

ADDIS ABABA UNIVERSITY
INSTITUTE OF BIOTECHNOLOGY



**Ethnobotanical knowledge of lima bean (*Phaseolus lunatus* L.,
Fabaceae) in Gurage zone and genetic diversity study of collections
from oromiya and SNNPR regions of Ethiopia**

By Leyila Nasir Basit



February, 2020

Addis Ababa

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A Thesis submitted to the Institute of Biotechnology in partial fulfillment
of the degree of Master of Science in Biotechnology

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This is to certify that the thesis prepared by Leyila Nasir, entitled “Diversity in farmer’s varieties of lima bean (*Phaseolus lunatus* L., *Fabaceae*) in Gurage zone and genetic diversity study of collections from oromiya and SNNPR regions of Ethiopia” submitted in partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology complies with the regulations of the University and meets the standard with respect to originality and quality.

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Abstract

Ethnobotanical knowledge of lima bean (*Phaseolus lunatus* L., *Fabaceae*) in Gurage zone and genetic diversity study of collections from oromiya and SNNPR regions of Ethiopia

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Msc Thesis, 2019
Addis Ababa University

Lima bean is one of the five most important legume crops of the world in the genus Phaseolus. However, in Ethiopia it is one of the orphan crops and there is no improvement strategy of this crop. The main objective of this study was to investigate the ethnobotanical importance of lima bean in Gurage zone and the genetic diversity of collections from Ethiopia using ISSR marker. The field study was carried out in December 2018 focusing on 6 kebeles distributed in 3 Woredas within the zone by interviewing a total of 84 informants comprising 72 general informants and 12 key informants aged between 19 and 75 years. For molecular genetic diversity study, a total of 96 samples were collected from five administrative zones of Ethiopia. Genomic DNA was isolated using CTAB method and 13 ISSR primers of which eight amplified a total of 106 clear and reproducible bands. A total of 7 lima bean farmer varieties were found and all of the farmers give names to their varieties based on seed color and seed size. Lima bean was mainly cultivated as a sole crop in the field and intercropped in the home gardens in Gurage zone. Informants indicated that lima bean is an important food item mainly consumed in the form of 'kik' (split grains for sauce making) and 'nifro' (cooked grains). The farmers produce lima bean mainly for home consumption. The genetic diversity among lima bean populations indicated that West Wellega showed the highest genetic diversity (0.1864) whereas Jimma showed the lowest (1.614). AMOVA demonstrated highly significant ($P=0.00$) genetic diversity among and within populations. A considerable proportion, 66 % of the total genetic diversity was distributed among populations and 34% within population. In addition, the calculated F_{st} value was high ($F_{st}=0.66$), associated with a low gene flow value ($N_m=0.27$), indicating lower differentiation of the populations, which, in turn, implied no significant exchange of planting materials among farmers in the studied populations. Unweighted Pair-Group Methods Using Arithmetic Mean Averages (UPGMA), STRUCTURE and Principal Coordinate (PCoA) analysis showed very strong grouping among individuals collected from the same zone and geographically distinct zones. Overall, ethnobotanical knowledge and genetic diversity achieved from this study could be used as base line datafor lima bean conservation and improvement strategy in Ethiopia.

Key word's: Accessions, AMOVA, genetic diversity, farmers' variety, Principal Coordinate Analysis

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ACRONYMS

AMOVA	Analysis of molecular variance
CTAB	Cetyltrimethylammonium bromide
EBI	Ethiopian Biodiversity Institute
GIS	Geographical information system
FAO	Food and Agriculture Organization of the United Nations
ISSR	Inter simple sequence repeat
ODK	Open data kit
SH2	Warm sub-humid lowland
SH3	Tepid sub-humid mid-highland
UPGMA	Unweighted Pair-Group Methods Using Arithmetic Mean Averages

1. INTRODUCTION

Legume seeds are important staple food and source of dietary minerals that potentially provide all of the 15 essential minerals required by humans (Heuze *et al.*, 2013). Lima bean (*Phaseolus lunatus* L.) is one of the five domesticated species of *Phaseolus* with the family fabaceae and the second most cultivated species of this genus after the common bean (*P. vulgaris* L.) (Baudoin *et al.*, 2004). It is believed to have originated in the Neotropics in the general area of Guatemala, Mexico and Peru. The distribution routes were from the Pacific foothills of Mexico, through the Central and South Americas to Southern Canada (Rafael, 2001). It was later spread to Southern Asia, extended to West and Central Africa. Van der Maeseen and Sadikin (1989) asserted that lima bean entered the African continent through the Portuguese explorers, particularly to the Central and Western parts of Africa from Brazil, during the slave trade (Amoatey *et al.*, 2000).

Lima bean is a true diploid ($2n=22$) and seed-propagated species (Martinez *et al.*, 2004). It is an annual or short-lived perennial species, with a mixed mating system that is predominantly autogamous but with a high out crossing levels reaching up to 48% (Baudoin *et al.*, 1998). The wild forms of *Phaseolus lunatus* are characterized by a prolonged flowering period, production of large number of pods and indeterminate climbing growth (Zorobi *et al.*, 2003). The seeds are different from each other in their size, shape, color and appearance of eye. They are herbaceous plants that are either perennial or annual in nature, but usually grown as annuals and a vegetative period within the range of 80 - 90 days or 120 days and beyond depending on the variety (Ibeawuchi, 2007).

Lima bean is generally grown in a wide range of ecological conditions and there are types that are suitable to low altitudes, humid and sub-humid tropical climates (Freytag *et al.*, 2002). Its seeds have high protein (210 - 260 g/kg) and high carbohydrate contents (550 - 640 g/kg), low fat (10 - 23 g/kg)

and fiber levels (32 - 68 g/kg), high levels of minerals such as K, Zn, Ca and Fe, and low levels of Na and P (Oshadi, 1993). It is an important source of protein for rural populations in South America and Africa and a subsistence crop in the humid tropics of America (Ibeawuchi, 2007).

Lima bean (butter bean) is one of the important grain legumes grown in South Western Ethiopia. The dried seeds are eaten after getting them cooked with other cereals and pulses known as 'nifro'. It is cultivated the home gardens or intercropped with cereals in the field. Seeds are variable in size, shape and color (EBI, 2012).

Ethiopia is among the major bean producers in Sub-Saharan Africa. However, national average bean yield is still declining behind the global average. This can be attributed largely to low-yielding capacity of cultivars under use, biotic and abiotic stresses, and low soil inorganic nutrients (Asfaw *et al.*, 2009).

Genetic erosion, which is defined as the reduction or loss of genetic diversity within and among populations of the same species over time (Jarvis *et al.*, 2000), is a major concern today particularly in the cases of neglected and underutilized orphan crops like lima bean. To the basis of our knowledge there have not been such studies on this crop in Ethiopia. Understanding of the molecular genetic diversity of lima bean populations and studies on farmers' varieties diversity are important steps to initiate the improvement and management of the crop.

This research is designed to combine information of farmers' knowledge from the field including the diversity of farmers' varieties (landraces), local use and management along with its ethnobotanical knowledge in Gurage Zone of Southern Nations, Nationalities and Peoples Region (SNNPR) of Ethiopia and genetic diversity analysis of collections from five zones of Ethiopia using a suitable marker, inter simple sequence repeat (ISSR) Such assessment of the genetic diversity of lima bean is an

important input for the genetic improvement and better use of the crop. This will ultimately support the farmers by improving different traits like grain yield, fodder quality, and household nutrition among many other traits.

1.1 Research Questions, Hypotheses and Research Objectives

1.1.1 Research Questions

- Are there different types of lima bean in Gurage zone? What are they, and which ones are more frequently cultivated by farmers and which ones are most preferred for specific purposes and commonly used by the user community in the study area?
- What are the local names of the landraces of lima bean and what are their respective meanings as understood and explained by the local language?
- Which agro ecological zone in the study area is very important for the growth and productivity of *Phaseolus lunatus*?
- What are the main production constraints of *Phaseolus lunatus* in the study area (pests, diseases, environment, others)?
- What is the genetic variation within and among the Ethiopian *Phaseolus lunatus* populations using ISSR markers?
- What is the genetic distance and similarity among different lima bean populations?

1.1.2 Research Hypotheses

The major hypotheses to be tested include:

- Farmers in Gurage Zone are not knowledgeable about the farmers' varieties diversity, the local uses and management of lima bean (Null hypothesis).
- There is a genetic distance and genetic similarity of *Phaseolus lunatus* present in Ethiopia using ISSR marker (Alternative hypothesis).

1.1.3 Objectives of the Study

1.1.3.1 General Objective

- To assess the diversity in farmers' varieties of lima bean (*Phaseolus lunatus* L.) and associated ethnobotanical knowledge in Gurage zone and to study genetic diversity of collections from Ethiopia.

1.1.3.2 Specific objectives

- To document the occurrence of lima bean to produce a checklist of farmers' varieties types cultivated in Gurage Zone of SNNPR using farmers' criteria and nomenclature;
- To identify and document the farmers' varieties diversity and uses of lima bean by recording vernacular names reported by farmers together with their uses;
- To investigate the distribution of lima bean farmers' varieties in Gurage zone, agroecological zones, administrative units and socio cultural areas;
- To study molecular genetic diversity of lima bean populations growing in different parts of Ethiopia (Gurage, Gamo Gofa, West Wellega, Kelem Wellega and Jimma);
- To investigate genetic diversity among different accessions of lima bean in Ethiopia.

2. LITERATURE REVIEW

2.1 Origin and Distribution of Lima Bean

Lima bean (*Phaseolus lunatus* L.) is one of the five cultivated crops from the *Phaseolus* species, with its origin in the Neotropics. It is believed to have originated from Guatemala, Mexico and Peru. Lima bean comprises of two botanical varieties; *Phaseolus lunatus* var. *silvester* (wild types) and *Phaseolus lunatus* var. *lunatus* (domesticated types). The *Phaseolus lunatus* var. *silvester* belongs to the Mesoamerican and Andean origin. The *Phaseolus lunatus* var. *lunatus* comprises of a smaller group of intermediate genotypes (Lioi and Gallasso, 2001). The wild lima bean is one of the few species for which multiple domestication events have been documented, giving rise to two cultivars, namely the 'Sieva' and 'Potato' which are small- seeded and the large- seeded types called 'Big lima' (Fofana *et al.*, 2001).

The distributions of both types were from the Pacific foothills of Mexico, through the Central and South Americas to Southern Canada (Rafael, 2001). It was later spread to Southern Asia and through the slave trade, extended to West and Central Africa. Van der Maeseen and Sadikin, (1989) asserted that lima bean entered the African continent through the Portuguese explorers, particularly to the Central and Western parts of Africa from Brazil during the slave trade (Amoatey *et al.*, 2000).

2.2 Botanical Classification

The genus *Phaseolus* is strictly concentrated in tropical and warm temperate America. Lima bean is an important economic crop of the genus *Phaseolus* and one of the 12 primary grain legumes (Fofana *et al.*, 2001). It is consisted of approximately 70 species of which five are domesticated (Delgado, 1985). These include common bean (*P. vulgaris* L.), lima bean (*P. lunatus* L.), scarlet runner bean (*P. coccineus* L.), tepary bean (*P. acutifolius* A. Gray) and year bean (*P. polyanthus* L.). Lima bean belongs to the family *Leguminosae/Fabaceae*, tribe *Phaseoleae* and Sub tribe *Phaseolinae*.

2.2.1 Botanical Description and Morphology

The lima bean is a warm season plant propagated through its seed (Vander Maeseen and Sadikin, 1989). They are twining vines or herbaceous plants that are either perennial or annual in nature, but usually grown as annuals and depending on the variety it has a vegetative period within the range of 80 days - 90 days or 120 days and beyond (Ibeawuchi, 2007). Baudoin *et al.* (1998) stated that it is an annual or short-lived perennial species. It exhibits two kinds of growth habits. The annuals are pseudo determinate which appear in a bush form, having terminal and axillary flowers and thin roots (Baudoin, 2006).

The perennial types are indeterminate and are mainly climbing and trailing in nature with axillary flowering and has swollen and fleshy roots (Ibeawuchi, 2007). The climbing types have vines or stems aided by stakes to provide support. The leaves are alternate and tri foliate in nature and have ovate to lanceolate leaflets. Inflorescences are 15 cm long and bear white to yellowish or violet bisexual flower which are usually less than 1 cm in length (Heuzé *et al.*, 2013). Beyra and Artiles (2004) reported that the flower is pale green, occasionally violet in color. Lima bean pods are flat, oblong and generally curved. Depending on the cultivar type, they are of 5-15 cm in length and are either glabrous or sparsely pubescent and set 2-4 seeded (Baudoin, 2006). Pods are uniformly green in color for the immature bean and light brown in mature ones. Seeds vary in size, shape and color (Baudoin, 2006) inferred that cultivar groups have been distinguished according to seed differences; the large seeded (*Phaseolus limensis*) and the smaller seeded 'baby Lima' or 'Sieva' bean (*Phaseolus lunatus*). Seeds have varied color ranging from white with black spots, green, brown, red, speckled and mottled, cream with speckled red or stripped red. The average size of these seeds ranges from 1 - 1.5 cm in length and 0.8-1.5 cm in width, and are flat kidney- like to rhomboid in shape (Baudoin, 2006).

Lima bean germination and emergence take 4 -10 days after sowing usually under favorable rainfall. The seed undergoes epigeal germination (Ecoport, 2011). Vegetative phase of growth hastens usually after a month and its growth cycle is completed in about 100 days. The outer two flowers develop at the nodes simultaneously, with the middle structure lagging in development. The latter flower albeit continues to develop at a faster rate if one or both outer structures abscise and have to develop again (Kee *et al.*, 2004). The flowering period takes 35 - 70 days and pods ripe 80-120 days after sowing with short day length (Van der Maeseen and Sadikin, 1989). However, other lima bean cultivars have two cycles per year depending on rainfall distribution. Flowering and fruition occur throughout the rainy season. The crop undergoes self and cross pollination processes. Pollen and stigma of the plant mature at the same time and due to closer proximity to unopened bud, self-pollination ensues. Cross pollination, however, often occurs and 75-80 % of the flower buds and young pods are shed under field conditions (Ibeawuchi, 2007). Seeds of the crop are dispersed through explosive dehiscence of pods in the wild.

2.3 Lima Bean Production and Utilization

2.3.1 Climatic Adaptation

Lima bean (*Phaseolus lunatus* L.) is generally a hardy species suitable to low altitude, humid and sub humid tropical climates, although it can be grown in a wide range of ecological conditions (Freytag *et al.*, 2002). Lima bean is found from sea level up to altitudes higher than 2000 m. It is usually found in warm temperate zones as well as arid and semi-arid tropical regions. Ustimenko- Bakumovsky (1983) reported that the lima bean is more sensitive to temperature conditions than the common bean (*Phaseolus vulgaris*) and temperatures below 16-17° C and above 35° C are unfavorable for its growth. Average rainfall that supports the crop is 900-1500 mm per year. However, once the crop is established, it can tolerate rainfall as low as 500-600 mm (Baudoin, 2006). The crop thrives well in

drained and aerated soils that are sandy loam. Despite its affinity for rainfall, lima bean can survive in marginal areas that experience severe abiotic stress like drought (FAO, 1984). The crop shows resistance to high salinity and can be short-day in its light requirement. Fofana *et al.* (2001) maintained that lima bean comprises photoperiod-insensitive types that flower in day lengths up to 18 hours, and short-day types that require a day length as short as 11.0-12.5 hours to initiate flowering. The crop also thrives in soils with pH of 6-6.8 (Lyman *et al.*, 1985) although it is tolerant in acid soils with pH of 4.4 (Baudoin *et al.*, 2006).

2.3. 2 Production and Crop Yield

Countries noted largely for production of the crop include the USA, Madagascar and Peru (Akande and Balogun, 2007). USA is the largest producer while production in Africa is rather low. In the past two decades, the total annual production was 50,000-100,000 tones in countries such as Ghana, Cote d'Ivoire, Nigeria and DR Congo altogether (Baudoin, 2006). Lima bean is grown mostly in gardens or backyards, usually intercropped with other crops (Broughton *et al.*, 2002). Optimum yield of fresh seeds ranges from 2.0 t ha⁻¹ to 8.0 t ha⁻¹ Eco crop (2011). In the tropical regions, the average dry seed production is 1.0 t ha⁻¹ to 1.5 t ha⁻¹. Yields of dry seed may reach 2 -2.5 t ha⁻¹ for annual lima bean types (bush types) and 3- 4 t ha⁻¹ from climbing types (Baudoin, 2006). However, the type of cultivar and cultivation practices can affect yield of lima significantly (FAO, 2002). Kee *et al.* (2004) reported that on the production yield of lima bean in Delaware, weather conditions affected flower bud development, pollination and pod maturation. Baudoin (2006) stated that climbing types may yield 3.0 t ha⁻¹ to 4.0 t ha⁻¹ under experimental conditions in the tropics (Eco crop, 2011).

2.3.3 Utilization of Lima Bean

Lima bean is a pulse crop usually grown for its enlarged seeds (IBPGRI, 2005) and cultivated primarily for its immature and dry seeds (Van der Maeseen, 1989). The lima bean is an important source of protein and vitamin and has the potential to alleviate malnutrition in rural areas of

developing countries. According to Oliveira *et al.* (2004), the lima bean is actually an alternative food source for human alimentation in the Northeast of Brazil. It can be dried and shelled, ground or as whole green beans and can be canned or frozen. It can also be baked, boiled and fried in oil. The young pods and leaves are consumed as vegetables in countries such as Ghana and Malawi (Broughton *et al.*, 2002). Seeds can be processed into porridges, puddings and cakes by the Yorubas, from Nigeria. The seeds and leaves can also be taken as beverages. In most parts of Asia, the young plants or leaves are consumed (Daisy, 1979 and Van der Maeseen, 1989). It is also used in soups and stew and in a variety of dishes such as succotash (Floridata, 2012).

Various parts of the crop have some medicinal properties. Juice from the leaves is used in nasal instillations against headache and as eardrops against otitis in Senegal and DR Congo (Baudoin, 2006). In traditional Asian medicine, both seeds and leaves are valued for their astringent qualities and consequently used as a diet for fever (Roecklein, 1987). In other parts of Africa, seeds are processed into powder and applied into small cuts on tumours to promote suppuration (Baudoin, 2006).

Lima bean straw (dried vines left after harvest) provides fodder to cattle and sheep. Dairy cows can be fed on young vines which are highly nutritive (Ishler and Adams, 2010). Vines cut at stage where leaves are still green. The vines can easily be converted into silage (Ishler and Adams, 2010) and fed to growing and milking cows. Ajayi (2011) indicated that silage made from young vines before flowering, incorporated with fresh Napier grass (*Pennisetum purpureum*) and pineapple increased dietary protein content, nutrient digestibility, nitrogen absorption and retention and reduction in weight loss of goats during the dry season. In addition, silage made from vines of lima bean, as compared to other crops such as Pigeon pea (*Cajanus cajan*) or African yam bean, produced optimal growth rate and weight gain in goats (Ajayi *et al.*, 2012).

2.4 Potential Constraints to Lima Bean Production

2.4.1 Diseases and Pests

One of the major diseases in lima bean is the stem anthracnose which affects the aboveground portions of the plant (Baudoin, 2006). Symptoms of the disease include severe pod blight and discoloration of the bloom. Other fungal diseases of notable interest are the damping off which hinders successful germination stands of plants and root rots which cause small sunken lesions and reddish-brown coloration on roots (Long *et al.*, 2014). Pests also cause serious damage to yield and quality of the crop. Root knot nematodes tend to affect the underground portions of the plant and aphids suck fluids from the plant creating a honey dew substance behind which result in pale yellowish color on leaves (Long *et al.*, 2014).

2.4.2 Cyanogenic glucosides

Leaves and seeds of lima bean contain toxic substances such as cyanogenic glucosides and linamarase, which have the potential to cause rapid respiration, drop in blood pressure, headache, vomiting and convulsions (Heuzé *et al.*, 2013). Baudoin (2006) reported that domesticated species contain a lower amount of cyanoglucosides (100-120 ppm) as compared to wild species (2000-2400

ppm). Cyanogenic potential varies with respect to maturity, stage of development, genotype and cultivation conditions. Young leaves and seeds have higher HCN (hydrogen cyanide) potential as compared to older leaves (Ballhorn *et al.*, 2005). It is important, therefore, to soak seeds overnight and cook lima beans properly to ensure most of the HCN is removed.

2.4.3 Anti-nutritional factors

Raw beans of lima bean contain anti nutritional factors mainly protease inhibitors and lectins which act negatively on the digestive tracts (Adeparusi, 2001) and affect growth and metabolism in broilers (Achi *et al.*, 2007). The foliage also contains factors such as phytic acid, saponins and tannins (Ajayi *et al.*, 2009). These factors can be eradicated using heat or moist treatment (Adeparusi, 2001).

2.5 Farmers' varieties diversity

Farmers' varieties have originated together with agriculture and horticulture during the past 10,000 years or so. Hence, Farmers' varieties of many crops have probably been grown for several millennia. Though Farmers' varieties are commonly considered as endemic to a particular region, they have always been moved over short or even long distances and thus brought into competition with autochthonous Farmers' varieties if present (Zeven, 1998). Farmers' varieties have been recognized by farmers for adaptation to specific sets of field conditions as well as particular uses within the food system. They are often highly variable in appearance, but they can be identified and almost always have different local names. They have, as their particular characteristics, a reputation for adaptation to local climate and cultural practices, and resistance or tolerance to diseases and pests (Harlan, 1992). Major advantages of Farmers' varieties are adaptation to their specific agro-systems, low input requirements, and ethnic reasons are also present in traditional agricultural practices. The genetic diversity of Farmers' varieties is very important for global biodiversity conservation for future world production (Wood and Lenne, 1997). Farmers' varieties generally provide high yield stability and intermediate average yields under a low input agricultural system (Zeven, 1998).

2.6 Genetic diversity

Genetic variation is essential for the development of improved cultivars, as well as survival of the species. Knowledge, access, and use of the available diversity in domesticated and wild relatives are essential for broadening the genetic base of cultivars to sustain improvement (Singh, 2005). Biological markers could have many different types but mainly classified into morphological, biochemical and molecular types. The morphological (classical or visible, agronomic traits) markers are phenotypic traits while biochemical markers are isozymes, and are based on gene product. The DNA markers which reveal sites of variation in DNA are marker with better resolution of diversity. Each type of marker system has its own advantages and disadvantages. The type of marker is selected based on the species types, availability and cost benefit analysis (Semagn *et al.*, 2006). Morphological markers are usually visually described phenotypic characters such as flower color, seed shape, growth habits or pigmentation (Winter and Kahl, 1995). Morphological markers are phenotypic markers with distinguishable trait that have evident to human eyes (Bagali *et al.*, 2010).

Isozymes markers are differences in enzymes that are detected by electrophoresis and specific staining (Winter and Kahl, 1995). Biochemical analysis is based on the separation of proteins in to specific banding patterns. It is a fast method which requires only small amount of biological material; however, only a limited number of enzymes are available and thus, the resolution of diversity is limited (Mondini *et al.*, 2009).

DNA markers are the most widely used types of markers predominantly due to their abundance. They arise from different classes of DNA mutations such as substitution mutation, point mutation, rearrangements, insertion and deletion; or errors in replication of tandemly repeated DNA (Paterson, 1996)

2.6.1. DNA markers

DNA markers are based on naturally occurring polymorphisms in DNA sequences (i.e.: base pair deletions, substitutions, additions, etc). They are superior to both morphological and biochemical markers because they are relatively simple to detect, abundant throughout the genome even in a highly inbred cultivars, completely independent of environmental conditions and can be detected at virtually any stage of plant development (Gupta *et al.*, 1999). According to Vithanage *et al.* (1995) molecular markers are "landmarks" which can be identified on the genome and therefore, offer the best possible means of identifying individuals from biological samples. Molecular markers can be applied in the identification of cultivars and clones, genetic mapping, marker assisted selection (MAS), population genetics, molecular systematic, etc. (Weising *et al.*, 2005). In recent years different marker systems were applied.

RAPD was the first PCR based molecular marker technique developed and it is by far the simplest (Williams *et al.*, 1990). Short PCR primers (approximately 10 bases) are randomly and arbitrarily selected to amplify random DNA segments throughout the genome. The resulting amplification product is generated at the region flanking a part of the 10 bp priming sites in the appropriate orientation. RAPD products are usually visualized on agarose gels stained with ethidium bromide and/or other nucleic acid staining chemicals. AFLP technique combines the power of RFLP with the flexibility of PCR-based technology by ligating primer recognition sequences to the restricted DNA (Semagn *et al.*, 2006). The first step in AFLP analysis involves restriction digestion of genomic DNA (about 500 ng) with a combination of rare cutter (EcoRI or PstI) and frequent cutter (MseI or TaqI) restriction enzymes. Double-stranded oligonucleotide adaptors are then designed in such a way that the initial restriction site is not restored after ligation. Such adaptors are ligated to both ends of the fragments to provide known sequences for PCR amplification (Saeed *et al.*, 2009). Complexity of

the bands can be reduced by adding selective bases to the primers during PCR amplification. Microsatellites, Simple Sequence Repeats (SSRs), or Short Tandem Repeats (STRs), are repeating sequences of 1-6 base pairs of DNA. SSR allelic differences are, therefore, the results of variable numbers of repeat units within the microsatellite structure (Francesco *et al.*, 2013). SSR are becoming the most important molecular markers in both plants and animals. SSR are very polymorphic due to the high mutation rate affecting the number of repeat units (Francesco *et al.*, 2013). PCR reactions for SSRs is run in the presence of forward and reverse primers that anneal at the 5' and 3' ends of the template DNA, respectively. SSR markers are highly informative due to co-dominance, multiallelism, heritability, abundance and wide coverage of the genome.

ISSR involves amplification of DNA segments present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. ISSR are semi-arbitrary markers amplified by polymerase chain reaction (PCR) in the presence of one primer complementary to a target microsatellite. Each band corresponds to a DNA sequence bordered by two inverted microsatellites (Tsumara *et al.*, 1996). 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 amplified products are usually 200–2000 bp long and amenable to detection by both agarose and polyacrylamide gel electrophoresis. ISSRs have been used in genetic diversity studies in different crop plants (Brenner, 2011), including, the crop lima bean (Martinaz, 2008): common bean (Sadeghi and Cheghamirza, 2012) It does not require genome sequence information; it leads to multi locus, highly polymorphous patterns and produces dominant markers (Mishra *et al.*, 2003). ISSRs exhibit the specificity of microsatellite markers, but need no sequence information for primer synthesis enjoying the advantage of random markers (Joshi *et al.*, 2000). The technique is simple, quick, and the use of radioactivity is not essential. ISSR markers usually show high polymorphic. However, like RAPDs, whereas reproducibility, dominant inheritance and homology of co-migrating

amplification products are the main limitations of ISSRs. A Single Nucleotide Polymorphisms (SNP) is a genetic variation when a single nucleotide (i.e., A, T, C, or G) is altered and kept through heredity. SNP's, represent sites in the genome where DNA sequence differs by a single base when two or more individuals are compared. They may be individually responsible for specific traits or phenotypes, or may represent neutral variation that is useful for evaluating diversity in the context of evolution. SNPs are the most widespread type of sequence variation in genomes discovered so far (Carla *et al.*, 2015).

3. MATERIALS AND METHODS

3.1 Description of the study area

The study of lima bean diversity was undertaken in five administrative zones distributed in SNNPR and Oromia regions of Ethiopia. For molecular genetic diversity study include a total of 96 accessions were collected from five zones namely Gurage, Gamo Gofa, Kelem Wellega, West Wellega and Jimma zones (Fig. 1). Seeds of lima bean were obtained from farmers' traditional seed storage containers, local markets and farmers' fields by using the seed collecting format of Ethiopian Biodiversity Institute (EBI) and planted at Addis Ababa University. The ethnobotanical survey was conducted at Gurage zone. The zone has 15 Woredas (districts); from those three Woredas namely Enemorna Ener, Abeshegea and Kebena were purposively selected based on widespread production of lima bean and the presence of multiple agro-ecological areas which provide opportunities for comparison of the ethnobotanical knowledge of different farmers' varieties. Samples from the collected seeds were deposited at the EBI for conservation purpose.

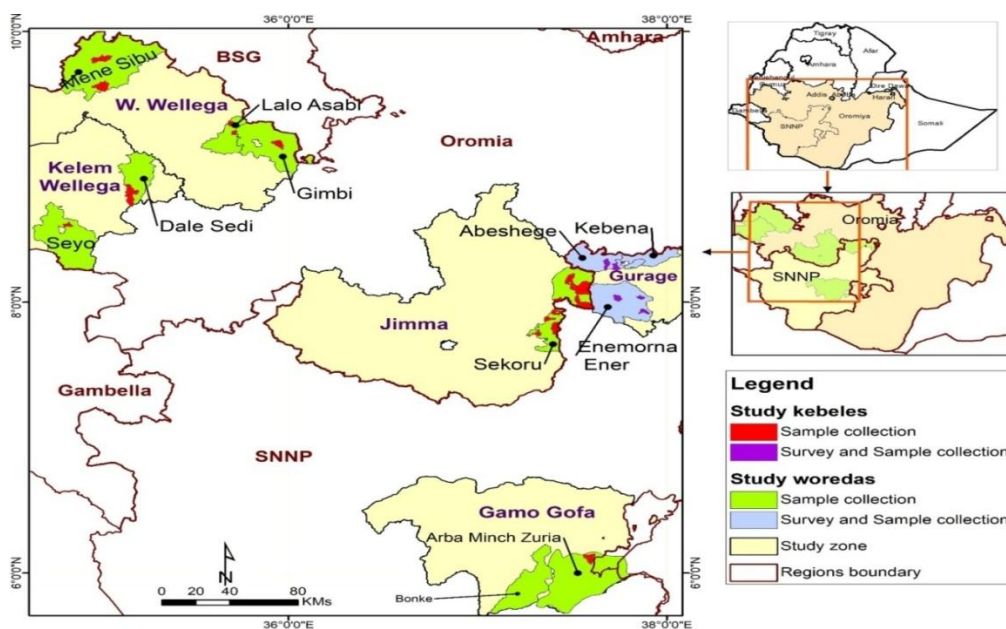


Figure 1: Map of Ethiopia showing the survey and sample collection areas

Global Positioning System (GPS) using android phone were used to collect georeferenced data of interviewee and seed collection sites. Digital camera was used to document pictures of the landraces and landscapes and android phone was used to conduct structured interview. The Open data kit (ODK) was used to record informant responses to the structured and semi-structured interviews. Seed sample bags were used for collecting different farmers' varieties of lima bean seeds.

3.1.1. Site selection

After purposively selecting the three Woredas/districts (Enemorna Ener, Abeshegea and Kebena) within the Gurage zone, study sites (sub-districts/kebeles) were selected to get areas that show greater diversity and production potential. This was done by visiting different parts of the area and farmers' fields and discussing with DAs and knowledgeable people of the area. Three kebeles each were selected randomly from each of the two agroecological zone for ethnobotanical study.

3.2. General informant selection for structured interview

The survey was done based on the information from local Agricultural Development Agent on the farmers who produce lima bean in the study area. Twelve randomly selected farmers (6 low-income and 6 middle/high-income households (3 women from high-income households, 3 men from high income households, 3 women from low-income households and 3 men from low-income households) from each kebele. A total of 72 general informants (12 informants x 3 kebeles x 2 strata = 72 general informants) considering differences in gender and age were interviewed (Fig. 2).

3.3. Key informant selection for semi-structured interview

The key informants were selected from the farmers who have already responded to the structured interview by ODK based on the recommendations made during the interviews. One man and one woman farmers who are knowledgeable about lima bean (*Phaseolus lunatus*) were selected from each kebele as key informants. A total of 12 key informants from 6 kebeles, i.e., 2 key informants from each kebele (2 x 6=12). (Figure 2)

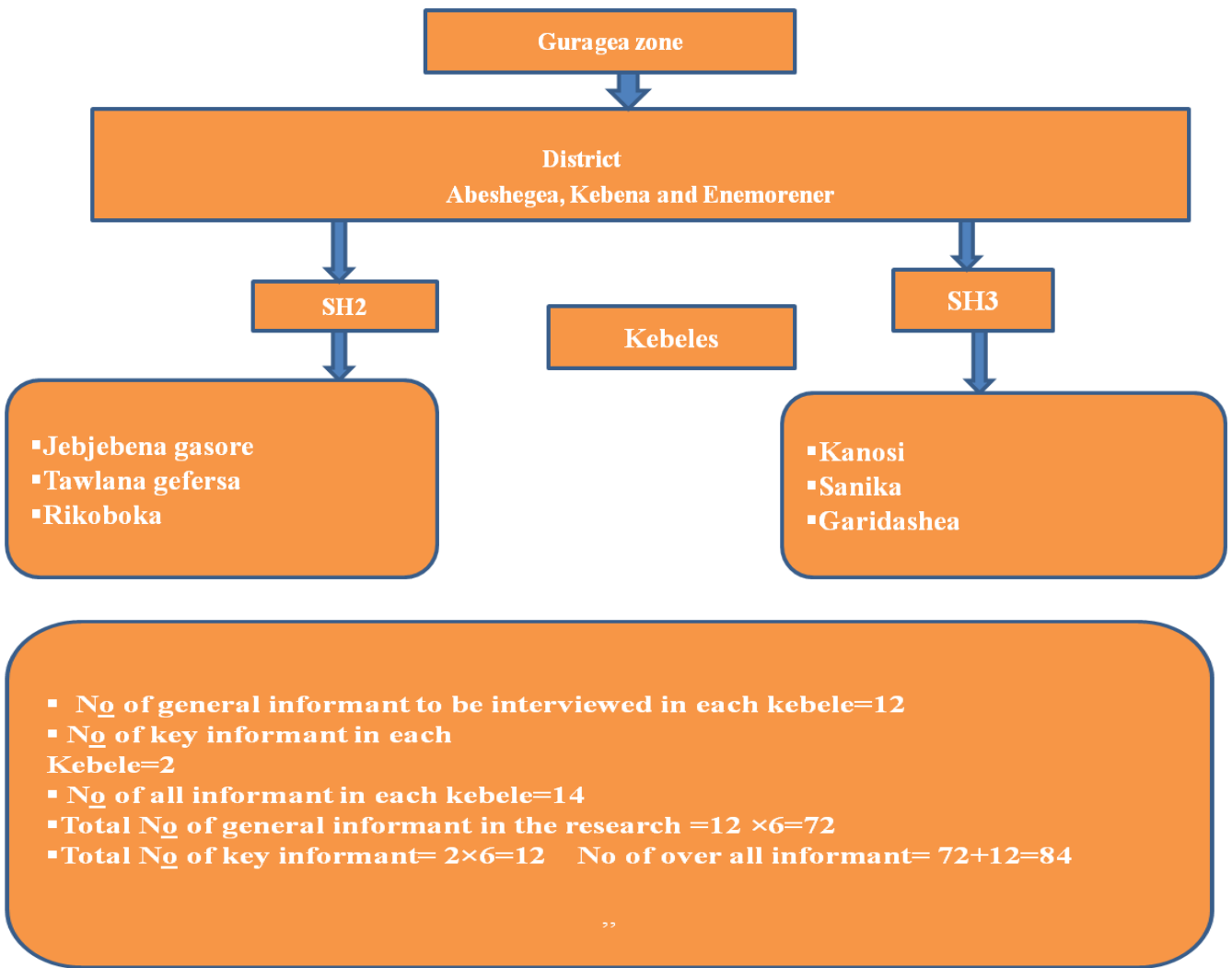


Figure 2: Schematic diagram of sampling kebeles within districts and different categories of informants

3.4. Field observation and guided field walk

Field observations were supported by local guides, language translators and participating informants to obtain the necessary data in the study area. The necessary information on how lima bean is cultivated, intercropped, its use and market value was systematically recorded. The local perspectives on vegetation type, dominant crops produced, landscapes and soil type of the study area were identified through personal observation and discussion with local experts (Development Agents) and elders.

3.5. DNA extraction

DNA extraction was performed at Plant Molecular Laboratory, Institute of Biotechnology, Addis Ababa University using modified CTAB extraction methods (Borche *et al.*, 2003). About 300 mg of single young leaves was ground in liquid nitrogen with mortar and pestle.

3.6. Genomic DNA quality and quantity detection

Agarose gel (50ml, 0.5xTBE and 0.8% agarose) was prepared and 4 µl of each genomic DNA samples mixed with 2µl of 6x loading dye was loaded on to the gel and electrophoresed at constant voltage of 85 V for 45 min. Gel picture was taken under UV transilluminator by Biodoc Analyses 2.0 with digital canon camera. Detection and visualization of isolated genomic DNA was tested on 0.8% (w/v) agarose gel.

Furthermore, the concentration and purity of the isolated genomic DNA was quantified by using NanoDrop (NanoDrop™2000/2000c) spectrophotometer at absorbance of 260 nm, 230 nm and 280 nm. OD ratios A260/A280, A260/A230 were used to estimate the quality and quantity of the DNA. Finally, DNA samples were adjusted to concentration of 100 ng /µl by diluting with sterilized distilled water and later it was stored at –20° C until used for PCR.

3.7. Primer screening

The ISSR marker assay was conducted at the Plant Genetics Laboratory of the Microbial, Cellular and Molecular Biology Department, College of Natural Sciences, Addis Ababa University. A total of 13 primers were screened for polymorphism and reproducibility using DNA of two individuals from each population (Table 1).

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

Primer	Annealing temperature	Primer sequence (5' to 3')	Repeat motives
DPISR1	55° C	AGGAGGAGGAGGAGGAGG	Tri-nucleotide
DPISR2	52° C	AGAGAGAGAGAGAGAGAGAGG	Di-nucleotide
DPISR3	52° C	AGAGAGAGAGAGAGAGAGAGC	Di-nucleotide
DPISR4	57° C	AGAGAGAGAGAGAGAGAGAGT	Di-nucleotide
DPISR6	52° C	CTCTCTCTCTCTCTCTCTG	Di-nucleotide
DPISR7	57° C	CTCTCTCTCTCTCTCTCTT	Di-nucleotide
DPISR8	55° C	GACACGACACGACACGACAC	Penta-nucleotide
DPISR9	45° C	ACTGACTGACTGACTG	Tetra-nucleotide
DPISR11	55° C	AGAGAGAGAGAGAGAGAGAG	Di-nucleotide
DPISR12	45° C	GACAGACAGACAGACA	Tetra-nucleotide
UBC834	45° C	AGAGAGAGAGAGAGAGAGYT	Di-nucleotide
UBC810	42° C	GAGAGAGAGAGAGAGAT	Di-nucleotide
UBC812	45° C	GAGAGAGAGAGAGAGAA	Di-nucleotide

3.8. PCR and gel electrophoresis

PCR amplification was carried out in a 20 µl reaction mixture containing 100 ng/ µl of 1.5 µl template DNA, 12.2 µl H₂O, 0.4 µl dNTP mix (0.25 mM), 2µl PCR buffer (Mg Cl₂ free), 0.5 µl Taq buffer (25 mM), 1.6µl Mg Cl₂ (50 mM), 1.3 µl primer (50 pM) and 0.5 µl Taq Polymerase (5U/µl). The amplification program was initial denaturation at 94° C for 4 min followed by 94° C denaturation for 15 sec, annealing at 45° C/ 60° C depending on the primers used for 1 min, extension at 72° C for 1 min and 30 sec for 40 cycles and the final extension at 72° C for 7 min. using Biometra 2003 T3 Thermo cycler. The PCR products were stored at 4° C. The PCR products (8 µl of amplified DNA mixed with

2 µl of 6x loading dye) were electrophoresed using 1.6% agarose gel that was stained with 2 µl of 10mg/ml of ethidium bromide at constant voltage of 100 V for 1 h and 30 min.

3.9. Data analyses

3.9.1. Ethnobotanical data analysis

Descriptive statistics

Ethnobotanical data were first entered into the excel spreadsheet and summarized using descriptive statistics to identify the most widely used lima bean farmers' varieties in the study area. In order to determine proportions of different farmers' varieties, cultivation methods, importance as a fodder, food and income generation, specific use, plant parts used and others, such as various ethnobotanical ranking methods, percentage frequency methods and inferential statistics in order to test the consistency of respondents' information and to get scientifically more valid results.

Spatial diversity

According to Whittaker (1960), diversity is divided into various components. The best known are diversity in one spot that he called alpha diversity, and the diversity along gradients that he called beta diversity. But almost everyone understands that as a measure of general heterogeneity (Tuomisto, 2010): how many more species/variety is there in a collection of sites compared to an average site. The third type of diversity is Gamma diversity, which is the total number of landraces within a region or among farmers of a certain ethnic group. In the same way, the diversity of lima bean landraces was analyzed by calculating the average number of landraces listed by each farmer as alpha diversity and obtaining beta diversity by dividing gamma diversity by alpha diversity. This indicates the degree to which farmers within the same ethnic group or region share the same landraces.

3.9.2. Molecular genetic diversity data analyses

Each fragment that is amplified using ISSR primers was treated as a unit character and scored as '0' for absence, '1' for presence and '?' for missing data. Based on recorded bands different software were used for analysis. POPGENE 3.0 was used to calculate genetic diversity and Shannon's diversity index for each population and for each primer as number of polymorphic loci and percent of polymorphism.

The Pair wise Nei's genetic distances between populations were analysed. Based on such data the geographic location-based population cluster was constructed. Unweighted Pair-Group Methods Using Arithmetic Averages (UPGMA) cluster analysis was also constructed for 96 accessions by using NTSYS pc 2.0 version. The genetic relatedness among accessions was further investigated using Principal Coordinate Analysis (PCoA) using GenAlEx 6.503.

To examine the genetic structure of the 96 accessions, the genotypic data for ISSRs marker was analyzed using a model-based approach implemented in STRUCTURE. Analysis of molecular variance was carried out by computation of the distance between "haplotypes", each individual's data pattern as one "haplotype" and computing variance components for each level (Excoffier *et al.*, 2006). The Analysis of Molecular Variation (AMOVA) was used to analyze among and within population genetic diversity by using Arlequin 3.5.2.2 version.

4. RESULTS

4.1 Ethnobotanical study

4.1.1 Diversity of lima bean compared to other legume crops in the study area

In the study area farmers grow different legume crops, common bean (53% and 47%), faba bean (8% and 36%) and chickpea (75% and 15%) in SH2 and SH3. However, there were a few farmers (8%) who plant grass pea in SH2 and lupine in SH3 (Fig. 3).

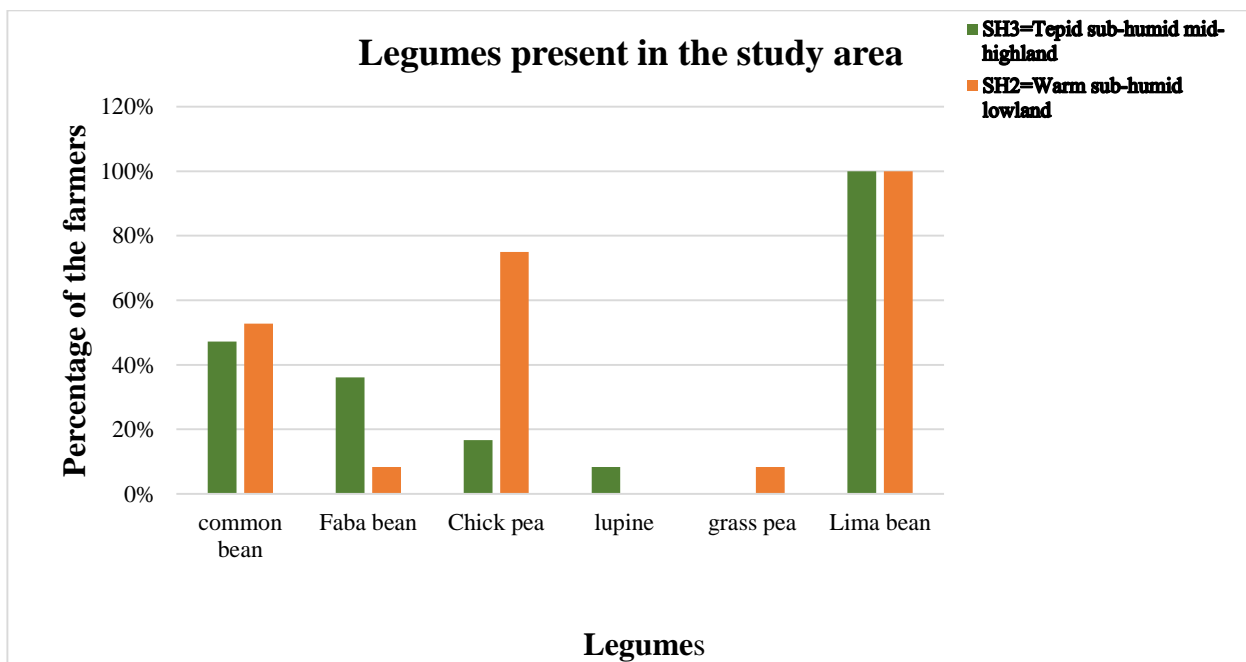


Figure 3. Farmers responses about lima bean cultivation as compared to other legumes in Gurage zone of SH2 and SH3 agro-ecology (n=72)

4.1.2 Distribution of farmers' varieties

A total of seven lima bean farmers' varieties were found to be cultivated by farmers in Gurage zone (Table 2). Of these, Guad/neche adengualo/adenguare, Burabure/bure adengualo/adenguare, Denjirye adengualo/adenguare, Zengurgure adengualo/adenguare are cultivated in both SH2 and SH3 agro-ecology. However, two varieties (Besha adenguare and Enseye adenguare) are only cultivated in SH2 and one variety (Genbenayegwad) in SH3. All varieties are planted mixed together in one field. The

result of the study indicates that there is a significant difference between SH2 and SH3 in the number of varieties (P value = 0.00). Not only the types of varieties are different, but also those varieties grown in the two agroecological zones differ in the amount of cultivation. More than 70% of farmers plant Buraburea/burea in SH2 and more than 50% of farmers grow Denjirye in SH3. A few farmers in SH3 grow a variety known as Enseye and Guad whereas farmers in SH2 grow Genbenayegvad variety (Fig. 4).

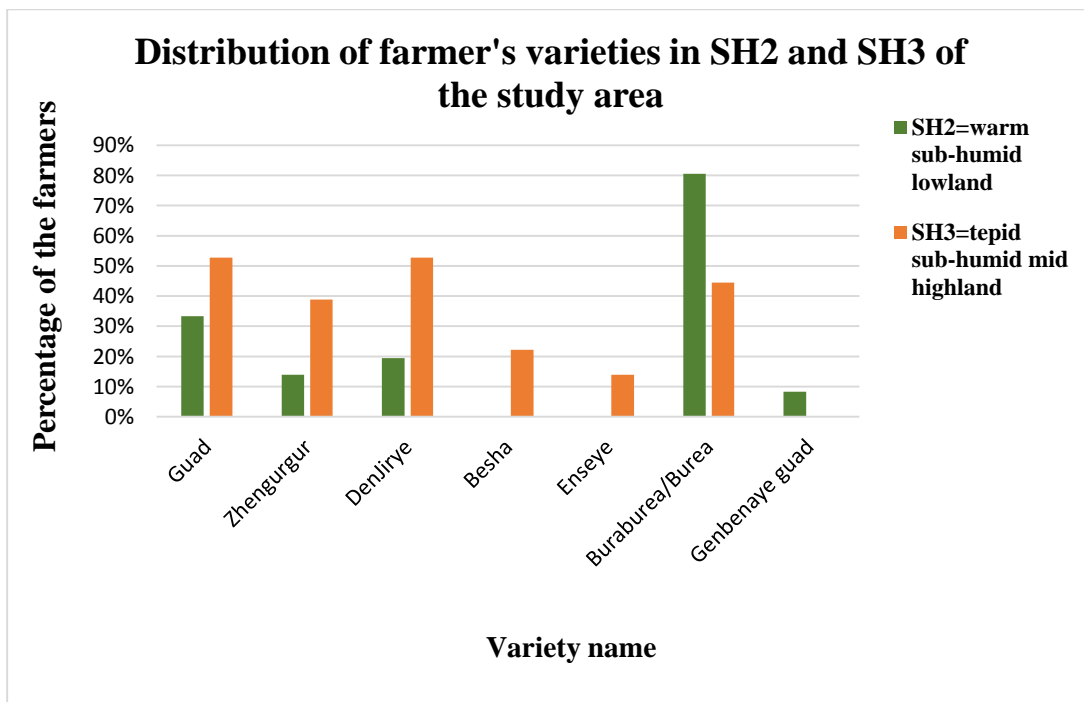


Figure 4. Distribution of farmer's varieties of lima bean in the study area (n=72)

Table 2: Vernacular names of lima bean landraces in Gurage zone and their meanings

Name	Zone	Woredas	Meaning
Guad/Nech Adenguarea	Guragea	Enemorna Ener, Abeshegea and Kebena	White-seeded lima bean
Denjirye/Telku Adenguarea	Guragea	Enemorna Ener, Abeshegea and Kebena	Large seeded lima bean
Enseye/Tenesu Adenguarea	Guragea	Enemorna Ener	Small seeded lima bean
Buraburea/Burea Adenguarea	Guragea	Enemorna Ener, Abeshegea and Kebena	White with red seeded lima bean
Genbenye Guad Adenguarea	Guragea	Abeshegea and Kebena	White with black seeded lima bean
Besha/Key Adenguarea	Guragea	Enemorna Ener	Red seeded lima bean
Zhingurgur Adengurea	Guragea	Abeshegea and Kebena	White with maroon seeded lima bean



Figure 5: Farmers' varieties of lima bean collected from Gurage zone

4.1.3 Landraces diversity and richness

The results of the study revealed that the diversity of farmers' varieties is different among the agroecological zones and study kebeles (Fig. 6). The total number of varieties (gamma diversity) is the highest in Jebjebenagasori (6) followed by Garidashe (5), Kanosi, Rikoboka and Sanika (4) and the lowest in Tawlanagefersa (3). The average number of varieties per household (alpha diversity) is the highest in Kanosi (2.33) followed by Sanika (2.25) and lowest in Rikoboka (1.5). Beta diversity was highest in Jebjebenagasori (3.43), and the lowest in Kanosi and Sanika (1.7). (Table 3)

Table 3: Diversity of the farmers' varieties within each Kebeles

Kebeles	Variety	Number of respondents	Alpha diversity	Gamma Diversity	Beta Diversity	
Garidashe		23	12	1.916667	5	2.63
Jebjebenagasori		21	12	1.75	6	3.43
Kanosi		28	12	2.333333	4	1.7
Rikoboka		18	12	1.5	4	2.6
Sanika		27	12	2.25	4	1.7
Tawlana gefersa		20	12	1.666667	3	1.88

4.1.4 Lima bean cultivation, cropping systems and management practices

4.1.4.1 Lima bean cultivation

Lima bean is grown along fence line and sometimes field margin in both SH2 and SH3 agro-ecologies. More than 95% lima bean growing farmers cultivated lima bean along a fence line in both SH2 and SH3 (Warm sub-humid lowland and Tepid sub-humid mid-highland) agro-ecology of Gurage zone, while 29% cultivated it both on their main farm fields and field margin in SH2 and less than 10% in SH3, however cultivating lima bean on home garden (6%) was practiced only in SH3 (Fig. 6).

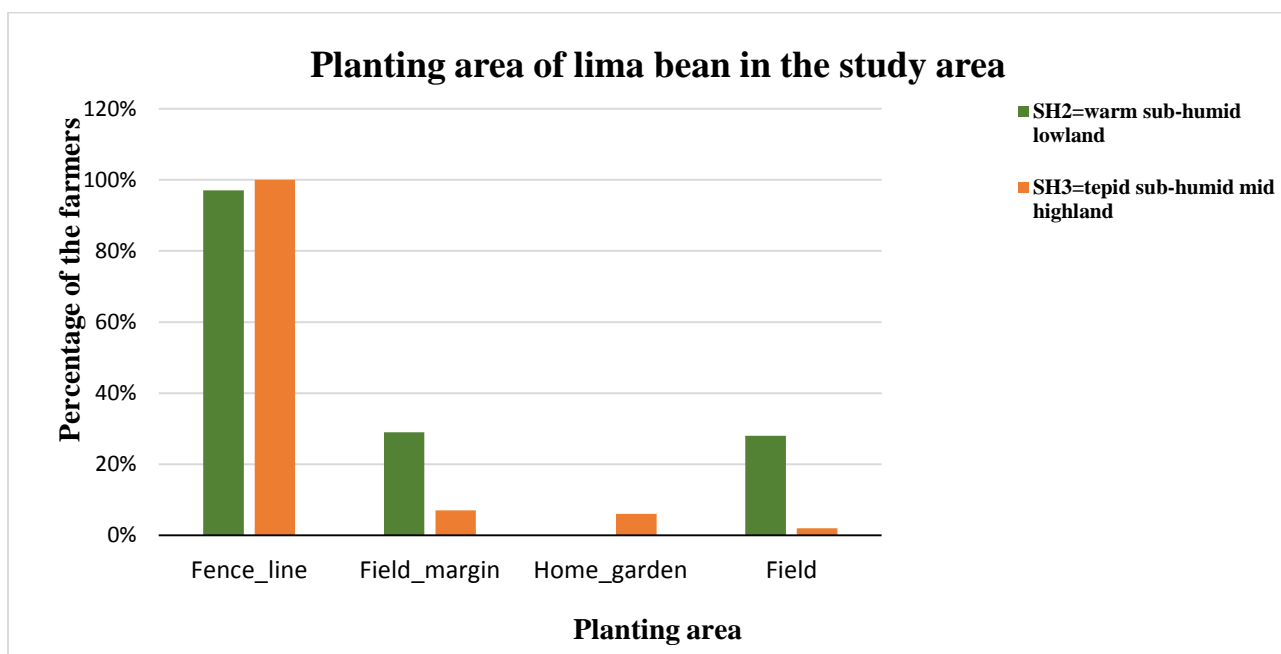


Figure 6: Lima bean planting area in Gurage zone (n=72)

4.1.4.2. Cropping system

In the study area, all lima bean varieties were mainly established as a sole crop and somewhat inter-cropping systems. Inter-cropping was practiced in the entire agro-ecological zones (SH2 and SH3) of Gurage administrative zone. In SH2, yam (*Dioscorea* spp.) (31%) was mostly used for intercropping with lima bean followed by maize, whereas in SH3 farmers intercropped lima bean with cassava

(*Manihot esculenta*) (25%) followed by yam (22%). In addition, 50% of the farmers in SH2 and 22% of the farmers in SH3 grow lima bean as a sole cropping (Fig. 7).

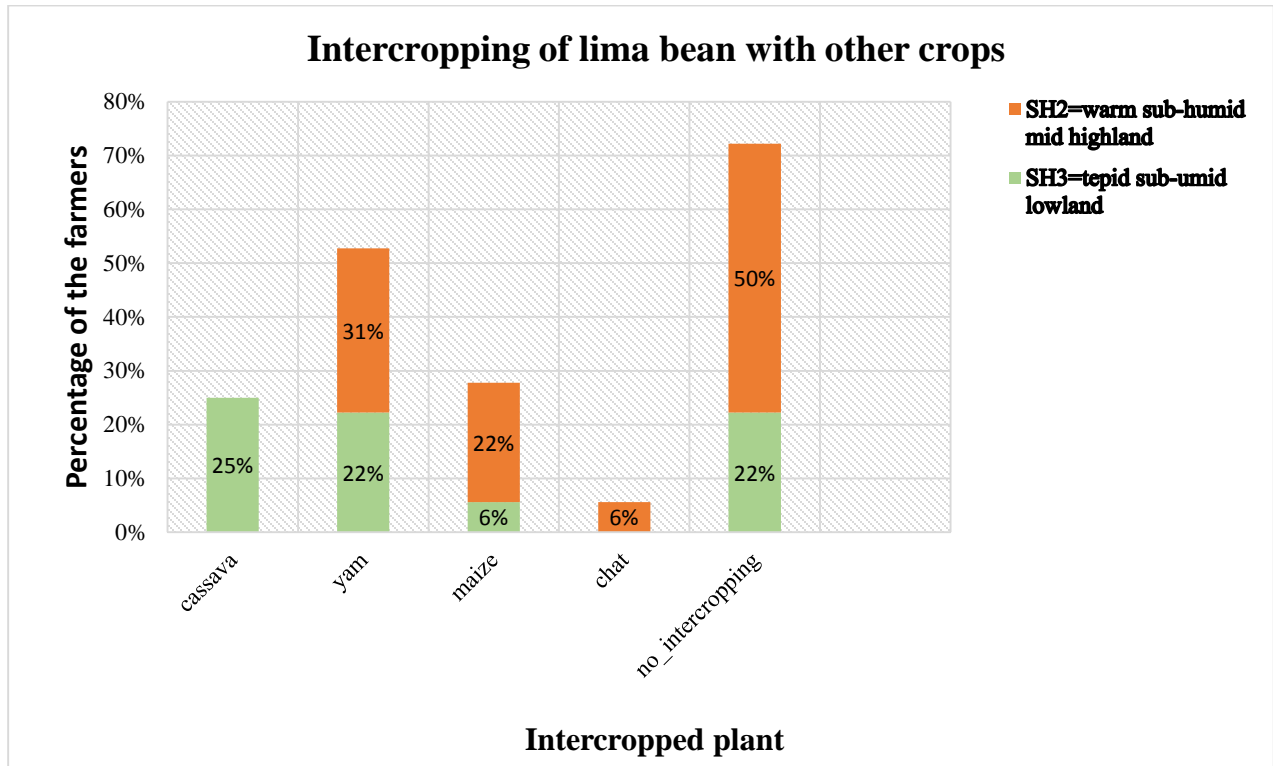


Figure 7: Intercropping of lima bean with other crops (n=72)

4.1.4.3 Management practices

Traditional management was practiced in the study area. As mentioned by informants, lima bean is a crop that needs minimum agricultural practices. Sowing of lima bean was done by direct broadcasting method to control seed quality and to get good yields, seed selection was undertaken for sowing purpose. Land ploughing is not done for cultivation of lima bean instead the farmers prepare the soil by hand with a hoe. Only some farmers who grow lima bean in main farm land plough their land. The crop was harvested by handpicking as soon as the seed feels hard within the pod, pod gets dried and change to yellow, before it starts to dehisce and scatter the seeds.

As key informants mentioned, 11 out of 12 (83%) didn't use fertilizer. Lima bean is weeded at least three times throughout the growing year. As the key informants mentioned, farmers prevent weeds by hand weeding. The majority of the farmers (94%) don't use herbicides to control weed and pesticide to control pest. However, some farmers use ash with animal urine and spread it on the lima bean plant to control pests.

4.1.4.4 Yield of lima bean under drought, water logging and high rain fall

In the study area the yield of lima been fluctuates according to different conditions. Under drought condition, the majority of the farmers (66%) get high yield, 25% get medium, 6% get very high yield and only 3% get low yield. However, under water logging condition, it's between high (2%) medium (23%), low (54%) and very low (1%). Therefore, under high rain fall, the majority of the farmers (85%) get medium yield (80%) (Fig. 8).

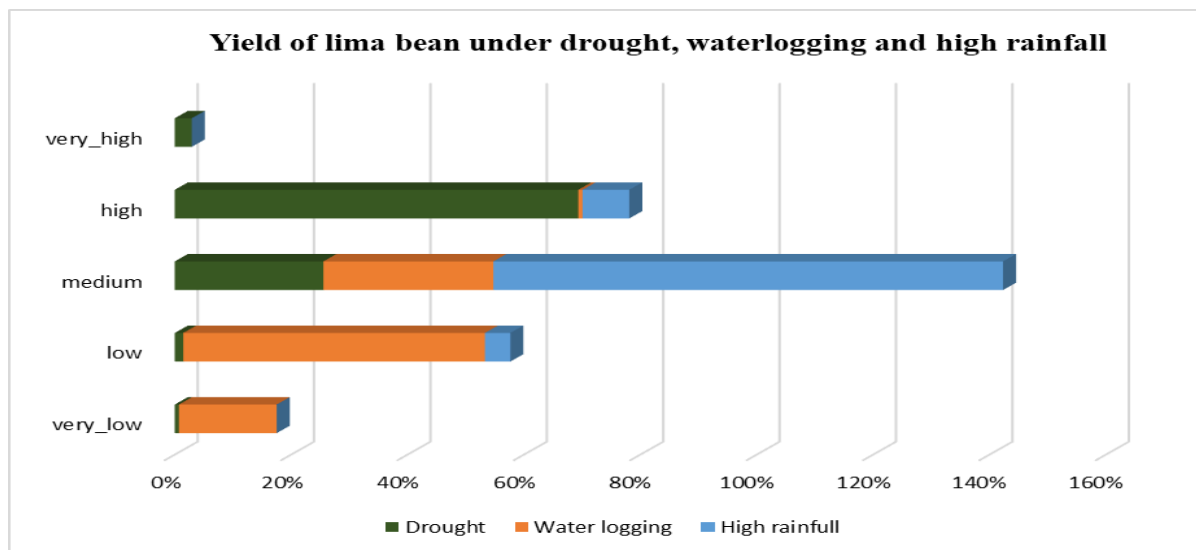


Figure 8: Yield of lima bean under drought, water logging and high rain fall

4.1.5. Importance of lima bean in the study area

4.1.5.1. Importance of lima bean as a food, feed and source of income

Traditionally, the local people have their own way of categorizing important values according to the value they provide like food, feed, income generation and medicinal value. Informants were asked to express their impression on how lima bean production contribution to improvement of their livelihood; food, fodder in addition to socio-economic and agroecological value of the landrace. Based on agro ecology and Woredas level lima bean has different uses (Fig. 9).

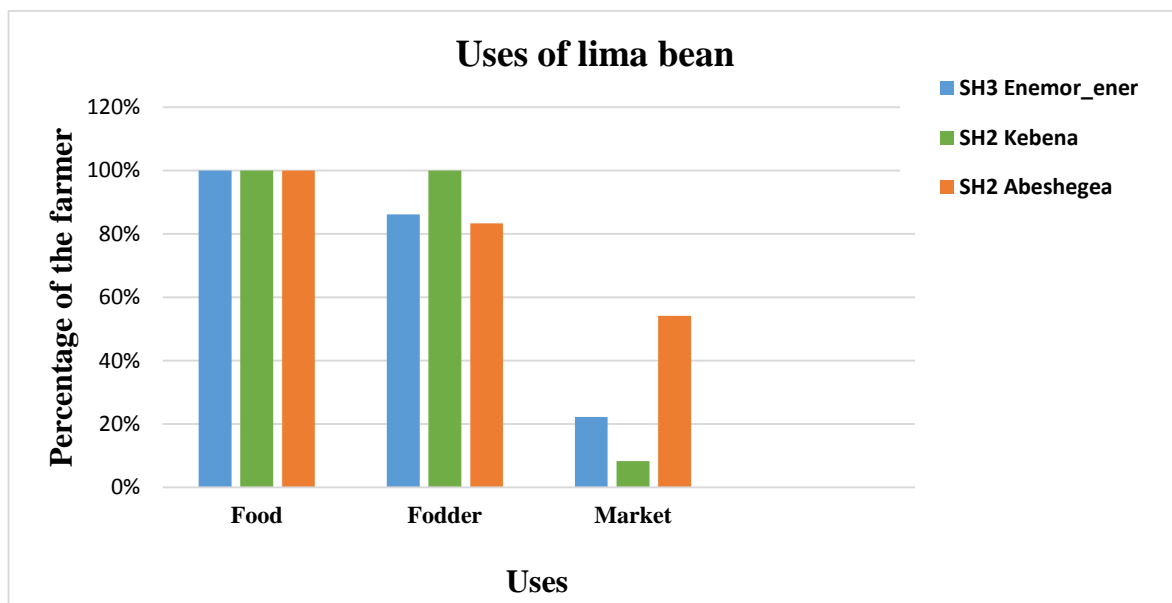


Figure 9: Use of lima bean within Woredas and agro-ecological zones

Table 4: The type of food prepared from lima bean varieties in Guragea Zone

Name of food	Common English name	Parts used	Mostly Preferred variety	The reason why this variety was preferred
<i>Kiki</i>	Stew of split grain	Seed	<i>All Variety</i>	
<i>Ayeb debelek</i>	Stew of split grain combine with cheesh	Seed	Guad/neche adenguarea/adengu alo	Good test The color of the seed is similar to cheese
<i>Nifro</i>	Boiled grain	Seed	Any Variety	

4.1.6. Local farmers perception about lima bean

In this study, key informants were asked what they think about the status of the production of lima bean in their area. All respondents said that they use lima bean just like meat or cow milk because it builds the body but its production has decreased over the years. According to the respondents, the reason is because of the nature of the crop, no improved new varieties, shortage of land, shortage of seed and damage by wild animals such as vervet monkey, .

4.1.7. Gender role in lima bean production

Men and women have different roles in lima bean production in the study area. All informants (n=72) reported that except collection of fodder for animals and harvesting, all production activities are performed by males. Thirty-six female informants also confirmed that lima bean production activities from sowing up to harvesting are performed by men. All twelve key informants said that it is the

custom of the place that crop production activities are done by men. However, in female headed households, productions of lima bean are done sometimes by female and neighboring men.

4.2 Molecular genetic diversity

4.2.1 Banding patterns and ISSR primers

Out of the 13 screened primers, eight of them resulted in clear banding pattern and were polymorphic so that they were used in this study (Table 5). The molecular weight of the fragments amplified using the eight primers were in the range of 200 bp to 3400 bp. A total of 106 fragments were amplified by the eight ISSR primers of which 95 (88.79%) were polymorphic. The highest number of bands (19) were exhibited by primer UBC 812 followed by DPISR1 and DPISR9 which generated 17 and 16 scorable bands, respectively. The least number of bands (8) were amplified by primer DPISR12.

Table 5: List of selected primers, amplification pattern, number of scorable band and repeat motives

Primers	Repeat motif	Amplification pattern	Number of scorable bands
DPISR1	(AGG)6	Excellent	17
DPISR9	(ACTG)4	Excellent	16
DPISR12	(GACA)4	Excellent	8
DPISR4	(AG)10T	Good	14
DPISR3	(AG)10C	Good	12
DPISR2	(AG)10G	Good	11
UBC812	(GA)8A	Good	19
UBC834	(AG)10YT	Excellent	9
Total			106

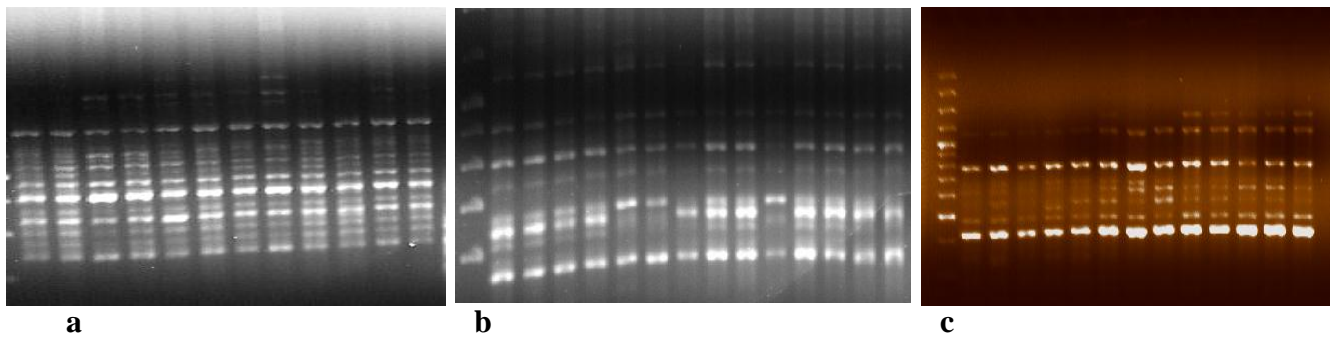


Figure 10. ISSR bands generated by DPISR1 (a), DPISR12 (b) and UBC834 (c) with 100 bp DNA ladder

4.2.2 Polymorphism

The number of polymorphic loci ranged from 8 for DPISR2, to 19 for UBC-812 and DPISR12 showed the least (50%) polymorphism whereas UBC 812, UBC 834, DPISR1 and DPISR9 showed the highest (100%) polymorphism. Among all the populations studied, West Wellega showed the highest percentage (48.95%) of polymorphism followed by Gamo Gofa (34.5%). Kelem Wellega showed 31.7% of polymorphism and Gurage zones showed 29.4%. The least percent polymorphism was exhibited by accessions from Jimma with 24.3% polymorphism. Generally, lima bean populations in Oromiya region showed higher percent polymorphism (34.9%) than that of SNNPR (32.15%).

Table 6: Number of polymorphic loci (NPL), percent polymorphism (PP), genetic diversity (H) and Shannon Information Index (I) of 96 lima bean accessions based on all primers used.

Population	With all primer			
	NPL	PP	H+SD	I+SD
West Wollega	53	49.53	0.1864	0.2753
Kelem Wollega	34	31.78	0.1677	0.2468
Jimma	26	24.3	0.1614	0.2328
Guragea	32	29.91	0.1848	0.2660
Gamo Gofa	37	34.58	0.1703	0.2480
Sum	182	170.1	0.8706	1.2689
Average	36.4	34.02	0.17412	0.25378
For individual primer				
DPISR1	17	100	0.1482	0.1908
DPISR9	17	100	0.1482	0.1908
DPISR12	4	50	0.1532	0.2179
DPISR4	11	78.67	0.1642	0.2384
DPISR3	11	91.67	0.1886	0.2498
DPISR2	8	72.73	0.2100	0.2936
UBC812	19	100	0.0906	0.1015
UBC834	9	100	0.1230	0.1439
Total	96	693.07	1.226	1.627
Average	12	86.63	0.1532	0.203338

4.2.3. Genetic diversity and Shannon Weaver's diversity indices

West Wellega accessions exhibited the highest gene diversity (0.1864) and Shannon information index (0.2753). The least gene diversity (0.1614) and Shannon information index (0.2328) was shown by accessions from Jimma. Primer DPISR2 showed highest gene diversity (0.21) and Shannon information index (0.29) and primer 812 showed the least gene diversity (0.09) and Shannon information index (0.10) (Table 6).

4.2.4. Analysis of molecular variance (AMOVA)

Partitioning of genetic diversity by analysis of molecular variance using grouped populations revealed that out of the total genetic diversity, most of the diversity was due to among populations diversity (66%), while the remaining is due to within populations' diversity (34%). Generally the results of AMOVA indicated larger genetic diversity among the populations rather than within populations and having F_{st} value of 0.66 and a gene flow of $N_m=0.2739$ (Table 7).

Table 7: AMOVA of lima bean populations

Source of variation	d.f	Sum of squares	Variance Components	Percentage of variation	Fixation Indices	P
Among Populations	4	1008.346	12.80809 Va	66.00	0.66000	0.00
Within Populations	91	600.425	6.59808 Vb	34.00		0.00
Total	95	1608.771	19.40617			

4.2.5. Genetic similarity

UPGMA dendrogram of lima bean accessions constructed based on Jaccard's similarity coefficient showed the highest similarity between West Wellega and Kelem Wellega populations (0.741) followed by between Gurage and Jimma populations (0.70). As it is shown on the UPGMA tree, Kelem Wellega and West Wellega are isolated from the same tree and this is also correlated with the close Zones of Wellega of Oromia region. The same is true for Gurage zone, Jimma and Gamo Gofa that formed their own group confirming the correlation of genetic distance with geographic distance (Fig. 11). Similarly the popgene result showed highest similarities between Kelem Wellega and West Wellega (0.8611) and the least similarity was observed between Kelem Wellega and Jimma (0.6011) (Table 8).

Table 8: Similarity matrix based on Jaccard's coefficients for 5 lima bean populations based on bands obtained using eight ISSR primers

Population name	Gurage	Jimma	Gamo Gofa	West Wellega	Kelem Wellega
Guragea	1.000				
Jimma	0.8536	1.000			
Gamo Gofa	0.7711	0.7384	1.000		
West Wellega	0.6487	0.6030	0.6800	1.000	
Kelem Wellega	0.6621	0.6011	0.7087	0.8611	1.000

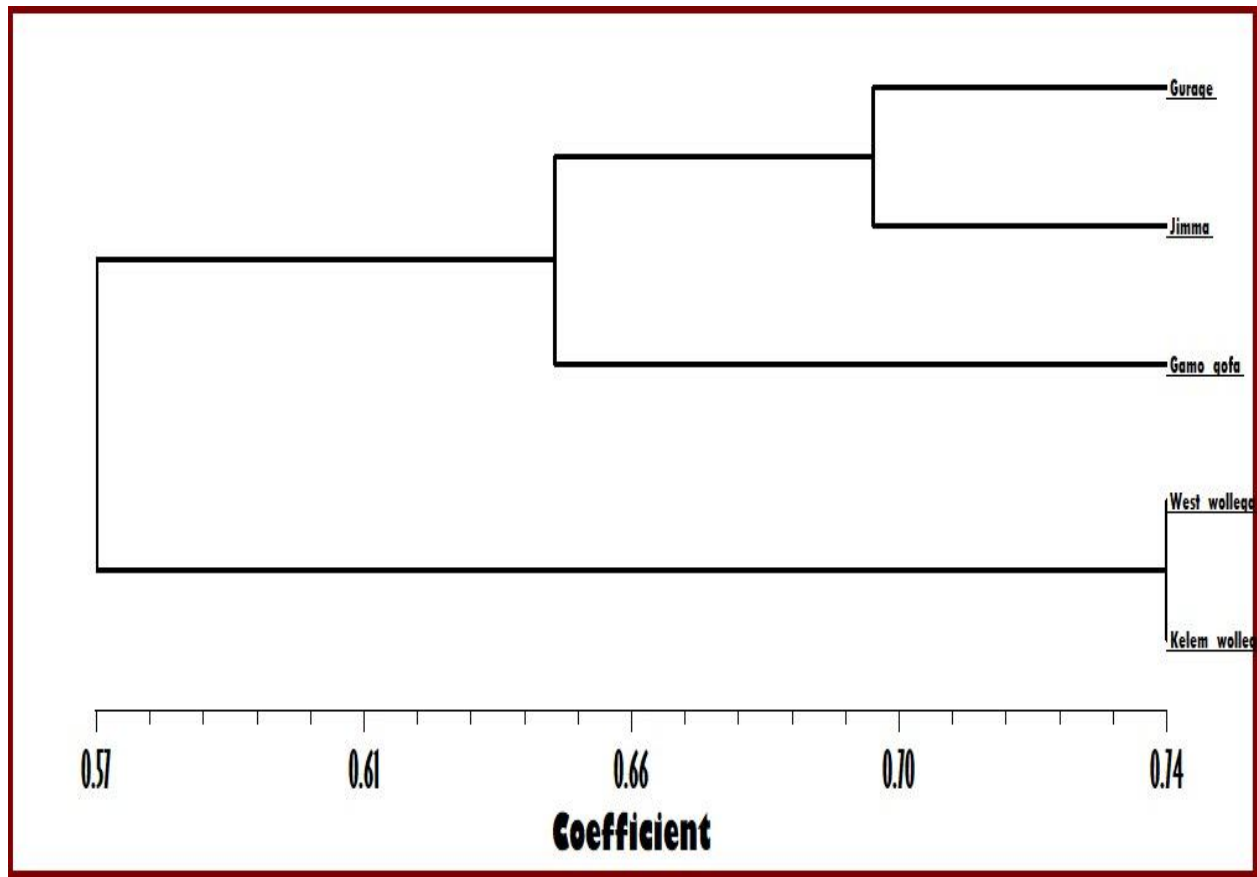


Figure 11: UPGMA based dendrogram for 5 lima bean populations using 8 ISSR primers

4.2.6. Genetic distance

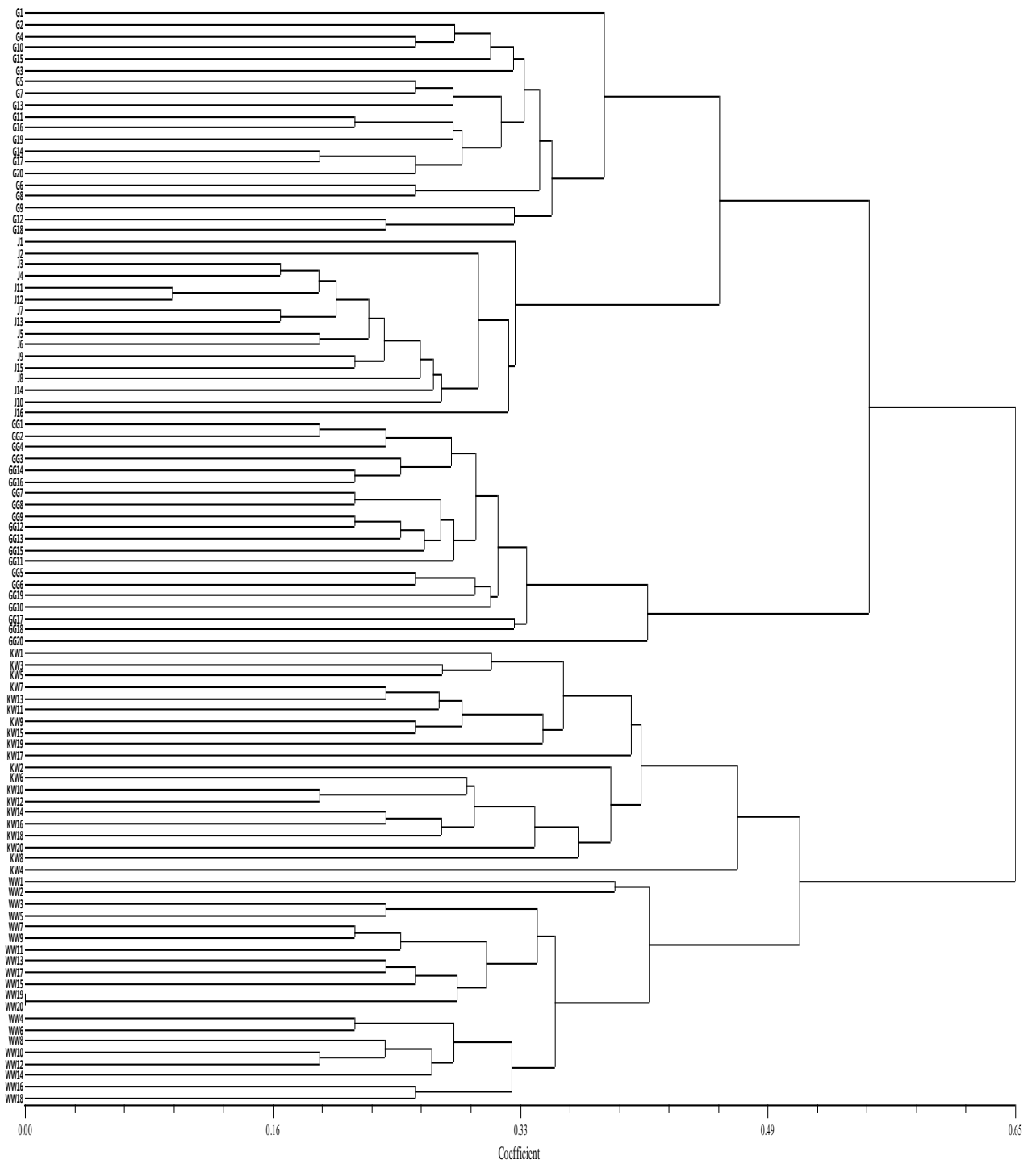
Genetic distance matrix based on Jaccard's coefficients revealed that the highest genetic distance (0.5089) was observed between Kelem Wellega and Jimma populations followed by West Wellega and Jimma (0.5059). The least genetic distance was observed between Kelem Wellega and West Wellega (0.1496) (Table 9).

Table 9: Genetic distance matrix based on Jaccard's coefficients for 5 lima bean populations using eight ISSR primers.

Population name	Gurage	Jimma	Gamo Gofa	West Wellega	Kelem Wellega
Guragea	0.00				
Jimma	0.1583	0.00			
Gamo Gofa	0.2599	0.3033	0.00		
West Wellega	0.4328	0.5059	0.3857	0.00	
Kelem Wellega	0.4123	0.5089	0.3444	0.1496	0.00

4.2.7 Cluster analysis

Jaccard's similarity coefficients were also used to construct UPGMA dendrogram for 96 individuals based on the bands obtained with the eight primers. Accordingly, individuals collected from West Wellega and Kelem Wellega tended to form strong separate group from the rest in UPGMA. However, populations collected from Gurage, Gamo Gofa and Jimma formed the same grouping based on their place of origin (Figs. 12 and 13). Generally, both individual UPGMA and population UPGMA trees revealed almost the same tree topology with similar groupings.



G= Gurage J= Jimma GG= Gamogofa KW= Kelem Wellega WW= West Wellega

Figure 12: UPGMA dendrogram depicting clustering patterns for 96 individuals of Lima bean based on Jaccard's similarity coefficient

4.2.8. Structure analysis

The result obtained from STRUCTURE software using eight ISSR primers for the 96 individuals of the five populations formed three groups, Kelem Wellega and West Wellega grouped together, individuals from Gurage and Jimma formed another group and individuals of Gamo Gofa also formed their own group (Fig. 13).

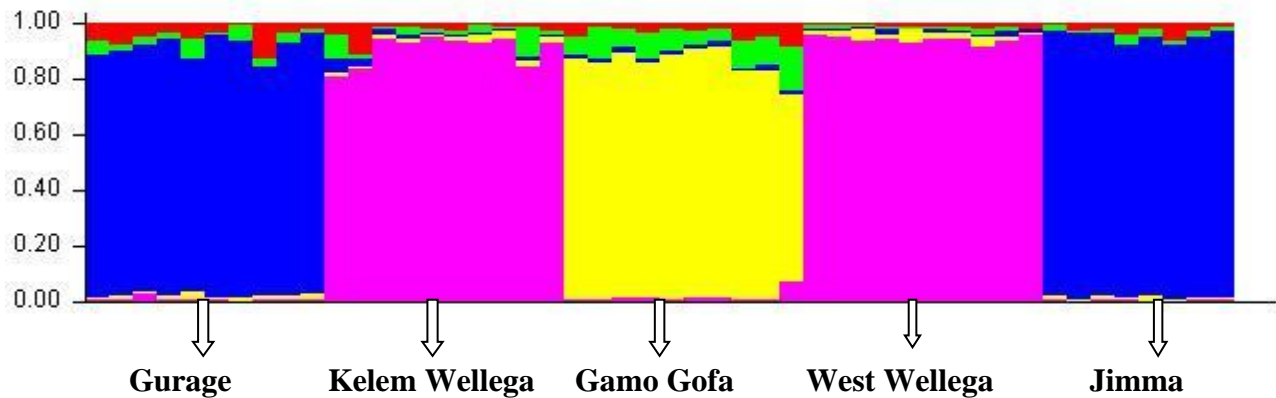


Figure 13: Structure analysis of 96 individuals of five populations of lima bean

4.2.9. Principal coordinates (PCO) analysis

Generally, individuals collected from West Wellega and Kelem Wellega clustered in the same group. However, populations collected from Guragea and Jimma were observed to form another grouping and Gamo Gofa form another based on their place of origin. (Fig 14).

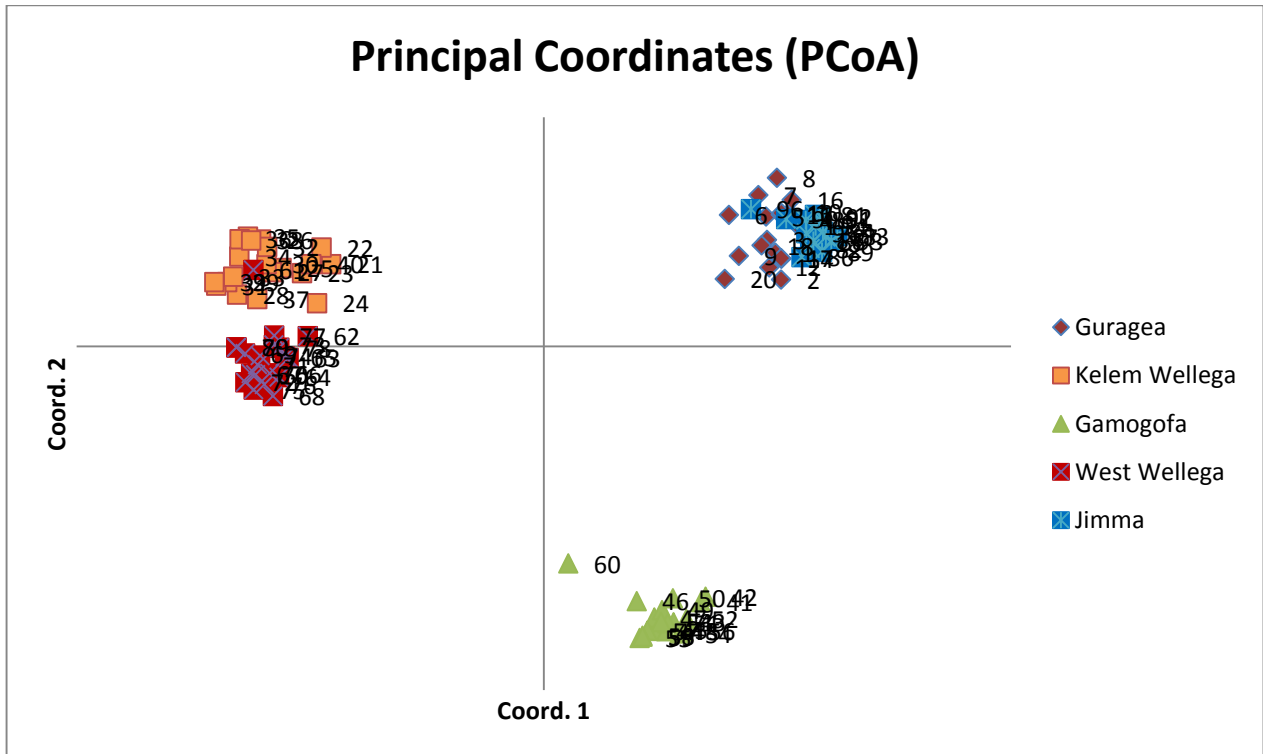


Figure 14: Two Dimensional representations of individuals belonging to five lima beans Populations based on Jaccard's similarity coefficient

5. DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

5.1.1 Diversity of farmers' varieties

Farmers' varieties have a high 'stability of their characteristics' and great 'resistance capacity to tolerate adverse influences'. Their production capacity, however, is less than that of cultivars and when grown outside their home region, their genetic composition will change (Zeven, 1998). In this study, informants' characterization and descriptions were used as means of distinguishing lima bean landraces. The majority of farmers who live in Gurage zone gave names for each lima bean variety and also they use some collective (generic) names. Informants identified and described a total of seven lima bean landraces based on the color and size of the seed because each variety has its own special use. For example, white lima bean has its own cultural importance. People in the Gurage zone eat kocho made from enset (*Ensete ventricosum*) dominantly with cheese. Since the white lima bean is similar with cheese in color and differ in test from the other varieties. To increase the mass and to give more taste, the local people use white lima bean by combining with cheese in the dish named as Aybe deblek (combined with cheese).

5.1.2 Cropping systems

In the study area assessed through survey the bush type of lima bean was never found, the pole type of all lima bean varieties were mainly established as a sole crop along a fence line and small number of farmers grow it by intercropped with other crops by providing them with good stalks probably because of the climbing nature of the crop. The main cash crops in the study area are chat (*Catha edulis*), coffee (*Coffea Arabica*) and enset. These crops are perennial in nature and some informants mentioned that when lima bean is intercropped with enset, it is not suitable for its growth. This may be due to climbing of the whole part of the plant and consequently inhibition of the transfer of water from the root to other

part. As a result, according to the responses of almost all (93%) of the informants who participated in the study, lima bean is not commonly rotated with other crops. This may be because of the perennial nature of the crop.

5.1.3 Management practices

Farmers' variety and seed management practices in traditional farming are often effective in achieving goals of adaptation and genetic gain in terms of yield but also in terms of maintaining formal, culinary and market traits (Clevel and Soleri, 2007). This study showed that local farmers played a great role in maintaining the landraces of lima bean by applying traditional farming systems, seed selection, seed storage and seed exchange practices. The traditional farming systems are followed by lima bean growing farmers in the study area. The field of lima bean land is not ploughed for sowing and fertilizer was not applied by most lima bean growing farmers. This may be because of the climbing nature of the crop and the nitrogen fixing ability of the crop to grow effectively without application of either manure or fertilizer. In addition to that lima bean is not a cash crop in the study area due to that it is not given much attention by farmers.

As respondents mentioned, they practice seed selection for the next season. Farmers' variety and seed management practices are based on farmers' experiences built from generation to generation. They have been the core elements of agricultural development since the domestication of crop plants and continue to be important for the future of agricultural crops (Margaret *et al.*, 2014).

5.1.4 Importance of lima bean in the study area

5.1.4.1 Food

In the study area, key informants informed us that lima bean is used as food, fodder, medicine and source of income. The seed is used in the form of green bean or as dry seed, cooked seed (*nifro*), combined with cheese (*Ayeb debelek*) and split seed (*kik*) but these uses vary based on agro ecology.

Lima bean is a pulse crop usually grown for its enlarged seeds (IBPGRI, 2005) and cultivated primarily for its immature and dry seeds (Van der Maeseen, 1989). The lima bean is an important source of protein and vitamin and has the potential to alleviate malnutrition in rural areas of developing countries (Oliveira *et al.*, 2004). In general, dry bean has high nutrient contents, commercial potential and atmospheric nitrogen fixing ability. Thus, it holds a great promise for increasing income, improving soil fertility as well as ensuring food security in Sub Saharan Africa (Margaret *et al.*, 2014).

The use of lima bean for food in Ethiopia seems to vary considerably with other countries, as some uses reported from other countries were not recorded in Ethiopia. For instance, it can be dried and shelled, ground or as whole green beans and can be canned or frozen. It can also be baked, boiled and fried in oil. The young pods and leaves are consumed as vegetables in countries such as Ghana and Malawi (Broughton *et al.*, 2002). The same source Shows that seeds can be processed into porridges, puddings and cakes by the Yoruba people in Nigeria. The seeds and leaves can also be taken as beverages. In most parts of Asia, the young plants or leaves are consumed (Van der Maeseen, 1989). It is also used in soups and stew and in a variety of dishes such as succotash (Floridata, 2012).

5.1.4.2. Feed

In this study, it was found that lima bean is used as fodder. Farmers feed livestock the straw, the seed, the fresh forage and the seed chaff as a nutritive feed. Lima bean straw (dried vines left after harvest) provides fodder to cattle and sheep. Dairy cows can be fed on young vines (cut at the stage when leaves are still green), which are highly nutritive (Ishler and Adams, 2010). The vines can easily be converted into silage (Ishler and Adams, 2010) and fed to growing and milking cows. Ajayi (2011) indicated that silage made from young vines before flowering, incorporated with fresh Napier grass (*Pennisetum purpureum*) and pineapple (*Ananas comosus*) increased dietary protein content, nutrient digestibility, nitrogen absorption and retention and reduction in weight loss of goats during the dry season. The straw is commonly used for fodder. As many respondents mentioned, it is very important for their livestock, it helps to make the animals very fat so that farmers get better income by selling them.

5.1.5 Molecular genetic diversity of lima bean population

In the present study, ISSR was used for the first time to assess genetic diversity of lima bean populations from Ethiopia. This method provides an alternative choice to other system for obtaining highly reproducible markers without any necessity for prior sequence information for various genetic analyses. Because of the abundant and rapidly evolving SSR regions, ISSR amplification has the potential of illuminating much larger number of polymorphic fragments per primer than any other marker system used such as RFLP or microsatellites. ISSRs are regions that recline within the microsatellite repeats and offer great potential to determine intra-genomic and inter-genomic diversity compared to other arbitrary primers, since they reveal variation within unique regions of the genome at several loci simultaneously. Several properties of microsatellites such as high variability among taxa, ubiquitous occurrence and high copy number in eukaryotic genome make ISSRs extremely useful markers for variability analysis (Morgante *et al.*, 2002).

Generally, populations from West Wellega and Gamo Gofa showed higher diversity than the other populations. AMOVA analysis resulted in higher genetic diversity among populations (66%) than within population (34%). This could be due to low seed exchange among different regions and markets which could lead to low intermix of populations between regions. Unlike other landraces of cultivated plants, lima bean in Ethiopia is restricted to a given area because the farmers grow lima bean along a fence line and use as home consumption. This agrees with the survey result in Gurage zone where the majority of the farmers use this crop for home consumption and very low number of farmers uses it for income generation in the local market. It is not widely exchanged among local community and markets. This shows that there is low gene flow between populations and regions. Martinez *et al.* (2006) Structure and Genetic Diversity of Wild Populations of Lima Bean (*Phaseolus lunatus* L.) from the Yucatan Peninsula, Mexico.. Luciana *et al.* (2013) who studied on Genetic structure of Lima bean (*Phaseolus lunatus* L.) landraces grown in the Mayan area. Gezahegne Girma *et al.* (2009) studied wild and cultivated rice species of Ethiopia using six ISSR primers and reported 38.3% and 28.3% polymorphism, respectively. These authors recommended that gene flow, genetic drift and evolutionary history might have important influence on genetic structure and diversity of a given population.

Lima bean is both self and cross pollinated plant hence, the proportion of genetic variation is dependent on the type of pollination that the species undergoes. If the species has large proportion of cross pollination, then we expect high genetic diversity within population and less divergence among populations. In addition to pollination, behavior of insects and germplasm exchange could facilitate gene flow among regions which could result in higher percent variation within population and less genetic structure. This is also supported with the spread of individual accessions on UPGMA, STRUCTURE and PCoA graphs with less intermix among populations. Dendrogram of the present

study by using UPGMA of Jaccard's coefficient of similarity showed West Wellega and Kelem Wellega populations of lima bean were closely related and Gurage, Jimma and Gamo Gofa form their own group indicating the correlation of genetic distance with geographical distance.

5.2 CONCLUSIONS

This research has confirmed the essential role that traditional farmers play in the development and maintenance of lima bean landraces. Local farmers in the study area have traditional cultivation, cropping, seed storage, seed selection and management practices. The majority of the farmers use sole cropping and somewhat intercropping for lima bean landrace cultivation. Even though the majority of farmers don't use selection criteria, some informants applied selection criteria based on seed color which revolved around the fulfillment of certain household needs.

Farmers in the study area and at market places indicated a decreasing trend in lima bean production because of the climbing habit of the crop which demands some kind of support. Farmers use lima bean as a source of food, feed and limited role in income generation. More than 95% of lima bean growing farmers in Gurage zone cultivated lima bean only on fence line whether grown in home garden or farm field while 8% of the respondents cultivated it both on their main farm field and home garden and the rest 3% cultivated it in home garden only. The present study assessed the extent of genetic diversity among Ethiopian accessions of lima bean using Inter Simple Sequence Repeat marker and found that West Wellega and Gamo Gofa populations showed the highest genetic diversity. Analysis of molecular variance for the accessions showed that the highest proportion of genetic diversity was attributed to among population than within population. It is also highly significant. This confirms that there was low level of gene flow and high level of genetic differentiation.

Further grouping of the genotypes into five groups (of varying constitution) was also evident in all the cluster analyses. Similarly, structure analysis using Bayesian models also identified $K=5$, as the optimum cluster number in the collection of lima bean accessions studied. The similarity in the

aforementioned results confirmed the presence of three groups Kelem Wellega and West Wellega in one group, Gurage and Jimma in the other group and Gamo Gofa form their own group. This result showed the correlation of genetic distance with geographic distance of the locations where seed samples were collected.

Seed collections of lima bean landraces, characterization and evaluation of local farmers' varieties must be applied. This will help the available landraces to be conserved. Analysis of genetic diversity in crop species using more than one method helps to better understand the levels of genetic variation and the genetic structure of populations. No single method is adequate for assessing genetic variation in germplasm collections, because different methods of sampling genetic variation allow sampling at different levels and differ in their power of genetic resolution as well as the quality of information content. The result of this study revealed that lima bean germplasm analyzed are genetically closely related; regardless of their origin from diverse geographical locations. However, generating more information on the genetic diversity is an important parameter in the future efforts of lima bean genetic resources conservation and sustainable utilization.

5.3 RECOMMENDATIONS

The results from the field survey and genetic diversity study on lima bean grown in Ethiopia, generated the following plausible recommendations:

- The practices of lima bean farmers need to be backed up and enhanced for effective conservation of the genetic resources in this crop and its landraces.
- The indigenous knowledge of lima bean producing farmers has to be documented in more details and from different localities in the country including West and Kelem Wellega, Jimma, Gamo Gofa and other zones.
- Experiences sharing on the ways of landrace seed selection, intercropping & other cropping practices should be encouraged among farmers of different locations and ethnic backgrounds and households in order to optimize conservation and utilization of the crop.
- Education and awareness creation of the local farmers and further research are needed in order to maintain the diversity and the genetic resources of lima bean.
- More collection is needed to increase the number of lima bean germplasm in the gene bank.
- Germplasm from West Wellega and Gamo Gofa zones should be targeted for improvement of this crop by breeders.
- Moreover, genetic diversity and population structure should be studied by including additional accessions from all over the country and using other powerful molecular markers.

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APPENDICES

Appendix 1: Structured interview and informed oral consent

“Hello, first I will introduce myself my name is Leyila Nasir and I am a student at Addis Ababa University in the department biotechnology this is my colleague [name], who is assisting me as a local guide and translator. We are conducting a study of the crop lima bean as part of my education at the university.” The purpose of this study is to identify different types of lima bean and to understand their use and management by farmers. With your permission, I would like to ask you questions about lima bean. Of course, your participation is entirely voluntary. These interviews require less than one hour. I do not anticipate that the interview will pose any risks to you. Because I am a student, I cannot pay you. We hope that our research will benefit farmers in Ethiopia by promoting the diversity of lima bean. If you agree to an interview, you do not have to answer all of my questions, and you can tell me at any time if you would like to stop. I would record your answers to my questions using my cell phone and my notebook. I would include this information in my thesis, and it would be shared with other researchers who are interested in lima bean. Do you have any questions? In case you have any questions in the future, here is my contact information. Leyila Nasir phone number Do you agree to participate in this study of the crop lima bean? If yes, proceed with the interview. If no, thank the farmer and continue to the next randomly-selected household.

Section 1: Basic information

<i>Date of interview:</i>	<i>Start time of interview:</i>
Name of interviewer:	
Region:	Zone:
Woreda/District:	Kebele/Sub-District:
<i>Latitude (in decimal degrees):</i>	<i>Longitude (in decimal degrees):</i>
<i>Altitude (in meters):</i>	
Agro ecological classification	
Random number of households from farmers’ association list:	
Relative wealth on farmers’ association list: <input type="checkbox"/> Low income <input type="checkbox"/> Middle/High income	

Section 2: General information about informant

Name of informant:
Language spoken by informant during interview: <input type="checkbox"/> Amharic <input type="checkbox"/> Other (specify): _____
Age (observed): <input type="checkbox"/> 18 to 30 <input type="checkbox"/> 30 to 45 <input type="checkbox"/> 45 to 60 <input type="checkbox"/> 60+
Gender (observed): <input type="checkbox"/> Male <input type="checkbox"/> Female
Is the informant the household head? <input type="checkbox"/> Yes <input type="checkbox"/> No
If no, household-head gender: <input type="checkbox"/> Female-headed <input type="checkbox"/> Male-headed

Section 3: Market access

How far is the closest market from your home? In minutes on foot
Do you go to any other larger market? <input type="checkbox"/> Yes <input type="checkbox"/> No
If yes, how far is this larger market from your home? In minutes (on foot)
How far is the nearest road with vehicle transport from your home? In minutes (on foot)

Section 4: Interspecific diversity of legume crops

Are you growing any legumes this year? Yes No If
yes, how many hectares.

Which legumes did you plant in 2010 (Meskerem to Pagume)?

For each of the legumes you listed, how many hectares of land did you plant in 2010 (Meskerem to Pagume) including any areas under intercropping? *Convert any local units to hectares (e.g. 1 timad or qurt = 0.25 hectare).*

Section 5: Use of Lima bean (*Phaseolus lunatus*)

How do you use Lima bean (*phasules lunatus*)?

Food Spice Medicine Fodder/Forage Fuel

Market Bee forage Other (specify): _____

Section 6: Cropping practices

Do you rotate Lima bean with other crops? Yes No

If yes, with which crops do you rotate Lima bean?

If yes, how often do you plant Lima bean within the crop sequence?

Do you intercrop Lima bean with other crops? Yes No

If yes, with which crops do you plant lima bean in the same field?

Section 7: Intraspecific diversity of Lima bean (*phasules lunatus*)

Note: Be sure to include all varieties, including landraces and released/improved types.

What varieties of Lima bean have you grown in the past three years (for the 2009, 2010, and 2011 harvests)?

Are there any other varieties that you have grown in the past, prior to 2009?

Are there any other varieties of Lima bean grown by other farmers in your community, but not by yourself?

Are there any other varieties of Lima bean that you remember from a long time ago, or have heard about from Elders, that are no longer grown by your community?

If you run out of a particular variety, whom would you ask to replenish your seeds? (*Record name as a potential key informant*).

Section 8: Key attributes of varieties

Instructions: At the top of each column, write the names of all varieties harvested in 2009, 2010, and 2011.

Use additional sheets if necessary

Question	Variety 1	Variety 2	Variety 3	Variety 4
Local vernacular name of variety				
Is this a traditional or a new variety?				
Where did you first obtain this variety? (e.g. family, neighbors*, DA, market, food aid, research center)				
For how many years have you planted this variety?				

Where do you plant this variety? (e.g. main fields, field margins, home gardens, fence lines, other places)				
Are the places where you grow this variety rain fed, irrigated, or both?				
Do you plant this variety on soils with low, moderate, and/or high fertility?				
How many times per year do you sow this variety?				
When do you usually sow this variety?				
When do you usually harvest this variety?				
Is this variety grown mainly for home use or mainly for the market?				
How many hectares of these varieties did you harvest in 2009 and 2010, including area under intercropping? <i>Make sure you convert any local units to hectares (e.g. 1 timad or qert = 0.25 hectare).</i>	2010: 2009:	2010: 2009:	2010: 2009:	2010: 2009:
How much of this variety did you harvest in 2008 and 2007? <i>Record with local units to be converted later. Be sure to include the name of the units. Later, go to a local market to determine the conversion factor from local units to kilograms.</i>	2010: 2009:	2010: 2009:	2010: 2009:	2010: 2009:
Based on your experience, was the yield for this variety in 2010 and 2011 a very high yield, a high yield, a medium yield, a low yield, or a very low yield?	2010: 2009:	2010: 2009:	2010: 2009:	2010: 2009:
What is the current price of this variety at your local market? (use farmer units)				

* If a particular variety came from friends or family, ask for the name of the individual who provided it as a potential key informant. Record that person's name in your notebook.

Section 9: Rating of attributes for varieties of Lima bean (*Phaseolus lunatus*)

Ask the informant to rate the varieties of Lima bean (*Phaseolus lunatus*) planted for the 2009, 2010, and 2011 harvests. This is **NOT** a ranking activity, so multiple varieties may receive the same score.

Rating criteria and scale	Var. 1	Var. 2	Var. 3	Var. 4	Var. 5
Local name (copied from previous pages)					
Best yield this variety has ever given you (5=very high, 4=high, 3=medium, 2=low, 1=very low)					
Yield under drought conditions (5=very high, 4=high, 3=average, 2=low, 1=very low)					

Yield when excessive rain causing water logging (5=very high, 4=high, 3=average, 2=low, 1=very low)					
Yield when rainy season begins late (5=very high, 4=high, 3=average, 2=low, 1=very low)					
Yield when rainy season ends early (5=very high, 4=high, 3=average, 2=low, 1=very low)					
Resistance to frost (5=never affected by frost, 4=rarely affected by frost, 3=sometimes affected by frost, 2=often affected by frost, 1=always affected by frost)					
Resistance to common diseases (5=never affected by diseases, 4=rarely affected, 3=sometimes affected, 2=often affected, 1=always affected by diseases)					
Tolerance to common insect pests (5=never affected by insect pests, 4=rarely affected, 3=sometimes affected, 2=often affected, 1=always affected)					
Tendency for seeds to detach/shatter (5=seeds never detach/shatter 4=rarely detach/shatter, 3=detach/shatter about half the time, 2=often detach/shatter, 1=always detach/shatter)					
Importance as food for the household (5=extremely important, 4=very important, 3=somewhat important, 2=not so important, 1=not at all important)					
Importance as a source of income (5=extremely important, 4=very important, 3=somewhat important, 2=not so important, 1=not at all important)					
Importance as fodder for livestock (5=extremely important, 4=very important, 3=somewhat important, 2=not so important, 1=not at all important)					
Effect on soil fertility (5=very positive effect on soil fertility, 4=some positive effect, 3=no effect, 2=some negative effect, 1=very negative effect on soil fertility)					
Ease of harvesting (5=Very easy to harvest, 4=easy, 3=neither easy nor difficult, 2=somewhat difficult, 1=very difficult to harvest)					
Ease of preparing as food (5=very easy to harvest, 4=easy, 3=neither easy nor difficult, 2=somewhat difficult, 1=very difficult to harvest)					
Taste (5=very good taste, 4=good taste, 3=neither good nor bad taste, 2=bad taste, 1=very bad taste)					

Section 10: Gender roles in production and management of Lima bean (*Phaseolus lunatus*)

Which gender and age groups live in your household (including the respondent)?	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No

When it comes to Lima bean (*Phaseolus lunatus*), who within the family usually participates in the following activities? Check any that apply.

Activity	Gender and age groups*			Is this true for all varieties? *
Ploughing with animals (mares)	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No

Preparing the soil by hand with a hoe (mekofer)	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No
Leveling soil and removing uprooted weeds (gulgualo)	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No
Sowing (zer mezerat)	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No
Weeding (marem) and cultivation (kutkuato)	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No
Fertilizer application (madaberia, fig, compost...)	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No
Harvesting (mached ena mesebseb)	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No
Threshing (mewkat)	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No
Storage (makemachet)	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No
Marketing (meshet)	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No
Seed selection (zer memret)	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No

Food preparation (megib mazegajet)	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No
Collection of fodder for animals (meno mesebebe)	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No
Other activity (specify):	<input type="checkbox"/> M-children	<input type="checkbox"/> M-adults	<input type="checkbox"/> M-Elders	<input type="checkbox"/> Yes
	<input type="checkbox"/> F-children	<input type="checkbox"/> F-adults	<input type="checkbox"/> F-Elders	<input type="checkbox"/> No

* Note: For this classification only, **children** are individuals 14 and younger, **adults** are ages 15 to 59, and **Elders** are 60 or older.

** Take detailed notes of any exceptions for particular varieties.

Section 11: Closing and follow-up questions

We are looking for both men and women who have a lot of knowledge about different lima bean. Is there anyone from your community who you recommend? (<i>Ask this if I haven't recorded any names of potential key informants in my notebook</i>)	
Thank you very much for answering my questions. Do you have any comments and/or questions you would like to raise at this time? (Record farmers' questions in my notebook, if relevant.)	
At any point during the interview, did the informant indicate that s/he had gained some new knowledge?	
<input type="checkbox"/> Yes <input type="checkbox"/> No	
If yes, what knowledge did he/she report to have gained as a result of the interview (Record farmers' statements in your notebook)	
<i>Time interview was completed:</i>	<i>Duration of interview (minutes):</i>

Appendix 2: Semi-structured interview with key informants

Note: You must take additional notes in your notebook. Informants will raise their own points during the interview – not necessarily as responses to questions - and these must be recorded. Second, interviewers must dig deeper by asking additional questions whenever she or he senses interesting information or ideas. This approach generates a richer, more complete understanding.

Section 1: Basic information about each Key Informant

Date of interview:	Start time of interview:
--------------------	--------------------------

Name of interviewer:	
Region:	Zone:
Woreda/District:	Kebele/Sub-District:
Language spoken during interview: <input type="checkbox"/> Amharic <input type="checkbox"/> Other (specify): _____	
Name of informant:	
Age (observed): <input type="checkbox"/> 18 to 30 <input type="checkbox"/> 30 to 45 <input type="checkbox"/> 45 to 60 <input type="checkbox"/> 60+	
Gender (observed): <input type="checkbox"/> Male <input type="checkbox"/> Female	

Section 2: Knowledge of varieties listed within the same kebeles

Compile a list of all of the varieties that have been mentioned by the general informants within the same kebele. For each variety, ask the following questions of the key informant. Remember to note if the key informant says that two or more local names refer to the same variety.

Are there any other varieties that you know that we can add to our list? You may include any that you remember or have heard about from a long time ago, but are no longer planted in the kebele.

Names of varieties (from ODK survey)	Have you heard of this variety?	Is this variety planted in this kebele?	Have you planted this variety yourself?
1.	<input type="checkbox"/> Yes <input type="checkbox"/> No	<input type="checkbox"/> Yes <input type="checkbox"/> No	<input type="checkbox"/> Yes <input type="checkbox"/> No
2.	<input type="checkbox"/> Yes <input type="checkbox"/> No	<input type="checkbox"/> Yes <input type="checkbox"/> No	<input type="checkbox"/> Yes <input type="checkbox"/> No
3.	<input type="checkbox"/> Yes <input type="checkbox"/> No	<input type="checkbox"/> Yes <input type="checkbox"/> No	<input type="checkbox"/> Yes <input type="checkbox"/> No
4.	<input type="checkbox"/> Yes <input type="checkbox"/> No	<input type="checkbox"/> Yes <input type="checkbox"/> No	<input type="checkbox"/> Yes <input type="checkbox"/> No

Section 3: Attributes/characteristics of varieties

Instructions: Ask the key informants the following questions about each of the varieties with which s/he is familiar. Record the information in your notebook. The following table is intended to keep track of the questions you have asked.

Question	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
What is the meaning of the local name? (e.g. color, shape, nutritional quality, origin, etc.)										
Are there alternative names for this variety?										

How do you recognize this variety? How does it differ from similar varieties? (By appearance or other features?)										
What are the ideal growing conditions for this variety (e.g. amount and timing of rainfall, sunlight, temperature)?										
Characterize the soil types on which the Variety is planted.										
What fraction of the farmers in your community plants this variety? (e.g. almost all, half, one out of ten)										
Over the past 5 years, has this variety become more commonly planted or less commonly planted in your community?										
If applicable...What is the main reason that this variety is becoming more or less common?										

Section 4: Use of Lima bean *Phaseolus lunatus* as food

What foods do you prepare using Lima bean?

Note: Add a note if foods are of special cultural value. You may include foods that the key informant does not prepare her/himself.

Name of food	Which parts are used?	Which variety is preferred?	Why is this variety preferred?

*For example, color, taste, ease of preparation, etc.

Section 5: Nutritional value of foods prepared with lima bean *Phaseolus lunatus* Do any of the foods you prepare with lima bean have any special health benefits?

Name of food	Health benefit	Specific group who use it or for whom it is recommended*?	Which variety is preferred?	Why is this variety preferred?

--	--	--	--	--

*For example: children, elderly, pregnant women, lactating women, sick people, people with broken bones, people who have lost a lot of blood.

Section 6: Use of f lima bean (*Phaseolus lunatus*) as medicine

Do you use lima bean as medicine?

Name of ailment treated with lima bean	Which parts used?	Which variety is Preferred?	Why is this variety preferred?

Section 7: Use of Lima bean (*Phaseolus lunatus*) as fodder Do your animals consume lima bean?

Which animals consume Lima bean?	Which part(s) do they consume?	When (in which season) do they consume Lima bean?

Section 8: Other specific uses of Lima bean (*Phaseolus lunatus*)

Do you use Lima bean for any other purpose?

Description of use	Which parts do you Use?	Which variety do you Prefer?	Why do you prefer this variety?

Section 9: Crop Management

1. Do the people in your community apply fertilizers to Lima bean? Which fertilizers are used
(Including manure, compost, crop residues)?
2. Do people in your community use pesticides or herbicides on Lima bean?
3. What else do people in your community do to prevent and control pests, weeds, and diseases affecting Lima bean?

4. Are there any varieties of Lima bean that produce more residues (stems, leaves, etc.) than others?
5. What do people in your community do with the residues from Lima bean? after the harvest?
6. Does anyone in your community ever use inoculants for Lima bean?
7. How do people in your community store Lima bean?
8. What are the major production constraints for Lima bean in your community (e.g. weeds, pests, diseases, drought, low fertility etc.)?

Section 10: Closing and follow-up questions

I have asked all of the questions I had for you. Thank you very much for spending this time with me and sharing your knowledge. Do you have any comments and/or questions you would like to raise at this time? (Record farmers' questions below)

(During the interview, did the informant indicate s/he had gained some new knowledge? Yes

No)

If yes, what knowledge did he/she report to have gained as a result of the interview?

Time interview was completed:

Duration of interview (in minutes):

Additional notes:

Appendix 3: Lima bean (*Phaseolus lunatus*) Seed Passport Descriptors

**Ethiopian Biodiversity Institute
Sheet**

Accession No _____ **Collection Record**

_____	_____	Collection No
_____	Genus _____	No
_____	Species _____	Date
_____	Local/Vernacular Name _____	Country
_____	Ethnic Group _____	Region
_____	Language _____	Zone
		Woredas
Village /Site _____	Keble _____	
Farmer's Name _____		
Latitude _____	Longitude _____	
	_____ Altitude	(M)

Topography

1. Swampy
2. Flood plain
3. Plain level
4. Undulated
5. Hilly
6. Hilly dissected
7. Steeply dissected
8. Mountaineer
9. Other (specify)

Sample

1. Single line
2. Pure line/clone
3. Population/mixture

- Site:**
1. Level
 2. Sloppy
 3. Summit

Genetic Status

1. Wild
2. Weed
3. Primitive cultivar/Landrace
4. Breeding line
5. Advanced cultivars

Source of Collection

1. Field
 2. Backyard
 3. Farm store/Threshing place
 4. Agricultural Institute
 5. Natural vegetation
 6. Other (specify)
4. Depression

Soil Texture

- 1. Sand
- 2. Sandy loam
- 3. Loam
- 4. Clay loam
- 5. Clay
- 6. Silt
- 7. Highly organic

Nature of Samples

- 1. Seed
- 2. Spikes
- 3. Pods
- 4. Cherry
- 5. Tuber
- 6. Rhizomes

Herbarium Specimen Yes, No

Photographs Yes, No

Soil Color

Sowing Month:

- 1. Black 1 2 3 4 5 6 7 8 9 10 11 12
- 2. Brown Early/Mid/Late

3. Orange

Harvesting Month

- 4. Red 1 2 3 4 5 6 7 8 9 10 11 12
- 5. Yellow Early/Mid/Late

Stoniness

Origin of Seed

- 1. None 1. Local
- 2. Low 2. Elsewhere
- 3. Medium
- 4. Rocky

Usage (specify) _____

Drainage

Disease & Pests _____

- 1. Poor **Note** (Associated wild weedy species, crop, local flora, disturbance factor) _____
- 2. Moderate

3. Well drained

Shade Yes No

Remark

Collectors Name (s): _____

Appendix 4: Geo-referenced data of the sample

Zones	Woredas	Kebeles	Latitudes	Longitudes	Altitudes	Collecting provenance		
Gurage	Enamor Ener	Sanika	N8 ⁰² '44''	E37 ⁰⁴⁹ '35''	2026.8m	Farmers' varieties		
			N8 ⁰² '35''	E37 ⁰⁴⁹ '50''	2038.9m	Farmers' varieties		
			N7 ⁰⁵⁶ '2''	E37 ⁰⁵² '1''	2192.8m	Farmers' varieties		
				N7 ⁰⁵⁶ '2''	E37 ⁰⁵² '10''	2253.3m	Farmers' varieties	
		Garidashe		N8 ⁰¹ '39''	E37 ⁰⁴⁵ '21''	1969.9m	Farmers' varieties	
				N8 ⁰¹ '48''	E37 ⁰⁴⁵ '20''	1971.2m	Farmers' varieties	
				N8 ⁰¹ '18''	E37 ⁰⁴⁵ '20''	1968.8m	Farmers' varieties	
		Kochira		N8 ⁰² '44''	E37 ⁰⁴⁹ '35''	2026.8m	Farmers' varieties	
				N7 ⁰⁵² '1''	E37 ⁰⁵² '1''	2192.8m	Farmers' varieties	
				N7 ⁰⁵⁶ '2''	E37 ⁰⁵² '10''	2253.3m	Farmers' varieties	
		Abeshegea	Tawlanagefersa	N8 ⁰¹⁶ '54''	E37 ⁰⁴¹ '11''	1765m	Farmers' varieties	
				N8 ⁰¹⁷ '8''	E37 ⁰⁴¹ '18''	1750.2m	Farmers' varieties	
				N8 ⁰¹⁶ '2''	E37 ⁰⁴¹ '29''	1767.8m	Farmers' varieties	
			Jebjebenagasori	N8 ⁰¹⁶ '24''	E37 ⁰⁴³ '21''	1814.5m	Farmers' varieties	
				N8 ⁰¹⁶ '21''	E37 ⁰⁴³ '22''	1794.6m	Farmers' varieties	
	N8 ⁰¹⁶ '34''			E37 ⁰⁴³ '15''	1866m	Farmers' varieties		
			N8 ⁰¹⁶ '38''	E37 ⁰⁴³ '11''	1859.8m	Farmers' varieties		
	Kebena	Rikoboka	N8 ⁰¹⁷ '9''	E37 ⁰⁴⁶ '13''	1842.7m	Farmers' varieties		
			N8 ⁰¹⁷ '13''	E37 ⁰⁴⁶ '5''	1844.3m	Farmers' varieties		
			N8 ⁰¹⁷ '13''	E37 ⁰⁴⁶ '17''	1891.9m	Farmers' varieties		
Gamo Gofa	Arba minche zuriya	Chano millea	N6 ⁰⁰ '0.''	E37 ⁰³⁴ '59''	1220.1m	Farmers' varieties		
			N6 ⁰⁰ '7''	E37 ⁰³⁴ '53''	1224.3m	Farmers' varieties		
			N6 ⁰⁰ '5''	E37 ⁰³⁴ '52''	1233.4m	Farmers' varieties		
			N6 ⁰⁰ '14''	E37 ⁰³⁴ '53''	1235.4m	Farmers' varieties		
			N6 ⁰⁰ '17''	E37 ⁰³⁴ '57''	1244.3m	Farmers' varieties		
			N6 ⁰⁰⁵ '21''	E37 ⁰³⁴ '64''	1258.1m	Farmers' varieties		
			N6 ⁰⁰⁴ '21''	E37 ⁰³⁴ '61''	1255.8m	Farmers' varieties		
			N6 ⁰⁰⁴ '26''	E37 ⁰³⁴ '62''	1253.5m	Farmers' varieties		
				Chano dorga	N6 ⁰⁰⁸ '25''	E37 ⁰³³ '67''	1273.2m	Farmers' varieties
					N6 ⁰⁰⁸ '26''	E37 ⁰³³ '69''	1276.2m	Farmers' varieties
					N6 ⁰⁰⁸ '29''	E37 ⁰³² '77''	1281.3m	Farmers' varieties
					N6 ⁰⁰⁸ '31''	E37 ⁰³³ '71''	1287.6m	Farmers' varieties
					N6 ⁰⁰⁸ '35''	E37 ⁰³³ '73''	1290.3m	Farmers' varieties
					N6 ⁰⁰⁷ '35''	E37 ⁰³³ '71''	1292.2m	Farmers' varieties
					N6 ⁰⁰⁷ '33''	E37 ⁰³³ '81''	1294.9m	Farmers' varieties

			N60°7'34''	E37°33'79''	1298.0m	Farmers' varieties
			N60°7'37''	E37°33'83''	1295.1m	Farmers' varieties
Jimma	Sekoru	Widaye	N8°11'21''	E37°06'31''	1955.9m	Farmers' varieties
			N8°11'27''	E37°06'37''	1950.7m	Farmers' varieties
		Cheka	N8°38'89''	E37°31'36''	1822.2m	Farmers' varieties
			N8°38'96''	E37°31'41''	1831.4m	Farmers' varieties
		Sadeka	N8°3'763''	E37°12'413''	1925.8m	Farmers' varieties
			N8°3'767''	E37°14'407''	1929.8m	Farmers' varieties
		Yero sekoru	N7°55'590''	E37°25'147''	1853.5m	Farmers' varieties
			N7°58'584''	E37°27'142''	1845.2m	Farmers' varieties
		Tiro afeta	N7°55'597''	E37°25'143''	1847m	Farmers' varieties
			N7°53'591''	E37°25'141''	1855m	Farmers' varieties
		Gebesiro	N7°12'31''	E37°33'89''	1865.5m	Farmers' varieties
			N7°12'36''	E37°33'97''	1876.1m	Farmers' varieties
		Liban bore	N8°11'38''	E37°30'54''	1902.1m	Farmers' varieties
			N8°12'36''	E37°30'62''	1914.1m	Farmers' varieties
		Horo kaki	N8°59'11''	E37°49'62''	1858.5m	Farmers' varieties
			N8°61'14''	E37°49'55''	1874.2m	Farmers' varieties
		Gonzeletta	N7°55'42''	E37°25'393''	1878.4m	Farmers' varieties
			N8°57'52''	E37°33'401''	1897.5m	Farmers' varieties
		Kumbi	N8°07'63''	E37°29'448''	1906.6m	Farmers' varieties
			N8°10'71''	E37°31'59''	193.2	Farmers' varieties
West Wellega	Lalo asabi	Haroji agemsa	N9°15'33'	E35°42'17''	1869.3m	Farmers' varieties
			N9°17'32'	E35°44'19''	1861.2m	Farmers' varieties
			N9°15'35'	E35°42'24''	1854.4m	Farmers' varieties
		Dengoro keta	N9°19'30'	E35°41'25''	1860.5m	Farmers' varieties
			N9°19'30'	E35°42'25''	1859.2m	Farmers' varieties
			N9°19'37'	E35°41'36'	1871.4m	Farmers' varieties
			N9°19'41'	E35°41'38''	1970.6m	Farmers' varieties
	Gimbi	Wedesa werika	N9°11'40'	E35°57'26''	1868m	Farmers' varieties
			N9°11'44'	E35°57'34''	1873.2	Farmers' varieties
			N9°11'47'	E35°57'28''	1871.1m	Farmers' varieties
			N9°11'30'	E35°57'22''	1822.7m	Farmers' varieties
	Mene sibu	Wama tobera	N9°36'17'	E35°0'2''	1411.9m	Farmers' varieties

			N9 ⁰ 36'23'	E35 ⁰ 0'7''	1418.4m	Farmers' varieties
			N9 ⁰ 36'10'	E35 ⁰ 0'00''	1430.9m	Farmers' varieties
			N9 ⁰ 36'19'	E35 ⁰ 0'25''	1423.1m	Farmers' varieties
			N9 ⁰ 34'19'	E35 ⁰ 2'13''	1324.1m	Farmers' varieties
		Guyo teyiba	N9 ⁰ 17'57'	E35 ⁰ 0'12''	1578.4m	Farmers' varieties
			N9 ⁰ 18'37'	E35 ⁰ 0'32''	1594.3m	Farmers' varieties
			N9 ⁰ 18'48'	E35 ⁰ 0'42''	1587.2m	Farmers' varieties
			N9 ⁰ 17'30'	E35 ⁰ 0'25''	1579.3	Farmers' varieties
Qelem Wellega	Dali-sedi	Chamo	N8 ⁰ 50'37'	E35 ⁰ 9'18''	1495.05m	Farmers' varieties
			N8 ⁰ 49'37'	E35 ⁰ 9'20''	1489.05m	Farmers' varieties
			N8 ⁰ 47'37'	E35 ⁰ 9'28''	1498.05m	Farmers' varieties
			N8 ⁰ 51'36'	E35 ⁰ 9'13''	1501.1m	Farmers' varieties
			N8 ⁰ 51'36'	E35 ⁰ 9'13''	1501.84m	Farmers' varieties
			N8 ⁰ 50'32'	E35 ⁰ 9'32''	1500.05m	Farmers' varieties
			N8 ⁰ 50'35'	E35 ⁰ 9'16''	1496.56m	Farmers' varieties
		Mender 14	N8 ⁰ 48'38'	E35 ⁰ 11'24''	1476.56m	Farmers' varieties
			N8 ⁰ 48'33'	E35 ⁰ 11'24''	1470.73m	Farmers' varieties
			N8 ⁰ 48'37'	E35 ⁰ 11'14''	1474.73m	Farmers' varieties
			N8 ⁰ 48'33'	E35 ⁰ 11'32''	1468.1m	Farmers' varieties
			N8 ⁰ 48'45'	E35 ⁰ 11'25''	1481.94m	Farmers' varieties
			N8 ⁰ 48'38'	E35 ⁰ 11'24''	1471.06m	Farmers' varieties
	Seyo	Ano Michael	N8 ⁰ 33'32'	E34 ⁰ 51'30''	1543.4m	Farmers' varieties
			N8 ⁰ 48'39'	E35 ⁰ 51'27''	1550.3m	Farmers' varieties
			N8 ⁰ 34'52'	E34 ⁰ 50'28''	1601.91m	Farmers' varieties
			N8 ⁰ 34'53'	E34 ⁰ 50'27''	1603.54m	Farmers' varieties
			N8 ⁰ 33'54'	E34 ⁰ 50'26''	1587.15m	Farmers' varieties
			N8 ⁰ 33'41'	E34 ⁰ 50'16''	1658.66m	Farmers' varieties
			N8 ⁰ 33'57'	E34 ⁰ 50'22''	1589.88m	Farmers' varieties

Appendix 5: Plant DNA Isolation Protocol

CTAB immersed leaf samples

1. Take 50-100 mg of fresh leaf tissue, clean it with distilled water, dry and then place it in autoclaved pestle mortar.
2. Add 2-3 ml CTAB buffer (100 mM Tris-HCl pH 8.0, 2% (w/v) CTAB, 20 mM EDTA, 1.4 M NaCl, 4% PVP and 2% (v/v) β -mercaptoethanol) (the tissue should be covered with buffer before the start of crushing) and crush the tissue completely.
3. Transfer 700 μ l of the crushed tissue (try to avoid solid material) with a broad mouth tip (the tip can be cut by surgical blade to make its bore wider) to a sterile eppendorf tube.

Silica gel dried samples

A. Weigh in 100 mg fresh leaf material (50mg dry material) per sample. (Pulverize thoroughly using a clean mortar and pestle). For fresh material add liquid nitrogen and for dry material use quartz sand.

First grind down slightly, then more powerful (cells have to be crashed).

Use mixer and miller for grinding leafs by the help of bids (2-3 bids per 2ml eppendorf tube along with leaf samples)

B. Transfer the powder into an Eppendorf cap (use a new, clean spatula for each sample)

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

D. Centrifuge for 5 minutes at 16000 rpm.

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

Optional (2nd Extraction): Add new CTAB solution (700 μ l) to the tissue pellet and stir slightly with a new 1000 μ l pipette tip, incubate 30 min at 65°C.

F. Proceed with step 4

4. Add 700 μ l of chloroform and mix thoroughly and centrifuge the tube at 16000 xg for 10minutes with 260C.

5. Transfer 600µl of the supernatant to new fresh eppendorf tube and add 60µl of 3Msodium acetate (pH 5.2) and mix thoroughly
6. Add 600µl of ice cold isopropanol and gently mix by inverting the tubes 3 - 5l times, Place in refrigerator (-20 0C) for 15 – 30 minutes (Sometimes can stay for long)
7. Centrifuge at 16000g for 5 minutes to pellet down the DNA @ 4⁰C.
8. Discard the supernatant and wash the DNA by 70% ethanol (1ml) by dissolving thepallet completely in the wash buffer (vortexing), Re pellet by centrifuge at 16000 xg for 3 minutes @ 4⁰C.
9. Repeat the wash step again by absolute (100%) ethanol (1ml) and dry the pellet(1:00 to 1:30 hr) (Aspiration) (Upside down the eppendorf under dry and cleannapkin/tissue paper)
10. Suspend the pellet in 0.1X TE buffer with RNase 60µl (slight vortexing)

