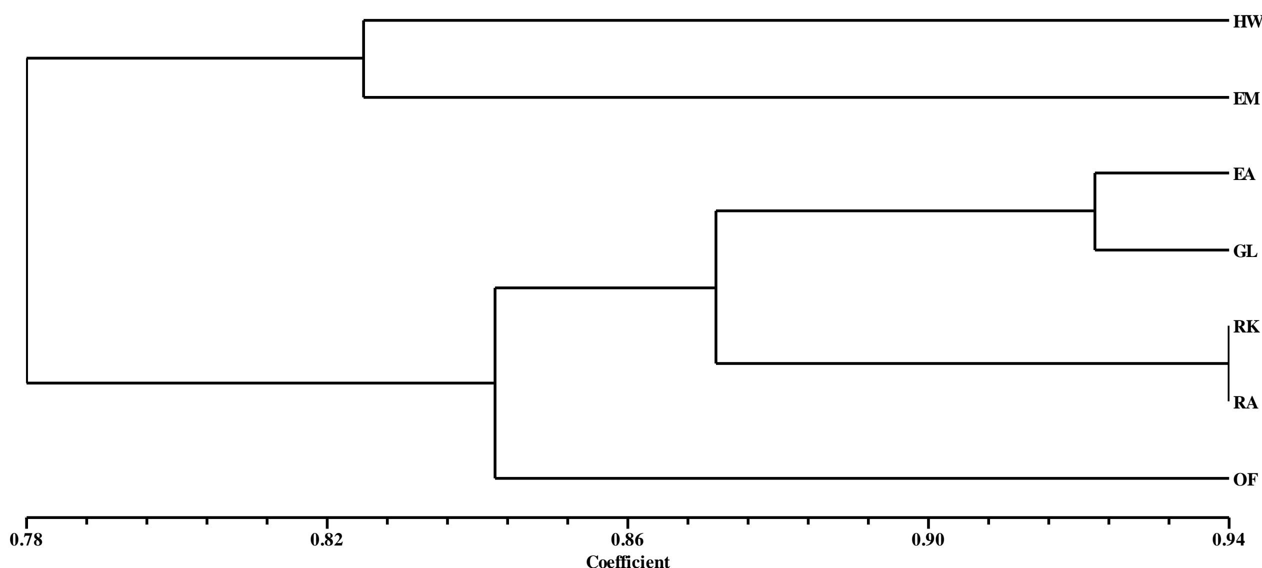

Figure 4. Population based UPGMA dendrogram for the seven populations.

HW= Hintalo Wejerat, EM= Endamekoni, EA= Emba Alaje, OF= Ofla, RK= Raya Kobo, RA=Raya Azebo, GL= Guba Lafto.

The dendrogram constructed from UPGMA on individual basis clearly identified six clusters (I, II, III, IV, V and VI) with average Jaccard's similarity coefficient of around 0.88 (Figure 5). The first cluster was composed of 13 accessions collected from Hintalo Wejerat. The second major cluster consisted of 5 accessions which were collected from Hintalo Wejerat and Endamekoni. The third major cluster consisted of 20 accessions collected from Endamekoni and Emba Alaje. The forth cluster consisted of 5 accessions collected from Emba Alaje. The fifth cluster consisted of 12 accessions from Emba Alaje and Ofla and the last cluster consisted of 22 accessions collected from Ofla, Raya Azebo, Raya Kobo, Guba Lafto and EBI. Most individuals from all populations tended to form their own cluster, while few individuals of some populations were mixed with each other on the tree.

In addition to this, individual based UPGMA clustering of an overall analysis, individuals showed a tendency of clustering with respect to their population except few intermixed populations. The highest level of genetic similarity (1.00) was observed between the accessions listed in each box of Table 7.

Table 7. List of accessions which are 100% similar in UPGMA clustering analysis.

HW-01 HW-04 HW-012	HW-05 HW-011 HW-013	HW-07 HW-08 HW-09 HW-010	HW-014 HW-016	EM-03 EM -04 EM -05 EM -08
EM -011 EM -014 EM -015	EA-02 EA-03 EA-04	EA-015 EA-016 EA-017 EA-018 EA-019	GL-01 GL-03 GL-04 GL-05	RK-06 GL-02

Very high degree of similarity (0.96) was also found between (EA-010 & EA-011, EA-08, EA-012, EA-013 and EA-014). In contrast the least degree of similarity 0.73 was found between accessions 23774 & 327509 and 0.87 between RK-07 & RK-08 respectively. Clustering analysis classified pea genotypes into groups that were somewhat consistent with their geographical origins. Cluster VI was cosmopolitan genotypes from a range of origins. Members of this group might share a common parent or may have been introduced from other regions that resulted from germplasm exchange among different populations.

The range of Jaccard's genetic similarity coefficient among the total accessions was in the range of 0.78 to 1.00. This result is comparable with Simioniuc *et al.* (2002) reported that ranged from 0.80 to 0.94 with RAPD markers and 0.85 to 0.94 with AFLP markers. Ahmed *et al*. (2015) also reported comparable genetic similarity ranging from 0.075 to 0.875 using SSR marker in 35 Pisum accessions. Kapila *et al*., (2012) also reported a relatively moderate similarity coefficient that ranged from 0.58 to 0.85 using RAPD and ISSR markers in31 pea genotypes. The result in this study is also in contrast with the result by Baranger *et al.* (2004), who obtained a very wide range of similarity (0.00 -1.00) in 148 Pisum genotypes using protein and PCR-based markers. Choudhury *et al*., (2006) found a genetic similarity ranging from 0.559 to 0.873 in 24 Indian released varieties of *P. sativum* found by employing 60 RAPD primers. The divergences could be attributed to divergences between pea accessions of deferent origin, different varieties, marker type and number of genotypes used.

Cluster analysis in this study classified pea genotypes into various groups and this can aid to plan possible breeding program. Two accessions collected from EBI (32774 and 237509) for comparison positioned themselves separately from any sub-cluster in the phylogenic tree

formed by UPGMA with the lower similarity coefficient of 0.58. Two accessions from Raya Kobo (RK-07 and RK-08) also showed a relatively lower genetic similarity (0.78) from the whole populations. These accessions might be morphologically different from others due to their geographical location. RK-07 and RK-08 were collected and morphological selected out of 24 accession collected from nine woredas in south Tigray and North Wollo areas by Alamata Agricultural Research Center. The accessions collected from EBI also showed some kind of difference in leaf morphology as observed at the early stage of germination in the green house. Genetically distinct pea gene pools can lay the foundation of genetic improvement and can be useful in future breeding programs (Zong *et al.,* 2009). Local materials like accessions 32774 and 237509 (collected from EBI) are also advantageous in breeding because they could probably have better adapted traits to the local environmental conditions than the exotic ones. Therefore, these unique accessions of EBI can be targeted for conservation and for further breeding programs as genetic variation plays key role for successful breeding program and designing effective conservation strategies. The low diversity populations' are also recommended for breeding to broaden the genetic base and maximize genetic gains by accumulating favorable alleles.

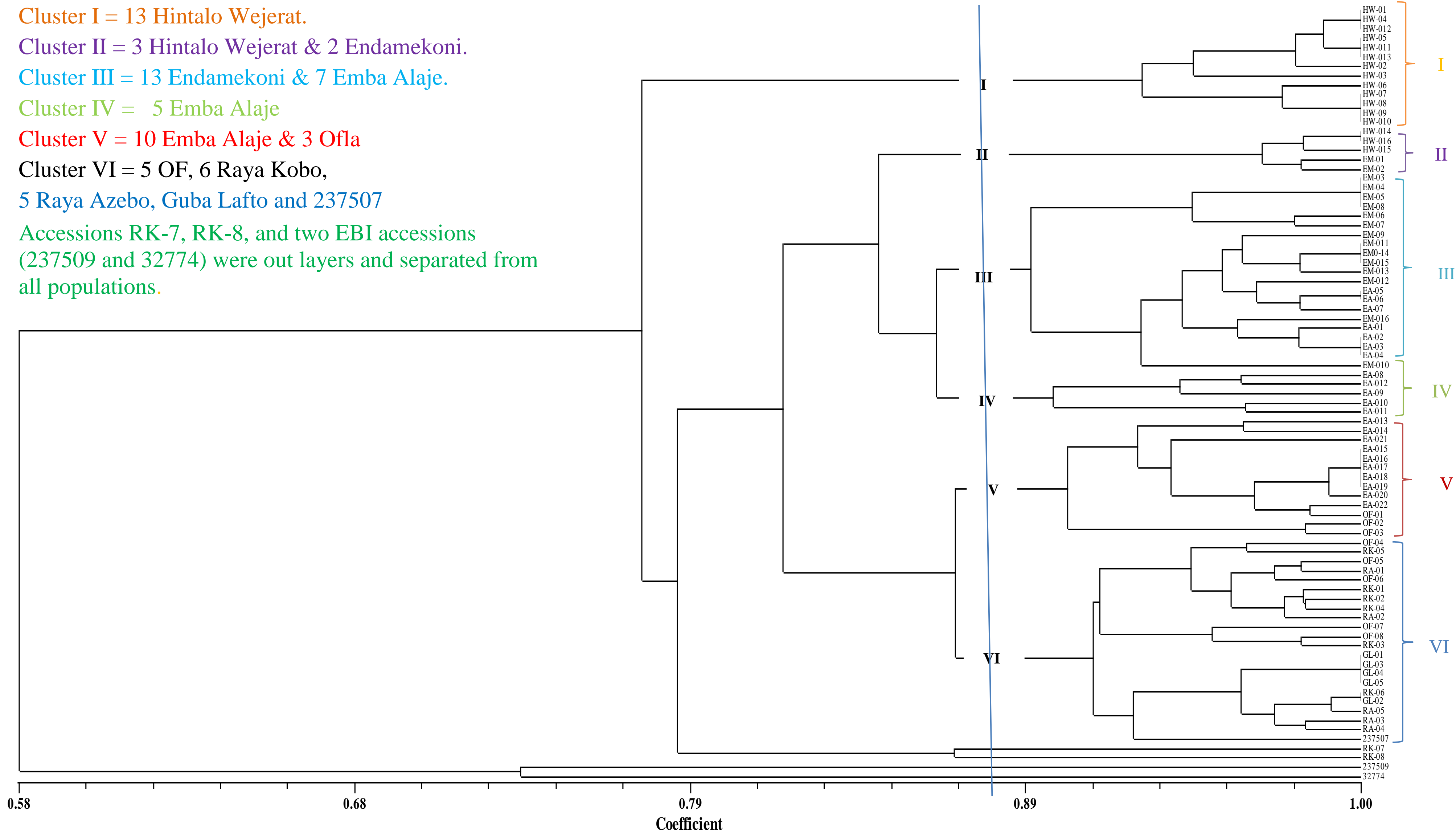

Figure 5. Individuals based UPGMA dendrogram from the seven populations.

HW = Hintalo Wejerat, EM = Endamekoni, EA = Emba Alaje, OF = Ofla, RA =Raya Azebo, RK = Raya Kobo, GL = Guba Lafto

4.4.2. Principal coordinates (PCO) analysis

Principal coordinate analysis was carried out on all the ISSR data obtained from 83 accessions. The analysis was carried out using Past and Statistica software by employing Jaccard's coefficients of similarity. The first three coordinates of the PCO had Eigen values of 23.82, 19.52 and 11.93 with percentage variation of 20.87 %, 17.10 % and 10.45 %, respectively. These were used to show the grouping of all of the accessions using two and three coordinates (Figure 6 and 7). The two-dimensional and three-dimensional plots were prepared using the first 2 and 3 PCOs and accounted for 37.97% and 48.42% of the total variation respectively. In both PCO plots, the genotypes were grouped into six clusters. Like in the cluster analysis, individuals from each population had the tendency to form a separate plot except accessions collected from EBI and few other accessions were distributed separately in the plot.

Using three coordinates (3D) and two coordinates (2D), almost similar result was observed like that of UPGMA tree. Thus, the clustering patterns generated by UPGMA and PCO were mostly congruent. This result is in agreement with the results reported by different researchers who have evaluated the genetic diversity of pea using UPGMA clustering and PCO for their molecular marker data. Baranger et al., (2004) performed PCA on pooled data from several molecular markers (RAPD, ISSR, SSR, sequence tagged sites, Allozymes, storage protein) in pea. Simioniuc *et al*. (2002); Taran *et al*. (2005) and Kumari *et al.* (2012) also obtained similar clustering pattern between PCO and UPGMA using AFLPs, SSR, RAPDs and morphological markers.

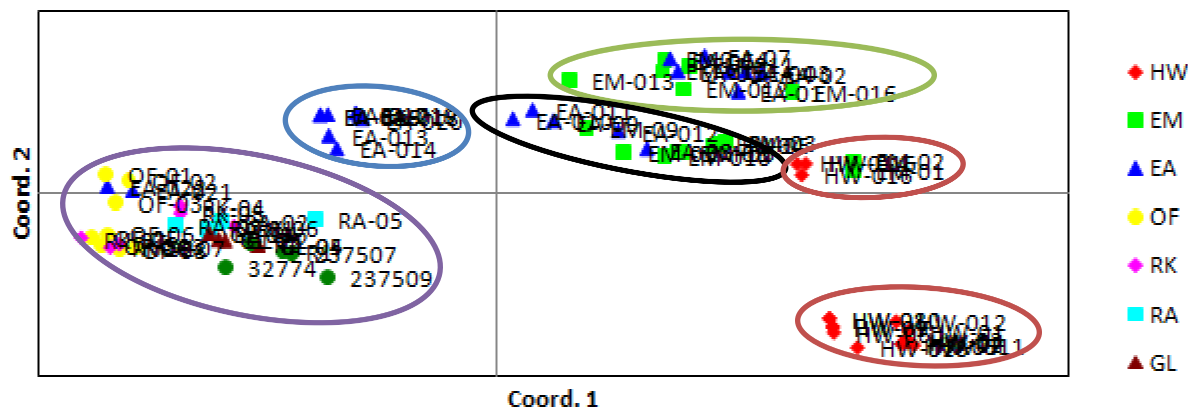

Figure 6. Two dimensional principal coordinate analyses of the seven populations.

HW= Hintalo Wejerat, EM= Endamekoni, EA= Emba Alaje, OF= Ofla, RK= Raya Kobo, RA=Raya Azebo, GL= Guba Lafto

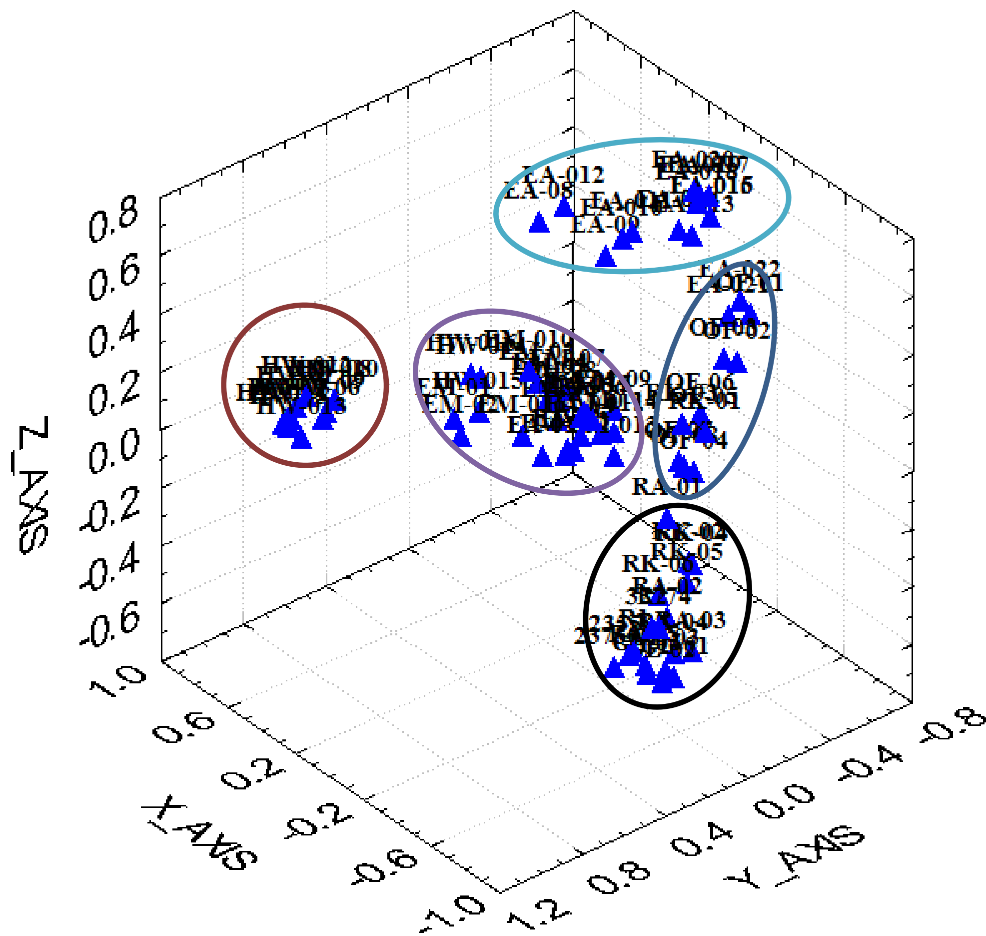

Figure 7. Three dimensional principal coordinate analyses of the seven populations.

HW= Hintalo Wejerat, EM= Endamekoni, EA= Emba Alaje, OF= Ofla, RK= Raya Kobo, RA=Raya Azebo, GL= Guba Lafto

5. SUMMARY, CONCLUSSIONS AND RECOMMENDATIONS

5.1. Summary

Sustainable utilization of crop genetic material and ensuring food security depends on the wise use and conservation of genetic resources. The genetic variability of crops provides the raw material for breeding new crop varieties. But these days, the local landraces of *P. sativum L. var. abyssinicum* in Ethiopia have become marginalized and as a consequence the crop's production has been decreasing due to several biotic and abiotic factors. Despite the fact that, *P. sativum L. var. abyssinicum* is currently under-utilized and at risk of genetic erosion, Ethiopia is one of the largest producers of field pea (*P. sativum*) in Africa. The present study aimed to estimate the genetic diversity within and among populations and to determine the population structure of *P. sativum L. var. abyssinicum* using inter-simple sequence repeat (ISSR) marker system. Accordingly, a total of 80 accessions were collected from their main natural growing regions in different areas of southern Tigray and North Amhara regions and 3 accessions were obtained from EBI. The germplasms were germinated in green house in Haramaya University for about three weeks and young leafs from five plants per accession were collected and dried in silica-gel.

Total genomic DNA was extracted by using modified CTAB method and purified using Ethanol (70 %), 7.5 M NH4Ac-solution, 3 M NaAC-solution and finally dissolved in TE buffers to store at -20°c. The quality and quantity of the genomic DNA was tested using 1% agarose and Nano Drop. Out 20 primers screened, six primers were selected based on their polymorphism, reproducibility and clarity of bands and PCR amplification was done on a Biometra 2003 T3 Thermo cycler. Gel electrophoresis was run for about 3 hours at constant voltage of 100 V; the bands were visualized under UV light and photographed using Gel Doc system (Biosens SC750). Scoring of Band was performed based on Presence '1' or absence '0' and '?' for ambiguous manually and different softwares like POPGENE (NPL, PPL, h & I), GeneAlex (AMOVA), NTSYS (UPGMA), Free Tree (NJ), GeneAlex and STATISTICA (PCO) was used for data analysis. The 6 primers led to amplification of 91 Scorable bands within the range from about 200 bp (881) to 3000 bp (834). Among the polymorphic loci, maximum (68.75 %) and minimum (50%) PPL was generated by primers 881 and 835 respectively. The gene diversity is ranged from 0.141 to 0.236 was obtained from primers 826 and 857 respectively. Shannon's information index ranged from 0.225 to 0. 352 also obtained from primers 826 and 857 respectively. The overall gene diversity and Shannon's information index were 0.176 and 0.258 respectively.

Out of the 91 scorable bands generated 55 (64.44%) bands were found to be polymorphic with the highest PPL of 24.18 (EM, EA and OF) and the least PPL of 0.00 % (GL). The gene diversity (h) indexes of the seven populations with overall primers ranged from 0.00 (GL) to 0.096 (OF). The Shannon's information index of the seven populations with overall primers were also ranged from 0.00 (GL) to 0.141 (OF). The overall gene diversity and Shannon's information index was 0.176 and 0.258 respectively. Analysis of molecular variance (AMOVA) also resulted in higher percentage (54 %) within population variation than among population variation (46 %) indicating that relatively moderate genetic exchange or gene flows, which actually have a more homogenizing effect on the genetic variation among populations by the dispersal of the seeds and seed exchange via market channels. The degree of genetic relatedness among populations varied considerably (0.697- 0.943) with the Highest similarity (0.943) measured between RA and RK and the least similarity (0.697) was found between RK and HW.

Using UPGMA tree, two coordinates (2D) and three coordinates (3D) PCOs (Figure 5, 6, & 7) almost similar results were obtained where individuals tend to form separate cluster each except few accessions that were found to get intermixed with each other. The three accessions collected from EBI and few others are distributed separately in the plot without forming a group. The result of each genetic diversity parameters in this study confirms that, there is a considerable gene diversity in *P. sativum L. var. abyssinicum*.

5.2. Conclusions

The results of this study indicated that, the genetic diversity of *P. sativum L. var. abyssinicum* was considerable at the species level. A relatively low genetic diversity was found among populations, which is attributed to limited geographic distance, moderate gene flow through market channels and selective pressure from environmental heterogeneity whereas, the relatively high genetic variation within populations could be affected by the biological characters, mating system and lifespan of *P. sativum L. var. abyssinicum.* Both UPGMA cluster analysis and PCA supported the grouping of all seven populations into six groups. Based on these findings, strategies can be proposed for the conservation and breeding programs of the species. Moreover, ISSR markers could be successfully used to assess within and among population genetic diversity of *P. sativum L. var. abyssinicum.*

5.3. Recommendations

In this study the genetic diversity analysis of *P. sativum L. var. abyssinicum* accessions was done using ISSR markers. Therefore, further study with different molecular markers such as SSRs is needed to confirm the result of the present study as a single molecular marker cannot fulfill all of the desirable properties of molecular markers. Further study is also recommended by collecting more germplasms in such a way that the collected germplasm should proportionally represent each locality of the *P. sativum L. var. abyssinicum* growing areas. Based on the result of this study, the high genetic diversity among the seven populations leads to possible germplasm conservation system of all the existing genetic resource of the species by the Ethiopian Biodiversity Institute. Since the need for the conservation of plant genetic resources has been widely accepted, germplasm characterization and evaluation complemented by molecular studies to generate adequate information for more efficient utilization of this economically important and endemic species should be encouraged. The accessions collected from EBI can also be targeted for further evaluation of their unique character for breeding purposes. Collecting the *P. sativum* L. var. *abyssinicum* accessions from different world collections like United States Department of Agriculture in USA and the John Innes Centre in UK to enrich the existed genetic diversity should also be done.

6. REFERENCES

Abbot, P. 2001. Individual and population variation in invertebrates revealed by Inter simple Sequence Repeats (ISSRs). Journal of Insect Science, 1(8):1-3.

Abel Teshome, Bryngelsson, T., Kifle Dagne and Mulatu Geleta. 2015. Assessment of genetic diversity in Ethiopian field pea (*Pisum sativum* L.) accessions with newly developed EST-SSR markers. *Biomedical and Conservation Genetics*, 16:102.

Ahmad S., Kaur S., Lamb, P.N.D., Lefsrud M., Singh J. 2015. Genetic diversity and population structure of Pisum sativum accessions for marker-trait association of lipid content. *The crop journal,* 3:238-245.

Ana, M. J., Ankica, K. S., Dejana, S.P., Radavan, M. and Nikola, H. 2009. Phenotypic and Molecular evaluation of genetic diversity of rapeseed (*Brassica napus* L.) genotypes. *African journal of biotechnology,* 8 (19): 4935-4844.

Arif, I.A., Bakir, M.A., Khan, H.A., Farhan, A.H., Homaidan, A.A., Bahkali, A.H., Sadoon, M. and Shobrak, M. 2010. Application of RAPD for molecular characterization of plant species of medicinal value from an arid environment. *Genetics and Molecular Research,* 9(4): 2191-2198.

Baranger, A., Aubert, G., Arnau, G., Lain, A.L., Deniot, G., Potier, J., Weinachter, C., Lejeune, H.I., Lallemand, J. and Burstin, J. 2004. Genetic diversity within *Pisum sativum* using protein and PCR-based marker. *Theoretical and Applied Genetics,* 108(7): 1309-1321.

Becher, A.S., Steinmetz, K., Weising, K., Boury, S., Peltier, D., Renou, P.J., Kahl, G. and Wolff, K. 2000. Microsatellites for cultivar identification in Pelargonium. *Theoretical and Applied Genetics*, 101:643–651.

Beckmann, J. S and Soller, M. 1986. Restriction Fragment Length Polymorphisms and Genetic Improvement of Agricultural Species. Euphytica 35: 111-124.

Begbulem, V. N. and Ita, U. R. 2016, Conservation of Genetic Diversity: It's Relevance in Poultry Production. Animal molecular Breeding, 6 (3): 1-5.

Berhanu Abraha and Berhane Gebresilassie. 2014. The Effect of Salinity (NaCl) on germination and early seeding growth of *Lathyrus sativum* and *Pisum sativum* var. *abyssinicum*. *African Journal of Science*, 8(5): 225-231.

Borsch, T., Hilu, K.W., Quandt, D., Wilde, V., Neinhuis, C. and Barthlott, W. 2003. Noncoding plastid trnT-trnF sequences reveal a well resolved phylogeny of basal angiosperms. *J. EVOL. BIOL*, 16 (2003) 558 – 576.

Botstein, D., White, R.L., Skolnick, M. and Davis, R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics,* 32: 314–31.

Burstin, J., Deniot, G., Potier, J., Weinachter, C., Aubert, G., and Baranger, A. 2001. Microsatellite polymorphism in *Pisum sativum*. *Plant Breeding*, 120:311–217.

Chakraborty, R. and Leimar, O. 1987. Genetic variation within a subdivided population. N. Ryman and F.M. Utter (Eds.), Population Genetics and Fishery Management. Washington: University of Washington: 89-120.

Choundhury, P.R., Tanveer, H. and Dixit, G.P.2006.Identification and detection of genetic relatedness among important varieties of pea (Pisum sativum L.) grown in India. *Genetica*, 130:183–191.

Cieslarova, J., Smykal, P., Dockalova, Z., Hanacek, P., Prochazka, S., Hybl, M. and Griga, M. 2011. Molecular evidence of genetic diversity changes in pea (*Pisum sativum* L.) germplasm after long-term maintenance. *Genetic Resource and Crop Evolution*, 58:439–451.

Çiftci, Y. and Okumu, B. 2002. Fish Population Genetics and Applications of Molecular Markers to Fisheries and Aquaculture: I- Basic Principles of Fish Population Genetics. *Turkish Journal of Fisheries and Aquatic Sciences,* 2: 145-155.

Collard, C.B.Y., Jahufer, M.Z.Z., Brouwer, J.B. and Pang, E.C.K. 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica,* 142: 169–196.

Conicella, C & Errico, A. 2014. Karyotype Variations in Pisum Sativum Ect. Abyssinicum. *International Journal of Cytology, Cytosystematics and Cytogenetics*, 43:1, 87-97.

Depeiger, A., Goubely, C., Lenoir, A., Cocherel, S., Picard, G., Rayanl, M., Greltel, F. and Delseny, M.1995. Identification of the most represented repeat motif in Arabidopsis thaliana microsatellite loci, *Theor. Appl. Genet.,* 91: 160- 168.

Deshpande, A. U., Apte, G. S., Bahulikar, R. A., Lagu, M. D., Kulkarni, B. G., Suresh, H. S., Singh, N. P., Rao, M. K. V., Gupta, V. S., Pant, A. and Ranjekar, P. K. 2001. Genetic diversity across natural populations of three montane plant species from the Western Ghats, India revealed by inter-simple sequence repeats. *Molecular Ecology*, 10: 2397—2408.

Duminil, J. and Michele, D.M. 2009. Progress in morphological and molecular systematics: Plant species delimitation: A comparison of morphological and molecular markers. *Plant Biosystems,* 1–15.

Duminil, J., Caron, H., Scotti, I., Cazal, S.O., Petit, R.J. 2006. Blind population genetics survey of tropical rainforest trees, *Molecular Ecology,* 15: 3505–3513.

Duran, C., Appleby, N., Edwards, D., and Batley, J. 2009. Molecular Genetic Markers: Discovery, Applications, Data Storage and Visualization. Current Bioinformatics, 4: 16-27.

Dyachenkoa, E.A., Ryzhova, N.N., Vishnyakova, M.A. and Kochieva, E.Z. 2014. Molecular Genetic Diversity of the Pea (*Pisum sativum* L.) from the Vavilov Research Institute Collection Detected by the AFLP Analysis. *Russian Journal of Genetics, 50(9): 916–924.*

Excoffier L, Smouse PE, Quattro JM, 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. Genetics 131: 479–491.

FAOSTAT. 2015. Food and Agriculture Organization of the United Nations. http://faostat.fao.org/site/567/Desktop_Default.aspx*? Page ID=567# ancor. [March 27, 2015].*

Gemechu Keneni, Mussa Jarso, Tezera Wolabu and Getnet Dino. 2005. Extent and pattern of genetic diversity for morpho - agronomic traits in Ethiopian highland pulse landraces: I. Field pea (*Pisum sativum* L.*). Genetic Resources and Crop Evolution,* 52: 539–549

Georgieva N., Nikolova I., and Kosev V. 2016**.** Evaluation of genetic divergence and heritability in pea (*Pisum sativum* L.). *Journal of Bioscience and Biotechnology,* 5(1): 61-67.

Godwin, I. D., Aitken, E. A. B. & Smith, L. W.1997. Application of inter simple sequence repeat (ISSR) markers to plant genetics. *Electrophoresis,* 18: 1524–1528.

Haddis Yirga and Dargie Tsegay. 2013. Characterization of *Dekoko* (*Pisum sativum* var. *abyssinicum*) accessions by qualitative traits in the highlands of Southern Tigray, Ethiopia. *African Journal of Plant Science,* 7(10): 482-487.

Hammer O., Harper, D.A.T. and Ryan, P.D. 2001. PAST-Paleontological Statistics. http://www.toyen.uio.no/~ohammer/past.

Hamrick, J. L., Blanton, H. M. and Hamrick, K. J.1989. Genetic Structure of Geographically Marginal Populations of Ponderosa Pine1. Amer. J. Bot. 76(11): 1559-1568.

Ipek, A., Barut, E., Gulen, H. and Ipak, M. 2009. Genetic diversity among some blackberry cultivars and their relationship with boysenberry assessed by AFLP Markers. *African journal of biotechnology*, 8 (19):4930-4834.

Jing, R., Vershinin, A., Grzebyta, J., Shaw, P., Petr Smykal, P., Marshall, D., Ambrose, M.J, Ellis, T.N, Flavell, A.J. 2010. The genetic diversity and evolution of field pea (Pisum) studied by

high throughput retrotransposon based insertion polymorphism (RBIP) marker analysis. *Evolutionary Biology*, 10 (44): 1471-2148.

Joshi, S.P., Gupta, V.S., Aggarwal, R.K., Ranjekar, P.K. and Brar, D.S. 2000. Genetic diversity and phylogenetic relationship as revealed by inter simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. *Theoretical and Applied Genetics*, 100:1311–1320.

Kapila, R. K., Naryal, S. & Dhiman, K. C. 2012. Analysis of genetic diversity among garden- and field-pea genotypes of higher Indian Himalayas. *J. Plant Biochem. Biotechnol*, 21(2):286.

Kebour, D., Ammar, B., Mefti, M., Alia, S. and Abdul, H. 2012. Use of the ISSR markers for the study of Genetic Polymorphism of the Pstachio fruit *pistacia Vera* L. in Algeria. *P. b u. sci. bull., series b,* 74: 1454-2331.

Kephart, S.R. 1990. Starch gel electrophoresis of plant isozyme: a comparative analysis of techniques. *Amer Journal of Biotechnology,* 77:693–712.

Kosterin, O.E. and Bogdanova, V.S. 2008. Relationship of wild and cultivated forms of Pisum L. as inferred from an analysis of three markers, of the plastid, mitochondrial and nuclear genomes. Genet Resour Crop Evol, 55:735–755.

Kosterin, O.E. and Bogdanova, V.S. 2015. Reciprocal compatibility within the genus *Pisum* L. as studied in F1 hybrids: Crosses involving *P. Sativum* L. *subsp. Sativum*. *Genetic Resources and Crop Evolution, 62*(5): 691-709.

Kumari, P., Basal, N., Singh, A.K., Rai, V.P., Srivastava, C.P. and Singh, P.K. 2013. Genetic diversity studies in pea (*Pisum sativum* L.) using simple sequence repeat markers. *Genetics and Molecular Research,* 12 (3): 3540-3550.

Kundan, K.M., Fougat, R.S., Ballani, A., Vinita, T., Yachana, J. and Madhumati, B. 2014. Potential and application of molecular markers techniques for plant genome analysis. *International Journal of Pure & Applied Bioscience,* 2 (1):169-188.

Kwon, S.J., Brown, F.A., Hu, J., McGee, R., Chasity Watt, C., Kisha, T., Gail, T.V., Michael Grusak, M., McPhee, K.E., Coyne, C.J.2012. Genetic diversity, population structure and genome-wide marker-trait association analysis emphasizing seed nutrients of the USDA pea (Pisum sativum L.) core collection. *Genes and Genomics*, 34: 305-320.

Lazaro, A. and Aguinagalde, A. 2006. Genetic variation among Spanish pea landraces revealed by Inter Simple Sequence Repeat (ISSR) markers: its application to establish a core collection. *Journal of Agricultural Science*, 144: 53–61.

Li, P., Wang, Y., Chen, Y. and Zhang, S. 2009. Genetic diversity of ISSR markers with the eleostreac content in tung tree (*Vernicia fordii). African journal of biotechnology,* 8 (19):4782-4788.

Liu, J., Shi, S., Chang, E., Yang, W. and Jiang, Z. 2013. Genetic Diversity of the Critically Endangered Thuja sutchuenensis Revealed by ISSR Markers and the Implications for Conservation. *International Journal of Molecular Sciences,* 14: 14860-14871.

Ma, L., Ji, Y.J., Zhang, D.X. 2015. Statistical measures of genetic differentiation of populations: Rationales, history and current states**.** *Current Zoology,* 61 (5): 886–897.

Maxted, N., and Ambrose, N. 2000. Peas (Pisum L.) Chapter 10. pp. 181–190. In: Maxted N and Bennett SJ (ed.), *Plant Genetic Resources of Legumes in the Mediterranean*. Dordrecht: *Kluwer Academic Publishers.*

McPhee, K. 2003. Dry pea production and breeding – A mini-review. Food, Agriculture & Environment, 1(1): 64-69.

Messiaen, C.M., Seif, A.A., Musa Jarso & Gemechu Keneni. 2006. PROTA 1: Cereals and pulses/Céréales et légumes secs. PROTA, Wageningen, Netherlands.

Mikic, A., Mihailovic, V., Dimitrijevic, M., Petrovic, S., Cupina, B., Đordevic, V., Kosev, V., Milosevic, B., Jovanovic, Z. and Milovac, Z. 2012. Evaluation of seed yield and seed yield components in red–yellow (*Pisum fulvum*) and Ethiopian (*Pisum abyssinicum*) peas. *Genetic Resource and Crop Evolution,* 60:629–638.

Mohan, M., Nair, S., Bhagwat, A., Krishna, T.G., Yano, M., Bhatia, C.R. and Sasaki, A. 1997. Genome mapping, molecular markers and marker-assisted selection in crop plants. *Molecular Breeding,* 3:87–103

Mondini, L., Noorani, A. and Pagnotta, M. A. 2009. Assessing Plant Genetic Diversity by Molecular Tools. *Diversity*, 1:19-35

Mulivi, G.M., Sprent, J.I., Soranzo, N., Provan, J., Odee, D., Folkard, G., Mcnicol, J.W. and Powell, W. 1999. Amplified length polymorphism (AFLP) analysis of genetic variation in *Moringa oliefera Lam*. *Molecular Ecology*, 8:463-470.

Mulusew Fikere, Tadele Tadesse, Setegn Gebeyehu and Bekele Hundie. 2010. Agronomic performances, disease reaction and yield stability of field pea (*Pisum sativum* L.) genotypes in Bale highlands, Ethiopia. *African Journal of Crop Science,* 4(4): 238-246.

Saitou, N. and Nei, M. 1987. The Neighbor-joining Method: A New Method for Reconstructing Phylogenetic Trees': Center for Demographic and Population Genetics, the University of Texas Health Science Center at Houston, *Molecular Biology and Evolution.* 4(4):406-425.

Neel, M. and Ellstr and, N. 2003. Conservation of genetic diversity in the endangered plant *Eriogonum oval folium* var. *vineum (Polygonaceae*). *Conservation Genetics,* 4:337–352.

Nei, M. 1973. Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences of the United States of America 70: 3321–3323.

Nei, M. 1987. Molecular Evolutionary Genetics. New York: Columbia University Press.

Nei, M. 1975. Molecular Population Genetics and Evolution. North-Holland, Amsterdam, the Netherlands.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, 89: 583-590.

Pavlicek, A., Hrda, S. and Flergr, J. 1999. Free - freeware program for construction of phylogenetic tree on the basis of distance data and bootsrap/Jackknife analysis of the tree Robustness: application in the RAPD analysis of Genus Frenkelia. *Folia Biologica (Praha),* 45:97-99.

Peakal, R. and Smouse, E.P. 2012. GeneAlex 6.5: genetic analysis in Excel. Population genetic software for teaching and research - an update. *Bioinformatics*, 28(19): 2537-2539.

Petit, R.J., Mousadik, A.E. and Pons, O. 1998. Identifying populations for conservation on the basis of genetic markers. *Conservation Biology,* 12: 844– 855.

Reddy, P.M., Sarla, N. and Siddiq, E.A. 2002. Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica*, 128(2): 9-17.

Rizkalla, S.W., Bellisle, F. and Slama, G. 2002. Health benefits of low glycaemic index foods, such as pulses, in diabetic patients and healthy individuals. *British Journal of Nutrition,* 3: 255–262.

RodinoÆ, A.P., Josefina, H.N., HermidaÆ, M., SantallaÆ, M., Antonio, M. and Ron, D. 2009. Sources of variation for sustainable field pea breeding. *Euphytica*, 166:95–107

Rohlf, J.F. and Corti, A.M. 2000. Use of Two-Block Partial Least-Squares to Study Co-variation in Shape. *System Biology*, 49(4):740 –753.

Sadeghi, A. and Cheghamirza, K. 2012. Efficiency of RAPD and ISSR marker systems for studying genetic diversity in common bean (*Phaseolus vulgaris* L.) cultivars. *Annals of Biological Research***.** 3 (7):3267-3273.

Samir, O. 2016. Population Genetics: *The Stanford Encyclopedia of Philosophy.2016 ed. Metaphysics Research Lab*, Stanford University, USA.

Schulman, A.H. 2007. Molecular markers to assess genetic diversity. *Euphytica,* 158:313–321

Semagn, K., Bjørnstad, A. and Ndjiondjop, M. N. 2006. An overview of molecular marker methods for plants. *African Journal of Biotechnology,* 5 (25): 2540 - 2568.

Simioniuc, D., Uptmoor, R., Friedt, W. and Ordon, F. 2002. Genetic diversity and relationships among pea cultivars revealed by RAPDs and AFLPs. *Plant Breeding,* 121:429–435.

Sirelkhatem, R. and Gaali, E.E.I. 2009. Phylogenic analysis in Acacia Senegal using AFLP molecular markers across the Gum Arabic Belt in Sudan. *African journal of biotechnology,* 8 (19):4817-4823.

Smykal, P., Aubert, G., Burstin, J., Coyne, C.J., Ellis Neel, T.H., Flavell, A.J., Ford, R., Hybl, M., Macas, J., Neumann, P., McPhee, K.E., Redden, R.J., Rubiales, D., Weller, J.L. and Warkentin, T.D. 2012. Pea (*P. sativum* L.*)* in the genomic era. *Agronomy,* 2: 74-115.

Smykal, P., Horacek, J., Dostalova, R. and Hybl, M. 2008a. Variety discrimination in pea (*Pisum sativum* L.) by molecular, biochemical and morphological markers. *Journal of Applied Genetics,* 49(2):155-166.

Smykal, P., Hybl, M., Corander, J., Jarkovsky, J., Flavell, A.J. and Griga, M. 2008b. Genetic diversity and population structure of pea (*Pisum sativum* L.) varieties derived from combined retrotransposon, microsatellite and morphological marker analysis. *Theoretical and Applied Genetics*, 117(3): 413-424.

Smykal, P., Kenicer, G., Flavell, J.A., Corander, J., Kosterin, O., Redden, O.J., Ford, R., Coyne, C.J., Maxted, N., Ambrose, M.J. and Ellis, N.T.H. 2011. Phylogeny, phylogeography and genetic diversity of the *Pisum* genus. Characterization and Utilization. *Plant Genetic Resources,* 9(1):4–18.

Sneath, P.H.A Sokal, R.R. 1973. Numerical taxonomy: the principles and practice of numerical classification. W.H. Freeman and Company, San Francisco.

Southern E.M.1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology, 98(3):503-508.

Southern, E. Detection of specific sequences among DNA fragments separated by gel-electrophoresis. *J. Mol. Biol.* **1975**, *98*, 503.

Spooner, D., Treuren, R. and Vicente, M.C. 2005.Molecular Markers for Genebank Management. *Biodiversity International*, Rome, Italy.

Studier, A.J., and Keppler, J.K. 1988. A note on the neighbor-joining algorithm of Saitou and Nei. Molecular biology and evolution, 5(6): 729-731.

Sun, M., Wong, K. C. and Joe, S.Y. L. 1998. Reproductive Biology and population genetic Structure of Kandelia candel (Rhizophoraceae), a Viviparous mangrove species. *American Journal of Botany,* 85(11): 1631–1637.

Tamirat Redae Gebreselassie. 2014. Effect of moisture on physical properties of *Dekoko* (*Pisum sativum* var. *abyssinicum*) seed. *Agric Eng Int: CIGR Journal*, 16 (2): 143-150.

Taran, B., Zhang, C., Warkentin, T., Tullu, A. and Vandenberg, A. 2005. Genetic diversity among varieties and wild species accessions of pea (*Pisum sativum* L.) based on molecular markers, and morphological and physiological characters. *Genome,* 48(2): 257-272.

Tiwari, G. and Lavanya, G.R. 2012. Genetic variability, character association and component analysis in F4 generation of field pea (*Pisum sativum* var. *arvense* L.). *Karnataka Journal of Agricultural Science, 25 (2): 173-175.*

Upadhyaya, H.D., Dwivedi, S.L., Ambrose, M., Ellis, N., Berger, J., Smykal, P., Debouck, D., Duc, G., Dumet, D., Flavell, A., Sharma, S.K., Mallikarjuna, N. and Gowda, C.L.L. 2011. Legume genetic resources: management, diversity assessment, and utilization in crop improvement. *Euphytica,* 180(1): 27- 47.

Vavilov, N.I. 1926. Studies on the origin of cultivated plants. *Bulletin of Applied Botany,* vol 26. Leningrad, USSR.

Vershinin, V.A., Allnutt, R.T., Knox, R.M., Ambrose, J.M. and Ellis-Noel, H.T. 2003. Transposable Elements Reveal the Impact of Introgression, Rather than Transposition, in *Pisum* Diversity, Evolution and Domestication. *Molecular Biology and Evolution, 20(12):2067–2075.*

Vijayan, K. 2005. Inter Simple Sequence Repeat (ISSR) Polymorphism and its Application in Mulberry Genome Analysis. *International Journal of Industrial Entomology,* 10(2):79-86.

Vijayan, K., Anuradha, H.J., Nair C.V., Pradeep AR, Awasthi, A.K., Saratchandra, B., Rahman, S.A.S., Singh, K.C., Chakraborti, R, Urs, S.R. 2006. Genetic diversity and differentiation among populations of the Indian eri silkworm, *Samia cynthiaricin* revealed by ISSR markers. *Journal of Insect Science,* 6:30.

Wang, M., Li, J., Zhang, L., Yang, R.W., Ding, C.B., Zhou, Y.H. and Yin, Z.Q. 2011. Genetic diversity among *Salvia miltiorrhiza Bunge* and related species using morphological traits and RAPD markers. *Journal of Medicinal Plants Research,* 5(13):2687-2694.

Wangari, N.P., Gacheri, K.M., Theophilus, M.M. and Lucas, N. 2013. Use of SSR markers for genetic diversity studies in *mulberry* accessions grown in Kenya. *International Journal for Biotechnology and Molecular Biology Research,* 4(3):38-44

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research,* 18: 6231-6535.

Wright, S. 1951. The genetic structure of populations. Annals of Eugenics 15: 323–354.

Wright, S. 1965. The interpretation of population structure by *F* statistics with special regard to systems of mating. Evolution 19: 395–420.

Yeh, F.C., Yang, R.C. and Boyle, T. 1999. PopGene Version 1.31: Microsoft Window-based freeware for population genetic analysis. *University of Alberta and Centre for International Forestry Research,* 11-23.

Yemane Asgedom and Skjelvag, A.O. 2003. Physico - chemical Traits of *Dekoko* (*Pisum sativum* var. *abyssinicum*) Seeds. *Plant Foods for Human Nutrition,* 58:275–283.

Zhang, Y., Deng, K., Xie, L., Li, X., Wang, B. and Chen, L. 2009. Genetic diversity analysis and conservation of the Chinese herb *saliva miltiorrhiza* collected from different geographic origins in china. *African journal of biotechnology*, 8(19):4949-4855.

Zhou, Y., Zhou, C., Yao, H., Liu, Y. and Tu, R. 2008. Application of ISSR markers in detection of genetic variation among Chinese *yam* (*Dioscorea opposite Thunb*) cultivars. *Life Science Journal*, 5(4):6 – 12.

Zietkiewicz, E., Rafalski, A., and Damian, L. 1994.Genome fingerprinting by simple sequence repeats (SSR) anchored polymerase chain reaction amplification. *Genomics,* 20: 176-183.

Zong, X., Redden, R.J., Liu, Q., Wang, S., Guan, J., Liu, J., Xu, Y., Liu, X., Gu, J., Yan, L., Ades, P. and Ford, R. 2009. Analysis of a diverse global *Pisum sp.* Collection and comparison to a Chinese local *P. sativum* collection with microsatellite markers. *Theoretical and Applied Genetics*, 118:193–204.

APPENDICES

Appendix Table 1. List of the 83 *P. sativum L. var. abyssinicum* accessions used in this study

Acc. No.	Region	Locality	Acc. Code	Elevation		Latitude		Longitude	
				Low	High	From	To	From	To
1.	Tigray	Hintalo Wejerat	HW-01	2129	3027	12^{0}41'10" N	13^{0}2'10" N	39^{0}38'40" E	39^{0}56'10" E
2.	Tigray	Hintalo Wejerat	HW-02	2129	3027	12^{0}41'10" N	13^{0}2'10" N	39^{0}38'40" E	39^{0}56'10" E
3.	Tigray	Hintalo Wejerat	HW-03	2187	3537	12^{0}56'40" N	13^{0}3'20" N	39^{0}34'0" E	39^{0}39'20" E
4.	Tigray	Hintalo Wejerat	HW-04	2187	3537	12^{0}56'40" N	13^{0}3'20" N	39^{0}34'0" E	39^{0}39'20" E
5.	Tigray	Hintalo Wejerat	HW-05	2187	3537	12^{0}56'40" N	13^{0}3'20" N	39^{0}34'0" E	39^{0}39'20" E
6.	Tigray	Hintalo Wejerat	HW-06	2187	3537	12^{0}56'40" N	13^{0}3'20" N	39^{0}34'0" E	39^{0}39'20" E
7.	Tigray	Hintalo Wejerat	HW-07	2187	3537	12^{0}56'40" N	13^{0}3'20" N	39^{0}34'0" E	39^{0}39'20" E
8.	Tigray	Hintalo Wejerat	HW-08	2187	3537	12^{0}56'40" N	13^{0}3'20" N	39^{0}34'0" E	39^{0}39'20" E
9.	Tigray	Hintalo Wejerat	HW-09	2187	3537	12^{0}56'40" N	13^{0}3'20" N	39^{0}34'0" E	39^{0}39'20" E
10.	Tigray	Hintalo Wejerat	HW-010	1208	3405	12^{0}44'40" N	13^{0}8'0" N	39^{0}35'10" E	39^{0}52'40" E
11.	Tigray	Hintalo Wejerat	HW-011	1208	3405	12^{0}44'40" N	13^{0}8'0" N	39^{0}35'10" E	39^{0}52'40" E
12.	Tigray	Hintalo Wejerat	HW-012	1218	2784	12^{0}56'0" N	13^{0}12'0" N	39^{0}40'0" E	39^{0}52'0" E
13.	Tigray	Hintalo Wejerat	HW-013	1218	2784	12^{0}56'0" N	13^{0}12'0" N	39^{0}40'0" E	39^{0}52'0" E
14.	Tigray	Hintalo Wejerat	HW-014	2129	3027	12^{0}41'10" N	13^{0}2'10" N	39^{0}38'40" E	39^{0}56'10" E
15.	Tigray	Hintalo Wejerat	HW-015	2129	3027	12^{0}41'10" N	13^{0}2'10" N	39^{0}38'40" E	39^{0}56'10" E

Acc. No.	Region	Locality	Acc. Code	Elevation		Latitude		Longitude	
				Low	High	From	To	From	To
16.	Tigray	Hintalo Wejerat	HW-016	2129	3027	$12^0$41'10" N	$13^0$2'10" N	$39^0$38'40" E	$39^0$56'10" E
17.	Tigray	Endamekhoni	EM-01	2258	3677	$12^0$38'50" N	$12^0$45'50" N	$39^0$27'0" E	$39^0$32'50" E
18.	Tigray	Endamekhoni	EM-02	2258	3677	$12^0$38'50" N	$12^0$45'50" N	$39^0$27'0" E	$39^0$32'50" E
19.	Tigray	Endamekhoni	EM-03	2258	3677	$12^0$38'50" N	$12^0$45'50" N	$39^0$27'0" E	$39^0$32'50" E
20.	Tigray	Endamekhoni	EM-04	2258	3677	$12^0$38'50" N	$12^0$45'50" N	$39^0$27'0" E	$39^0$32'50" E
21.	Tigray	Endamekhoni	EM-05	2258	3677	$12^0$38'50" N	$12^0$45'50" N	$39^0$27'0" E	$39^0$32'50" E
22.	Tigray	Endamekhoni	EM-06	2556	3910	$12^0$47'0" N	$13^0$52'50" N	$39^0$28'10" E	$39^0$32'50" E
23.	Tigray	Endamekhoni	EM-07	2556	3910	$12^0$47'0" N	$12^0$52'50" N	$39^0$28'10" E	$39^0$32'50" E
24.	Tigray	Endamekhoni	EM-08	2556	3910	$12^0$47'0" N	$12^0$52'50" N	$39^0$28'10" E	$39^0$32'50" E
25.	Tigray	Endamekhoni	EM-09	2556	3910	$12^0$47'0" N	$12^0$52'50" N	$39^0$28'10" E	$39^0$32'50" E
26.	Tigray	Endamekhoni	EM-010	2556	3910	$12^0$47'0" N	$12^0$52'50" N	$39^0$28'10" E	$39^0$32'50" E
27.	Tigray	Endamekhoni	EM-011	2412	3349	$12^0$48'10" N	$12^0$51'40" N	$39^0$31'40" E	$39^0$34'0" E
28.	Tigray	Endamekhoni	EM-012	2412	3349	$12^0$48'10" N	$12^0$51'40" N	$39^0$31'40" E	$39^0$34'0" E
29.	Tigray	Endamekhoni	EM-013	1823	2710	$12^0$43'30" N	$13^0$49'20" N	$39^0$31'40" E	$39^0$36'20" E
30.	Tigray	Endamekhoni	EM0-14	1823	2710	$12^0$43'30" N	$13^0$49'20" N	$39^0$31'40" E	$39^0$36'20" E
31.	Tigray	Endamekhoni	EM-015	1823	2710	$12^0$43'30" N	$13^0$49'20" N	$39^0$31'40" E	$39^0$36'20" E

Acc. No.	Region	Locality	Acc. Code	Elevation		Latitude		Longitude	
				Low	High	From	To	From	To
32.	Tigray	Endamekhoni	EM-016	1823	2710	12^{0}43'30" N	13^{0}49'20" N	39^{0}31'40" E	39^{0}36'20" E
33.	Tigray	Emba - Alaje	EA-01	2120	3858	13^{0}0'0" N	13^{0}9'20" N	39^{0}34'0" E	39^{0}40'40" E
34.	Tigray	Emba - Alaje	EA-02	2120	3858	13^{0}0'0" N	13^{0}9'20" N	39^{0}34'0" E	39^{0}40'40" E
35.	Tigray	Emba - Alaje	EA-03	2120	3858	13^{0}0'0" N	13^{0}9'20" N	39^{0}34'0" E	39^{0}40'40" E
36.	Tigray	Emba - Alaje	EA-04	2120	3858	13^{0}0'0" N	13^{0}9'20" N	39^{0}34'0" E	39^{0}40'40" E
37.	Tigray	Emba - Alaje	EA-05	2301	3909	12^{0}51'40" N	12^{0}56'20" N	39^{0}27'0" E	39^{0}30'30" E
38.	Tigray	Emba - Alaje	EA-06	2301	3909	12^{0}51'40" N	12^{0}56'20" N	39^{0}27'0" E	39^{0}30'30" E
39.	Tigray	Emba - Alaje	EA-07	2301	3909	12^{0}51'40" N	12^{0}56'20" N	39^{0}27'0" E	39^{0}30'30" E
40.	Tigray	Emba - Alaje	EA-08	2301	3909	12^{0}51'40" N	12^{0}56'20" N	39^{0}27'0" E	39^{0}30'30" E
41.	Tigray	Emba - Alaje	EA-09	2301	3909	12^{0}51'40" N	12^{0}56'20" N	39^{0}27'0" E	39^{0}30'30" E
42.	Tigray	Emba - Alaje	EA-010	2301	3909	12^{0}51'40" N	12^{0}56'20" N	39^{0}27'0" E	39^{0}30'30" E
43.	Tigray	Emba - Alaje	EA-011	2301	3909	12^{0}51'40" N	12^{0}56'20" N	39^{0}27'0" E	39^{0}30'30" E
44.	Tigray	Emba - Alaje	EA-012	2492	3442	12^{0}52'50" N	12^{0}57'30" N	39^{0}34'0" E	39^{0}37'30" E
45.	Tigray	Emba - Alaje	EA-013	2492	3442	12^{0}52'50" N	12^{0}57'30" N	39^{0}34'0" E	39^{0}37'30" E
46.	Tigray	Emba - Alaje	EA-014	2492	3442	12^{0}52'50" N	12^{0}57'30" N	39^{0}34'0" E	39^{0}37'30" E
47.	Tigray	Emba - Alaje	EA-015	2492	3442	12^{0}52'50" N	12^{0}57'30" N	39^{0}34'0" E	39^{0}37'30" E

Acc. No.	Region	Locality	Acc. Code	Elevation		Latitude		Longitude	
				Low	High	From	To	From	To
48.	Tigray	Emba - Alaje	EA-016	2492	3442	$12^0$52'50" N	$12^0$57'30" N	$39^0$34'0" E	$39^0$37'30" E
49.	Tigray	Emba - Alaje	EA-017	2492	3442	$12^0$52'50" N	$12^0$57'30" N	$39^0$34'0" E	$39^0$37'30" E
50.	Tigray	Emba - Alaje	EA-018	2492	3442	$12^0$52'50" N	$12^0$57'30" N	$39^0$34'0" E	$39^0$37'30" E
51.	Tigray	Emba - Alaje	EA-019	2492	3442	$12^0$52'50" N	$12^0$57'30" N	$39^0$34'0" E	$39^0$37'30" E
52.	Tigray	Emba - Alaje	EA-020	2492	3442	$12^0$52'50" N	$12^0$57'30" N	$39^0$34'0" E	$39^0$37'30" E
53.	Tigray	Emba - Alaje	EA-021	2492	3442	$12^0$52'50" N	$12^0$57'30" N	$39^0$34'0" E	$39^0$37'30" E
54.	Tigray	Emba - Alaje	EA-022	2492	3442	$12^0$52'50" N	$12^0$57'30" N	$39^0$34'0" E	$39^0$37'30" E
55.	Tigray	Ofla	OF-01	1887	2900	$12^0$31'50" N	$12^0$36'38" N	$39^0$30'30" E	$39^0$34'0" E
56.	Tigray	Ofla	OF-02	1887	2900	$12^0$31'50" N	$12^0$36'38" N	$39^0$30'30" E	$39^0$34'0" E
57.	Tigray	Ofla	OF-03	1887	2900	$12^0$31'50" N	$12^0$36'38" N	$39^0$30'30" E	$39^0$34'0" E
58.	Tigray	Ofla	OF-04	1887	2900	$12^0$31'50" N	$12^0$36'38" N	$39^0$30'30" E	$39^0$34'0" E
59.	Tigray	Ofla	OF-05	1887	2900	$12^0$31'50" N	$12^0$36'38" N	$39^0$30'30" E	$39^0$34'0" E
60.	Tigray	Ofla	OF-06	1887	2900	$12^0$31'50" N	$12^0$36'38" N	$39^0$30'30" E	$39^0$34'0" E
61.	Tigray	Ofla	OF-07	1887	2900	$12^0$31'50" N	$12^0$36'38" N	$39^0$30'30" E	$39^0$34'0" E
62.	Tigray	Ofla	OF-08	1074	2640	$12^0$27'10" N	$12^0$30'40" N	$39^0$37'40" E	$39^0$34'0" E
63.	Amhara	Raya Kobo	RK-01	1229	2947	$11^0$47'0" N	$12^0$28'20" N	$39^0$20'40" E	$39^0$45'20" E

Acc. No.	Region	Locality	Acc. Code	Elevation		Latitude		Longitude	
				Low	High	From	To	From	To
64.	Amhara	Raya Kobo	RK-02	1229	2947	11^{0}47'0" N	12^{0}28'20" N	39^{0}20'40" E	39^{0}45'20" E
65.	Amhara	Raya Kobo	RK-03	1229	2947	11^{0}47'0" N	12^{0}28'20" N	39^{0}20'40" E	39^{0}45'20" E
66.	Amhara	Raya Kobo	RK-04	1229	2947	11^{0}47'0" N	12^{0}28'20" N	39^{0}20'40" E	39^{0}45'20" E
67.	Amhara	Raya Kobo	RK-05	1229	2947	11^{0}47'0" N	12^{0}28'20" N	39^{0}20'40" E	39^{0}45'20" E
68.	Amhara	Raya Kobo	RK-06	1229	2947	11^{0}47'0" N	12^{0}28'20" N	39^{0}20'40" E	39^{0}45'20" E
69.	Amhara	Raya Kobo	RK-07	1100	3000	11^{0}47'0" N	12^{0}28'20" N	39^{0}20'40" E	39^{0}45'20" E
70.	Amhara	Raya Kobo	RK-08	2129	3027	11^{0}47'0" N	12^{0}28'20" N	39^{0}20'40" E	39^{0}45'20" E
71.	Tigray	Raya Azebo	RA-01	1540	2947	12^{0}41'10" N	12^{0}52'50" N	39^{0}34'0" E	39^{0}43'20" E
72.	Tigray	Raya Azebo	RA-02	1540	2947	12^{0}41'10" N	12^{0}52'50" N	39^{0}34'0" E	39^{0}43'20" E
73.	Tigray	Raya Azebo	RA-03	1540	2947	12^{0}41'10" N	12^{0}52'50" N	39^{0}34'0" E	39^{0}43'20" E
74.	Tigray	Raya Azebo	RA-04	1079	2085	12^{0}41'10" N	13^{0}2'10" N	39^{0}38'40" E	39^{0}56'10" E
75.	Tigray	Raya Azebo	RA-05	1079	2085	12^{0}41'10" N	13^{0}2'10" N	39^{0}38'40" E	39^{0}56'10" E
76.	Amhara	Guba Lafto	GL-01	1372	3746	11^{0}26'20" N	12^{0}7'40" N	39^{0}14'20" E	39^{0}45'20" E
77.	Amhara	Guba Lafto	GL-02	1372	3746	11^{0}26'20" N	12^{0}7'40" N	39^{0}14'20" E	39^{0}45'20" E
78.	Amhara	Guba Lafto	GL-03	1372	3746	11^{0}26'20" N	12^{0}7'40" N	39^{0}14'20" E	39^{0}45'20" E
79.	Amhara	Guba Lafto	GL-04	1372	3746	11^{0}26'20" N	12^{0}7'40" N	39^{0}14'20" E	39^{0}45'20" E

Acc. No.	Region	Locality	Acc. Code	Elevation		Latitude		Longitude	
				Low	High	From	To	From	To
80.	Amhara	Guba Lafto	GL-05	1372	3746	$11^{0}26'20''$ N	$12^{0}7'40''$ N	$39^{0}14'20''$ E	$39^{0}45'20''$ E
81.	Tigray	EIB/Alamata	237507	1500		No. information is available for the accessions collected from Ethiopian Biodiversity Institute.			
82.	Tigray	EIB/Alamata	237509	1500					
83.	Amhara	EIB/Kobo	32774	3100					

HW= Hintalo Wejerat EM= Endamekhoni, OF= Ofla, EA= Emba Alaje, RK= Raya Kobo, RA= Raya Azebo, GL= Guba Lafto, EBI= Ethiopian Biodiversity Institute

Appendix Table 2. The ND measurement of the quality and quantity of DNA from the 83 accessions

Acc. No.	Acc. code	N.A. Con.(ng/µl)	A260	A280	A260/280	A260/230
1.	HW-01	3037.2	60.744	30.181	2.01	2.25
2.	HW-02	447.1	8.943	4.383	2.04	2.11
3.	HW-03	394.2	7.885	3.913	2.02	2.27
4.	HW-04	486.2	9.724	4.730	2.06	2.08
5.	HW-05	440.3	8.806	4.220	2.09	2.08
6.	HW-06	557.0	11.139	5.542	2.01	2.11
7.	HW-07	243.8	4.876	2.380	2.05	2.26
8.	HW-08	392.3	7.847	3.759	2.09	1.98
9.	HW-09	443.8	8.875	4.338	2.05	2.02
10.	HW-010	304.5	6.090	2.998	2.03	2.28
11.	HW-011	2136.9	42.738	21.223	2.01	2.16
12.	HW-012	1639.4	32.788	16.033	2.05	2.30
13.	HW-013	1145.4	22.908	11.224	2.04	2.29
14.	HW-014	1272.0	25.439	12.634	2.01	2.20
15.	HW-015	1414.8	28.295	14.215	1.99	2.23
16.	HW-016	2110.7	42.213	20.697	2.04	2.19
17.	EM-01	2522.6	50.452	25.246	2.00	2.15
18.	EM-02	4779.0	95.579	48.266	1.98	2.12
19.	EM-03	2408.7	48.175	23.956	2.01	2.13
20.	EM-04	3578.0	71.560	36.159	1.98	2.21

Acc. No.	Acc. code	N.A. Con.(ng/µl)	A260	A280	A260/280	A260/230
21.	EM-05	2508.9	50.178	25.151	2.00	2.17
22.	EM-06	4724.3	94.487	47.467	1.99	2.09
23.	EM-07	403.9	8.077	3.882	2.08	2.10
24.	EM-08	2140.3	42.806	20.952	2.04	2.20
25.	EM-09	3391.2	67.823	32.830	2.07	2.26
26.	EM-010	1802.2	36.043	17.844	2.02	2.21
27.	EM-011	3008.9	60.177	29.098	2.07	2.23
28.	EM-012	414.9	8.299	3.906	2.12	2.11
29.	EM-013	2524.6	50.492	24.369	2.07	2.19
30.	EM0-14	1448.7	28.975	14.397	2.01	2.18
31.	EM-015	1456.5	29.130	14.313	2.04	2.21
32.	EM-016	3746.1	74.922	36.726	2.04	2.25
33.	EA-01	1462.3	29.245	15.165	1.93	2.26
34.	EA-02	1242.6	24.852	12.648	1.96	2.30
35.	EA-03	2892.9	57.857	29.440	1.97	2.09
36.	EA-04	2406.9	48.138	24.048	2.00	2.20
37.	EA-05	1707.9	34.158	16.805	2.03	2.28
38.	EA-06	1759.0	35.180	17.546	2.01	2.13
39.	EA-07	848.7	16.974	8.123	2.09	2.24
40.	EA-08	1768.1	35.362	17.407	2.03	2.21

Acc. No.	Acc. code	N.A. Con.(ng/µl)	A260	A280	A260/280	A260/230
41.	EA-09	4055.6	81.112	40.787	1.99	2.21
42.	EA-010	2065.6	41.312	20.849	1.98	2.16
43.	EA-011	2246.8	44.936	22.446	2.00	2.15
44.	EA-012	791.2	15.823	7.678	2.06	2.26
45.	EA-013	507.9	10.158	5.104	1.99	2.0.0
46.	EA-014	1298.5	25.970	12.729	2.04	2.18
47.	EA-015	1106.3	22.126	10.654	2.08	2.25
48.	EA-016	1228.4	24.568	11.923	2.06	2.27
49.	EA-017	2971.4	59.429	29.604	2.01	2.27
50.	EA-018	2215.8	44.317	22.632	1.96	2.27
51.	EA-019	4309.3	86.185	44.500	1.94	1.57
52.	EA-020	1954.3	39.085	19.649	1.99	2.13
53.	EA-021	1292.9	25.858	12.683	2.04	2.21
54.	EA-022	1018.9	20.378	9.911	2.06	2.25
55.	OF-01	1928.0	38.561	19.000	2.03	2.29
56.	OF-02	1112.3	22.245	10.790	2.06	2.29
57.	OF-03	1888.4	37.768	18.967	1.99	2.24
58.	OF-04	1897.2	37.944	19.661	1.93	2.18
59.	OF-05	1873.0	37.460	18.095	2.07	2.25
60.	OF-06	2592.6	51.853	27.224	1.90	2.11

Acc. No.	Acc. code	N.A. Con.(ng/µl)	A260	A280	A260/280	A260/230
61.	OF-07	2295.5	45.910	22.883	2.01	2.18
62.	OF-08	2251.7	45.034	23.223	1.94	2.10
63.	RK-01	1379.9	27.599	13.679	2.02	2.26
64.	RK-02	1503.9	30.079	14.916	2.02	2.24
65.	RK-03	4412.8	88.257	44.907	1.97	2.22
66.	RK-04	3631.7	72.634	36.159	2.01	2.26
67.	RK-05	3376.2	67.524	33.672	2.01	2.14
68.	RK-06	1115.4	22.308	10.568	2.11	2.25
69.	RK-06	3362.9	67.258	33.321	2.02	2.09
70.	RK-08	3379.9	67.597	33.071	2.04	2.26
71.	RA-01	4921.6	98.432	51.384	1.92	2.16
72.	RA-02	4125.3	82.506	41.968	1.97	2.21
73.	RA-03	1610.0	32.200	16.223	1.98	2.28
74.	RA-04	2220.5	44.409	22.234	2.00	2.28
75.	RA-05	2075.2	41.504	20.594	2.02	2.23
76.	GL-01	3186.1	63.722	32.786	1.94	2.18
77.	GL-02	1360.8	27.216	13.237	2.06	2.21
78.	GL-03	3707.7	74.155	37.211	1.99	2.24
79.	GL-04	3773.7	75.474	37.606	2.01	2.25
80.	GL-05	3901.7	78.033	38.401	2.03	2.24

Acc. No.	Acc. code	N.A. Con.(ng/µl)	A260	A280	A260/280	A260/230
81.	237507	2134.6	42.692	20.497	2.08	2.23
82.	237509	3638.4	72.768	35.505	2.05	2.24
83.	32774	2488.9	49.778	24.972	1.99	2.16

HW= Hintalo Wejerat EM= Endamekhoni, OF= Ofla, EA= Emba Alaje, RK= Raya Kobo, RA=Raya Azebo, GL= Guba Lafto