SCHOOL OF APPLIED NATURAL SCIENCE

DEPARTMENT OF APPLIED BIOLOGY GRADUATE PROGRAM

GENETIC DIVERSITY OF SELECTED ACCESSIONS OF GROUNDNUT (*Arachis hypogaea* L.) IN ETHIOPIA USING INTER SIMPLE SEQUENCE REPEAT MARKER

BY

MOHAMMED ABDELLA

A Thesis Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Degree of Master of Science in Applied Biology (Biotechnology)

November - 2018

Adama, Ethiopia

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GSR/0196/09

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APPLIED BIOLOGY DEPARTMENT

APPROVAL OF BOARD OF EXAMINERS

We, the undersigned, members of the Board of Examiners of the final open defence by Mohammed Abdella have read and evaluated his thesis entitled "**Genetic Diversity of Selected Accessions of Groundnut (*Arachis Hypogaea* L.) In Ethiopia Using Inter Simple Sequence Repeat Marker**" and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfilment of the requirement of the Degree of MSc in Applied Biology (Specialisation in Biotechnology).

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STATEMENT OF THE AUTHOR

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Date of submission: _____

DEDICATION

I dedicate this thesis to my family and all Ethiopian people who are in need of peace and development. GOD BLESS ETHIOPIA!!

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ABBREVIATIONS AND ACRONYMS

AFLP - Amplified Fragment Length Polymorphism

CTAB - Cetyl Trimethyl Ammonium Bromide

EBI - Ethiopian Biodiversity Institute

ICRISAT - International Crops Research Institute for the Semi-Arid Tropic

ISSR - Inter Simple Sequence Repeat

NJ - Neighbor Joining

PCoA - Principal Coordinate Analysis

RAPD - Random Amplified Length Polymorphism

RLFP - Restriction Fragment Length Polymorphism

PCoA - Principal Coordinate Analysis

SSR - Simple Sequence Repeats

UPGMA - Unweighted Pair group Method with Arithmetic Averages

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ABSTRACT

Groundnut (Arachis hypogaea L.*) belongs to the family Leguminoseae and it is the world's 4th most important source of edible oil and 3rd most important source of vegetable protein. In Ethiopia their morphological variations have already been documented but molecular variations was not studied for this valuable crop. The main objective of this study was to determine the genetic diversity of A.* hypogaea *accessions from Ethiopia using ISSR marker. Using four ISSR primers, 56 reproducible bands were generated of which 29 were polymorphic. The band size ranged from 120 bp to 1100 bp. The number of amplified bands varied from 12 in primer 841 to 18 in primer 881. The maximum percent of polymorphic bands (84.6 %) was obtained using primer 857, whereas the minimum percent of polymorphic bands (27.8 %) was obtained in Primer 881 with an average value of 51.78% polymorphism. Average number of bands and polymorphic bands per primer were 14 and 7.25, respectively. The polymorphic information content (PIC) value ranged between 0.29 and 0.76 with an average of 0.49. The mean Nei's gene diversity and Shannon's information index were 0.245 and 0.325, respectively. Genetic relationship between A.* hypogaea *accessions on the basis of Jaccard's pair wise similarity coefficients varies from 44% to 83% with an average value of 63.5%. The UPGMA dendrogram based on cluster analysis grouped the forty three A.* hypogaea *accessions into five distinct clusters at 63.5% similarity coefficient, and the principal coordinate analysis revealed similar grouping. In both UPGMA and PCoA analysis accessions clustered into individual groups, where most of the accessions were grouped in separate clusters irrespective of their geographic origins. According to Jaccard's similarity, accessions GOBG-1 (Bale/Ginir) with GOB-14 (Babile/Tofic-2), GOG-1 (Gursum /Llalemi-1) with GAW-1 (Amhara /Wangua) and GOB-17 (Babile/Gemechu) with GOBG-1 (Bale/ginir) accessions are most genetically distant ones, so that the accessions should be considered as the primary sites in designing conservation areas for this crop. Results of this study would be promising as a genetic marker for the identification of A.* hypogaea *accessions and an important source of knowledge for subsequent A.* hypogaea *researches such as genetic conservation and plant germplasm improvement.*

KeyWords: *Ethiopia, Genetic diversity, Neighbor Joining, Nei's gene diversity, Shannon diversity index.*

1. INTRODUCTION

1.1 Background of the Study

Groundnut or peanut (*Arachis hypogaea* L.) belongs to the family Leguminoseae and genus *Arachis.* Cultivated groundnut (*Arachis hypogea* L.) is a highly self-pollinated, allotetraploid annual legume with 2n=4x=40 with a basic chromosome number of x=10 (Stalker, 1997). *A. hypogaea* is an important oil-seed crop, which is cultivated and grown throughout the tropics and sub-tropics between 40° south and 40° north of the equator where the annual rainfall ranges between 500 to 1200 mm and with average daily temperature of higher than 20°C (Mastewal *et al.,* 2017). *A. hypogaea* seeds are valued both for their oil and protein contents. Groundnut kernels contain 42 - 50 % oil, 26% protein, 18% carbohydrates, and are also rich source of riboflavin, thiamine, nicotinic acid and vitamin E (Kathirvelan and Kalaiselvan, 2007). Being a legume, this plant improves soil by fixing nitrogen biologically without consuming non-renewable energies and without disturbing agro-ecological balance (Jiaramraja and Fantahun, 2014).

Arachis hypogaea is the 13th most important food crop of the world. It is the world's 4th most important source of edible oil and 3rd most important source of vegetable protein. Globally, *A. hypogaea* is grown in 26.4 million ha worldwide with a total production of 37.1 million metric ton and an average productivity of 1.4 metric t/ha (Hamakareem *et al.,* 2016). Major *A. hypogaea* growing countries include China, India, the United States and Nigeria (Taru *et al.,* 2010). *A. hypogaea* is one of the four economically important oilseed crops along with noug, flax and sesame in Ethiopia (Mastewal *et al.,* 2017). Besides, this crop helps small scale producers in getting significant revenue and also helps Ethiopia in getting foreign currency earnings through export (Jiaramraja and Fantahun, 2014).

In Ethiopia *A. hypogaea* is grown and covered nearly 80,000 hectares (Fredu *et al.,* 2015) of arable land per annum and the major producing regions which account for most of *A. hypogaea* production in Ethiopia are; Eastern Hararghe in Oromia and Metekel in Benishangul-Gumuz regional state (Fredu *et al.,* 2015; Addisu and Ermias, 2017). Despite its importance, the improvement of *A. hypogaea* productivity is stagnant in the country. Research result showed that yields of more than 2.0 tons/ha can be obtained but the national average yield produced by the farmers in Ethiopia is considerably low, 1.3 tons/ha, indicating the need of maximum effort to improve productivity (Gebbreselassie *et al.*, 2014).

Assessment of genetic diversity is an important step in any crop improvement program and it plays an important role because of hybrids between genetically diverse parents manifest greater heterosis and/or genetic recombination (Bhandari *et al.,* 2017) for potential yield increase and food production than those between more closely related parents. Evaluation of genetic diversity based on morphological features may not be efficient as they are highly influenced by environments. Molecular markers are fixed marks in the genome found at specific locations of the genome which are used to identify specific genetic differences. In order to precisely identify traits of interest, the marker must be close to the gene of interest so that the allele of both the marker and the gene could be inherited together (Semagn *et al.,* 2006). The molecular markers offer many advantages over morphological markers as they are phenotypically neutral, occur throughout the genome, neither influenced by environments nor by pleotropic and epistatic interactions, and expression is not dependent on plant age (Molosiwa, 2012). The development of marker protocols such as RFLP, AFLP, ISSR, SNP and SSR have revolutionized the genetic analysis by detecting level of polymorphism /genetic diversity (Raina *et al.,* 2001).

Genetic diversity in *A. hypogaea*, based on morphological, biochemical and molecular markers, has been reported by many researchers worldwide (Patel and Galakiya, 2014; Roomi *et al.,* 2014; Peng *et al.,* 2016; Dhwani *et al.,* 2017; Yusuf *et al.,* 2017). However, molecular marker-based genetic diversity study of *A. hypogaea* accessions is still lacking in Ethiopia. ISSR marker is more reproducible and cost effective for researchers in developing countries like Ethiopia. The technique does not need any prior information about DNA sequence. ISSR-PCR has been reported as a rapid, reproducible, and cheap fingerprinting technique based on the variation found in the regions between microsatellites (Zhang *et al.,* 2006; Golkar *et al.,* 2011). The ISSR techniques have been used in Ethiopia to detect genetic diversity and population structure of Oromo Potato (Ijara, 2015), Tef, Coffee, Lentils, Rice and Sesame. The objective of this study is therefore to analyze the genetic diversity of *A. hypogaea* accessions grown in different regions of Ethiopia using ISSR markers in an effort to provide some information for future research to improve and conserve this important crop.

1.2 Statement of the Problem

Ethiopia is home to many crop cultivars and landraces. These varieties were developed through selection based on agronomic traits. This resulted in a wide spectrum of varieties that are highly valued both in domestic and foreign market (Gezahagn, 2013).

Morphological diversity is evaluated with reference to yield and quality, using traditional field plot techniques. However, these techniques are tedious and time consuming. Furthermore, the morphological characters may be unstable and influenced by environmental conditions. Therefore, cultivars do not assure the accurate genetic identity and extent of distribution (Patel and Galakiya, 2014).

Phenotypic/morphological characterizations of genotypes of *A. hypogaea* were made (Yusuf *et al.,* 2017), but the molecular genetic diversity has not been studied. Moreover, morphological variability is often restricted; characters may not be obvious to study its genetic diversity since morphological characters may be affected by environment. Nowadays, a variety of different genetic markers have been proposed to assess genetic variability as a complementary strategy to more traditional approaches in genetic resources management. The use of molecular marker will be helpful for the collection of advanced and novel genotypes. Genomic research can provide new tools and resources to revolutionize crop genetic improvement and production. It also provides accurate knowledge at molecular level which is not possible with phenotypic markers (Johan *et al.*, 2011). However, genomic research in *A. hypogaea* is far behind in our country due to the shortage of essential infrastructure, tools and resources. For this reason, landraces remain the major source of planting materials currently used by farmers.

Molecular characterization using PCR-based ISSR markers provides a suitable method, which can be used for varietal identification in *A. hypogaea* supplies and to differentiate between the various grades of *A. hypogaea*. This research generated basic information that could be useful for *A. hypogaea* breeding programs. Better understanding on the diversity of this crop is important for improvement and exploitation of *A. hypogaea* at a national level. This study will help to narrow and fill the wide research gap currently observed between molecular genetic studies and improvement programs of *A. hypogaea* in Ethiopia.

1.3 Objectives

1.3.1 General Objective

- The main objective of this study is to determine the genetic diversity of selected *A. hypogaea* accessions from Ethiopia using ISSR marker.

1.3.2 Specific Objectives

- To evaluate the potential informativeness of ISSR markers for identifying *A. hypogaea* accessions.
- To determine the level and pattern of genetic diversity and degree of polymorphism among selected *A. hypogaea* accessions.
- To identify accessions with higher diversity for genetic improvement and conservation of *A. hypogaea*.

2. LITERATURE REVIEW

2.1 Groundnut (*Arachis hypogaea* L.) Crop Origin and Distribution

Cultivated *A. hypogaea* is an ancient crop of the New World, which originated in South America (southern Bolivia/north west Argentina region) where it was cultivated as early as 1000 B.C. Dissemination of the crop to Africa, Asia, Europe and the Pacific Islands occurred presumably in the sixteenth and seventeenth centuries with the discovery voyages of the Spanish, Portuguese, British and Dutch (Krapovickas, 1969, 1973; Gregory, 1980). Seven primary centers of diversity have been described for *A. hypogaea*: (1) Guaraní region (Paraguay Paraná river basins and southwestern Brazil) for var. *fastigiata* and var. *vulgaris*; (2) Goiás and Minas Gerais region of Brazil (Jocantis-São Francisco river basin) also for var. *fastigiata* and var. *vulgaris*; (Susana, 2003) (3) Rondonia and northwestern Matto Grosso region of Brazil (head waters of the Amazon River) for var. *hypogaea*; (4) Bolivian region (eastern slopes of the Andes) for var. *hypogaea*; (5) Peruvian region (upper Amazon and west coast) for vars. *hirsuta, fastigiata* and *peruviana*; (6) northeastern Brazil for var. *fastigiata*; and (7) Ecuadorian region for var. *aequatoriana*. Africa has been described as a secondary center of diversity for cultivated groundnut (Krapovickas and Gregory, 1994).

Natural hybridization among types introduced to Africa from Brazil followed by selection is thought to be responsible for the variation in the African collection (Susana, 2003). *A. hypogaea* spread was; through Portuguese in the late 15th century, carried two-seeded varieties from the east coast of South America (Brazil) to Africa, to the Malabar Coast of southeastern India and possibly to the Far East. In the early 16th century, Spaniards took three-seeded Peruvian runner types (including var. *hirsuta*) to Indonesia, China up to Madagascar from the west coast of South America via the Western Pacific (Krapovickas and Gregory, 1994). It was later in the 1700s that the 'Spanish' types were taken to Europe where they were grown for oil and human consumption (Stalker, 1997). By the nineteenth century, *A. hypogaea* became an important food crop in West Africa, southeast and south Asia, and USA. More than 100 countries now grow *A. hypogaea* (Singh and Nigam, 2016).

2.2 Taxonomy and Botanical Description

Arachis hypogaea is a member of the family *Leguminoseae*, subfamily *Fabaceae*, tribe *Aeschynomeneae*, subtribe *Stylosanthenae*, genus *Arachis* and species *hypogaea* (Isleib *et al.*, 1994). *A. hypogaea* is an allotetraploid with genome AABB**,** 2n = 4x = 40 (Krapovickas and Gregory, 1994). Based on differences in branching pattern and in the presence of reproductive nodes on the main stem, *A. hypogaea* is subdivided into two subspecies: subsp. *hypogaea* and subsp. *fastigiata*. Subspecies *hypogaea* has alternate branching pattern, no reproductive nodes on the main stem, spreading or erect growth habit, a longer maturation period and fresh-seed dormancy (Susana, 2003). This subspecies is subdivided into botanical varieties *hypogaea* (Virginia and Runner) and *hirsute* (Peruvian humpback or Chinese dragon type). Subspecies *fastigiata* has a sequential branching pattern, reproductive nodes on the main stem; erect growth habit, earlier maturity and little or no seed dormancy (Krapovickas and Gregory, 1994). The two botanical varieties within subspecies *fastigiata* are var. *fastigiata* (Valencia type) and var. *vulgaris* (Spanish type). Krapovickas and Gregory (1994) later revised the classification of cultivated *A. hypogaea* to include the two botanical varieties, var. *peruviana* (Valencia type) and *aequatoriana* (Zaruma type), which are classified with vars. *fastigiata* and *vulgaris* within subspecies *fastigiata* (Susana, 2003).

The plant is an annual herbaceous plant, with an undetermined mode of growth and a number of varieties belonging to either of the subspecies *A. hypogaea* ssp. *hypogaea* or *A. hypogaea* ssp. *fastigiata* (Stalker, 1997). The species and varieties are classified according to the location of the flowers on the plant, patterns of reproductive nodes on the branches, number of trichomes, as well as pod morphology (Krapovickas and Gregory, 1994). Cultivated *A. hypogaea* is generally self-pollinating although little out-crossing does occur with the assistance of bees, which pollinate the flowers. The geographic habit of *A. hypogaea* is a unique characteristic and could be responsible for dispersal and thus population structure. Much of the dispersal is by water and therefore the species distribution matches to a great extent the flow of major rivers (Holbrook and Stalker, 2003). For this reason, discoverers observed that most ancient species were found in higher elevations, their immediate descendants occupied the next lower eroded surfaces, while the distantly evolved species occupied still lower and more recently eroded surfaces (Singh *et al.*, 2004).

A. hypogaea seed consists of two cotyledons, a stem axis and leaf primordial, hypocotyls and primary root. Seed germination is of the epigeal mode and the cotyledons tend to change color to green after emergence. The primary root system is a tap root system and numerous lateral roots are visible on the third day after germination. *A. hypogaea* roots do not have the normal root hair, but tuffs of hair can be seen on the lateral root (Figure 1). The former is only restricted to a root zone of 35 cm below the soil surface. Although *A. hypogaea* has a symbiotic relationship with bradyrhizobium, root hairs are not the primary invasive sites in contrast to most other legumes (Stalker, 1997). Stems are initially solid, but as the plant grows they tend to become somewhat hollow. The main stem develops from a terminal bud of the epicotyl and two cotyledonary laterals grow on opposite sides near the soil level. The main stem can be uptight or prostrate and ranges from 12 to 65 cm in length (Holbrook and Stalker, 2003). Lateral branches can be prostrate and run along the ground or be upright. Leaflets on the main stem differ in shape and size from those on lateral branches. Branching patterns of reproductive to vegetative nodes on the cotyledonary laterals is one of the primary traits dividing the subsp. *hypogaea* (alternating pairs of vegetative: reproductive nodes) and subsp. *fastigiata* (sequential patterns of reproductive nodes). However, intermediate types are commonly observed (Stalker, 1997).

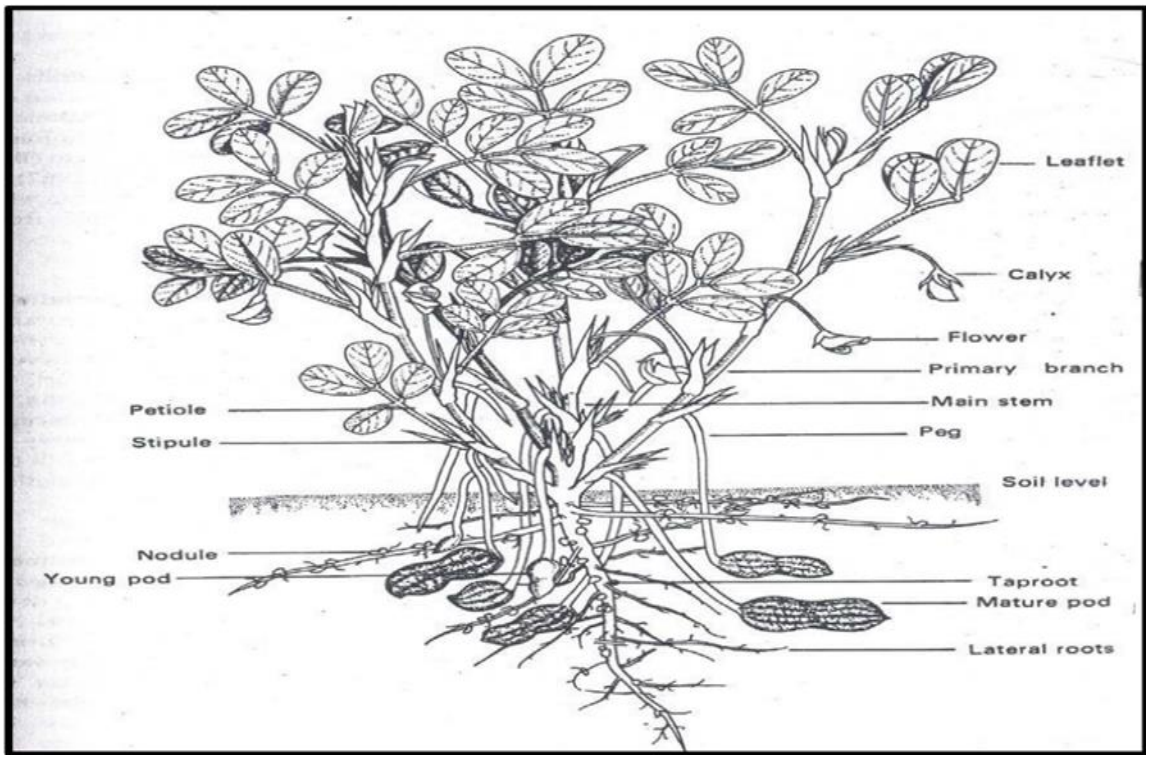


Figure 1. A stylized *A. hypogaea* plant (Nigam, 2015).

2.3 Importance of *Arachis hypogaea* L.

A. hypogaea and its products have been a component of the world's diet for years. The geographical location, cultivar type and cultivation conditions influence the nutritional profile of the nuts. *A. hypogaea* is the 13th most important food crop and 4th most important oilseed crop of the world. The seeds contain oil and protein (Ingale and Shrivastava, 2011). *A. hypogaea* contains 18% carbohydrates with a starch content of 0.5 – 5% and sucrose content of 4 – 7%. The oil content of *A. hypogaea* is 36 - 54%, of which about 76 – 80% is unsaturated fatty acids. Oleic acid makes up 40 – 45% and linoleic acid makes up 30 – 35% of the composition of unsaturated fatty acid (Woodroof, 1983b). *A. hypogaea* is an excellent source of mono and poly unsaturated fatty acids, with levels exceeding that of soybean (Nwokolo, 1996). Though the oils have a high caloric value, they have been shown to have links to improved cardiovascular health. In addition to mono and poly unsaturated fatty acids, *A. hypogaea* are a rich source of magnesium, fiber, folate, vitamin E, copper and arginine, all of which have cardiovascular disease risk reducing properties (Mattes, 2003). It is also reported, the vitamin profile of *A. hypogaea* to include riboflavin, thiamin (which is destroyed to a great degree by roasting and blanching**)**, nicotinic acid and Vitamin E, with appreciable amounts of B complex vitamins and Vitamin K, but practically no Vitamins A, C or D (Woodroof, 1983b) .

The uses of *A. hypogaea* are diverse; all parts of the plant can be used. Groundnut kernels contain 42 - 50 % oil, 26% protein, 18% carbohydrates, and are also rich source of riboflavin, thiamine, nicotinic acid and vitamin E (Kathirvelan and Kalaiselvan, 2007). The *A. hypogaea* kernels are consumed directly as raw, roasted or boiled kernels or oil extracted from the kernel is used as culinary oil. It is also used as animal feed (oil pressings, seeds, green material and straw) and industrial raw material (oil cakes and fertilizer). The crop plays an important role in the dietary requirements of resource poor women and children and haulms are used as livestock feed (Ingale and Shrivastava, 2011). About two thirds of world production is crushed for oil, which makes it an important oilseed crop. It is also used in soap making, medicine, pharmaceuticals, emulsion for insect control, manufacturing cosmetics and lubricants, fuel for diesel engines, oleic steering and their salts can be made from *A. hypogaea*. The residual oilcake contains 7 to 8% nitrogen, 1.5% P_2O_5 and 1.2% K_2O and is used as a fertilizer**.** It is an important protein supplement in cattle and poultry rations. It is also consumed as confectionary (sweet) product. The cake can be used for manufacturing artificial fiber. The haulms (plant stalks)

are fed (green, dried or silage) to livestock. *A. hypogaea* shell is used as fuel for manufacturing of coarse boards and cork substitutes (Jasani, 2009).

In folk medicine, *A. hypogaea* is used for aphrodisiac purposes, inflammation, cholecystosis, nephritis and decoagulant. These multiple uses of *A. hypogaea* plant make it an excellent cash crop for domestic markets as well as for foreign trade in several developing and developed countries (Mattes, 2003). *A. hypogaea* is a legume crop; therefore it is good in nitrogen fixation. *A. hypogaea* should be grown in rotation with cereals (e.g., maize and sorghum), which have been well fertilized, because *A. hypogaea* respond well to fertilizer applied to the previous crop rather than to the *A. hypogaea* themselves (Jasani, 2009). It also further reduces the mono-culture of maize and other crop production in different regions (Ingale and Shrivastava, 2011).

2.4 Major Constraints in *Arachis hypogaea* L. Production

A. hypogaea suffers from several biotic and abiotic production constraints. Some of them are global in nature; and others are either regional or local (Singh and Nigam, 2016). *A. hypogaea* breeders and physiologists have been working across the world to improve the yield of the crop under various biotic and abiotic stresses. Biotic stresses include diseases such as rust (*Puccinia arachidis*), early leaf spot (*Cercospora arachidicola*), late leaf spot (*Phaseoisariopsis personata*), crown rot (*Aspergillus niger*), stem and pod rot, stem necrosis, rosette disease and pests like termites (*Odontotermes* sp.), white grubs (*Lachnosrerna consanguiea*), jassids (*Empoasca kerri*), aphids (*Aphis craccivora*), leaf miners (*Aproaerema modicella*), red hairy caterpillars (*Amsacta albistriga*), tobacco caterpillars and thrips etc, whereas among abiotic stresses, drought is predominant (Prasad *et al.,* 2010). Drought can occur at any stage during early-season, mid-season, end-of-season and intermittent. Drought also predisposes *A. hypogaea* pods to aflatoxin contamination by *A. flavus*. Other abiotic constraints include low soil fertility, salinity, iron chlorosis, aluminum toxicity, cold temperature at germination, and high temperature at podding and harvest (Singh *et al.,* 2004).

2.5 *Arachis hypogaea* L. Distribution and Production Areas in Ethiopia

After its first introduction to Eritrea in the 1920 and then to Harer, *A. hypogaea* is grown in many lowland areas of Ethiopia. *A. hypogaea* in Ethiopia is produced mainly by small holder farmers in the lowlands of the country (Fredu *et al.,* 2015). In Ethiopia *A. hypogaea* is grown and covered nearly 80,000 hectares (Fredu *et al.,* 2015) of arable land per annum.

In terms of area distribution and production in Ethiopia, *A. hypogaea* is produced in Oromia around (52, 921.26 ha) (East and West Welega, Illubabor, East and West Hararghe), Benishangul-Gumuz (Metekel, Assosa, Kemashi, Pawe, Mao Komo) (18,592.72 ha), Harari (2874.09 ha), Amhara (Awi, Oromia special zone) (2,380.15 ha), and SNNP (South Omo, Gamo Gofa) (376.66 ha). However, Oromia and Benishangul-Gumuz regions are the major producing regions which account for most of *A. hypogaea* production in Ethiopia (Fredu *et al.*, 2015; Addisu and Ermias, 2017). Oromia region is the leading region in both area cultivation and production in Ethiopia. It accounts for more than 60 percent of area cultivation and production. Next to Oromia, *A. hypogaea* is widely cultivated in Benishangul Gumuz (CSA, 2015; Fredu *et al.*, 2015). Though the crop is grown different regions in Ethiopia, Oromia and Benishangul-Gumuz regions account for nearly 90% of the production in the country (Figure 2). Within these regions, Eastern Hararghe zone in Oromia region and Metekel zone in Benishangul-Gumuz region are the main centers for *A. hypogaea* production (Fredu *et al.*, 2015).

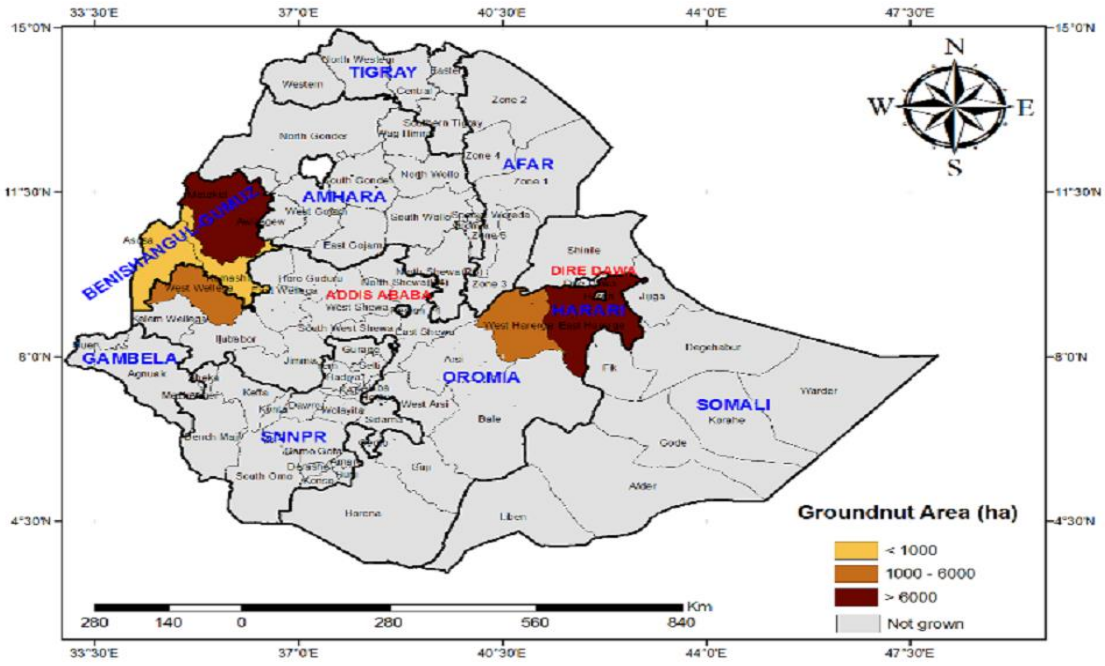


Figure 2. *A. hypogaea* producing areas in Ethiopia based on the data from CSA agricultural sample survey (CSA, 2015).

2.6 Genetic Resources of *Arachis hypogaea* L. Gene Pool.

After its introduction to different part of the world, the cultivated *A. hypogaea* evolved considerable genetic diversity. While the wild *Arachis* species constitute genetic reservoir of useful characteristics, particularly resistance to biotic and abiotic stresses. *A. hypogaea*

genetic resources have been classified into different gene pools (Radhamani and Singh, 2008). The gene pool concept was proposed in order to provide a genetic perspective and relationship of cultivated plant species to other components of genetic diversity, to the wild relatives, based on cross-compatibility into (1) primary gene pool (GP-1), (2) secondary gene pool (GP-2) and (3) tertiary gene pool (GP-3) (Singh and Nigam, 2016). Application of this principle facilitates clearer understanding of phylogenetic relationships between the wild and cultivated species and helps to identify appropriate breeding strategies for incorporating desired genes into conventionally usable form of cultivated species for designing new cultivars. This helps to facilitate conventional cytogenetic manipulations to establish fertile hybrids, improve genetic recombination for incorporation of desirable genes into a usable form and hybridization using pre- or post-fertilization manipulations to establish hybrids (Nigam, 2015).

Alternatively, biotechnological approaches may be applied to access genes through sexual or parasexual means of genetic transformation. This approach has been used in *A. hypogaea* classifying the genetic diversity into four gene pools: (1) The primary gene pool consists of landraces and traditional cultivars of *A. hypogaea* from primary and secondary centers of genetic diversity in South America and other groundnut growing countries and wild *A. monticola* found in northwest Argentina having free crossability with *A. hypogaea* producing normal segregants, (2) The secondary gene pool consists of diploid species from section *Arachis*, cross-compatible with *A. hypogaea*, despite ploidy differences, producing sterile to partially fertile hybrids, (3) The tertiary gene pool includes species of section *Procumbentes*, which have crossed with diploid species of section *Arachis* and probably coevolved with series *perennes* of section *Arachis*; *Erectoides*, whose species are weakly cross-compatible with diploid species of section *Arachis* and *A. hypogaea*; and *Rhizomatosae*, whose tetraploid species can be crossed both with diploid species of section *Arachis* and *A. hypogaea*, (4) The quaternary gene pool of the remaining *Arachis* species that are cross-incompatible or very weakly cross-compatible to species of section *Arachis* and are classified into five other sections (Radhamani and Singh, 2008).

2.7 Germplasm Collections of *Arachis hypogaea* L.

After the establishment of International Crops Research Institute for Semi-Arid Tropics (ICRISAT); in the last few decades, a significant progress has been made in the collection of *A. hypogaea* germplasm from various centers of genetic diversity (Radhamani and Singh, 2008). Cultivated *A. hypogaea* accessions were collected from *A. hypogaea*

growing areas of various countries, included landraces, material developed by the breeders and/or released varieties, and the genetic stocks identified with special features or sources of resistance to biotic and abiotic stresses, representing different botanical varieties and cultivar groups. *A. hypogaea* germplasm is conserved as pods or seeds, except for wild *Arachis* species belonging to section *Rhizomatoseae* (Singh *et al.,* 2004), which rarely produce seed and if produce, progenies are highly heterogeneous and therefore are conserved as live plants under controlled conditions providing an environment close to their habitat (Singh and Nigam, 2016).

Globally, centers maintaining major collection of *A. hypogaea* genetic resources includes: Argentina – IBONE/INTA (3534 accessions); Brazil - CENARGEN/EMBRAPA, Brasilia (3340 accessions); China - Oil Crops Research Institute, Wuhan / National Gene Bank, Chinese Academy of Agricultural Sciences, Beijing (8349 accessions); ICRISAT - RS Paroda Gene Bank, ICRISAT, Patancheru, India (15,418 representing 95 countries); India - ICAR - Directorate of Groundnut Research, ICAR, Junagadh (9024 accessions); National Bureau of Plant Genetic Resources, ICAR, New Delhi (14,585 accessions); USA - Southern Regional Plant Introduction Station, Griffin, GA (USDA collection - 9917 accessions); National Seed Storage Laboratory, Ft. Collins, Co (duplicate collection under long - term storage); NC State University, Raleigh, NC (740 accessions). Other centers are: Senegal - Senegalese Institute of Agricultural Research (ISRA), Bambey and USA - Texas A and M, College Station, TX (Nigam, 2015). One of the most significant outcomes of this broad collection effort was the publication of a new monograph of the genus *Arachis*. The monograph represents a major milestone for the study and conservation of *A. hypogaea* genetic resources, and provides a sound scientific foundation upon which future work can be based (Singh and Nigam, 2016).

2.8 Types of Markers and Their Application in Genetic Diversity Studies

2.8.1. Morphological Markers

The morphological method is the oldest and considered the first step in description and classification of plants (Molosiwa *et al.,* 2015). Morphological markers are the earliest classical methods of genetic markers for assessment of variation based on highly heritable phenotypic traits such as flower color, seed shape, growth habits and pigmentation (Vithanage *et al.,* 1995). These measures have the advantage of being readily available, do not require sophisticated equipment and are the most direct measure of phenotype, thus

they are available for immediate use, an important attribute (Praneet and Pankaj, 2015). Phenotypic traits have the advantage that they may be directly related to the fitness of the populations and usefulness for plant breeding (Upadhaya *et al.*, 2008). The use of morphological and agronomic traits is a standard way of assessing genetic variation for many species, especially under-researched crops such as groundnut (Molosiwa *et al.*, 2015). These analyses are carried out by raising germplasm lines, pure lines, improved varieties etc. in a particular experimental design. This involves morphological characterization of different entries grown in the field as the morphological characteristics are the strongest determinants of the agronomic value and taxonomic classification of plants (Bhandari *et al.*, 2017). If phenotypic observations are based on adequately large sample sizes and the physical traits measured show significant differences among populations, they can provide a reasonable representation of overall genetic performance (Upadhaya *et al.*, 2008).

However, morphological determinations need to be taken by an expert in the species, they are subject to changes due to environmental (show continuous variation) factors and may vary at different developmental stages and their number is limited (Praneet and Pankaj, 2015). They are mostly dominant/recessive, have some biological effect and some morphological variants cannot survive. Different sets of morphological traits are taken into consideration for different group of crop plants (Bhandari *et al.*, 2017). In addition to these shortcomings they show lower polymorphism, complex inheritance, epistatic interaction, pleotrophic effect, dominant intra-allelic interactions and need extra time as well as resources for evaluation in field and greenhouse. This limits their application in taxonomy and breeding, can be overcome by the application of biochemical and molecular markers. But it does not mean that any of the biochemical or molecular or both replace morphological markers, rather they are used in combination (Robertson *et al.*, 1997).

2.8.2 Protein (biochemical) Markers

To overcome the limitations of morphological traits, other markers have been developed at both the protein level (phenotype) and the DNA level (genotype). Protein markers are usually named biochemical markers but, more and more; they are mistakenly considered as a common class under the so-called ‘molecular markers (Praneet and Pankaj, 2015). Biochemical markers (seed storage proteins and isozymes) are markers based on protein polymorphisms through electrophoretic separation of protein molecules. The assays require the extraction of proteins from plant tissues, followed by their separation using gel

electrophoresis based on their net charge and size (Schulman *et al.,* 2004) taking advantage of the migrational properties of proteins and enzymes, and revealed by histo-chemical stains specific to the enzymes being assayed (Praneet and Pankaj, 2015). Detecting polymorphisms i.e. detectable differences at a given marker occurring among individuals in protein markers is a technique that shares some of the advantages of using morphological ones. However, protein markers are also limited by being influenced by the environment and changes in different developmental stages. Even so, isozymes are a robust complement to the simple morphometric analysis of variation (Praneet and Pankaj, 2015).

Therefore, depending up on number of loci, state of homozygosity or heterozygosity, and enzyme configuration, from one to several bands are visualized. The positions of these bands can be polymorphic and considered as informative loci. Isozymes have provided a valuable tool for the study of genetic variation in crop populations and to investigate species relationships (Johan *et al.,* 2011). Seed protein and isozyme profiles as revealed by polyacrylamide gel electrophoresis are successfully used to resolve taxonomic problems and describing the genetic diversity within many crops. Seed proteins are considered as practical and reliable methods because seed storage proteins and nucleotide sequences are largely independent of environmental fluctuations. The main advantages of protein markers are their co-dominant inheritance, very little environmental influence and low cost of assay. While disadvantages include restricted number of suitable allozyme (isozyme) loci in the genome, highly biased genomic sampling, requirement of fresh tissue, and sometimes limited variation (Weising *et al.,* 2005). Therefore, their major shortcomings are lower polymorphism, lower genome coverage, technical difficulties and the need for specific developmental stage for certain enzymes (Schulman *et al.,* 2004).

2.8.3 Molecular Markers

Diversity based on phenotypic and morphological characters, usually varies with environments and evaluation of traits requires growing the plants to full maturity prior to identification, but now the rapid development of biotechnology allows easy analysis of large number of loci distributed throughout the genome of the plants. Molecular makers have proven to be powerful tools in the assessment of genetic variation and in elucidation of genetic relationships within and among species (Chakravarthi and Naravaneni 2006). Understanding the molecular basis of the essential biological phenomena in plants is crucial for the effective conservation, management, and efficient utilization of plant

genetic resources (PGR) (Linda *et al.,* 2009). DNA-based molecular markers have acted as versatile tools and have found their own position in various fields like taxonomy, plant breeding, and genetic engineering etc. Collecting DNA marker data to determine whether phenotypically similar cultivars are genetically similar would therefore be of great interest in crop breeding programs (Duzyaman, 2005).

Molecular markers are fixed marks in the genome found at specific locations of the genome, there are used to identify specific genetic differences. In order to precisely identify traits of interest, the marker must be close to the gene of interest so that the allele of both the marker and the gene could be inherited together (Semagn *et al.,* 2006). DNA markers are passed on from one generation to another through the laws of inheritance. Several markers are available to choose for genetic diversity studies. The selection criteria could be based on cost, technical labour, level of polymorphism, reproducibility, locus specificity and genomic abundance (Molosiwa, 2012). DNA polymorphisms can be detected in nuclear and organelle DNA, which is found in mitochondria and chloroplasts. Molecular markers concern the DNA molecule itself and, as such, are considered to be objective measures of variation (Praneet and Pankaj, 2015). Molecular markers may or may not correlate with phenotypic expression of a genomic trait (Linda *et al.,* 2009).

Molecular markers are the method of choice for genetic diversity assessment and offer numerous advantages over conventional, phenotype-based alternatives on account of their hyper variability, high reproducibility, amenability to automation, neutral, stable, (Bhandari *et al.,* 2017), not subject to environmental pleiotropic and epistatic effects, tests can be carried out at any time during plant development regardless of growth, differentiation, development, or defense status of the cell and best of all, they have the potential of existing in unlimited numbers, covering the entire genome (Praneet and Pankaj, 2015). Molecular markers can be applied in the identification of cultivars and clones, genetic mapping, marker assisted selection (MAS), population genetics, molecular systematics, etc. (Ijara, 2015). Molecular markers can be used for dissecting polygenic traits into their Mendelian components or Quantitative Trait Loci (QTL) and this increasing understanding of the inheritance and gene action for such traits allows the use of markers - selection procedures (Anderson *et al.,* 1993).

Molecular markers are also useful in the development of genetic and physical maps, and have increased the efficiency of indirect selection of marker linked traits (Molosiwa,

2012). These techniques provide opportunities to obtain high amplification of genetic traits for the development of genetic maps, variety identification and for the analysis of important morphological and agronomic traits. In addition, these markers reveal a high level of polymorphism on plant materials (Bhandari *et al.,* 2017). 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 (Linda *et al.,* 2009). Different marker systems such as Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNAs (RAPDs), Sequence Tagged Sites (STS), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) or microsatellites, inter simple sequence repeat (ISSR), Single Nucleotide Polymorphisms (SNPs) and others have been developed and applied to a range of crops. The DNA based marker systems are generally classified as hybridization-based (non-PCR) markers and (PCR)-based markers (Ijara, 2015).

2. 8.3.1 Hybridization (Sequence dependent) Non-Polymerase Chain Reaction (PCR) method

Molecular markers based on restriction- hybridization techniques were employed relatively early in the field of plant studies and combined the use of restriction endonucleases and the hybridization method (Ijara, 2015).

2.8.3.1.1 Restriction Fragment Length Polymorphism (RFLP)

A Restriction Fragment Length Polymorphism, or RFLP, (commonly pronounced "rif lip"), is a technique that exploits variations in homologous DNA sequences. It refers to difference between samples of homologous DNA molecules that come from differing locations of restriction enzyme sites (Tharachand *et al.,* 2012). This was the first molecular marker technique developed and used in MAS for plant breeding. Saturated molecular genetic maps based on RFLP markers have been developed for several crops. The technique centers around the digestion of genomic DNA digested with restriction enzymes (Mishra *et al.,* 2014). The possibility of comparing band profiles generated after restriction enzyme digestion in DNA molecules of different individuals (Praneet and Pankaj, 2015). Restriction endonucleases are bacterial enzymes able to cut DNA, identifying specific

palindrome sequences and producing polynucleotidic fragments with variable dimensions. Any changes within sequences (i.e., point mutations), mutations between two sites (i.e., deletions and translocations), or mutations within the enzyme site, can generate variations in the length of restriction fragment obtained after enzymatic digestion (Linda *et al.,* 2009). These differences in fragment lengths resolved by gel electrophoresis and western blotting, Specific banding patterns are then visualized by hybridization with labeled probe (Linda *et al.,* 2009).

The main advantages of RFLP markers are co-dominance, high reproducibility, no need of prior sequence information, and high locus-specificity (Lateef, 2015). RFLP analysis is a well-accepted method in plant breeding and is used for many different purposes: e.g. the selection of traits of agronomic importance linked to RFLP markers, quality testing of seeds segregation analysis of progenies, evaluation of diversity in a germplasm collection (Jaroslava *et al.,* 2002), also for the construction of linkage maps and gene tagging in many crop species. It has been successfully applied for genetic diversity assessments, particularly in cultivated plants. In self-pollinating legumes such as chickpea, lentil, peanut and soybean, a very low level of polymorphism has been reported (Tadele, 2014). However, RFLP technique has a problem of detecting low polymorphism and few loci per assay; it is also not amenable to automation (Molosiwa, 2012), requires relatively large amounts of purified DNA, time consuming, laborious and uses probe that is difficult to handle (Ijara, 2015). The limitations in terms of routine use of RFLP lead to the development of other markers such as RAPDs (Molosiwa, 2012).

2.8.3.2 Polymerase Chain Reaction (PCR)-based molecular markers

The Polymerase chain reaction (PCR) is a molecular biology technique for enzymatically replicating (amplifying) small quantities of DNA and useful in studying DNA sequence variation without using a living organism. It is used to amplify a short (usually up to 10 kb), well-defined part of a DNA strand (between two specific priming sites in the genome) from a single gene or just a part of a gene (Tharachand *et al.,* 2012). Polymerase chain reaction based markers require less DNA per assay than RFLP and are higher throughput (Molosiwa, 2012). The process involves two oligonucleotide primers that flank the DNA fragment of interest and amplification is achieved by a series of repeated cycles of heat denaturation of the DNA, annealing of the primer to their complementary sequences, and extension of the annealed primers with a thermophilic DNA polymerase. Since the extension products themselves are also complementary to primers, successive cycles of

amplification essentially double the amount of the target DNA synthesized in the previous cycle and the result is an exponential accumulation of the specific target fragment (Johan *et al.*, 2011).

After the invention of PCR technology, a large number of approaches for generation of molecular markers based on PCR were detailed, primarily due to its apparent simplicity and high probability of success. Usage of random primers overcame the limitation of prior sequence knowledge for PCR analysis and facilitated the development of genetic markers for a variety of purposes mainly in genetic diversity studies (Milee *et al.*, 2008).

2.8.3.2.1 Random Amplified Polymorphic DNA (RAPD)

In the last decade, the RAPD technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers (Firas and Abdulkareem, 2015). RAPDs were the first PCR-based molecular markers to be employed in genetic variation analyses (Linda *et al.*, 2009). The principle of RAPD is that, a single, short oligonucleotide primer (mostly ten bases long), which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that the amplified fragment generated by PCR depends on the length and size of both the primer and the target genome (Firas and Abdulkareem, 2015). The use of short primers is necessary to increase the probability that, although the sequences are random, they are able to find homologous sequences suitable for annealing. DNA polymorphisms are then produced by rearrangements or deletions at or between oligonucleotide primer binding sites in the genome. As this approach requires no prior knowledge of the genome analyzed, it can be employed across species using universal primers (Linda *et al.*, 2009).

In this reaction, a single species of primer anneals to the genomic DNA at two different sites on complementary strands of DNA template. If these priming sites are within an amplifiable range of each other, a discrete DNA product is formed through thermo cyclic amplification. On an average, each primer directs amplification of several discrete loci in the genome (relatively small number of primers can be used to generate a very large number of fragments) (Jaroslava *et al.*, 2012), making the assay useful for efficient screening of nucleotide sequence polymorphism between individuals (Ijara, 2015). With this technique, there is no specific target DNA, so each particular primer will adhere to the template DNA randomly. As a result, the nature of the obtained products will be unknown. The DNA fragments generated (usually within the 0.5–5 kb size range) are then separated

and detected by gel electrophoresis. RAPDs can be detected by running PCR products through electrophoresis on an agarose or acrylamide gel (Praneet and Pankaj, 2015) and view under ultraviolet light and presence and absence of band was observed (Ijara, 2015).

The power of RAPD is that it is a fast technique, easy to perform and comparatively cheap. It is immediately applicable to the analysis of most organisms because universal sets of primers are used without any need for prior sequence information (Jaroslava *et al.*, 2012). It is viewed as having several advantages compared to RFLP and fingerprint (Firas and Abdulkareem, 2015). RAPD markers offers many advantages such as higher frequency of polymorphism, use of fluorescence, requirement of only a few nanogram of DNA, no requirement of prior information of the DNA sequence and feasibility of automation. The use of such techniques for germplasm characterization facilitates the conservation and utilization of plant genetic resources, permitting the identification of unique accessions or sources of genetically diverse germplasm. The technique is widely used to analyze the genetic relatedness in several crop species including groundnut. It is also used in the identification and classification of accessions, identification of breeds and genetic diversity analysis (Mishra *et al.*, 2014). Nevertheless, disadvantages of RAPD markers are the fact that it predominantly provides dominant markers, and incapability to detect allelic differences in heterozygotes. Polymorphisms are detected only as the presence or absence of a band of a certain molecular weight, with no information on heterozygosity. Additionally, because of their random nature of amplification and short primer length, they are not ideal for genome mapping. Moreover, these markers do not exhibit dependable amplification patterns and differ with the experimental conditions (Lateef, 2015).

2.8.3.2.2 Amplified Fragment Length Polymorphism (AFLP)

AFLP was developed to overcome some of the shortcomings of reproducibility of RAPDs as the technique combines the digestion of DNA with some specific restriction endonucleases with a PCR-based technique (Molosiwa, 2012). It is a combination of RFLP and RADP methods (Ijara, 2015). In this approach the sample DNA is enzymatically cut up into small fragments (as with RFLP analysis) (Johan *et al.*, 2011), but Only DNA fragments with nucleotides that flank the restriction sites that match the selective nucleotides of the primer are amplified during PCR . The technique is amenable to high-throughput analysis which is an added advantage. It is also more efficient and reproducible as compared to the RAPD. AFLP analysis first uses the restriction digestion of genomic DNA with a combination of rare cutting (EcoRI or PstI) and frequent cutting (MseI or

TaqI) restriction enzymes digest genomic DNA (Molosiwa, 2012), followed by ligation of double-stranded adaptors to the sticky ends of the restriction fragment to generate template DNA. The nucleotide sequence of the adapters and the adjacent restriction sites serve as primer binding sites during selective PCR amplification of some of these fragments. The amplified fragments are separated by electrophoresis on polyacrylamide gels and visualized either through autoradiography or fluorescence methods (Tadele, 2014**).** Scoring AFLP data is easy since polymorphisms are recognized in the form of presence or absence of data rather than determination of sizes at various loci.

The primer pairs used for AFLP usually produce 50-100 bands per assay. The number of amplicons per AFLP assay is a function of the number of selective nucleotides in the AFLP primer combination, the selective nucleotide motif, GC content and physical genome size and complexity (Ahmed, 2012). The origins of AFLP polymorphisms are multiple and can be due to: (i) mutations of the restriction site which create or delete a restriction site; (ii) mutations of sequences flanking the restriction site, and complementary to the extension of the selective primers, enabling possible primer annealing; (iii) insertions, duplications or deletions inside amplification fragments. These mutations can cause the appearance /disappearance of a fragment or the modification (increase or decrease) of an amplified- restricted fragment (Linda *et al.,* 2009).

AFLP represents dominant marker system among the different marker systems available at present and also provides multi-locus and genome wide marker profiles. These features make the AFLP technology more suitable for molecular characterization and DNA fingerprinting of any germplasm collection. In addition, no prior sequence information is needed for amplification. As a result, AFLP has become extremely beneficial in the study of taxa including bacteria, fungi and plants, where much is still unknown about the genomic makeup of various organisms (Tharachand *et al.,* 2012). AFLPs can be applied in studies involving genetic identity, phenotyping, population genetics, quantitative trait loci (QTL) mapping, parentage and identification of clones and cultivars, and phylogenetic studies of closely related species because of the highly informative fingerprinting profiles generally obtained. Their high genomic abundance and generally random distribution throughout the genome make AFLPs a widely valued technology for gene mapping studies (Ijara, 2015), these include establishing linkage groups in crosses, saturating regions with markers for gene landing efforts and assessing the degree of relatedness or variability among cultivars (Milee *et al.,* 2008). However, there are some limitations of AFLP

includeing: (1) It requires more number of steps to produce the result, (2) It requires template DNA free of inhibitor compounds that interferes with the restriction enzyme, (3) The technique requires the use of polyacrylamide gel in combination with AgNO3 staining, radioactivity, or fluorescent methods of detection, which will be more expensive and laborious than agarose gels, (4) It involves additional cost to purchase both restriction and ligation enzymes as well as adapters, and (5) Most AFLP loci are dominant, which does not differentiate dominant homozygotes from heterozygotes. This reduces the accuracy of AFLP markers in population genetic analysis, genetic mapping, and marker assisted selection (MAS) (Mishra *et al.,* 2014).

2.8.3.2.3 Simple Sequence Repeat (SSR)

Microsatellites, also known as simple sequence repeats (SSR), variable number tandem repeats (VNTR) and short tandem repeats (STR) are tandem repeats motifs of 1-6 nucleotides found at high frequency in the nuclear genomes of most taxa. It provided a choice for many genetic researches since they are amenable to low, medium and high-throughput approaches (Lateef, 2015). It consists of tandemly repeated 2-7 base pair units arranged in repeats of mono-, di-, tri-, tetra and penta-nucleotides (A,T, AT, GA, AGG, AAAG etc.) with different lengths of repeat motifs. These repeats are widely distributed throughout the plants and animal genomes that display high level of genetic variation based on differences in the number of tandemly repeating units of a locus (Johan *et al.,* 2011). PCR amplification protocols used for microsatellites employ either unlabeled primer pairs or primer pairs with one of the primers being radiolabelled or fluorolabelled. Electrophoresis of unlabelled PCR products can be carried out on smaller vertical polyacrylamide gels or on horizontal agarose gels (Molosiwa, 2012). 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. Polymerase slippage during DNA replication, or slipped strand mispairing, is considered to be the main cause of variation in the number of repeat units of a microsatellite, resulting in length polymorphisms that can be detected by gel electrophoresis (Ijara, 2015). Basically there are two strategies used for microsatellite development: microsatellites markers can be sourced based on DNA sequence information deposited in the databases (mining in public libraries/databases) or through screening of genomic DNA libraries specifically constructed for discovery of repeated sequences in the genome (Molosiwa, 2012). The development of microsatellite markers involves several distinct steps from obtaining the

library to developing a working set of primers that can amplify polymorphic microsatellite loci. These include: (1) Microsatellite library construction, (2) Identification of unique microsatellite loci, (3) Identifying a suitable area for primer design,(4) Obtaining a PCR product, (5) Evaluation and interpretation of banding patterns and (6) Assessing PCR products for polymorphism (Mishra *et al.,* 2014).

In natural plant populations, microsatellites have great potential for helping to understand what determines patterns of genetic variation, particularly when used in concert with chloroplast DNA (cpDNA) markers. Their utility has been demonstrated in studies of genetic diversity, cultivar identification, mating systems, pollination biology and seedling establishment (Ahmed, 2012). It is reported that among different classes of molecular markers, SSR markers are useful for a variety of applications in plant molecular biology (Mishra *et al.,* 2014), this includes integrating the genetic, physical and sequence-based physical maps in plant species, and simultaneously have provided breeders and geneticists with an efficient tool to link phenotypic and genotypic variation (Mishra *et al.*, 2014).

SSRs have several advantages over other molecular markers. For example, (1) microsatellites allow the identification of many alleles at a single locus, (2) they are evenly distributed all over the genome and they are co-dominant, (3) little DNA is required and the analysis can be semi-automated and performed without the need of radioactivity (Tharachand *et al.,* 2012), (4) microsatellites can offer more detailed population genetic insight than maternally inherited mitochondrial DNA (mtDNA) because of the high mutation rate and bi-parental inheritance (5) highly polymorphic and specific (Ahmed, 2012), (6) very repeatable, (7) so cheap and easy to run (8) need a small amount of medium quality DNA and with the advance of DNA isolation technology, it was possible to identify loci in highly degraded ancient DNA (aDNA), where traditional enrichment procedures have been unsuccessful, (9) with the development of high-throughput sequencing platforms, such as the GS-FLX (Roche, Branford, CT, USA) SSR has recently become fast and efficient (Ahmed, 2012). With the above mentioned features, microsatellites have become the genetic markers of choice in many plant systems (Trachand *et al.*, 2012). On the other hand, these markers have several disadvantages: expensive, laborious and time-consuming. The low frequency of SSRs in plants also hinders the large scale isolation of SSRs (Firas and Abdulkareem, 2015).

2.8.3.2.4 Inter Simple Sequence Repeat (ISSR)

Soon after the discovery of the polymerase chain reaction (PCR) in 1983, new PCR-based DNA marker systems were continuously being developed. In the early 1990's, the development of what would become today's 'inter-simple sequence repeat (ISSR)' markers was independently reported by several research groups (Ahmed, 2012). Inter Simple Sequence Repeat (ISSR) markers have been proposed as a new source of genetic markers that are inherited in Mendelian fashion and are scored as dominant markers. ISSR analysis offers breeders and geneticists with competent means to link phenotypic and genotypic variations and is rapidly being used by the research community in various fields of crop improvement (Rao *et al.,* 2007). ISSRs are first reported by Zietkiewicz *et al.* (1994). ISSRs are DNA fragments about 100 - 3000 bp in length which is located between flanking microsatellite regions (Figure 3) (Omondi *et al.,* 2016). ISSR marker technique is a PCR based method, which involves amplification of DNA present at an amplifiable distance in between two identical microsatellite repeats oriented in opposite direction. The technique uses microsatellites, usually 16-25 bp long as primers in a single primer PCR reaction targeting multiple genomic loci to amplify the inter simple repeat sequences of different sizes. The microsatellite repeats used as primers for ISSRs can be di, tri, tetra or penta-nucleotides (Mishra *et al.,* 2014). ISSRs use longer primers (15–30 mers) as compared to RAPD primers (10 mers), which permit the subsequent use of high annealing temperature leading to higher stringency. The annealing temperature depends on the GC content of the primer used and ranges from 45 to 65ºC. The amplified products are usually 100–3000 bp long and amenable to detection by both agarose and polyacrylamide gel electrophoresis (Mishra *et al.,* 2014). By single microsatellite primers, ISSRs of different sizes are amplified in a PCR reaction targeting multiple genomic loci. Thus, fragments of several loci are generated at once, separated by gel electrophoresis and scored for presence or absence (Omondi *et al.,* 2016). In contrast to other molecular markers, the target sequences of the ISSR primers are abundant throughout the eukaryotic genome and evolve quickly, which consequently helps reveal a much larger number of polymorphic loci than other dominant markers such as RAPD (Firas and Abdulkareem, 2015).

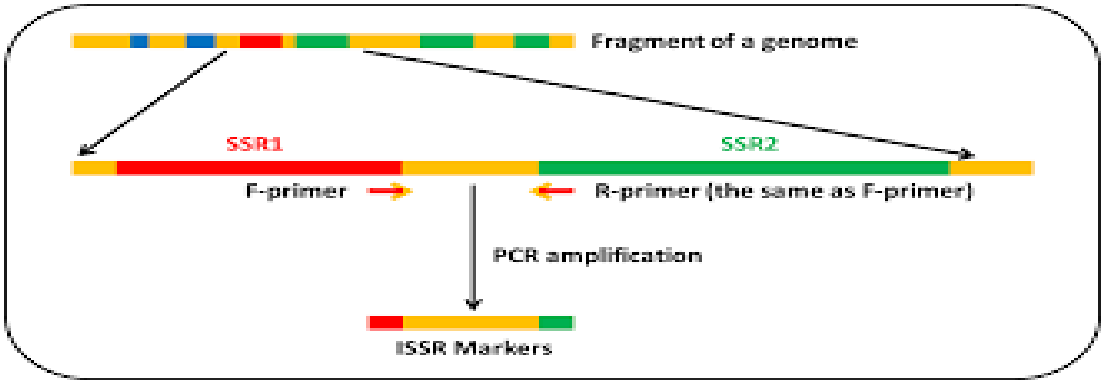


Figure 3. ISSR marker amplification region on the genome (Omondi *et al.*, 2016).

Generally, ISSR markers are unmapped but can be used to saturate RFLP and SSR linkage maps, and generate species specific, gene specific and trait specific markers. The evolutionary rate of change within microsatellites is considerably higher than most other types of DNA based markers, so the likelihood of polymorphism in these sequences is greater. The source of variability in the ISSRs can be attributed to template DNA, nature of primer used and detection method (Pradeep Reddy *et al.*, 2002). The ISSR method has several benefits over other techniques: first, it is known to be able to discriminate between closely related genotypes and second, it can detect polymorphisms without any previous knowledge of the crop's DNA sequence (Ahmed, 2012). In addition, it does not require genome sequence information; it leads to multilocus, highly polymorphous patterns and produces dominant markers (Mishra *et al.*, 2014). ISSR PCR is a fast, inexpensive genotyping technique based on variation in the regions between microsatellites (Ahmed, 2012). ISSRs segregate mostly as dominant markers following simple Mendelian inheritance. ISSR analyses offer breeders and geneticists with competent means to link phenotypic and genotypic variations in various fields of plant improvement (Tadele, 2014). The technique was subsequently applied to numerous plant genetics studies: identification of cultivars, genetic mapping/fingerprinting, assessment and characterization of genetic diversity, bio geographical studies, gene tagging, marker assisted selection, determining SSR motif frequency and studies on natural populations/speciation, detection of soma-clonal variation and molecular systematic. Despite these advantages, ISSRs can have problems in reproducibility, and their dominant inheritance and homology of co-migrating amplification products result in similar problems like for RAPDs (Omondi *et al.*, 2016).

3. MATERIALS AND METHODS

3.1 Plant Materials

Seeds of 43 *A. hypogea* accessions were obtained from Ethiopian Biodiversity Institute (EBI), Addis Ababa, Ethiopia which was originally collected from different geographical locations of Ethiopia; Gursum 19 (Llalemi 1, Llalemi 2, Awdal, Oda 1, Oda 2, Oda 3, Oda 4, Oda 5, Nur Selam 1, Nur Selam 2, Odaa 1, Odaa 2, Abader, Harobata 1, Harobata 2, Harobata 3, Harobata 4, Awdal 1, Awdal 2), Babile 21 (Berkele/s 1, Berkele/s 2, Babile/ Shek A., Awsherit 1, Awsherit 2, Lecole, Ifa Gende 1, Ifa Gende 2, Ifa Gende 3, Medigana 1, Medigana 2, Dendaro, Tofic 1, Tofic 2, Berkele, Gende, Gemechu, Tula, Tula About, Abdul 1, Abdul 2), Somalia 1 (Jigjiga/Beledka), Amhara 1 (Adoawe/Wangua), Bale 1 (Ginir) (Figure 4). The seeds of all the 43 *A. hypogaea* accessions were planted in plastic pots containing sandy loamy (composted) soil and maintained in a greenhouse (30°C/23°C) day/night controlled temperature for about four weeks at Awash Melkasa Agricultural Research Center. Watering was done once a day regularly. Fresh leaves from four week old plants were used for genomic DNA extraction. 50 mg of young leaves were collected from plants of each accession just before flowering and ground for genomic DNA extractions.

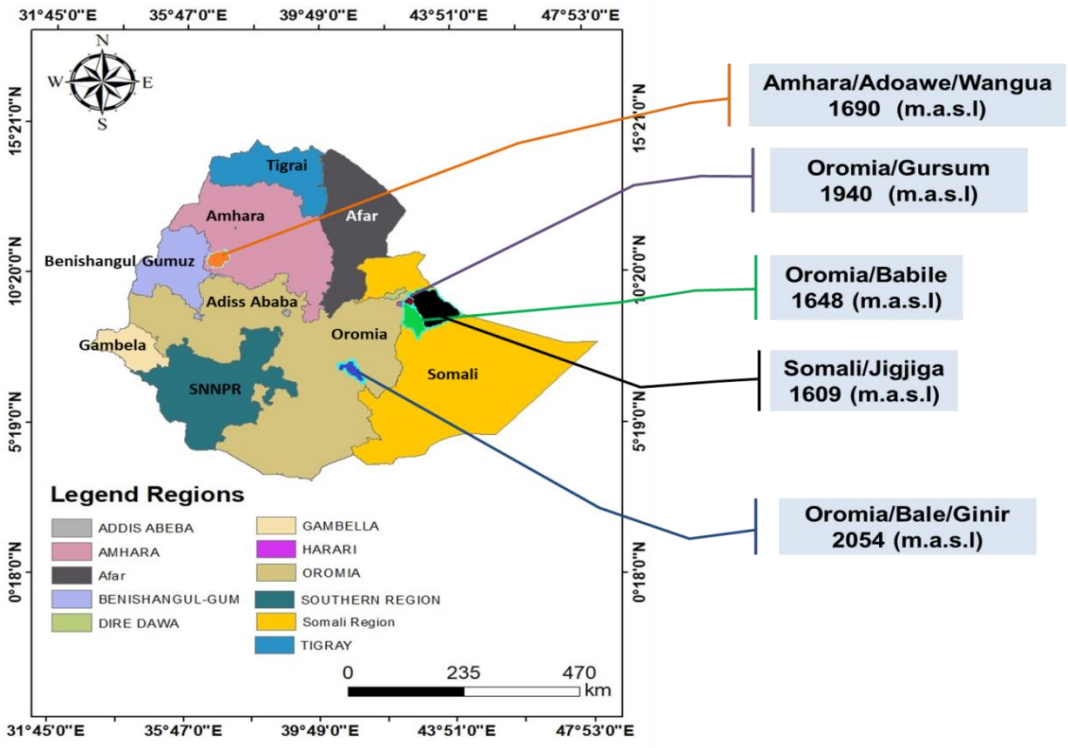


Figure 4. Locations and sites of Ethiopia from where the samples were collected by EBI.

3.2 DNA Extraction, Quantification and Purity Checking

Genomic DNA extraction was done based on the CTAB method described in Wang *et al.* (1994) which involves minor modification in the amount of CTAB solution used (1000 µl), as well as incubation and centrifugation time, to get optimal amounts of DNA. The detail for the DNA extraction procedure described in the Appendix. About 50 mg of silica-gel dried leaves for each accession were ground with mix and miller machine at Plant Molecular Genetics Research Laboratory, Department of Microbial, Cellular and Molecular Biology Department, Addis Ababa University. For optimum amount of DNA for ISSR-PCR reaction the second extraction was used, taken for polymerase chain reaction (PCR). The samples were stored at 4 ^{o}C until electrophoresis and subsequent dilution and PCR reaction. The yield of DNA isolated was measured/quantified using a Nano Drop ND-8000 UV spectrophotometer. Moreover, the purity of DNA was visually determined by agarose gel electrophoresis by running the samples on 1 % agarose gel.

3.3 Primer Selection and Optimization

A total of nine Inter simple sequence repeat primers obtained from the Genetic Research Laboratory (Primer kit UBC) (originally bought from University of British Columbia) were used for the initial testing of polymorphism and reproducibility. For optimization and screening of primers, the concentration of extracted DNA from each accession were adjusted to 50 ng/µl and used for PCR optimization process. All pre-selected 9 primers were screened for reproducibility and polymorphism. Finally, three di-nucleotide primers i.e. primer 810, 841 and 857, and one penta-nucleotide primer i.e. primer 881, were selected based on polymorphism and reproducibility (Table 1).

Table 1. List of ISSR Primers Screened for Polymorphism and the Reproducibility of the Amplified Bands.

NO	Primers	Annealing T^{o} (^{o}C)	Sequence of nucleotides (5'- 3')	Amplification pattern
1	810	45	GAGAGAGAGAGAGAGAT	Polymorphic, Reproducible
2	812	45	GAGAGAGAGAGAGAGAA	Not amplified
3	824	48	TCTCTCTCTCTCTCTCG	Not reproducible
4	840	45	GAGAGAGAGAGAGAGAYT	Not amplified
5	841	48	GAGAGAGAGAGAGAGAYC	Polymorphic, Reproducible
6	842	45	GAGAGAGAGAGAGAGAYG	Not amplified
7	848	45	CACACACACACACACARC	Not reproducible

8	857	48	ACACACACACACACACYG	Polymorphic, Reproducible
9	881	48	GGGGTGGGGTGGGGTG	Polymorphic, Reproducible

Source: Primer kit 900 (UBC 900); Y = Pyrimidines (C or T), R = Purines (A or G).

3.4 PCR Amplification and Gel Electrophoresis

The polymerase chain reaction was done using Biometra 2000 T3 Thermo-cycler. PCR amplification was carried out in a 25 µl total reaction mixture containing 50 ng/µl template DNA, 17.5 µl ddH20, 0.5 µl dNTP (1.25 mM), 2.5 µl PCR buffer (10xThermopol reaction buffer), 2.5 µl $MgCl_2$ (2 mM), 0.5 µl primer (20 pmol/µl) and 0.5 µl Taq Polymerase (5 U/µl). The amplification program was 4 min preheating and initial denaturation at 94^{o}C, then 39 cycles at 94^{o}C for 30 sec, 1 min primer annealing at 45/48 ^{o}C based on primers used, 90 sec extension at 72^{o}C with a final extension of 7 min at 72^{o}C. The PCR products were also stored at 4^{o}C until loaded on gel for electrophoresis.

The amplification products were checked first on test gel using 1% agarose gel using 1 X TBE for the presence of ISSR-PCR products. The amplified products were run on to ISSR gel using 1.67% agarose, with 1 X TBE using gel electrophoresis chamber. The ISSR gel (1.67 % TBE) was prepared using 300 ml TBE mixed with 5.01 g agarose using 500 ml Erlenmeyer flask and then boiled in micro oven for 3 minutes. After it was cooled for about 20 minutes at room temperature, ~12µl Ethidium Bromide (10 mg/ml) added for staining purpose and the gel poured on gel casting tray and allowed to solidify for more than 1 hr. Eight micro litter ISSR amplification products and 2µl (6X) loading dye (0.12% bromo-phenol blue and 30% glycerol) were mixed thoroughly and loaded on the gel. A 1200 base-pair ladder were used to estimate the molecular size of the DNA fragments. The gel was run on electrophoresis machine for 2 hours at constant voltage of 100 V. The ISSR band patterns were visualized and photographed under UV light using Biometra Biodoc analyzer**.**

3.5 Data Scoring and Statistical Analysis

Data Scoring and Statistical Analysis of ISSR bands were scored as present (1) and absent (0) representing the ISSR profile of each sample. The 0/1 matrix were used to calculate the Jaccard's similarity coefficient for all possible pairs of samples using Free Tree 0.9.1.50 (Pavlicek *et al.,* 1999) and NTSYS-pc version 2.02 Rohlf (2000) software. Jaccard's similarity coefficient which is calculated as:

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

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 numbers of bands present in individual j but not in individual i. The resulting similarity matrices were employed to construct UPGMA-based dendrogram. The unweighted pair group method with arithmetic average (UPGMA) was used in order to determine the genetic relationship among accessions and generates dendrogram using NTSYS- pc version 2.02 (Rohlf, 2000). Percent of polymorphism, Nie's pairwise gene diversity and Shannon's Weaver pairwise diversity index (I) with POPGENE software 1.32 (Yeh *et al.,* 1997). The value of polymorphism information content was calculated using software Power Marker version 3.2 (Liu and Muse, 2005). The matrix of genetic similarity was also used in a principal coordinate analysis (PCoA) to resolve the patterns of clustering among the accessions based on Jaccard's coefficient. The calculation of Jaccard's coefficient were made with PAST software version 1.18 (Hammer *et al.*, 2001).

4. RESULTS AND DISCUSSION

4.1 ISSR Polymorphism

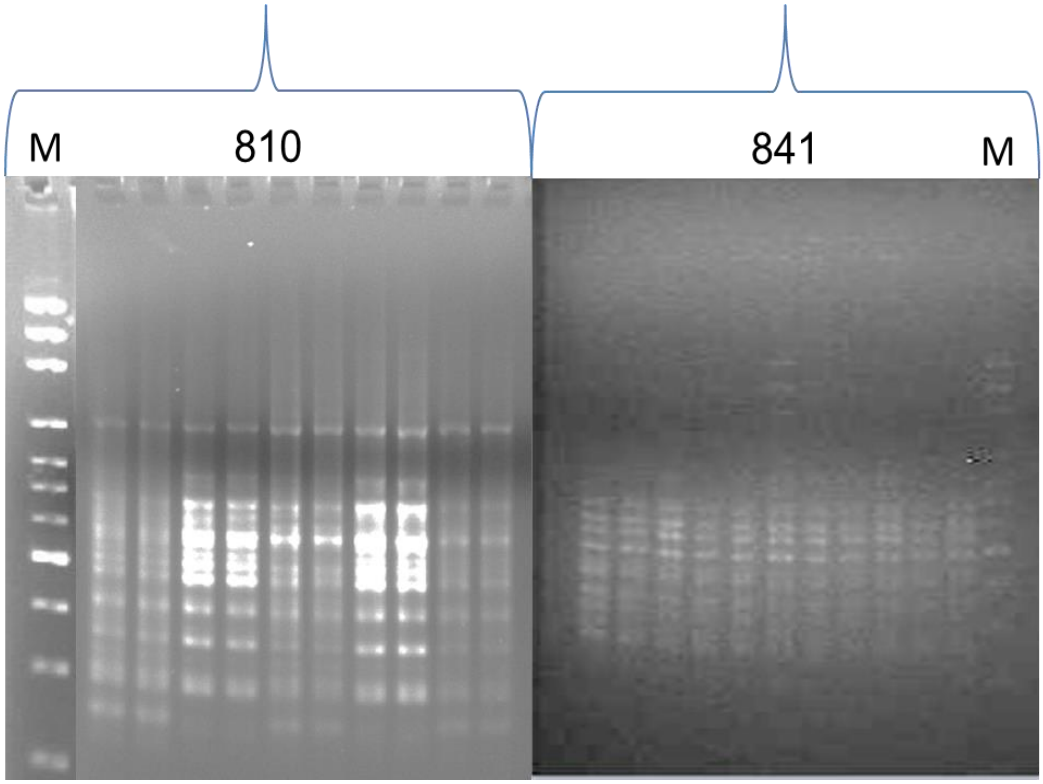
Out of the 9 primers screened initially, four of them with clear and reproducible banding pattern were selected and used in this investigation (Table 2). The size of the bands amplified using the four primers were in the range of 120 to 1100 bp (Figure 5). The four ISSR primers generated 56 bands across the primers, of which 29 were (Table 2). The amplified bands of the primers ranged from 12 (primer 841) to 18 (primer 881) across the accessions. The number of polymorphic bands of the primers ranged from 5 in primer 841 and 881 to 11 in primer 857. Average number of amplified bands and polymorphic bands per primer were 14 and 7.25, respectively. The percentage of polymorphism for primers ranged from 27.8% in primer 881 to 84.6% in primer 857, with an average polymorphism percent of 51.78% (Table 2).

Table 2. DNA Amplification Profile and Polymorphism Generated in *A. hypogaea* using 4 ISSR Primers.

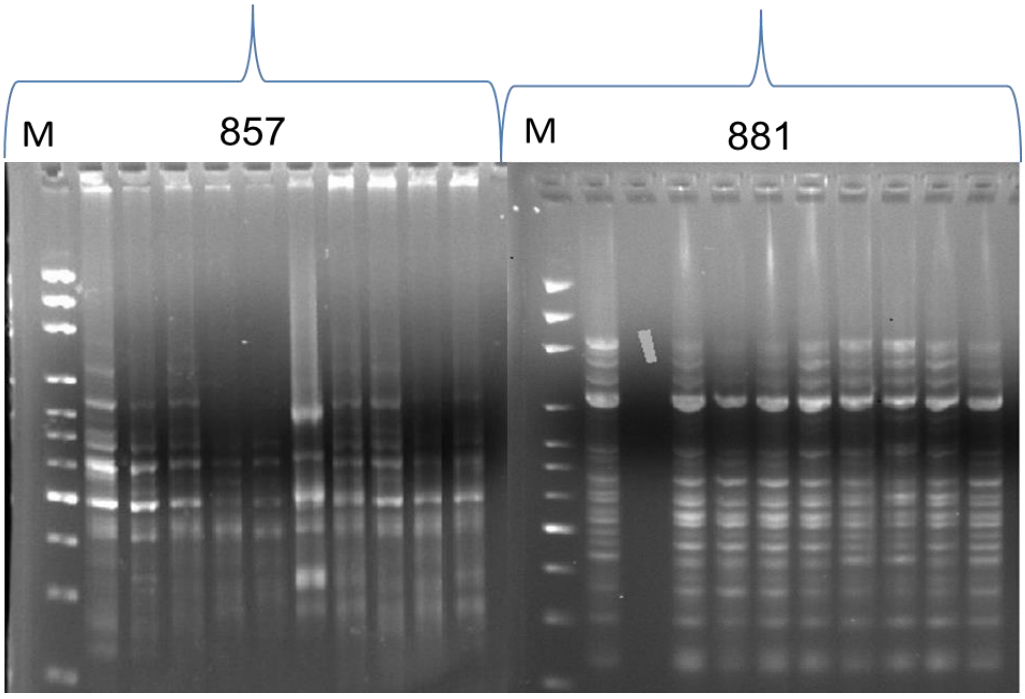
Primer	Sequence 5'-3'	Annealing T^o (^oC)	Total number of bands	Polymorphic bands	% of polymorphism
810	(GA)8T	45	13	8	61.5 %
841	(GA)8YC	48	12	5	41.7 %
857	(AC)8YG	48	13	11	84.6 %
881	(GGGGT)3G	48	18	5	27.8 %
Total			56	29	51.78 %

Source: Primer kit 900 (University of British Colombia); Y = Pyrimidines (C or T), R = purines (A or G).

In this investigation, the di-nucleotide primers, namely 810, 841 and 857 were observed to have 61.5%, 41.7% and 84.6% of polymorphism, respectively. The penta-nucleotides primer 881 was observed to have 27.8% polymorphism. A representation of the ISSR band profile obtained with primer 810 and 841 (A), 857 and 881 (B) are shown in (Figure 5).



(**A)**



(B)

Figure 5. ISSR band patterns generated from accessions of *A. hypogaea* from primers: 810 and 841 (A), 857 and 881 (B).

In this study, the di-nucleotide ISSR primers 810 with GA repeats and 857 with AC repeats, detected higher polymorphism among accessions compared with penta-nucleotide primer. Similar reports were recorded by Tadele *et al.* (2014) with (GA) primers and Tshilenge-Lukanda *et al.* (2014) with (AC) repeats, showed higher polymorphism in plants than primers with other nucleotides. The main reasons behind these variable results of molecular markers might be due to mutation at PCR priming site including changes in the repeat number of SSR motifs and insertion or deletion of nucleotides within amplified fragments. Moreover, the genetic background of *A. hypogaea* collections, and primer variation in quality and quantity might be the possible reasons for this result. Although it is likely that the major factor in this difference is the length of the repeat unit and type, the possibility that the sequence composition and distribution or frequency might be the reasons for this result. Moreover, the choice of appropriate primer motives in ISSR fingerprint is critical to detect high polymorphism and reveal relationship between different genetic resources.

The polymorphism of about 51.78 % observed in the present study represents moderate variability among accessions at the DNA level. The sort of genetic variability found in this crop is of the same type as reported by previous researchers. For example, Raina *et al.* (2001) reported 54% of polymorphism among 13 *A. hypogaea* accessions using ISSR markers. This level of genetic variation is relatively low when compared with other studies; Dhwani *et al.* (2017) reported a higher level of polymorphism (87%) in *A. hypogaea* using ISSR markers. Similarly, Suvendu *et al.* (2009) reported 74.7% polymorphism using ISSR markers among 20 *A. hypogaea* accessions. Various molecular markers showed different efficiency for evaluating DNA fingerprinting in *A. hypogaea*. For example, Herselman (2003) found a very low level of polymorphism (on average 2.78%) among 21 closely related cultivated South African *A. hypogaea* genotypes using AFLP marker. Dwivedi *et al.* (2001) reported a low genetic diversity (18%) among 26 accessions of cultivated *A. hypogaea*, including interspecific derivatives, landraces and released cultivars using RAPD markers. Kochert *et al.* (1991) found a very low level of RFLP variability among 8 U.S *A. hypogaea* cultivars and 14 wild *Arachis* species. Mace *et al.* (2007) observed a high level of polymorphism (99.4%) within germplasm of *A. hypogaea* which are resistant to bacterial wilt. Halward *et al.* (1992) used a single primer DNA amplification for genetic studies among 25 genotypes of *Arachis hypogaea* and found no variation in banding patterns. He and Prakash (1997) reported narrow

polymorphism (3%) in cultivated *A. hypogaea* by using a DAF (DNA amplification fingerprinting) approach.

This is the first report on genetic diversity study on selected *A. hypogaea* accessions found in Ethiopia based on molecular markers. The ISSR analysis used in the present study involved amplification of regions between adjacent, inversely oriented microsatellites using a SSR motif containing primers anchored at the 3' or 5' end by two or four arbitrary, often degenerate nucleotides (Zietkiewicz *et al.,* 1994). The ISSR method provides an alternative choice to obtain highly reproducible markers without any necessity for prior sequence information for various genetic analyses. It takes advantage of the ubiquitously distributed SSRs in the eukaryotic genomes. Because of these abundant and rapidly evolving SSR regions, ISSR amplification has the potential of revealing much larger numbers of polymorphic fragments per primer that any other marker system used such as RAPD or microsatellite (Suvendu *et al.,* 2009).

4.2 Polymorphism Information Content (PIC)

Polymorphism information content is the probability of detection of polymorphism by a primer/primer combination between two randomly drawn genotypes and depends on the number of detectable alleles and the distribution of their frequency. The PIC value, as a relative measure level of polymorphism varied from 0.29 (primer 881) to 0.76 (primer 857) with an average value of 0.49 (Table 3). The study of Cuc *et al.* (2008) in which an average of 0.46 PIC was calculated, are in support of our findings. Similarly, Roomi *et al.* (2014) observe an average of 0.548 PIC value among *A. hypogaea* accessions.

Table 3. DNA Amplification Profile, Polymorphism and Polymorphism Information Content Generated in 4 ISSR Primers.

Primer	Total number of bands	Polymorphic bands	% of polymorphism	PIC
810	13	8	61.5 %	0.49
841	12	5	41.7 %	0.42
857	13	11	84.6 %	0.76
881	18	5	27.8 %	0.29
Total	56	29	51.78 %	0.49

Markers with high PIC values are described as highly polymorphic and thus detect higher level of genetic variation in an organism. Hildebrand *et al.* (1992) asserted that PIC value is an important primary data which determines informativeness of a genetic marker and that a PIC value of 0.70 and above is highly informative whiles a value of 0.44 is moderately informative. PIC is a statistic that measures the usefulness of a genetic marker for diversity analysis. Therefore, primer 810 and 857 used in this study have moderate to high informativeness for diversity analysis in *A. hypogaea*, indicating that these primers are informative for determining *A. hypogaea* accessions. The primers 841 and 881 used in this study have lower PIC, indicating these primers are less informative for detecting the polymorphism of *A. hypogaea*. Therefore, further research on genetic diversity of *A. hypogaea* should be based on other informative and polymorphic primers. Comparison of the number of polymorphic bands with the PIC values revealed that a greater number of polymorphic bands were associated with higher values of PIC. However, the present study reveals the most informative primer was primer 857 with PIC value of 0.76 and the least informative primer was 881 with PIC value of 0.29. The observed variation in PIC values in this study could be attributed to the type of primers used and genetic background in the *A. hypogaea* materials used.

4.3 Genetic Diversity

The binary data matrix generated by the amplified fragments of the 43 *A. hypogaea* accessions in the ISSR-PCR analyses was used for the computations of Nei's genetic distances and Shanon's indices for pairwise comparison of the accessions for the analysis of ISSR data. The Nei's gene diversity (H) and Shannon's indices (I) ranged from 0.11 to 0.24 between GOG-6 (Gursum/Oda-3) and GOB-10 (Babile/Medigana-1) to 0.38 to 0.41 between GOBG-1 (Bale/Ginir) and GOB-14 (Babile/ Tofic-2) with a mean value of 0.245 and 0.325, respectively.

The different levels of genetic diversity for *A. hypogaea* may be explained, in part by the use of different ISSR molecular markers and in part by the different sampling strategies, such as the geographic distance between sampled accessions. The results revealed that GOG-6 (Gursum/Oda-3) and GOB-10 (Babile/Medigana-1) accessions were closely related, having the lowest genetic diversity, which could be attributed to the levels of management implemented and focus on frequent seed exchange between these areas by

different stakeholders. Additionally, the observed low genetic diversity between accessions could be associated with usage of large sample size and small geographical distance representing major *A. hypogaea* growing regions (Misrak Oromia - Grsum/Babile). Further, this low genetic diversity might be explained by the lack in access of genetically diverse relatives in these areas, existence of uniformity or monoculture possibly due to seed propagation prevailed in the area, homogeneity due to single or few seed sources during its introduction to these area. The highest genetic distance values belonged to GOBG-1 (Bale/Ginir) and GOB-14 (Babile/Tofic-2) accessions, which were genetically the most distant accessions and this, would be expected due to its geographical isolation which can be explained by topographic barriers preventing the dispersal of seeds and pollen that might limit the gene flow among *A. hypogaea* accessions. These values can be employed in a breeding program such that the accessions with the highest genetic divergence could be selected as parents to improve the *A. hypogaea* accessions.

4.4 Genetic Relationship

Knowledge about genetic constitute differences of genotypes is highly important in breeding programs. The importance is because of the need to identify parents with high genetic distances to produce progenies with higher heterosis. In this study, generated similarity matrix by ISSR based on the Jaccard's pairwise similarity coefficient matrices showed similarity ranged from 44 % to 83 %. The average similarity across the 43 accessions was found to be 63.5 %. Based on ISSR marker genetic relationship analysis different researchers found varietal level of genetic similarity between A. *hypogaea* genetic resources. For example, Tshilenge-Lukanda *et al*. (2012) reports lower level of similarity based on jaccard's coefficient between 40 *A. hypogaea* accessions which ranges between 0.11 and 0.37 with a mean value of 0.24 (24%). Baloch *et al.* (2010) found higher level of genetic similarity between 32 genotypes of *A. hypogaea* which ranges between 0.92 and 1.00 with a mean value of 0.98 (98%). Dhwani *et al.* (2017) found moderate level of genetic similarity between 24 *A. hypogaea* genotypes ranges between 0.54 and 0.90 with a mean value of 0.72 (72%). Additionally, other researchers also found different level of genetic similarity based on Jaccard's coefficient between *A. hypogaea* genetic resources. For example, Disha *et al.* (2016) reports 0.747 (74.7%) genetic similarity between six different bunch type groundnut varieties by using RAPD marker. Dwivedi et al. (2001) found 86.2% of similarity among selected *A. hypogaea* germplasms

based on RAPD marker. Shoba *et al.* (2010) found 77% of similarity among 11 *A. hypogaea* genotypes by using SSR marker.

In the present study, lowest genetic similarity value (i.e. maximum diversity) was found between accessions GOBG-1 (Bale/Ginir) and GOB-14 (Babile/Tofic-2) (44%) followed by that between GOG-1 (Gursum/Llalemi-1) with GAW-1 (Amhara/Wangua) and GOB-17 (Babile/Gemechu) with GOBG-1 (Bale/Ginir) at a similarity value of 46%, while highest similarity coefficient (i.e. minimum diversity) was found between the accessions GOG-6 (Gursum/Oda-3) with GOB-10 (Babile/Medigana-1) and GOB-7 (Babile/Ifa-gendi-1) with GOB-16 (Babile /Gende) 83% followed by that between GOG-12 (Gursum/Odaa-2) with GOB-9 (Babile/Ifa-gendi-3) at a similarity value of 82%.

Highest levels of distance between the accessions from GOBG-1 (Bale/Ginir) and GOB-14 (Babile/Tofic-2) could be attributed to the fact that the accessions have been popularly cultivated in the respective regions over time giving enough time for significant genetic differentiation along these particular geographical lines. At the same time, it could indicate genetic evidence of *A. hypogaea* being a diverse taxon as reported by Tang *et al*. (2007). Tang *et al*. (2007) reported that the genetic diversity in cultivated *A. hypogaea* was much richer at the molecular level when compared with that in the morphological and biochemical levels. Therefore, this would mean that the studied accessions from GOBG-1 (Bale/Ginir) and GOB-14 (Babile/Tofic-2) has the highest amount of diversity that can be used for *A. hypogaea* improvement in Ethiopia. In the same manner, it can be argued that GOG-1 (Gursum/Llalemi-1) with GAW-1 (Amhara/Wangua) and GOB-17 (Babile/Gemechu) with GOBG-1 (Bale/Ginir) has diversity that can be used for improvement of *A. hypogaea* at Babile, Gursum, Amhara/Wangua and Bale/Ginir. Generally, the higher level of genetic distance found in this study may be due to the fact that geographically isolated accessions in certain geographic locations could accumulate genetic differences and evolve unique traits as they adapt to different environment.

In particular, *A. hypogaea* is distributed across differential environmental conditions with respect to soil composition, altitude and annual rainfall, which could drive the acquisition of local adaptations. Moreover, the environmental conditions could influence flowering times and restrict the gene flow through pollination and seeds dispersion among geographical locations. Additionally, highest genetic distance observed between the above accessions could possibly attributed to their geographical isolation and limited gene flow

which might be resulted from having long geographical distance, therefore they might have a larger possibility of heterosis in a breeding programs aimed to improve productivity and food security in Ethiopia.

The comparison of the genetic distances between accessions from GOB-10 (Babile /Medigana-1) with GOG-6 (Gursum/Oda-3), GOB-7 (Babile/Ifa-gendi-1) with GOB-16 (Babile/Gende) and GOG-12 (Gursum/Odaa-2) with GOB-9 (Babile/Ifa-gendi-3) revealed a closer genetic relationship with each other than all other accessions. This high level of similarity could be due to having similar ecological environment and short geographical distance and or selecting similar traits during breeding programs in Gursum or Babile accessions. However, the genetic distances between the accessions were much lower indicating the genetic relatedness of the various geographic accessions which may be caused by the high rates of gene flow due to exchange of seed materials and *A. hypogaea* trades between this locations, and limited time for significant genetic differentiation along geographical lines. Further, the low genetic distances observed between these accessions could possibly reflect the initial bottleneck during domestication maintained by the inherent self-pollination mechanism of *A. hypogaea* crop. Thus the enhancement of diverse populations by using diverse genetic resources is needed in the future for various purposes such as population genetic structure, germplasm analysis, identification of cultivars, selection of parents and phylogenetic relationships.

Beside the observed highest genetic similarity between closely located geographical locations and lowest genetic similarity between distant geographical locations, the Jaccard's similarity matrices shows that there is also a significant level of genetic similarity between geographically distant accessions. For example, some of the accessions from Babile and/or Gursum show a similarity of 50% - 70% with Bale- Ginir, Somalia-Jigjiga and Amhara-Wangua. Unlike other landraces of cultivated plants, *A. hypogaea* in Ethiopia is not restricted to a given area rather it is widely exchanged among local community and markets. This shows that there might be a gene flow between different populations and regions of Ethiopia. This is also supported with the spread of individual accessions on UPGMA and PCoA analysis.

4.5 Cluster Analysis

Evaluation of similarity coefficients or dendrogram helps to select superior individuals to obtain hybrids with greater segregation and heterotic effects during recombination.

Jaccard's coefficients UPGMA and neighbor joining (NJ) analysis were used to construct dendrogram for 56 ISSR-PCR fragments scored from 43 accessions. UPGMA clustering analysis put the accessions into one cluster at 59.2 % similarity. They were however clustered in to five clusters based on the cut-off point of 63.5% similarity, equivalent to the mean genetic similarity of all the accessions (Figure 6). Similar result was reported by Dhwani *et al.* (2017) for similarity coefficient of clustering *A. hypogaea* genotypes using ISSR marker. Cophenetic correlation created from comparing similarity and output matrix of the dendrogram was 87% or 0.87, which indicated that the cluster generated by Jaccard's similarity was a good fit to the matrix, where $r > 0.9$ indicates a very good fit, r - 0.8 - 0.9 indicates good fit and $r < 0.8$ indicates a poor fit. Similar result was observed by Yousefi *et al.* (2015) reported 0.87 cophenetic correlation coefficient acquired for the clustering method representing a good fitness between the dendrogram clusters and the similarity matrices in *Thymus* collections using ISSR marker.

The dendrogram for the accessions (Figure 6) did not divide the accessions into distinct groups resembling the geographically-defined accessions. The accession clustering follows geographical proximity which shows the presence of gene flow among neighboring and distant regions or locations. The UPGMA analysis of the individuals in each accession revealed that almost all individuals were distributed and inter-mixed with individuals of another locality. While comparing the clusters or sub clusters within different groups of accessions, no specific/significant trend of grouping of accessions was observed. However, a loose clustering of accessions as per their geographical origin was observed. For example, accessions from Babile (62.5%) and Gursum (61.5%) were grouped in cluster I sub cluster I and II, respectively (Figure 6).

Additionally, in constructed dendrogram of *A. hypogaea,* accessions coming from the same geographical location were distributed in various clusters. For instance, the accessions from Gursum and/or Babile were distributed in all 5 distinct clusters. This observation could be an indication for the presence of within population genetic variation in *A. hypogaea*. Moreover, accessions from distant geographical locations were clustered together with other accessions from different regions. This fact becomes evident, for example, at the top of the cluster 'GOBG-1' accession from Bale/Ginir was clustered with accessions from Babile and Gursum. Similarly, at the bottom and middle of the clusters the accessions from GAW-1 (Amhara/wangua) and GSJ-1 (Somalia/Jigjiga/Beledka) were clustered with accessions from Babile and Gursum which is unexpected because they are

geographically far distant locations. Although such evidence has already been reported by (Raina *et al.*, 2001; peng *et al.*, 2016), suggesting that there was no obvious differentiation in the population structure of *A. hypogaea* from different geographic origins. From the evidence of the current study it would seem likely that the accession clustering is the result of the traditional ways that *A. hypogaea* have been distributed across the regions and also by frequent exchange of seeds which might be resulted from the migration of peoples through settlements in which local farmers might took seeds. The migration might often because of multitude of reasons including environmental degradation, vulnerability, food insecurity, natural disaster, land scarcity and other factors they faced. The analysis also revealed intermixing of individuals possibly due to admixture that may be resulted from the increasing demand for *A. hypogaea* by consumers in Ethiopia, short and long distance marketing of seeds of *A. hypogaea* or the movement of traders and exchange of germplasm across different regions could be a possible explanation for the spreading of *A. hypogaea* seeds (Fredu *et al.*, 2015; Jemal and Nick, 2015; Addisu and Ermias, 2017).

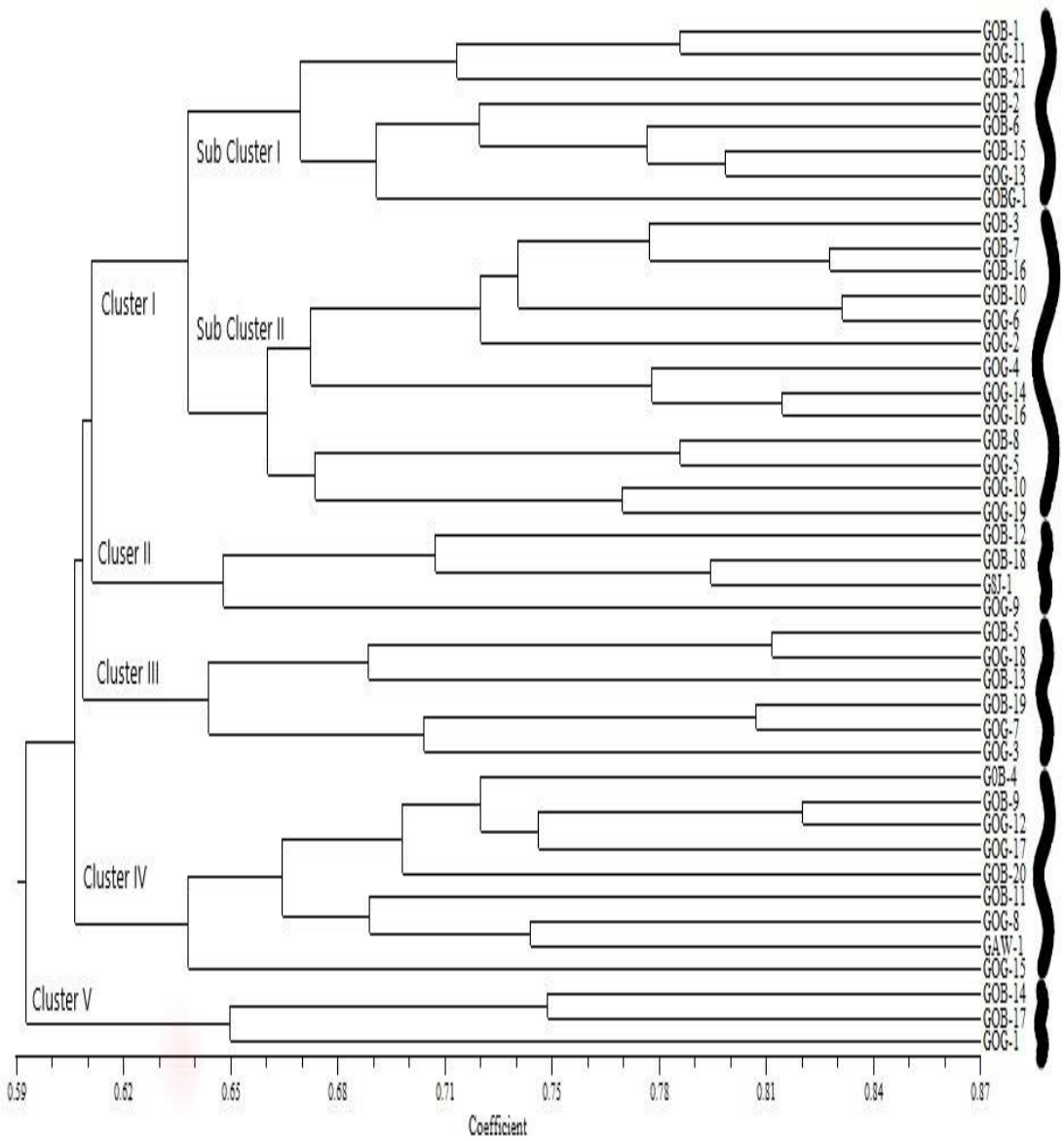


Figure 6. UPGMA based dendrogram obtained for 43 accessions using 4 ISSR primers.

Key: GOG – Oromia/Gursum, GOB – Oromia/Babile, GOBG – Bale/Ginir, GSJ – Somalia/Jigjiga, GAW – Amhara/Wangua

4.6 Principal Coordinate Analysis

Principal coordinate analysis (PCoA), as a complementary technique for cluster analysis, is one of the important multivariate statistical approaches to group based on similarity coefficients. The dependability of the dendrogram and the principal coordinate analysis intensely supports the reliability of the marker system. All the data obtained using four ISSR primers were used for PCoA analysis using Jaccard's coefficients of similarity. The first two components of the coordinates of the PCo having eigen values of 16.3 and 8.2 with variance of 33.2 % and 16.4 %, respectively, and together 49.6 % used to show the

grouping of individuals using two co-ordinates. Similar to the UPGMA clustering pattern, the 43 accessions of *A. hypogaea* were grouped into five groups (clusters) based on the principal co-ordinate analysis (Figure 7).

The PCoA result indicated that most of the accessions did not group together with other accessions from the same geographical region (Figure 7). The possible reasons for this could be the exchange of *A. hypogaea* seeds among farmers across regions. This result confirms the result obtained in UPGMA. However, most of the accessions that represent similar geographical regions were found to form distinct groups and spread all over the plot (Figure 7). Also accessions from distant geographical locations tend to form similar group or cluster. This could have led to a generally low coefficient of variation observed in *A. hypogaea* accessions, an indication of high level of uniformity and the presence of gene flow. This suggested that the source of these accessions could be the same due to seed exchange among the farmers and traders. The result of principal co-ordinate analysis (PCoA) was partially in accordance with the cluster analysis.

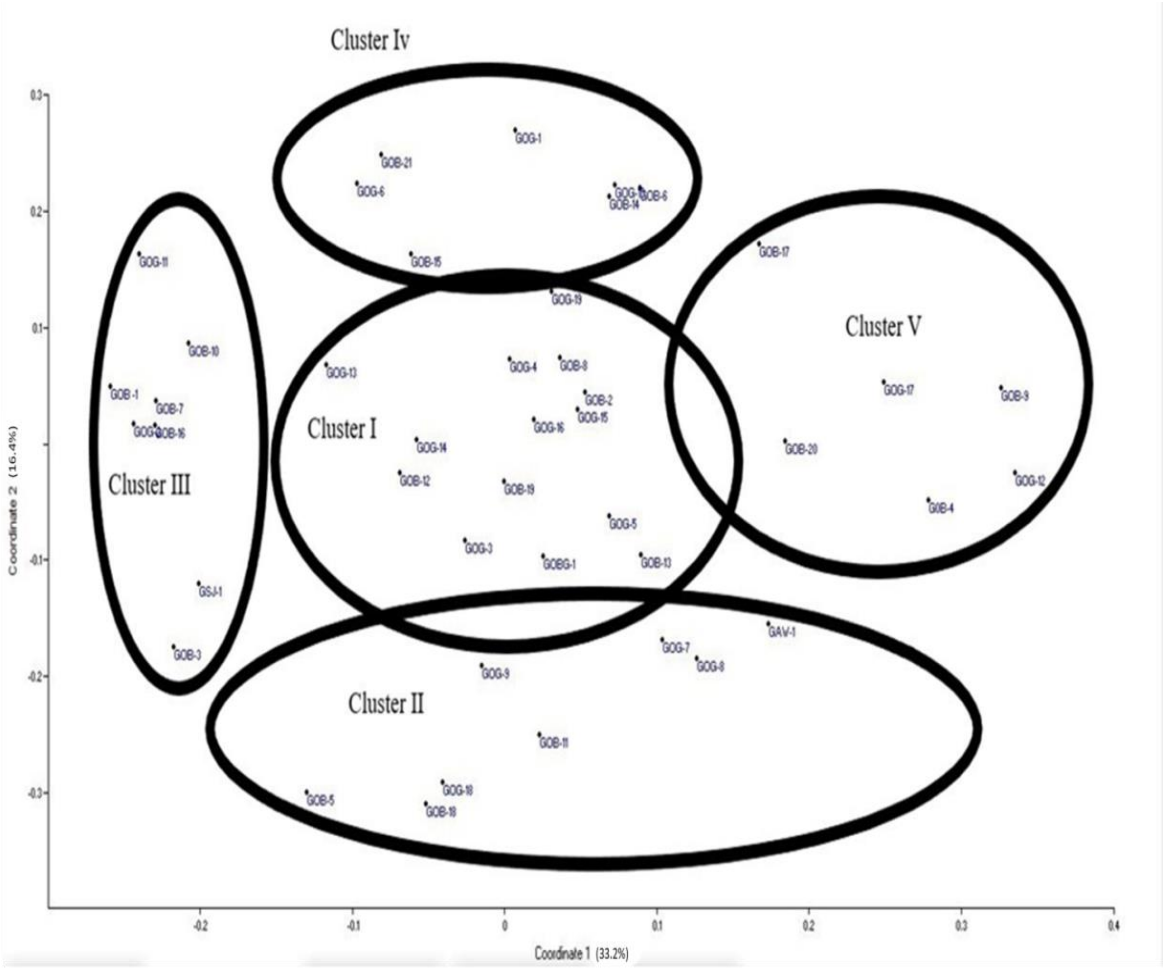


Figure 7. PCoA scatter plot diagram showing relationships among *A. hypogaea* accessions.

Though the PCoA shows random distribution of accessions on the plot, the Jaccard's similarity coefficient however shows high genetic distance between accessions. Improvement programs should, therefore, target selection of individual plants with desirable traits from these accessions, since the accessions are more diverse. However, this does not mean that less diverse accessions do not consist plants with important traits. But it only means that the probability of getting individual plants with good traits from such accessions is very low as compared to those that are more diverse. Therefore, a more detailed sort of selection should also be practiced in such accessions. Hybridization/crossing between any distantly related accessions is expected to yield more heterotic and vigorous plants constituting much of the different traits contained in the above distant accessions. Therefore, hybridization or crossing between distantly related accessions of the present study could be an appropriate strategy for improvement programs. Using both (genetic diversity and distance) parameters simultaneously for improvement programs would be the best approach. In this case, the best individuals selected and crossed with individuals selected from an accession that is/are comparatively distant from the former accessions could yield good results.

5. SUMMARY AND CONCLUSION

- In Ethiopia, the present study is the first report on genetic diversity of *A. hypogaea* using ISSR marker. The molecular analysis results using four ISSR primers revealed moderate level of 51.78 % of polymorphic loci among accessions.

- Based on Jaccard's similarity coefficient, highest genetic similarity observed between accessions suggest that the homogeneity among the accessions (geographic region based) could be due to gene flow or that they may had a common origin, alternatives that needs to be further explored. On the other hand, the least genetic similarity observed between accessions indicates a good amount of genetic divergence and would therefore be useful in broadening genetic base of accessions of *A. hypogaea* in Ethiopia. The genetic distance between populations, on the other hand, is a valuable parameter for genetic improvement programs. The most diverse accessions identified in this study should be given a due emphasis to include them in *A. hypogaea* breeding program and should be considered as the primary/valuable sites in designing conservation areas for this crop.

- Both PCoA and UPGMA cluster analysis shows the clustering of all 43 accessions into five clusters. Cluster analyses of the present study showed that most of the accessions did not group together with other accessions from the same geographical region and geographically distant accessions located in one cluster. The close relationship between accessions from the different geographical locations might be due to gene flow caused by the exchange of accessions through farmers and traders across regions of Ethiopia. This generally indicates the possibilities of both inter and intra population variation of *A. hypogaea* could occur in Ethiopia. To get an objective discrimination of genetic diversity of cultivated *A. hypogaea* and to make a wider classification of them, substantially more DNA markers and more samples would be needed from diverse geographical locations.

- Finally, this study reported a successful fingerprinting of *A. hypogaea* accessions using ISSR and demonstrated the usefulness of these markers in estimating the extent of genetic variation in *A. hypogaea* accessions. These molecular markers will greatly assist in the identification of new and different sources of diversity

which may help breeders to decide which seed materials to cross for making new genetic combinations and to determine which genetic resources should be retained in a collection in order to conserve maximum genetic diversity in the gene bank. The results also provide important milestone for future conservation and improvement programs of *A. hypogaea*. Furthermore, results from the above multivariate statistic estimates suggest that there could be a possibility of sampling plants with the same genetic constitution from different regions. Therefore, representing a population by as many collections as possible would be the best approach.

6. RECOMMENDATIONS

- Further study with more sample size and geographic range would give more clear patterns of diversity for the whole study underlying causes of the observed genetic differences noted in this study.

- Analysis with molecular markers, such as AFLP, SSR and other newly emerged molecular markers, which can be easily used for genetic screening and complete characterization of existing populations are recommended.

- The use of ISSR markers in *A. hypogaea* must be further continued in order to drive specific linkage between ISSR markers and genes controlling agronomically important characters.

- It is recommended that the present study be complemented with a parallel investigation on adaptive traits (drought, pest and insect resistance) and other economic traits (growth and form) before a definite strategy is adopted.

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8. APPENDIX

8.1 REAGENTS AND SOLUTIONS:

A. 1.0 M Tris-HCl (pH 8.0)

Dissolve 12.11gm Tris HCl in sterile de-ionized water, adjust pH to 8.0 with conc. HCl, and make up volume to 100 ml with de-ionized water and autoclave at 15 psi for 20 min.

B. 0.5 M EDTA (pH 8.0)

EDTA (dissolved salt; Mw = 372.3) = 18.61gm

Dissolve, 18.61gm EDTA in sterile de-ionized water, adjust pH to 8.0 with 5N NaOH, make up volume to 100 ml with de-ionized water and autoclaved at 15 psi for 20 min.

C. 5 M NaCl

NaCl = 29.2 gm

Dissolve 29.2gm NaCl in sterile de-ionized water, make up volume to 100 ml with de-ionized water and autoclave at 15 psi for 20 min

D. Extraction buffer

1M Tris-HCl (pH 8.0) = 10 ml

0.5 M EDTA (pH 8.0) = 2 ml

3 M NaCl = 46.6 ml

2% CTAB (w/v) = 2 g

0.2% ß-Mercaptoethanol = 0.2ml

Dissolve the above and make up to 100 ml with de-ionized water and autoclave at 15 psi For 20 min.

E. 10% working C-TAB

10% CTAB = 10 g

5 M NaCl = 14 ml

Dissolve the above in water, make up to 100 ml and autoclave at 15 psi for 20 min.

F. 3M NaOAC (pH 4.8)

Sodium Acetate = 24.61 gm

Dissolve 24.61 gm NaOAC in sterile de-ionized water, adjust pH to with glacial acetic acid, make up volume to 100 ml with de-ionized water and autoclave at 15 psi for 20 min.

G. Choloroform : Iso-amyl alcohol mixture (24:1) (100 ml)

Choloroform = 96 ml

Iso-amyl alcohol = 4 ml

H. 70% ethanol (100ml)

Absolute alcohol = 70 ml

Double distilled water = 30 ml

I. RNase stock

1 M Tris- HCL (pH 8.0) = 100 µl

5 M NaCl = 300 µl

RNase = 10 mg

Adjust volume to 1 ml with de-ionized water, boil for 15 min and allow to cool slowly and stored at -20ºC

J. TE (10:1)

1 M Tris-HCl (pH 8.0) = 1.0 ml

0.5 M EDTA (pH 8.0) = 0.2 ml

Dissolve the above and make up volume to 100 ml with de-ionized water and autoclave at 15 psi for 20 min.

K. 10 X TBE (pH 8.0) Tris base = 108.0 g Boric acid = 55.0 g EDTA (0.5M) = 20ml Dissolve the above and make up volume to 1000 ml with double distilled water.

8.2 LIST OF INSTRUMENTS USED IN:

A. DNA extraction, purification, quantification.

(a) Electronic balance

(b) Micropipette (2µl,20µl,100µl,1000µl)

(c) Water bath

(d) Layophilizer

(e) Deep Freezer (-20°C)

(f) Refrigerator

(g) Fume hood

(h) Table top centrifuge

(i) Vortex

(j) Eppendorf tubes (0.5ml, 2ml)

(k) Tips (200µl, 1000µl)

(l) Quant Fluorimeter

(m) Quartz Cuvette (n) pH meter

(n) Water purification system

(o) Hybridization oven

(p) Vertical autoclave

B. ISSR analysis

(a) Thin walled PCR tubes

(b) PCR

(c) Laminar air flow

(d) Micro centrifuge

(e) Pipett

(f) Pipette tips

C. Gel Electrophoresis

(a) Gel electrophoretic unit

(b) UV transilluminator

(c) Power pack (300)

(d) Microwave oven

(e) Laboratory shaker

(f) Gel Doc. System

8.3 DNA Extraction Procedure

1. Pour CTAB solution (700 µl per sample) in a 15ml-tube and add 0.2 vol % Mercapto-ethanol (use fume hood!). Mercapto-ethanol is stored at 4°C. Incubate the CTAB at 65°C.

2. Weigh in 100 mg fresh leave material (50mg dry material) per sample. Pulverise thoroughly using a clean mortar and pestle. For fresh material add liquid nitrogen or quartz sand for dry material. First grind down slightly, then more powerful (cells have to be crashed). Use safety goggles!

3. Transfer the powder into a sterile Eppendorf cap (use a new, sterile spatula for each sample.

4. Add 700 µl of warm (65°C) CTAB solution to the powdered sample (open the caps carefully), dissolve the powder and incubate the sample for 30 minutes at 65°C.

5. Centrifuge for 5 minutes at 15000 rpm, 20°C.

6. Transfer the supernatant (only clear liquid) in a new Eppendorf-cap. Use blue pipette tips which are cut.

7. Add new CTAB solution (700 µl – modified to 1000 µl) to the tissue pellet and stir slightly with a new 1000 µl pipette tip, incubate 30 min at 65°C. Step 6 and 7 are repeated. The same is carried out for a third extraction. Each fraction proceeds with step 9 and is treated separately.

8. Add 600 µl Chloroform to the cap with supernatant. (This chloroform step should be carried out immediately).

9. Shake the samples thoroughly by inverting the Eppendorf-caps for approximately 5 minutes.

10. Centrifuge for 5 min at 15000 rpm.

11. Transfer the supernatant (only clear liquid) in a new Eppendorf-cap. Use blue pipette tips which are cut. Work carefully; do not transfer suspended matter (normally the Chloroform is covered by a thin layer of fine sediment material). Chloroform has to be disposed of in a special waste bottle.

12. Repeat the chloroform extraction (step 8-11) to make sure that all impurities are removed and then proceed with step 13.

13. Add cooled Isopropanol (4°C), approximately **2/3** of the solution volume. Shake carefully by inversing the Eppendorf cap. In most cases DNA becomes visible as white threads. Freeze for more than 2 h at -20°C. (BREAK POSSIBLE).

14. Centrifuge 10 min at 15000 rpm in a cooled centrifuge.

15. Aspirate liquid using yellow tips (without touching pellet!). If pellet is solid enough the larger part of the liquid may be poured out. (Alternatively add TE and proceed with Qiagen kit).

16. Add 200 µl Ethanol 70 % to the pellet.

17. Centrifuge for 10 min at 15000 rpm in a cooled centrifuge.

18. Aspirate Ethanol using yellow tips. Dry the DNA-pellet at room temperature. (Usually 15 min are sufficient; after drying no liquid drops are to be seen)

19. Dissolve pellet in 100 µl TE (1x, p.a. grade) and store at 4°C. (BREAK POSSIBLE)

20. Add cooled 7.5 M NH4Ac-solution (4°C, half of the solution volume). Mix carefully.

21. Add cool Ethanol 100 % (double of the solution volume). Mix carefully. Freeze for more than 2 h at -20°C. (BREAK POSSIBLE)

22. Centrifuge 30 min at 15000 rpm in a cooled centrifuge. Aspirate fluid carefully.

23. Add 200 µl Ethanol 70%.

24. Centrifuge 10 min. at 15000 rpm in a cooled centrifuge. Aspirate liquid and dry pellet at room temperature. Dissolve the pellet in 100 µl TE (1x, p.a. grade)

25. Repeat steps 20 to 23 with 3 M NaAC-solution (instead of NH4Ac), then proceed with step 26.

26. Centrifuge 10 min. at 15000 rpm in a cooled centrifuge. Aspirate liquid and dry pellet at room temperature. Dissolve the pellet in 100 µl TE (1x, p.a. grade).

Appendix Table 1. Regions and sites of Ethiopia from where the samples were collected

NO	Gene bank accession no	Code	Collection region	locality	Geographical location	
					Latitude (N)	Longitude (E)
1	19739	GOB 1	Oromia/Misrak/ Babile	Berkele/s 1	9°12’25. 33”	42°21’26.31”
2	19740	GOB 2	Oromia/Misrak/ Babile	Berkele/s 2	9°12’25. 35”	42°21’27.34”
3	19741	GOB 3	Oromia/Misrak/ Babile	Babile/Shek A.	9°11’54. 45”	42°21’41.22”
4	19742	GOB 4	Oromia/Misrak/ Babile	Awsherit 1	9°09’21. 23”	42°22’21.34”
5	19743	GOB 5	Oromia/Misrak/ Babile	Awsherit 2	9°09’21. 29”	42°22’21.28”
6	19744	GOB 6	Oromia/Misrak/ Babile	Lecole	9°07’36. 32”	42°21’02.33”
7	19745	GOB 7	Oromia/Misrak/ Babile	Ifa Gende 1	9°13’42. 11”	42°18’22.18”
8	19746	GOB 8	Oromia/Misrak/ Babile	Ifa Gende 2	9°13’41. 11”	42°18’22.28”
9	19747	GOB 9	Oromia/Misrak/ Babile	Ifa Gende 3	9°15’30. 21”	42°18’21.12”
10	19748	GOB 10	Oromia/Misrak/ Babile	Medigana 1	9°16’06. 13”	42°18’12.43”
11	19749	GOB 11	Oromia/Misrak/ Babile	Medigana 2	9°16’06. 21”	42°18’24.28”
12	19750	GOB 12	Oromia/Misrak/ Babile	Dendaro	9°17’25. 21”	42°17’25.14”
13	19751	GOB 13	Oromia/Misrak/ Babile	Tofic 1	9°16’06. 36”	42°17’36.25”
14	19752	GOB 14	Oromia/Misrak/ Babile	Tofic 2	9°16’06. 41”	42°17’36.32”
15	19753	GOB 15	Oromia/Misrak/ Babile	Berkele	9°10’48. 27”	42°18’23.12”
16	19754	GOB 16	Oromia/Misrak/ Babile	Gende	9°09’37. 42”	42°18’50.10”
17	19755	GOB 17	Oromia/Misrak/ Babile	Gemechu	9°07’25. 34”	42°18’54.03”
18	19756	GOB 18	Oromia/Misrak/ Babile	Tula	9°13’05. 11”	42°19’28.32”
19	19757	GOB 19	Oromia/Misrak/ Babile	Tula About	9°12’17. 23”	42°19’38.42”
20	19758	GOB 20	Oromia/Misrak/ Babile	Abdul 1	9°11’49. 22”	42°19’43.37”

21	19759	GOB 21	Oromia/Misrak/ Babile	Abdul 2	9°11’49. 28”	42°19’58.23”
22	19760	GOG 1	Oromia/Misrak/ Gursum	Llalemi 1	9°19’33. 14”	42°26’05.26”
23	19761	GOG 2	Oromia/Misrak/ Gursum	Llalemi 2	9°19’33. 38”	42°26’05.38”
24	19762	GOG 3	Oromia/Misrak/ Gursum	Awdal	9°18’19. 23”	42°26’11.36”
25	19763	GOG 4	Oromia/Misrak/ Gursum	Oda 1	9°19’14. 11”	42°27’06.18”
26	19764	GOG 5	Oromia/Misrak/ Gursum	Oda 2	9°18’37. 42”	42°28’38.21”
27	19765	GOG 6	Oromia/Misrak/ Gursum	Oda 3	9°18’40. 13”	42°28’38.16”
28	19766	GOG 7	Oromia/Misrak/ Gursum	Oda 4	9°18’30. 32”	42°29’41.38”
29	19767	GOG 8	Oromia/Misrak/ Gursum	Oda 5	9°18’30. 28”	42°29’41.25”
30	19768	GOG 9	Oromia/Misrak/ Gursum	Nur Selam 1	9°19’30. 26	42°28’38.32”
31	19769	GOG 10	Oromia/Misrak/ Gursum	Nur Selam 2	9°19’30. 53”	42°28’38.45”
32	19770	GOG 11	Oromia/Misrak/ Gursum	Odaa 1	9°21’54. 13”	42°29’50.34”
33	19771	GOG 12	Oromia/Misrak/ Gursum	Odaa 2	9°21’54. 24”	42°29’50.46”
34	19772	GOG 13	Oromia/Misrak/ Gursum	Abader	9°17’51. 28”	42°24’14.33”
35	19773	GOG 14	Oromia/Misrak/ Gursum	Harobata 1	9°17’12. 43”	42°23’43.24”
36	19774	GOG 15	Oromia/Misrak/ Gursum	Harobata 2	9°17’12. 38”	42°23’43.28”
37	19775	GOG 16	Oromia/Misrak/ Gursum	Harobata 3	9°16’12. 18”	42°23’18.21”
38	19776	GOG 17	Oromia/Misrak/ Gursum	Harobata 4	9°16’22. 20”	42°23’35.12”
39	19777	GOG 18	Oromia/Misrak/ Gursum	Awdal 1	9°17’20. 56”	42°26’26.65”
40	19778	GOG 19	Oromia/Misrak/ Gursum	Awdal 2	9°17’20. 48”	42°26’26.51”
41	19779	GSJ -1	Somalia/Jigjiga	Beledka	9°17’51. 23”	42°39’08.34”
42	24208	GAW -1	Amhara/Adoawe	Wangua	10°48’4 5.43”	36°25’35.12”
43	28662	GOBG1	Oromia/Bale	Ginir	7°11’86. 21”	40°37’46.21”