Genetic Diversity and Population Structure of Ethiopian Barley [*Hordeum vulgare* (L.)] germplasm Using Inter Simple Sequence Repeat (ISSR) Markers

Sirnesa Feyen

A Thesis Submitted to

The Department of Applied Biology

School of Applied Natural science

Presented in Partial Fulfillment of the Requirement for the Degree of Master's in Applied Biology (Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

June, 2024

Adama, Ethiopia

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Sirnesa Feyen

Advisor: Seifu Juneidi (PhD)

Co-advisor: Tilehun Mekonin (PhD)

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DECLARATION

I hereby declare that this master thesis entitled "Genetic Diversity and Population Structure of Barley germplasms In Ethiopia as Revealed by Inter-Simple Sequence Repeat Markers" is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation

Sirnesa Feyen		
Name of student	Signature	Date

RECOMMENDATION

We, the advisors of this thesis, hereby certify that I/we have read the revised version of the thesis entitled "Title Genetic Diversity and Population structure of Barley germplasms in Ethiopia as revealed by inter-simple sequence repeat (ISSR) markers" prepared under our guidance by **Sirnesa Feyen** submitted in partial fulfillment of the requirements for the degree of Masters of Science in Applied Biology (Biotechnology).

Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

Major Advisor	Signature	Date
Co-advisor	Signature	Date

APPROVAL OF BOARD OF EXAMINERS

We, the advisors of the thesis entitled "Genetic Diversity and Population Structure of Barley germplasm in Ethiopia using ISSR Markers" developed by **Sirnesa Feyen** hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor	Signature	Date
Co-advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by Sirnesa Feyen have read and evaluated the thesis entitled "Genetic Diversity and Population Structure Analysis of Barley (*H. vulgare* L.) Germplasm in Ethiopia using inter-simple sequence repeat Markers" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Biology (Biotechnology).

Chairperson	Signature	Date
Internal Examiner	Signature	Date
External Examiner	Signature	Date

Final approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC)

Department Head	Signature	Date
School Dean	Signature	Date
Office of Postgraduate Studies, Dean	Signature	Date

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LIST OF ABBREVIATIONS AND ACRONYMS

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
CTAB	Cetyl-trimethyl Ammonium Bromide
DNA	Deoxyribonucleic acid
DNTPS	Deoxyribonucleotide triphosphate
Fst	Fixation index
I	Shannon information index
IBC	Institute of Biodiversity and Conservation
IPGRI	International Plant Genetic Resources Institute
ISSR	Inter simple sequence repeat
Na	Number of allele
Ne	Number of effective allele
NJ	Neighbor joining
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PPL	Percent of polymorphic loci
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SNNPR	South nation, nationality and people's region
SSR	Simple sequence repeat
STRs	Short tandem repeats
UPGMA	Unweighted pair group with arithmetic mean

ABSTRACT

Barley is a member of the grass family Poaceae (Gramineae), specifically the Triticeae tribe). The Triticeae tribe includes 350 species and several economically significant cereals and forages. The present study was designed to investigate the genetic diversity and population structure of 97 barley germplasms in Ethiopia representing 14 populations using 8 ISSR markers. To analyses the genetic diversity in subdivided populations, the total genetic diversity (Ht), intrapopulation genetic diversity (Hs) and gene differentiation (Gst) were determined. The POPGEN 32 software was used to measure the following parameters: observed number of alleles (na), effective number of alleles (ne), Nei's gene diversity (h), percentage of polymorphic loci (PPL) and Shannon's information index (I). A genetic dissimilarity matrix parameter was calculated using Mega software version 2.3.4. Population structure and admixture patterns were determined using the admixture model based on the Bayesian algorithm implemented in STRUCTURE software version 2.3.3). The admixture model with correlated allele frequencies was used, assuming that the genome of each individual resulted from the mixture of K ancestral populations. All the markers used in the present study shows polymorphic and less to moderate informative. The genetic diversity index (h) for all primers ranged from 0.119 to 0.373 and average mean value of 0.193 alleles per locus. Polymorphic information content ranged from 0.127 to 0.263 with overall mean of 0.178 and within-populations genetic diversity was confirmed with gene diversity values ranging from 0.28 to 0.43 with an overall mean of 0.36. Analysis of molecular variance revealed that most (63%) of the total genetic variation was accounted for within populations genetic variation and 37% was among population variation. Clustering and principal Co-ordinate analyses were sharply grouped the samples based on their genetic structure. Population structure analysis revealed the presence of four sub-populations with higher degree of genetic admixture. Among the studied populations, Jimma and Arsi populations showed relatively higher gene diversity than others indicating these sites could be targeted for breeding and conservation of barley. Therefore, the present study had successfully provided the genetic diversity and population structure of barley germplasms in the studied areas.

***Keywords**: Genetic diversity, Molecular marker, ISSR marker, Polymorphism, H. Hordeum vulgare.*

1. INTRODUCTION

1.1. Background of the study

Barley (*Hordeum vulgare*) (L. subsp. vulgare) is one of the oldest domesticated crops. *Hordeum* is a genus of approximately 32 species that range from diploid to polyploid, perennial to annual, and can be found all over the world. Barley is well-suited to a variety of agro-ecological conditions, and it tolerates soil salinity, drought, and cold to a large extent (Dejene & Le´on, 2010). It is the world's fourth most important cereal crop, after wheat, maize, and rice, in terms of planting areas and production, and is primarily used for animal feed, brewing malts, and human food (Almerekova et al., 2021).

Plant survival and agricultural improvement both rely on genetic diversity. Plant genetic diversity enables plant breeders to develop new and improved cultivars with desirable qualities such as high yield potential, large seed size, and traits that breeders prefer (pest and disease resistance, photosensitivity, and so on). Assessment of diversity can be done using morphological, cytological, biochemical, and molecular methods. Morphological markers were initially utilized for diversification study and are still in use today. These were natural variants of a single plant species. Later, cytological and biochemical differences seen in a species' genotypes were used to assess genetic diversity. With the development of genomic technology, molecular markers became the favored method for measuring genetic diversity (HR Bhandari et al., 2017).

The existence of variations in gene sequences among individual members of a species is known as genetic diversity. It's known as the diversity found in various genotypes of the same species. In the context of this work, genetic diversity refers to the heritable variation within and between populations of organisms, including plant species. Variations in morphology, anatomy, physiological behavior, or biochemical traits can be altered of heritable character variance. Diversity at several gene loci within an individual is known as genomic diversity. Agricultural workers have paid the most attention to genetic variety (HR Bhandari et al., 2017).

Genetic diversity is a critical component of biological diversity that is important for conservation efforts. Plant genetic differences influence the overall amount of biodiversity. Without genetic diversity, the world population cannot adapt to and endure environmental changes. Genetic variation studies are useful for germplasm conservation, population and variety identification,

and identifying alleles that may aid the organism's adaptation to changing environmental conditions (Hussain & Nisar, 2020). Assessing the genetic variability of a species within and among different populations is important to devise mechanisms for effective identification, conservation, and multiplication of suitable genetic materials (Oumer et al., 2015). Because of its unique geographic location, wide range of altitudes, fluctuating climate, copious amounts of rainfall, and diverse soil properties, Ethiopia may be recognized for its rich biodiversity and for serving as a center of origin for numerous crop species and diversities. Because of this environmental variance, there are many different types of vegetation, crop species, and landrace varieties of crops that have been preserved and used for beadwork by farmers (Tesfaye & Mengistu, 2017). Although Ethiopia is being to be suggested the center of diversity for barley, most of the country's farmers still achieve very low yields due to a combination of genetic, environmental, and socioeconomic constraints (Lakew, 2020).

Barley is a crop produced extensively throughout Ethiopia due to its numerous benefits and is cultivated by farmers. The primary evaluation criteria for barley varieties have been biological yield, which includes improved grain production, nutritional quality, and disease resistance. Ethiopia is known as a barley diversity center; nonetheless, most of the country's farmers continue to produce extremely poor yields due to a combination of genetic, environmental, and socioeconomic restrictions. In Ethiopia, barley is adapted to a broad range of agro-ecological environments and it is tolerant to soil salinity, drought and frost to a considerable level. Field observation in different highlands of Ethiopia showed that frost effect is one of the major constraints of barley production in long rainy season. The crop successfully grows in the arid climates of the Sahara, the Tibetan plateaus, the highlands of the Himalayas, and the Andean countries, the tropical plains of India and the mountains of Ethiopia (Tiegist & Le´on, 2010).

1.2. Statement of the problem

Barley (*Hordeum vulgare* L.) is one of Ethiopia's oldest cereal crops, and it ranks fifth in both area coverage and production, with approximately 1,013,623.72 ha and 18,155,830.29 qt, respectively. Furthermore, the significant variety of the environmental circumstances in Ethiopia that promote adaptive selection, and the cultivation of barley in two growing seasons each year have most likely influenced the structure of these landrace variants (Addisu Fekadu et al., 2017). In the Ethiopian highlands, barley is the only source of food and feed. Compared to wheat,

barley can survive more harsh growing circumstances, such as drought or low soil fertility (Firdissa et al., 2022). Despite its importance, the species is underestimated and underexploited. Its preservation and enhancement have received little attention. Furthermore, accurate knowledge about the production system, genetic diversity, and genetic potential are significant constraints of *Hordeum vulgare* variety Ethiopia. So yet, just a few molecular diversity studies have been studies for *Hordeum vulgare*. Because of the Lack of improved varieties for the different production systems, Shortage of high-quality breeder's seed and Pre-basic seed of improved varieties, Inadequate studies on Ethiopian landraces, especially for malting purposes, Weak coordination and linkage between barley breeders across centres and suitability for preparing different kinds of known Ethiopian traditional dishes and adaptability to different climatic condition of this crop genetic, diversity needed to be carried out using ISSR. However, no study using ISSR markers was conducted on *Hordeum vulgare* genetic diversity in Ethiopia or anywhere else in Ethiopia. This study is, therefore, aimed at assessing the genetic diversity of *Hordeum vulgare* germplasm using ISSR markers. Therefore, the present study was designed with the following general and specific objectives.

1.3. Objectives

1.3.1. General objective

- The general objective of this study was to evaluate and assess the extent and patterns of distributions of the existing genetic diversity of Barley populations in different region of Ethiopia using ISSR markers

1.3.2. Specific Objectives:

- To assess the extent of genetic diversity within and between populations of cultivated barley using ISSR markers.
- To determine population differentiation among population of Barley with respect to regions

1.4. Significance of the study

The result of this study:

- will provide information on the general genetic diversity of barley germplasm which may assist in the identification and selection of genetic material for conservation and improvement

- Will provide the possible application of inter simple sequence repeat markers for of *Hordeum vulgare* cultivars and selection of genetic material for conservation
- Can be used as a reference for other finding to do similar works.
- Enhance the knowledge of *Hordeum vulgare* germplasm diversity molecular marker

2. LITERATURE REVIEW

2.1. Origin, domestication and distribution of Barley

Barley is one of the cereal founder crops, domesticated around 10,000 years ago in the Fertile Crescent (Firdissa et al., 2022). Domestication of barley most likely began before 7000 B.C. in the Near Eastern region known as the "Fertile Crescent." The Fertile Crescent includes Jordan, Lebanon, Palestine, Syria, southeastern Turkey, Iraq, and western Iran. The wild progenitor of cultivated barley (*Hordeum vulgare*) subsp. *spontaneum* is still widely dispersed throughout the Fertile Crescent, notably in the driest (Dejene et al., 2023). Accordingly, after detailed molecular characterization of 317 wild and 57 cultivated barley lines, generally concluded that the Israel-Jordan area in the southern part of the Fertile Crescent has the highest probability of being the geographical area within which wild barley was domesticated; and wild populations found in the southern part of the Fertile Crescent in western Iran have also contributed germplasm to the cultivated barley on its way to the Himalayas (Mashilla Dejene, 2019). Barley agriculture covers around 27.3 million hectares in Europe and 12.16 million hectares in Asia. However, in other parts of the world, it is substantially smaller than in these two continents. For example, North and South America account for around 6.64 million hectares, Africa for 4.87 million hectares, and Oceania for 4.46 million hectares. Russia, Ukraine, Canada, Turkey, and Spain account for around 41.5% of the world's barley production (Dejene et al., 2023). The initial concentration is on Southeast Asia, mountainous Central and Western China, and the lowland areas that surround them. Naked barley is grown there mostly in mountainous locations at elevations of at least 2000 meters. The second concern is on Northeast Africa (Ethiopia's hilly highlands), where unique naked barley types are found. The third focus involves Western Asia: Turkey and Transcaucasia. Recent studies have been heavily focused on DNA markers. The findings can advance the concept of naked barley domestication and confirm or disprove existing hypotheses concerning the origin and distribution of naked barley. One of the possibilities regarding the origin of naked barley is that it originated in south-Western Iran and then spread to other locations. This notion is based on an examination of the dominant SCARsKT7 marker, which is closely related to the nud locus (Lukina et al., 2022)

Ethiopia was first thought to be the origin of cultivated barley, but due to the lack of wild relatives, it was later regarded as a secondary center of diversity. Some authors still confirm

Ethiopia as a centre of origin for cultivated barley. This is due to existence of very high phenotypic diversity and flavonoid patterns. Moreover, based on archaeological and historical studies, it has been suggested that in Ethiopia barley was cultivated as early as 3,000 BC probably after being introduced from Asia. Recently, barley accessions from three continents were studied using nuclear and chloroplast SSR markers, as Well as a 468-bp fragment from the non-coding region of chloroplast DNA (Dido et al., 2021).

They came to the conclusion that barley farmed in Eritrea (and Ethiopia) might not have originated from the wild variety found in the Fertile Crescent today since they could clearly distinguish Eritrean Ethiopian barley from barley from West Asia, North Africa, and Europe. Nevertheless, a number of endemic types of barley have arisen in Ethiopia, whether or not it is a place of origin for cultivated barley (Shimelis Tesfaye and Berhanu Sime, 2022). Various researchers postulated a centre of origin and/or diversification at the Horn of Africa (Firdissa et al., 2022).

Barley cultivation in Ethiopia dates back to ancient times. The long history of cultivation, as well as the various agro-ecological zones and cultural approaches, have resulted in a vast range of barley varieties. In Ethiopia, barley ranks fifth among all grains (teff, wheat, maize, and sorghum), with an area coverage of 1,018,752.94 hectares and an annual production of 1,781,652.208 tons. Shewa, Gojam, Arsi, Gonder, Wollo, and Bale are the primary production regions for the crop (Haile, 2018). Being one of the 12 Vavilovian Centers of Diversity, Ethiopia is well renowned for its varied agro-ecological and climatic features. There are many different ecological conditions throughout the nation due to the altitudinal variety, which varies from 110 meters below sea level in part us of the Kobar Sink to 4,620 meters above sea level at Ras Dashen. This variation is caused by variations in temperature and rainfall as well as edaphic variables. This complex topography and environmental heterogeneity provide sustainable environment for a wide range of life forms. As a result, Ethiopia is known as one of the world's most fertile genetic resource centers (Tiegist Dejene et al., 2023). Barley is well-suited to a variety of agro- ecological conditions and is very resistant to soil salinity, drought, and cold. The crop thrives in arid conditions such as the Sahara, Tibetan plateaus, Himalayan highlands, Andean countries, India's tropical plains, and Ethiopian mountains (Tiegist Dejene et al., 2023).

Barley (*Hordeum vulgare* L.) is one of the oldest domesticated crops, having been farmed in

Ethiopia for over 5000 years. It is grown in every region of the country. Barley can be grown at altitudes ranging from 1500 to 3500 meters, however it is most commonly grown between 2000 and 3000 meters. This wide distribution illustrates the country's wide ecological range (T. Dejene et al., 2023). Ethiopia has a special location for barley farming, which has been practiced there for at least the previous 5000 years, according to a number of reliable accounts. It is thought that the Agew people, who lived in approximately 3000 BC, were the first Ethiopians to plant barley (Haile, 2018). In Ethiopia, barley is known as gebs ye ehil nigus, which translates to "king of crops" due to its versatility and adaptability to many of Ethiopia's traditional foods and beverages (Jemal Mohammed et al., 2016).

2.2. Biology and genetics of barely

Barley is an annual grass that grows to 60-120 cm tall. Barley has two distinct root systems: seminal and adventitious. The quality, texture, and structure of the soil, as well as the temperature, all influence how deep the roots develop. The deepest roots are often seminal in origin, whereas the upper layers of soil are densely packed with later-developing adventitious roots. If the grain is thoroughly planted, a 'rhizomatous stem' emerges, producing leaves once it reaches the surface. The 'rhizome' can be one or more internodes long and may have adventitious roots (Australian Government, 2008). The *Hordeum* genus has 32 species, each with a basic chromosomal number of x=7. Thell.1 is a diploid species with 14 chromosomes (2n = 2x). Other *Hordeum* species are diploid, tetraploid (2n = 4x = 28), and hexaploid (2n = 6x = 42) (Australian Government, 2008).

With 23.3% of all accessions (129 000), the Ethiopian barley collection is among the top ten largest in the world. The barley collection has expanded to over 15400 accessions during the last 30 years, with landraces making up the majority and being housed at IBC. Barley (*Hordeum vulgare*) subsp. vulgare L. is the only species now included in the collection; 10277 accessions were gathered in Ethiopia, and 1101 were donated from nine different nations. However, 3982 accessions of unclear provenance necessitated detail investigation. Landraces are gaining popularity in numerous breeding projects, particularly for resilience to abiotic and biotic stressors, with a participatory research approach. More than 80% of Ethiopia's barley production comes from landraces (Masreshaw Yirga, 2018)

2.3. Taxonomy of Barely

The presence of a consistent and well-defined crop classification system is critical in crop and agricultural sciences. Plant classification occurs before the simplicity of plant collection, study, breeding, and specialized development initiatives. Standardized plant names make it easier to communicate, distribute, and retrieve scientific knowledge. In 1753, Linnaeus published Species Plantarum, which included the first botanical description of barley (Al-Khayri et al., 2019). Barley is a member of the grass family *Poaceae (Gramineae)*, specifically the *Triticeae tribe).*The medium-sized genus *Hordeum* is a member of the Triticeae grass tribe. The tribe comprises about 350 species, including many forage grasses (Elymus and Thinopyrum), ecologically significant temperate grassland taxa (Aegilops, Agropyron, Elymus, *Hordeum*, Pseudoroegneria, and others), and important cereals like wheat (Triticum spp.), rye (Secale cereale), and triticale (xTriticosecale; an artificial wheat x rye hybrid) (Blattner, 2018). The cultivated species *H. vulgare* L. is divided into two subspecies: multi-row (H. vulgare L. subsp. *vulgare* and two-row (H. vulgare L. subsp. distichon (L.) Koern.). They include the groups of covered and naked botanical varieties. Among naked barleys, the groups of multi-row naked barley (*convar. Celeste* (L.) A. Trof) and two-row naked barley (convar. nudum (L.) A. Trof.) were identified. Naked barley groups are characterized by a large number of endemic varieties from various foothill and highland areas. This classification mirrors the enormous polymorphism of species and botanical varieties within this genus, including naked barley. The group of multi-row naked barley, *H. vulgare* L. subsp. *vulgare convar. coeleste* (L.) A. Trof., includes 58 botanical varieties (Lukina et al., 2022).

Table 1: Taxonomic classification of cultivated barley Kingdom (Karanjeet Singh Sandhu, 2015)

Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* - Vascular plants
Super division	Spermatophyta - Seed plants
Division	Magnoliophyta - Flowering plants
Class	*Liliopsida* – Monocotyledons
Subclass	*Commelinidae*
Order	*Poales*
Family	*Poaceae (Gramineae)*
Genus	*Hordeum* L. – barley
Scientific name	*Hordum. vulgare L.*
Common	Cultivated barley

2.4. Economic importance of *Hordeum vulgare*

Barley (*Hordeum vulgare L.*) is one of the world's most ancient food crops. It has been an important cereal crop since the early stages of agricultural innovations 8,000–10,000 years ago (Addisu & Shumet, 2015). In Ethiopia, however, it is a major staple crop, and in the Ethiopian highlands, barley is the only source of food and feed. Highland farmers who manage the crop using ancient technologies use different parts of the barley crop for different purposes: the grain is used in a wide range of traditional food preparations and home-made beverages; the straw is used not only to feed livestock during dry seasons, but also as a material for thatching roofs and bedding (Firdissa et al., 2022). In Ethiopia, barley-based cuisine is served as main, side and ceremonial dishes at weddings and annual celebrations. They are sometimes prepared as restorative dishes and offered to breastfeeding moms in the assumption that they will increase breast milk production. Furthermore, certain dishes are said to be a cure for gastritis, while others are reported to be a decent alternative for breast milk or to repair broken bones and fractures (Jemal Mohammed et al., 2016).

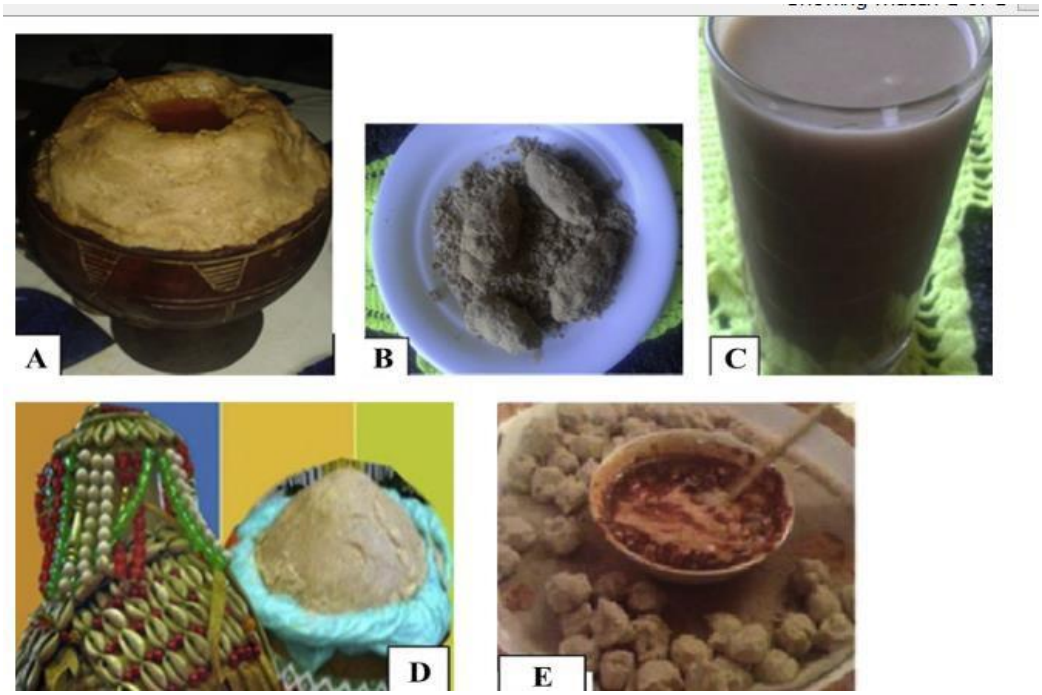

Figure 1: (A) Genfo, (B) beso in solid form, (C) beso in liquid form, and (D) chuko with its traditional (E) Tihlo (Jemal Mohammed et al., 2016).

The crop has a great value in the social and food habits of the Ethiopian people, being used for preparing various types of foodstuffs (injera or flattened pancake, bread, porridge, muq, beso, kinche, chiko, and qolo) and local drinks (tela, borde and araqee) and industrial beverages (beer and malt products) (M. Dejene, 2019).

Figure 2: Masaba apparatus for the distilling of areke liquor (Firdissa et al., 2022).

The straw is utilized as animal feed, particularly during the dry season. Cereals and their contents have recently been rediscovered and promoted as part of a healthy and balanced diet, particularly whole-grain products. Barley grain also contains 3 to 7% β-glucan, an important dietary fiber that has significant blood cholesterol lowering effects. Barley plays an important role in ensuring food security, as it requires relatively low input. Its yield stability is far better than other cereals, making it a dependable source of food in bad seasons (Azeb Hailu et al., 2016). Cereals and their constituents have recently been rediscovered and promoted as important components of a healthy and balanced diet, particularly whole-grain products. Cereal grains offer the human body with not just carbohydrates and protein, but also dietary fiber, minerals, vitamins, and antioxidants that are essential for human health (Firdissa et al., 2022). Although barley is not among the top cereal crops in Ethiopia, its importance is rapidly growing in terms of production, potential for poverty reduction, as well as for the country's coffers and the current balance of payment situation (Shahidur et al., 2015).

2.5. Production and productivity of Barely

The global area under barley cultivation, annual production, and productivity for the 2016-17 cropping season were 49.28 million hectares, 147.06 million metric tons, and 2.98 metric tons per hectare, respectively, with estimates for the 2017-18 cropping season at 47.81 million hectares, 143.68 million tons, and 3.0 metric tons per hectare. Likewise, the global area under barley cultivation, annual production, and productivity for the 2018-19 cropping season were 49.23 million hectares, 140.71 million metric tons, and 2.86 metric tons per hectare, respectively. (Mashilla Dajane, 2019). Throughout the study period, the average yield per hectare of the barley crop observed globally changed from year to year. The highest average yield per hectare of barley in the world was achieved in 2019 (3,108 kg/ha). In 2019, the average barley production increased by 4.85% compared to 2015. Europe in the analyzed period obtained the highest yields for barley production; they varied between 3,552 kg/ha and 3,948 kg/ha. In 2019, the average yield per hectare of barley in Europe increased by 4.63%, compared to 2015. America ranks second in terms of average yield per hectare achieved for the barley crop. The most significant average yield per hectare of barley was achieved in 2016 (3,822 kg/ha). Compared to 2015, the average barley production per hectare grew by 14.01% in 2019 (Elena et al., 2021). Africa occupies the last position at regional level in terms of yield per hectare for barley production. When it comes to barley output yield per hectare, Africa comes in lowest among the regional countries. 2018 was the greatest yield ever measured (1,690 kg/ha). Here, from 2015 to 2019, the barley crop's average yield per hectare improved by 6.76% (Elena et al., 2021).

Table 2: The evolution of the average yield per hectare for the barley crop worldwide, during 2015-2019 (kg/ha) (Elena et al., 2021).

Specification	2015	2016	2017	2018	2019	2015/2019
World-total	2,964	3015	3101	2910	3108	104.08
Africa	1567	1125	1264	1690	1673	106.76
America	3318	3822	3671	3616	3783	114.01
Asia	1965	2018	2048	1899	2031	103.35
Europe	3773	3740	3904	3552	3948	104.63
Oceania	2193	2249	2830	2304	2049	93.430

Ethiopia is Africa's second-largest barley grower, trailing only Morocco with about 26 percent of the continent's total production. About 4.5 million smallholder farmers cultivated barley on more than 1 million hectares of land in 2013–14. Over the last ten years, there has been a consistent rise in the overall production. From 1.1 million metric tons in 2003–2004 to 1.9 million tons in 2013–2014, the production has grown at an average growth rate of 6% annually. Due to the fact that yield growth (roughly 5%) is significantly greater than area growth (1% during the same period), it appears that yield growth has been the primary driver of production growth (Shahidur et al., 2015).

Cross-country historical data study unequivocally demonstrates Ethiopia's great potential for increasing barley yield. Ethiopia's average barley yields lag well behind those of Kenya, South Africa, and a large portion of the industrialized world, even though overall they are higher than the average yield across the continent (Figure 3). Ethiopia's average barley output over the last ten years has been 1.43 tons, which is less than half of what Kenya (3.26 tons/ha) and South Africa (2.93 tons/ha) have produced. The average barley yield in developed nations with excellent yields, such France, Germany, and the Netherlands, is more than six tons per hectare. Ethiopian barley yields are still much lower than regional and global averages, even with the subsector having experienced recent growth (Shahidur. et al., 2015).

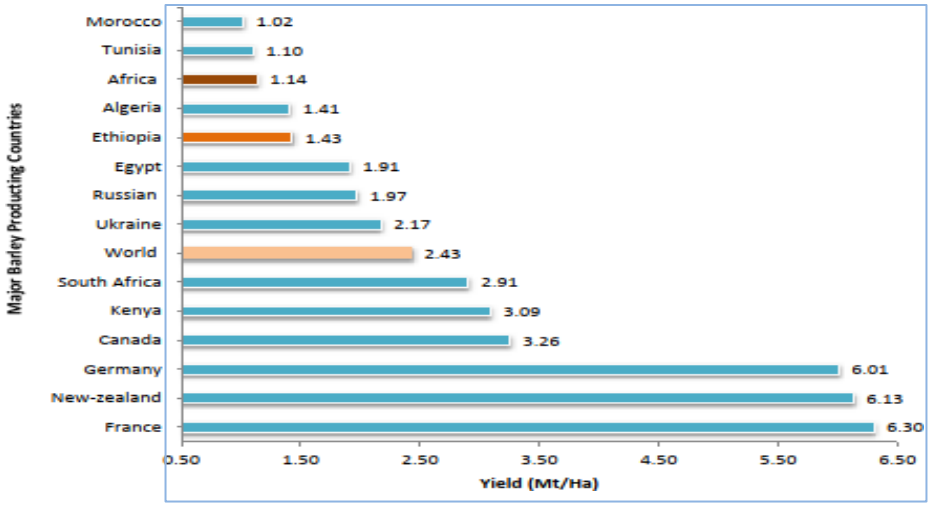

Figure 3: Average barley yield in Ethiopia, compared other countries averages (Shahidur. et al., 2015).

One of the most significant staple food crops in Ethiopia's mid- and highlands has always been barley. During the 2016–2017 main cropping seasons, it was grown on 44,929.97 hectares of land and yielded 110,813.15 tons of grain, with a productivity of 2.47 t ha–1. Similarly, during the 2017–18 main cropping seasons, it was grown on 951,993.15 hectares of land and yielded 2,052,996.372 tons of grain, with 2.16 t ha–1 (Mashilla Dajane, 2019).

Over the previous 25 years, Ethiopia's barley production area has fluctuated. In the late 1970s, it covered around 0.8 million hectares, and by the late 1980s, it had grown to over 1 million hectares. After then, it started to decrease and stayed in the range of 0.8 and 0.9 million hectares until the start of the third millennium. With the exception of the years when the area planted to barley expanded to more than one million hectares, the production of barley has generally been below one million tons annually throughout the majority of the previous 25 years. But productivity has never risen over 1.3 t/ha, or around half of the global average (Lakew, 2020). Over 1 million meher hectares of land were farmed by approximately 4.5 million smallholder farmers for barley in 2013–14. Over the last ten years, there has been a consistent increase in the overall production. From 1.1 million metric tons in 2003–2004 to 1.9 million tons in 2013–2014, the production has grown at an average growth rate of 6% annually. Given that yield growth roughly 5 percent is significantly more than area growth 1 percent during the same period, it suggests that yield growth was the primary driver of output growth (Shahidur. et al., 2015).

Table 3: Variability in barley production, areas covered and yield, by region (2003 -2013) (Shahidur. et al., 2015).

		Measure of Variability (2003-2013)		
Area Cultivated ('000 Ha)	Mean	Annual Compound Growth Rate CGR (%)	Coefficient of Variation (CV)	Cuddly La Valle Index (CDV)
Tigray	96	2.81	0.35	0.34
Amhara	349	0.93	0.29	0.29
Oromia	483	0.70	0.29	0.29
SNNPR	83	1.96	0.38	0.37
National	1014	1.14	0.25	0.25
Production ('000 Mt)				
Tigray	130	7.61	0.52	0.32
Amhara	451	5.42	0.38	0.32
Oromia	769	5.61	0.42	0.31
SNNPR	113	6.25	0.55	0.46
National	1473	5.72	0.40	0.21
Yield (Mt/Ha)				
Tigray	1.35	4.67	0.48	0.30
Amhara	1.29	4.45	0.34	0.22
Oromia	1.59	4.88	0.40	0.15
SNNPR	1.34	4.20	0.41	0.26
National	1.45	4.53	0.38	0.08

In Ethiopia 1,019,477.93ha of land is covered by both food and malt barley and 1908262411q with an average yield about 1872 kg/ha are produced at national level. In Tigray, for 2013/14 meher season barley production was 246551795 kg, produced from 185,337.29 hectares of land (Azeb Hailu *et al*., 2016). Ethiopian peasant farmers produce more than 90% of the country's barley as landrace. In fact, the nation's overall cereal production is made up of around 20% barley production. Ethiopia's output is only 1.37 tons per hectare on average, while the global yield average is approximately 5.5 tons per hectare, with 7 tons per hectare possible under ideal circumstances (Azeb Hailu *et al.*, 2016). The barley producing area expanded from 578,790 ha in 1993 to 959,273 ha in 2016, with a rise in production from 787,484 tons to 2,024,922 tons and a productivity increase from 1.36 tons to 2.11 tons per hectare. This demonstrates an

increase of 65.7%, 157.1%, and 55.1% in area harvested, production, and productivity, respectively (Demeke et al., 2019).

2.6. Genetic diversity study by different markers

It's also possible that the extremely varied geography, height, climate, soil types, and farming practices contribute to the genetic diversity. Studies on genetic diversity using various markers have shown that the farming system, elevation, and types of barley rows influence the population structure of Ethiopian barley. Social influences, such as a predilection for genotypes appropriate for various purposes, also made a substantial contribution to the diversification (Teklemariam et al., 20. The discovery of genetic potentials and their application in breeding programs require a thorough understanding of the genetic variety of crop plants. High-potential hybrids can be produced by selecting parents who are cultivars with appropriate genetic distance (Tarinejad et al., 2015).

2.6.1. Morphological markers

Morphological markers are a marker system based on phenotypic appearance. It remains a crucial genetic marker, having been the first to be employed for assessing variance. Morphological characters are also inexpensive and easy to score (Wodajo, 2012). Since the beginning of breeding, morphological marker techniques have been a crucial tool for choosing plants with desired characteristics. Observable features like leaf form and flower were among the most common markers used during the evolution of plant breeding. Proposed applications of molecular markers included crop color, pubescence color, pod color, seed color, seed shape, awn type and length, fruit shape, flesh color, stem length, etc. However, morphological assessments require the expertise of a species expert because the species is limited in number, its morphology might vary at different developmental phases, and it is susceptible to changes in environmental conditions (Arti et al., 2018). Although morphological characterization does not require expensive equipment, it may be more costly than molecular assessment because extensive tracts of land are occasionally needed for these experiments. Although phenotypic plasticity often affects these traits, this makes it easier to assess diversity when there is environmental variation (Mondini et al., 2009). Generally speaking, morphological indicators have less qualitative characteristics. These dominantly inherited markers are observable and do not require specialized methods to evaluate (Ramesh et al., 2020).

2.6.2. Biochemical Markers

Biochemical markers, also known as isozymes, are multi-molecular enzymes that are encoded by different genes but perform the same tasks. They are enzyme allelic variations, therefore biochemical markers can be used to assess gene and genotypic frequencies. Biochemical markers have been used successfully to detect genetic diversity, population structure, gene flow, and population subdivisions. They are co-dominant, simple to use, and affordable. However, they are fewer in number, detect less polymorphism, and are influenced by diverse extraction methodologies, plant tissues, and different plant growth stages (Nadeem et al., 2018).

Biochemical markers are those produced from the analysis of the chemical products of gene expression. It provides the ability to detect polymorphism of alleles at any specific locus based on protein mobility. Isozyme method is quick, inexpensive, and simple. However, isozyme markers are less common than DNA markers; they underestimate the level of genetic variation, and interpretation of bands can be challenging due to complex banding profiles caused by polyploidy or duplicate genes. Furthermore, proteins with identical electrophoretic mobility (comigration) are not be homologous (Balami, 2007). Several biochemical techniques have been used to complement morphological examination of barley. Characterization using these markers was inefficient for barley types due to limited allelic variation. Molecular markers have shown to be effective tools in the characterization and evaluation of genetic variation within and between species and populations. The advent of the polymerase chain reaction (PCR) favored the development of different molecular techniques such as inter-simple sequence repeat (Al Hadeithi, 2015).

2.6.3. Molecular markers

The era of molecular marker development and application began in the 1980s. This landmak in plant genomics was followed by the development of PCR-based DNA markers a decade later. Since then, numerous genetic markers have been used in various parts of plant molecular breeding and genomics (Amiteye, 2021). A molecular marker is a DNA sequence that is located in a known position on a chromosome or a gene and whose phenotypic expression is frequently discernible and can be used to detect an individual or as a probe to mark a chromosome, nucleus, or locus (Idrees, 2014). A molecular marker is a DNA sequence on a locus in the genome of an organism at which the DNA genomic sequence varies among different individuals of a

population. Molecular markers work by revealing variations (polymorphism) of the DNA sequences between different individuals in the population. These variations include insertions, deletions, point mutations, and translocations (Hussain & Nisar, 2020). DNA-based molecular markers are employed in genetic variation studies to provide an efficient link between genotypic and phenotypic variation. Depending on the approach employed for polymorphism identification, these markers are classified into three types: (1) hybridization, (2) PCR, and (3) sequencing. The majority of them were produced either using genomic libraries or by arbitrary amplification of the genomic areas using PCR, or a combination of both restriction fragmentation using enzymes following selective amplification using PCR (Ramesh et al., 2020). The PCR technique allows particular DNA sequences to be amplified from genomic DNA sections using specific or arbitrary oligonucleotide primers. Molecular markers are among the most useful technologies available for plant improvement research (Amiteye, 2021). Molecular markers are fragments of nuclear, mitochondrial, or chloroplast DNA that contain particular sequences and repetitive DNA (satellite, scattered, and tandem repeats). These technologies, which rely on DNA polymorphisms, can be regarded objective measures of variation and have catalyzed research in a wide range of areas, including phylogeny, taxonomy, ecology, genetics, plant and animal breeding, and so on. Genetic markers are detectable inherited genetic variants that can help us understand genetic components. There are numerous types of genetic markers with various available for assessing genetic variances among natural populations, each with its own set of advantages and disadvantages (Balami, 2007). In recent years different marker systems such as Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) or micro- satellites and others have been developed and applied to a range of crops genetic variation (Wodajo, 2012). Molecular markers have been utilized in recent years in the agronomic sector as powerful tools for the analysis of genetic variation as they offer an efficient way of linking phenotypic and genotypic variation (Garrido-Cardenas et al., 2018).

2.7. Non-PCR based molecular markers :- Restriction Fragment Length Polymorphisms

RFLP is the most common hybridization-based molecular marker. RFLP markers were originally employed in 1975 to discover DNA sequence polymorphisms for genetic mapping of adenovirus serotypes with temperature-sensitive mutations. It was then used to map the human genome before being used to plant genomes. The method is based on restriction enzymes, which reveal a pattern difference in DNA fragment sizes between organisms (Semagn et al., 2006). Restriction Fragment Length Polymorphisms (RFLPs) are markers detected by using restriction enzymes to cleave DNA at a specific sequence. For example, the EcoR1 restriction enzyme breaks DNA anytime the base sequence GAATTC is encountered. Differences in the lengths of DNA fragments will be observed if, for example, one individual's DNA carries that sequence at a certain portion of the genome (e.g., the tip of chromosome 3), but another individual has the sequence GAATTT (which is not cut by EcoR1). RFLPs were the first molecular markers to gain widespread use. Their use, however, is time-consuming and costly, thus simpler marker systems have now been created (Dejene et al., 2010). Detection of the marker is performed by hybridization techniques, labeling a DNA fragment to be used as a probe and carrying out a Southern blot analysis. What is done is to digest different DNA samples with restriction enzymes in the hope that the sequence differences will occur at the cleavage sites of these restriction enzymes, so that a different and characteristic digestion pattern is obtained of each DNA sequence (Garrido-Cardenas et al., 2018). The RFLP is a technique that is not widely used now, but it was one of the first techniques used for DNA analysis in forensic science and several other fields. The RFLP is defined by the existence of alternative alleles associated with restriction fragments that differ in size from each other. The molecular basis of the RFLP is that nucleotide base substitutions, insertions, deletions, duplications, and inversions within the whole genome can remove or create new restriction sites. Despite the fact that it is less commonly utilized now, RFLP analysis has provided significant benefits. It helps scientists map the human genome and provides information on hereditary disease. RFLP analysis is beneficial for determining where a disease-specific gene is located on a chromosome. This is accomplished by examining the DNA of a group of family members that suffer from a specific disease and then looking for RFLP alleles that have the same type of inheritance pattern for the condition. Using RFLP analysis,

scientists can identify those who may be at risk for the disease or carriers of the mutated gene (Al-Samarai & Al-Kazaz, 2015).

2.8. PCR- based molecular markers

2.8.1. Random Amplified Polymorphic DNA (RAPD)

RAPD were the first PCR- based molecular markers to be employed in genetic variation analysis. It is a type of PCR reaction, but the segments of DNA that are amplified are random. RAPD markers are useful DNA based method for initial assessment of genetic variation, especially the assessment of genetic diversity in plant species (Ahmed, 2020). The RAPD markers are small oligo nucleotide primers usually 10 bp in length of arbitrary sequence to generate bands profiles. These primers bind to the complementary sequences along the genome and PCR amplification occurs when the regions between the opposing primer sites are within amplifiable distances (Idrees, 2014). The objective through the use of RAPD markers is the obtaining of fragments of different sizes after carrying out a reaction of PCR on the genomic DNA that is being studied. In practice, what is done is to design random primers that are to be attached to different regions of the DNA, so that a given profile is to be obtained for each pair of primers. If, as a consequence of a mutation, the site to which the primer has to be attached changes, the amplification products will also change, obtaining a substantially different profile. As it is easy to understand, it is not necessary to know in advance the sequence of the DNA to which the primer is to be attached (Garrido-Cardenas et al., 2018).

2.8.2. Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism is a DNA fingerprinting technology that detects DNA restriction fragments by PCR amplification. AFLP provides a high level of resolution for delineating complicated genomic structures, distinguishing individuals in population gene flow experiments, and registering plant varieties (Ahmed, 2020). In this case, the template is DNA that has previously been digested with restriction enzymes. The second key distinction is that with AFLPs, the amplification is selective rather than random. As in the case of RAPDs, in this case, it is not necessary to know the sequence of the DNA to be amplified beforehand and by means of this technique a series of bands of 50–300 bp, known as fingerprints. One of the great advantages of AFLP markers is that they are easily multiplexable, which allows them to increase

their performance considerably. Their main drawback is that when a fragment with low sequence homology is presented between samples, the number of common AFLPs will be very low and the technique is no longer useful (Garrido-Cardenas et al., 2018). However, AFLPs are dominant bi-allelic markers that cannot distinguish between dominant homozygous and dominant heterozygous individuals. The AFLP method is an ideal molecular approach for population genetics and genome typing, it is consequently widely applied to detect genetic polymorphisms, evaluate, and characterize animal genetic resources (Al-Samarai & Al-Kazaz, 2015).

2.8.3. Microsatellites or Simple sequence Repeat (SSR)

Microsatellites are short, repeating DNA units that appear throughout the genome. They are often extremely variable between individuals, making them an appropriate marker for investigating population genetics concerns. Microsatellites or STRs are repetitive co-dominant sequences of 2–6 bp of DNA that are present throughout the entire genome. They are often used for identification or fingerprinting of DNA Microsatellites consist of moderately repeated tandem copies of simple sequence repeats (SSR; 2 to 6 bp in length) flanked by unique sequences. Microsatellite markers are short DNA fragments that are usually less than 100 bp. They consist of motifs of one to six nucleotides, repeated several times, and have a characteristic mutational behavior. They may be dinucleotide, trinucleotide, or tetranucleotide, and the majority of the repeats are found in noncoding regions. These are also called short tandem repeats, single sequence repeats, or single sequence length polymorphisms. The origin of such polymorphism is most likely due to slippage events occurring during DNA replication. Microsatellites are classified according to the type of repeat sequence as perfect, imperfect, interrupted, or composite. Microsatellites can be detected by PCR amplification of a single tandem repeat locus with primers that anneal to its flanking region. The alleles at each microsatellite locus are the PCR-amplified fragments that express the size polymorphism. Microsatellites are favored molecular markers due to their high amount of allelic variation, which can be easily examined, the fact that they are codominantly inherited, and their variety of application (Munaut *et al*., 2011). Microsatellite sequences are especially suited to distinguish closely related genotypes; because of their high degree of variability, they are favored in population studies and for the identification of closely related cultivars. If nucleotide sequences in the flanking regions of the microsatellite are known, specific primers (generally 20–25 bp) can

be designed to amplify the microsatellite by PCR (Ahmed, 2020). Unlike conserved flanking regions, microsatellite repeat sequences mutate frequently by slippage and proofreading errors during DNA replication that primarily change the number of repeats and thus the length of the repeat string. Because alleles differ in length, they can be distinguished by high-resolution gel electrophoresis, which allows rapid genotyping of many individuals at many loci for a fraction of the price of sequencing DNA (Idrees Muhammad, 2014).

2.8.4. Single Nucleotide Polymorphisms

Single nucleotide polymorphism (SNP) detection technologies are used to look for novel polymorphisms and identify the allele(s) of an existing polymorphism in target sequences. When two human genomes are compared side by side, they are 99.9% similar. However, with a 3.2 billion base pair genome, each person harbors some 3.2 million differences in his/her diploid genome. Most of the differences are due to single base substitution polymorphisms, popularly known as single nucleotide polymorphisms (SNPs). While the majority of the SNPs are of no biological consequence, a fraction of the substitutions have functional significance and are the basis for the diversity found among humans. SNPs, as genetic markers, can be used to track the inheritance patterns of chromosomal regions from generation to generation, making them useful tools for studying genetic factors associated with human diseases (Kwok & Chen, 2003). Single nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide (A, T, G or C) differs among members of a species. SNP is the most abundant marker system both in animal and plant genomes and has recently emerged as the new generation molecular markers for various applications. Because they are binary or co-dominant, they can effectively distinguish between homozygous and heterozygous alleles. Furthermore, unlike microsatellites, their power stems from the vast number of loci that can be examined (Idrees Muhammad, 2014).

2.8.5. Inter Simple Sequence Repeats (ISSR)

ISSR, a molecular marker technology, has been available since 1994. It is a glorified RAPD that has been used to examine polymorphisms based on the presence of microsatillites throughout the genome. Longer primers (14-16 base pairs) allow for more rigorous annealing conditions during PCR amplification. The inter-simple sequence repeat (ISSR) technique is a PCR-based method that entails amplifying a DNA segment located at an amplifiable distance between two identical microsatellite repeat regions orientated in opposite directions. In this method, microsatellites of

16-25 bp length are employed as primers in a single primer PCR reaction, targeting various genomic loci to amplify primarily inter-SSR sequences of varying widths (Tarinejad *et al.*, 2015). Inter-simple Sequence Repeat 16-25 bp long primers in a single primer PCR reaction that targets various genomic loci to amplify inter-SSR sequences of different sizes. The microsatellite repeats employed as primers might be di-or tri-nucleotide. ISSR segregates primarily as dominant markers through straightforward Mendelian inheritance. However, they have been demonstrated to segregate as codominant markers in some circumstances, allowing for the differentiation between homozygote and heterozygote. Since 1994, ISSR has been utilized for genome mapping, diversity analysis, and DNA fingerprinting of a broad variety of organisms (Idrees Muhammad, 2014). In the present research, we used the dominantly expressed multilocus DNA markers, and inter-simple sequence repeats which had been successfully used in advance for cereals in genetic variation assessment, cultivar and genotype identification, fingerprinting of interspecific hybrids and gene tagging of interesting agronomic traits (Tarinejad et al., 2015). The ISSR method has several benefits over other techniques: first, it is known to be able to discriminate between closely related genotypes, and second, it can detect polymorphisms without any previous knowledge of the crop's DNA sequence (Abdella et al., 2022).

Figure 4: Schematic illustration of the design and annealing of ISSR PCR primers (Samuel, 2021).

Inter simple sequence repeat (ISSR)-PCR is a technique that involves the use of microsatellite sequences as primers in a polymerase chain reaction to generate multi-locus markers. It is a simple and quick method that combines most of the advantages of microsatellites (SSRs) and amplified fragment length polymorphism (AFLP) with the universality of random amplified polymorphic DNA (RAPD). ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping, and evolutionary biology (Marwal et al., 2020). Inter-simple sequence repeat (ISSR), inter-retro transposon amplified polymorphism (IRAP) and retro transposon-microsatellite amplified polymorphism (REMAP) constitute a broad class of marker that exploits the existence of large numbers of copies of highly conserved repetitive sequences throughout the genomes of many plants. Amplification of inter-simple sequence repeats (ISSRs) is a relatively novel technique and has proven to be a powerful, rapid, simple, reproducible and inexpensive way to assess genetic diversity or to identify closely related cultivars in many species including fruit trees. The ISSR marker system detects polymorphisms in inter-microsatellite DNA regions without any prior sequence knowledge (Marwal et al., 2020).

3. MATERIALS AND METHODS

3.1. Plant materials and sampling strategy

The study included 97 Barley (*Hordeum vulgare*) L. germplasms from fourteen populations (Table 4 and Appendix 4) obtained from EBI. Seeds of the study materials were previously collected from Oromia, Amhara, Tigray, Benishangul-Gumuz and, Southern Nations Nationalities and Peoples Regional States which were the major barley (*Hordeum vulgare*) L.) growing regions of Ethiopia (Figure 5). For genomic DNA extraction, healthy young leaves were collected from two-three weeks old seedlings that were grown in pot at Adama Science and Technology University. The collected leaf samples were dried with silica gel in zip locked plastic bag at room temperature and preserved until genomic DNA extraction was executed. The dried leaf was delivered to the Plant Genetics Research Laboratory, College of Natural Sciences, Addis Abeba University, Ethiopia, for genotyping. The accessions were chosen based on their major growth regions.

Table 4: Number of Barley (*Hordeum vulgare* L.) genotypes used for molecular diversity analysis.

N0	Populations	No. of Genotypes	Latitude (degree)	Longitude (degree)	Altitude (meter)
1.	Jimma	4	08-17-00-N	35-33-00-E	2000
2.	Ilubabora	9	08-27-00-N	36-22-00-E	1920
3.	West Shewa	3	09-18-00-N	39-17-00-E	2670
4.	East Shewa	2	09-18-00-N	39-17-00-E	2930
5.	North Shewa	3	09-18-00-N	39-17-00-E	3190
6.	East Hararghe	3	09-19-00-N	41-37-00-E	3250
7.	Bale	8	07-08-00-N	40-42-00-E	1800
8.	Arsi	20	07-36-00-N	39-28-00-E	2750
9.	South Wollo	8	10-58-00-N	39-39-00-E	2450
10.	Hadiya	7	07-41-00-N	37-52-00-E	2800
11.	North Gonder	5	13-07-00-N	37-52-00-E	2720
12.	Metekel	5	10-30-00-N	35-50-00-E	2200
13.	Eastern Tigray	11	14-16-00-N	39-26-00-E	2650
14.	Southern Tigray	9	13-29-00-N	39-30-00-E	2400
	Total	97			

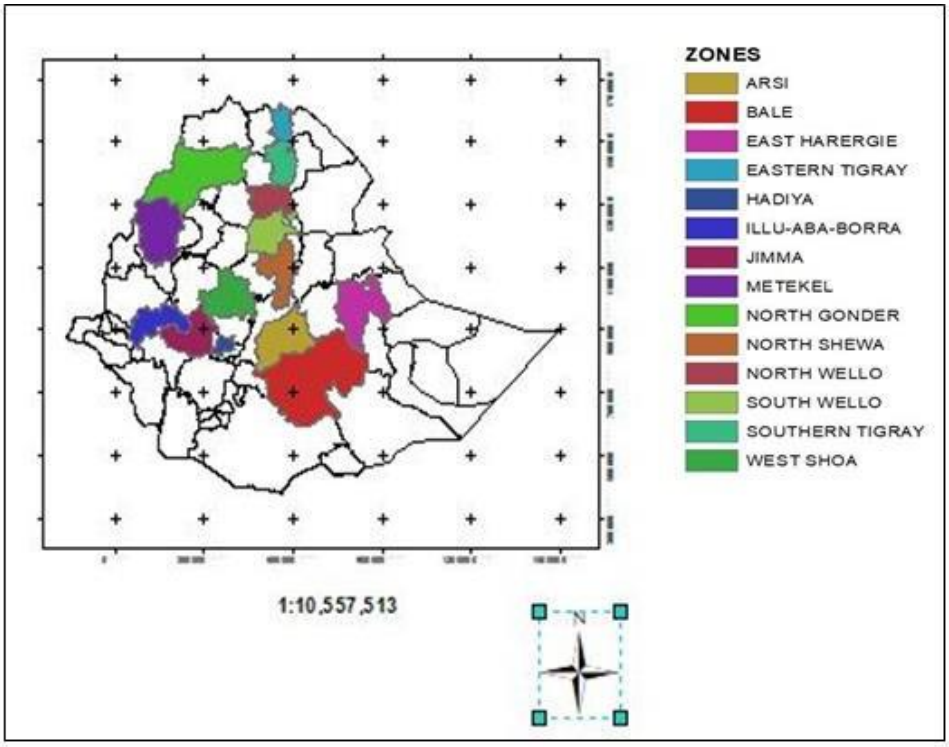

Figure 5: Map of Ethiopia showing collection sites for Hordeum vulgare populations

3.2. Genomic DNA extraction

A total of 97 barley (*Hordeum vulgare* L.) individuals were used in this study. The plant was grown in pot at Adama science and Technology University. The harvested plant leave were preserved in zip-lock plastic bag with the appropriate amount of silica gel. For genotyping, genomic DNA (gDNA) was extracted according to the cetyltrimethylammonium bromide (CTAB) protocol (Tahir et al., 2023). The quality and concentration of DNA were checked by gel electrophoresis in 1%agarose and using Nano Drop (Thermo Scientific Nano Drop 2000 Spectrophotometer) (Medhin et al., 2019).

3.3. Primer selection and optimization

A total of twelve available ISSR primers which were originally obtained from University of British Colombia (UBC) were screened for their polymorphism and reproducibility on 14 randomly selected samples. From the twelve available primers, eight primers were selected based on their reproducibility and polymorphism (Table 5). The master mix, which included Taq DNA polymerase, MgCl2, PCR buffer, and dNTPs, was provided by Adis Ababa University's plant genetics and molecular laboratory. Then the primers annealing temperature optimized.

Table 5: List of the 12 primers, sequence, amplification pattern, repeat motifs and annealing temperature, used for optimization and screening.

ISSR Primers	Sequence (5' - 3')	Annealing temp.	Amplification pattern	Remark
UBC-809	AGAGAGAGAGAGAGAGG	55	Reproducible and Polymorphic	selected
UBC-811	GAGAGAGAGAGAGAGAC	51	Reproducible and Polymorphic	selected
UBC-848	CACACACACACACACAGG	55	Reproducible and Polymorphic	selected
UBC-854	TCTCTCTCTCTCTCTCAG	55	Reproducible and Polymorphic	selected
UBC-873	5'-GACAGACAGACAGACA-3'	55	Reproducible and Polymorphic	selected
UBC-880	GGAGAGGAGGAGAGGAGA	55	Reproducible and Polymorphic	selected
UBC-881	(GGTG)3	55	Reproducible and Polymorphic	selected
UBC-895	AGAGTTGGTAGCTCTTGATC	55	Reproducible and Polymorphic	selected
UBC-872	5'-GATAGATAGATAGATA-3'.	55	Not Reproducible and polymorphic	Not selected
UBC-841	$(GA)_8TC$	51	Not Reproducible and polymorphic	Not selected
UBC-864	ATGATGATGATGATGATG	51	Not Reproducible and polymorphic	Not selected
UBC-810	$(CA)_8T$	55	Not Reproducible and polymorphic	Not selected

3.4. PCR Amplification and gel Electrophoresis.

The polymerase chain reaction was conducted using biometra 2003 Thermo cycler machine. Polymerase chain reaction amplification was carried out in a 20µl final reaction volume containing 2 µl template DNA (100 ng L^{-1}), 11.7 µl double distilled water (ultra-pure), 0.5 µl dNTPS mixture (10mM), 2.5 µl PCR buffer (10x), 2.5 µl $MgCl_2$ (25 mM), 0.5µl primer (20 pmol/µl), and 0.3 µl Taq polymerase(1 U/µL). The PCR program was set at an initial denaturation step for 4 minutes at 94^oC followed by 40 cycles of 1-minute denaturation at 94^oC, primers annealing at 51/55^oC (depending on the primers) for 30s, primer extension at 72^oC for 1-minute 30s and final extension at 72^oC for 8 minutes followed with holding temperature at 4°C. The PCR products were stored at 4oc until loaded on the gel for electrophoresis. The products were fractionated in 2% agarose gel using 1 X TBE buffer at 100 V for 2 hrs. The gel was stained in gel red (2 µl), visualized under UV light and subsequently photographed using a BIO-RAD Gel Doc TM EZ Imaging System. A 100 bp size ladder was used to estimate the size of the amplified products.

3.5. Data scoring and analysis

Each ISSR bands was treated as a unit character and clear bands were counted manually after taking gel image with gel documentation system and scored as binary data; '1' for band present, '0' for band absent, and '?' for missing data. The resulted binary data matrix was subjected to different molecular software to execute the various genetic diversity parameters. Number of alleles (Na), number of effective alleles (Ne), Shannon's information index (I) gene diversity (h), genetic identity and genetic distance (GD), analysis of molecular variance (AMOVA), percentage of polymorphic loci (% PPL) and principal coordinate analysis (PCoA) were calculated using GenAlex version 6.5 software. The polymorphism information content (PIC) was computed using the formula: PIC = 2f (1 − f) where f is the frequency of occurrence of polymorphic bands in different primers (Caetano et al., 2019). Locus based diversity indices including major allele frequency (MAF) and polymorphic information contents (PIC), Population differentiation (Fst) and gene flow (Nm) were determined using POPGENE version 1.32. A genetic dissimilarity matrix parameter Unweighted Pair Group Method with Arithmetic mean was calculated using GenAlex version 6.5. Bayesian model-based clustering with presumed K populations was employed for barley germplasm using STRUCTURE HARVESTER, version 2.3.4. The run parameters were 100, 000 interactions of burn in with 100,000 Monte Carlo Markov Chain (MCMC) interactions. Using web-based STRUCTURE HARVESTER, K values from 1 to 10 were used with 3 independent runs for each K. A bar plot for the optimum K was determined using the Clumpak beta version (http://pophelper.com/.).

4. RESULTS

4.1. ISSR primers and their banding patterns

In this investigation 14 populations obtained from five different regions were analyzed using ISSR primers. Eight selected ISSR primers were amplified a total of 105 scorable bands ranging in size from 450 to 1700bp, corresponding to an average of 13.125 bands per primer (Table 6) were used to analyzed 14 populations obtained from five different regions. The highest number of bands (14) was recorded for primer 881 while the lowest bands (7) were generated by primer UBC895. The major allele frequency (1.00) was recorded for the primer UBC880, UBC854, UBC895 while the lowest (MAF =0.732) was obtained by primer UBC809 with the overall mean of 0.928. The PIC values for the ISSR loci ranged from 0.127 (for the marker UBC811) to 0.263 for marker UBC 809, with an average mean value of 0.178 (Table 6). The gene diversity index (h) for all primers ranged from 0.119 (UBC811) to 0.256, 0.373 (UBC881 and UBC809) respectively and average mean value of 0.193 alleles per locus (Table 6). In addition, Shannon information index ranged from 0.187 (for the primer 811) to 0.401 (for the primer 881, with an average of 0.299 (Table 5). Fst values from 0.05 to 0.15 were taken to indicate moderate differentiation between populations, from 0.15 to 0.25 is high differentiation, and greater than 0.25 is very high differentiation (Dido et al., 2021).

The lowest genetic differentiation (Gst 0.019) and the highest gene flow (Nm=2.193) were scored for primer 809 and 880 respectively. Conversely, the highest genetic differentiation value of (0.402) and the lowest gene flow (Nm= 0.031) were recorded for the primer 880 and 873 respectively (Table 6).

Table 6: Summary of genetic diversity indices for the 8 ISSR loci across the 97 *Hordeum Vulgare* germplasm in Ethiopia.

Primer code	Na	Ne	MAF	h	I	PIC	Ht	Hs	Gst	Nm
UBC809	1.857	1.451	0.732	0.373	0.196	0.263	0.260	0.133	0.019	0.448
UBC811	1.50	1.193	0.946	0.119	0.187	0.127	0.118	0.072	0.112	1.149
UBC848	1.92	1.251	0.95	0.157	0.249	0.156	0.160	0.098	0.029	1.38
UBC854	1.714	1.326	1.00	0.190	0.292	0.220	0.201	0.113	0.020	0.427
UBC873	1.642	1.245	0.94	0.146	0.229	0.150	0.172	0.071	0.166	0.031
UBC880	2.00	1.414	1.00	0.236	0.354	0.183	0.237	0.090	0.402	2.193
UBC881	1.930	1.410	0.857	0.256	0.401	0.164	0.218	0.125	0.357	0.884
UBC895	1.857	1.26	1.00	0.190	0.317	0.156	0.211	0.0516	0.279	0.373
Max	2.00	1.451	1.00	0.373	0.401	0.263	0.260	0.133	0.402	2.193
Min	1.50	1.193	0.732	0.119	0.187	0.127	0.118	0.0516	0.019	0.031
Mean	1.800	1.323	0.928	0.193	0.299	0.178	0.196	0.0975	0.504	0.491

Na: Observed number of alleles; Ne: Effective number of alleles; MAF: major allelic frequency I: Shannon's Information index; h: Nei's gene diversity; Ht: Total genetic diversity Hs: genetic differentiation; Gst: Genetic differentiation statistics by locus; Nm: estimate of the number of migrants (gene flow) from Gst where Nm = 0.5 (1 − Gst)/Gst, PIC: polymorphic information content, Max: Maximum, Min: minimum

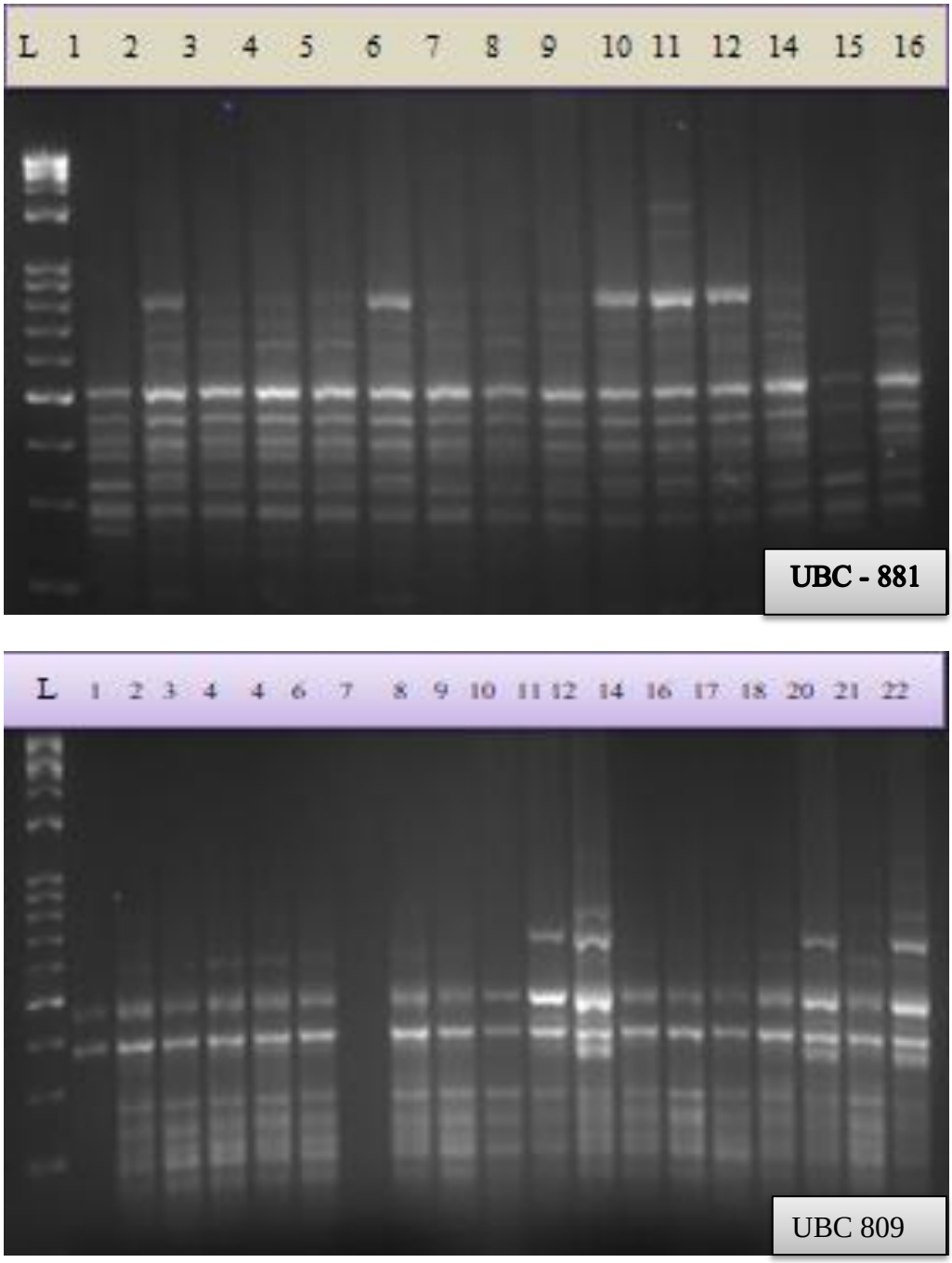

Figure 6: ISSR profiles of H. Vulgare genotypes generated for primer UBC 881and UBC 809 respectively

L: ladder, 1: Genotype 1, 2: Genotype 2, 3: Genotype 3, 4: Genotype 4, 5: Genotype 5, 6: Genotype 6, 7: Genotype 7, 8: Genotype 8, 9: Genotype 9, 10: Genotype 10, 11: Genotype 11, 12: Genotype 12, 14: Genotype 14, 15: Genotype 15, 16: Genotype 16, 17: Genotype 17, 18: Genotype 18, 20: Genotype 20, 21: Genotype 21, 22: Genotype 22.

4.2. Genetic diversity within and among populations

The ISSR markers exhibited a wide range of polymorphic information content (PIC) and Nei's gene diversity across genomes of Ethiopian barley (*Hordeum vulgare* L.). Frequency distribution of ISSR for gene diversity, and frequency of the major allele values of the genome is presented in (Table 6). The overall mean value of polymorphic information content was that ranged from 0.127% (UBC 811) to 0.263 (UBC 809). Nei's genetic diversity score was varied from 0.043 (North Gonder) to 0.177 (Jimma) with a mean value of 0.097. The highest number of bands was scored for sample collected from Jimma showing 68, followed by North Shewa, East Hararghe and Arsi with (60) bands. The least bands (40) were observed for sample collected from Metekel (figure 7). The PPL per population was between 10.48% (MT) and 48.57% (Jimma) with an overall mean of 27.28%. Therefore, among *Hordeum vulgare* populations, Jimma populations exhibited relatively the highest Nei's gene diversity (h = 0.177) and percentage of polymorphic loci (PPL = 48.57%), followed by the population of Arsi that resulted (h = 0.1448; PPL = 45.71%) (Table7). Shannon's Information index measure (I) ranged from 0.187 (Ilubbabora) to 1.241 (East Hararghe) and 1.245 (Arsi) with an overall mean of 0.145 (Table 7). Similarly, Arsi population was also reported by having of the highest Shannon's index (Allel et al., 2017).

The degree of genetic differentiation among the populations that was close to one another, such as Jimma with Ilubabora. On the other hand, regions like, West Shewa, East Shewa, North Shewa, East Haraghe, Bale with Jimma and Arsi with Ilubbabora exhibited a moderate level of genetic differentiation. There was higher genetic differentiation between East Shewa with Eastern Tigray, Metekel and South Wollo populations.

Pair wise population genetic distance matrix confirmed the presence of considerable genetic distance among tested *H. vulgare* populations collected from different regions of Ethiopia. The pair wise Nis's genetic distance between the populations ranges from 0.037 (between populations of Jimma and Ilubabora) to 0.245 between populations of West Shewa with the populations of Eastern Tigray, Metekel and South Wollo with West Shewa (Table 8).

Table 7: Summary of allelic patterns and diversity indices across populations over the eight ISSR loci.

Pop	N	Band freq.	Na	Ne	I	h	He	Uhe	PPL
JIM	3.94	0.391	1.133	1.304	0.265	0.177	0.177	0.204	48.57%
ILUB	8.839	0.340	0.857	1.220	0.187	0.125	0.125	0.133	36.19%
WSH	2.946	0.374	2.943	0.533	1.083	0.048	0.071	0.048	12.38%
ESH	2.000	0.333	0.533	0.505	1.081	0.047	0.069	0.047	11.43%
NSH	2.982	0.400	0.924	0.857	1.215	0.122	0.181	0.123	31.43%
EH	2.946	0.380	0.943	0.876	1.241	0.135	0.198	0.135	33.33%
BL	7.857	0.296	0.905	0.838	1.203	0.120	0.181	0.120	35.24%
AR	19.44	0.304	0.990	1.000	1.245	0.144	0.219	0.145	45.71%
SW	7.670	0.321	0.790	0.781	1.191	0.108	0.161	0.109	29.52%
NG	4.866	0.325	0.514	0.581	1.068	0.043	0.068	0.043	15.24%
HD	6.759	0.318	0.619	0.629	1.100	0.061	0.092	0.060	19.05%
MT	4.830	0.332	0.476	0.486	1.084	0.045	0.065	0.045	10.48%
ETG	10.607	0.345	0.648	0.657	1.149	0.085	0.124	0.084	21.90%
STG	8.920	0.327	0.800	0.810	1.176	0.102	0.154	0.102	31.43%
Mean	6.747	3.243	0.753	1.169	0.145	0.0977	0.098	0.109	27.28%

Key: N: sample size, Na: Observed number of alleles, p & q: Estimated Allele Frequency, h: Nei's gene diversity, I: Shannon's Information index, and PPL: percentage of polymorphic loci. Na: No. of Different Alleles, Ne: No. of Effective Alleles: 1/(p^2 + q^2), I: Shannon's Information Index: − 1* (p * Ln (p) + q * Ln(q)), He = Expected Heterozygosity: 2 * p * q, uHe: Unbiased Expected Heterozygosity: (2N/(2N − 1)) * He Where for Diploid Binary data and assuming Hardy–Weinberg Equilibrium, q: (1 − Band Freq.) ^0.5 and p: 1 – q.

In the banding pattern analysis of 97germplasms that were grouped into 14 populations, Jimma exhibited the highest number of bands (68), followed by North Shewa, East Hararghe and Arsi had similar number of bands (57). While, Metekel represent the lowest number of bands (39) (Figure7).

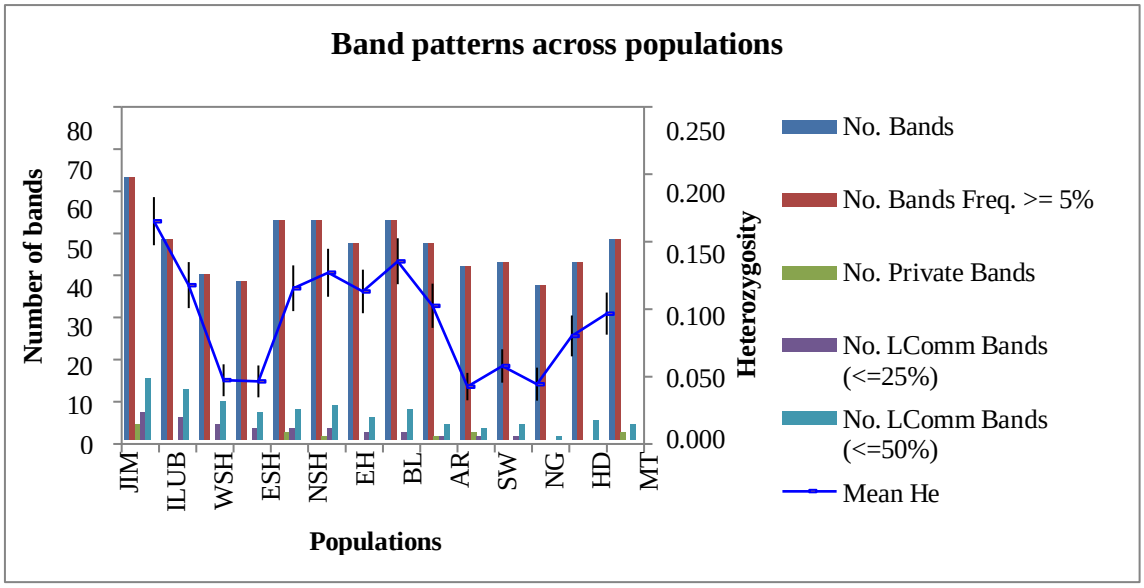

Figure 7: Band pattern across populations.

The pairwise coefficient of genetic differentiation between the populations ranged from 0.036 (between Hadia and south wollo) and 0.037 (between Ilubbabora and Jimma) to 0.245 (between ETG and WSH). The highest and statistically significant genetic differentiation, (PhiPT = 0.245, p = 0.001) was observed between populations of Southern Tigray and East Shewa, implying the lowest gene flow between them. The second highest genetic differentiation with a gene flow rate of 0.240 was observed between the populations of Metekel and South Wollo with West Shewa (Table 8).

Table 8: Pairwise Population Matrix of Nei Genetic Distance

Pop ID	JIM	ILUB	WSH	ESH	NSH	EH	BL	AR	SW	NG	HD	MT	ETG	STG
JIM	0.000													
ILUB	0.037	0.000												
WSH	0.106	0.081	0.000											
ESH	0.143	0.116	0.076	0.000										
NSH	0.143	0.140	0.132	0.155	0.000									
EH	0.142	0.145	0.151	0.150	0.074	0.000								
BL	0.110	0.114	0.139	0.134	0.070	0.074	0.000							
AR	0.131	0.134	0.211	0.178	0.103	0.067	0.047	0.000						
SW	0.186	0.212	0.240	0.209	0.152	0.062	0.095	0.061	0.000					
NG	0.183	0.191	0.230	0.200	0.146	0.092	0.091	0.073	0.081	0.000				
HD	0.192	0.194	0.216	0.182	0.159	0.086	0.105	0.092	0.036	0.098	0.000			
MT	0.182	0.185	0.240	0.214	0.124	0.111	0.118	0.086	0.077	0.129	0.072	0.000		
ETG	0.168	0.189	0.245	0.212	0.136	0.086	0.109	0.077	0.052	0.093	0.067	0.051	0.000	
STG	0.196	0.205	0.239	0.243	0.147	0.056	0.118	0.077	0.064	0.123	0.115	0.135	0.088	0.00

Nei's Original Measures of far genetic distance (0.243) was recorded among barley genotypes obtained from Southern Tigray with East Shewa, and (0.249) between Southern Tigray with West Shewa indicates the existence of high genetic distance among the genotypes (Table 9). The genetic distance ranged from 0.036 to 0.249. Besides, the genetic identity ranged from 0.78 to 0.964, and the highest genetic identity was found among Ilubbabora and Jimma, Bale and Arsi, Hadiya, and South Wollo. However, a lower (0.78) genetic identity value was observed between genotypes originating from East Shewa and southern Tigray (Table 9).

Table 9: Nei's Original Measures of Genetic Identity and Genetic distance

Pop	JIM	ILUB	WSH	ESH	NSH	EH	BL	AR	SW	NG	HD	MT	ETG	STG
JIM	****	**0.9641**	0.8996	0.8667	0.8666	0.8678	0.8957	0.8769	0.8301	0.8435	0.8472	0.8337	0.8408	0.8221
ILUB	0.0366	****	0.9226	0.8902	0.8695	0.8647	0.8920	0.8748	0.8093	0.8300	0.8305	0.8312	0.8233	0.8147
WSH	0.1058	0.0805	****	0.9270	0.8762	0.8600	0.8702	0.8097	0.7863	0.7930	0.8139	0.7865	0.8033	0.7872
ESH	0.1430	0.1163	0.0758	****	0.8560	0.8606	0.8746	0.8367	0.8117	0.8091	0.8335	0.8076	0.8086	**0.7841**
NSH	0.1432	0.1398	0.1322	0.1555	****	0.9289	0.9324	0.9025	0.8586	0.8744	0.8737	0.8835	0.8945	0.8633
EH	0.1418	0.1454	0.1508	0.1502	0.0738	****	0.9285	0.9350	0.9395	0.9075	0.9091	0.8946	0.9140	0.9454
BL	0.1101	0.1143	0.1390	0.1339	0.0700	0.0741	****	0.9539	0.9091	0.9068	0.9045	0.8885	0.9005	0.8883
AR	0.1314	0.1338	0.2110	0.1782	0.1026	0.0672	0.0472	****	0.9408	0.9311	0.9098	0.9176	0.9053	0.9256
SW	0.1862	0.2116	0.2404	0.2087	0.1524	0.0625	0.0953	0.0611	****	0.9115	0.9502	0.9257	0.9283	0.9377
NG	0.1702	0.1863	0.2319	0.2118	0.1342	0.0970	0.0978	0.0714	0.0926	****	0.9171	0.8887	0.8832	0.8750
HD	0.1658	0.1858	0.2059	0.1821	0.1350	0.0953	0.1004	0.0946	0.0511	0.0866	****	0.9510	0.9111	0.8740
MT	0.1818	0.1849	0.2401	0.2137	0.1239	0.1114	0.1182	0.0860	0.0772	0.1180	0.0502	****	0.9304	0.8737
ETG	0.1734	0.1945	0.2190	0.2124	0.1115	0.0899	0.1048	0.0995	0.0744	0.1242	0.0931	0.0722	****	0.9086
STG	0.1958	0.2049	0.2392	0.2433	0.1470	0.0562	0.1184	0.0773	0.0643	0.1335	0.1346	0.1350	0.0958	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

JIM: Jimma, Ilub: Ilubbabora, WSH; West Shewa, ESH East Shewa, NSH: North Shewa, EH: East Hararghe, SW: South Wollo, HD; Hadiya, NG: North Gonder, MT: Metekel, ETG: Eastern Tigray, STG: Southern Tigray. ****: 0000

4.3. Genetic similarity among the barley (*Hordeum vulgare* L.) populations

Summary of the genetic similarity above diagonal and genetic distance below diagonal of the fourteen populations computed based on Jaccard's similarity index. The largest (0.964) similarity coefficient was between Ilubabora and Jimma and smallest similarities coefficient (0.784) was between West Shewa and Eastern Tigray) populations (Table 9). The second highest genetic similarity was observed (0.953) between the populations of Arsi and Bale (Table 9).

4.4. Analysis of molecular variance (AMOVA).

Analysis of molecular variance (AMOVA) revealed the presence of significant ($p = 0.001$) and higher genetic differentiation (0.367) with highest gene flow (2.193) and the variability within populations accounted (63%) of the total genetic variation and 37% of the total variation among populations. Therefore, without grouping, (Table: 10) high variation is attributed within the 97 barley populations with high fixation index of (0.367) with least variation among populations.

Table 10: Analysis of molecular variance (AMOVA) showing distribution of genetic variation within and among populations of *H. vulgare* using ISSR markers.

Source of variation	Df	SS	MS	Est. Var.	PV %	F-Statistics	P-value
Among Pops	13	484.480	37.268	4.42	37	0.367	0.001
Within Pops	83	632.108	7.616	7.616	63		
Total	96	1116.588		12.525	100		0.001

DF = Degrees of Freedom SS = Sum of Squares; MS = Mean Square; Est. Var. = Estimated Variability, Nm=haploid populations= [(1 / Fst) - 1]/ 4, PV =percent of variation

4.5. Cluster and principal co-ordinates analysis

4.5.1. Cluster analysis

The UPGMA cluster analysis divided the 97 individuals into four main clusters (Cluster C1, C2, and C3) and C4 (Figure 8). The first cluster (C1) was 6 groups that composed of the samples from Jimma, Ilubbabora, North Shewa, Bale, East Hararghe and West Shewa. This population

may appear together due to long distance transfer of pollen & Migration individuals through different pollinators. The second cluster (C2) was consists of 5 genotype and includes sample from Jimma, Bale, East Hararghe, South Wollo and Arsi. Another cluster (C3) had 6 populations which were from Arsi, Bale, Eastern Tigray, southern Tigray, North Shewa and East Hararghe (Figure 8). Cluster (C4) had considered as the largest sub group which contains the Northern Gonder, Hadiya, Metekel, Eastern Tigray and Southern Tigray. This clustering analysis confirmed the presence of intermixing among the individuals of the various populations, with the exception of the Ilubbabora, West Shewa, and East Shewa populations, which form their own groups with no intermixing between them or with other populations at the sub-cluster levels.

Some of the individual accessions collected from the same region tend to spread all over other cluster and forming their own group, likely due to the presence of considerable gene flow among population (Figure 8).

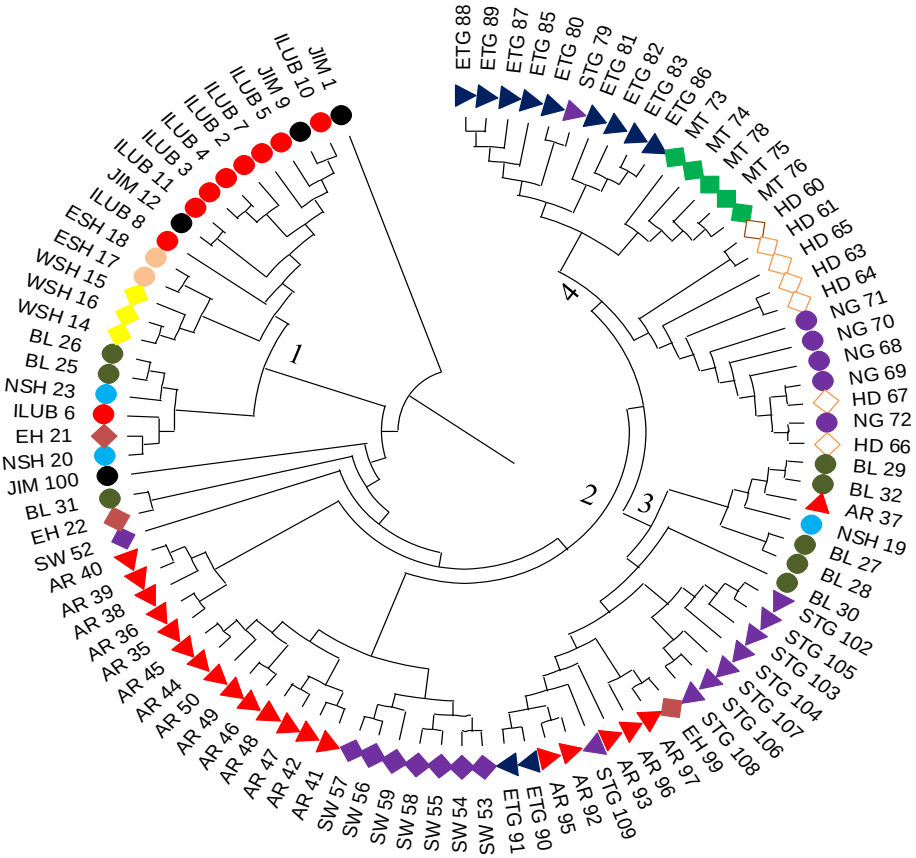

Figure 8: UPGMA based dendrogram showing genetic relationships among the eight Populations of H. vulgare using ISSR primers

4.5.2. Principal co-ordinates analysis

PCoA is a technique frequently used in multivariate statistics to display the pattern of genetic structure and similarly to determine the amounts of variance described per component and cumulatively (Atsbeha et al., 2023). The principal coordinate analysis (PCoA) conducted using the 97 *H. Vulgare* samples roughly grouped the populations into four groups (Figure 9). None of the clusters composed of exclusively individual of a single population. The percentages of principal coordinates accounted 36.25% of the total variation. The first, second, and third principal coordinate axes showed 18.42, 9.46% and 8.37%, respectively. It clustered the entire population into four subgroups with high genetic admixture. PCoA grouped the population and except of ESH, ILUB, WSH and MT, all populations are being distributed over second groups (Figure 9).

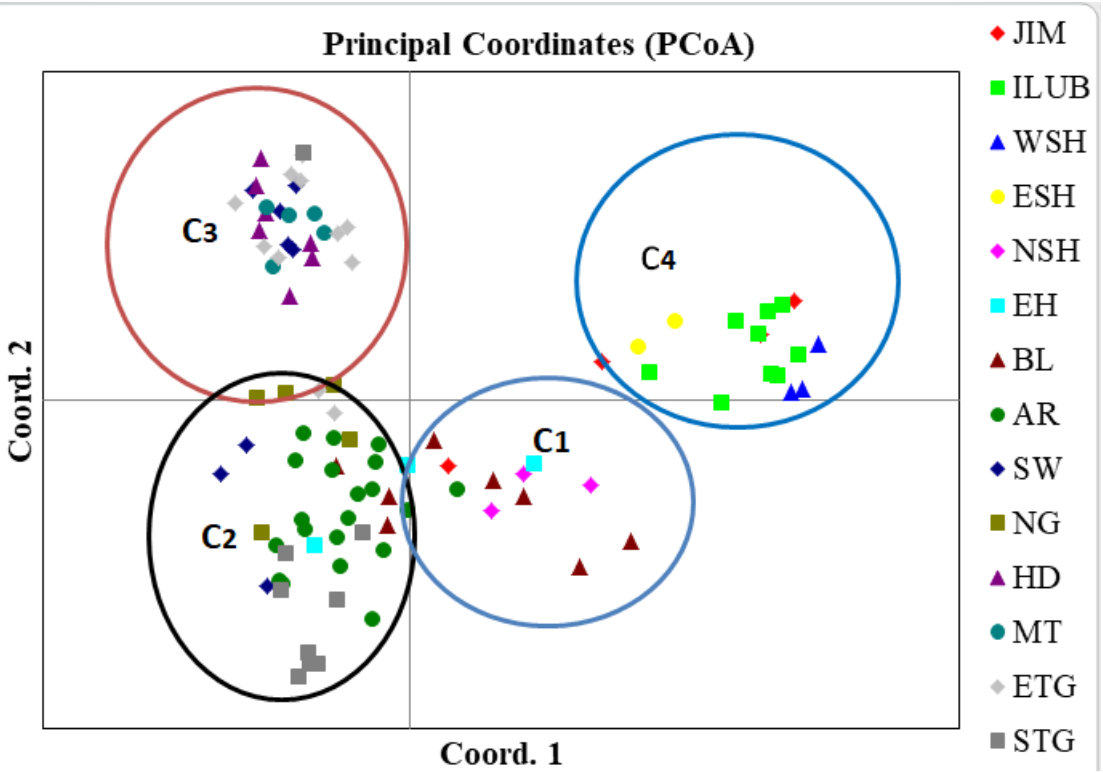

Figure 9: Principal coordinate analysis of H. Vulgare germplasm in Ethiopia accessions using ISSR markers.

4.6. Population structure analysis

To provide further evidence and infer population structure, a Bayesian algorithm implemented in the STRUCTURE 2.3.4 Software analyses was used. The result indicated a clear peak at K = 4 signifying the optimal sub-populations in the panel (Fig. 10-a). Based on this value, Clumpak result (bar-plot) detected high level of genetic admixture and hence there was low degree of geographic origin-based structuring of populations. The UPGMA based clustering analysis (Fig. 8) also identified four clear clusters and some are grouped based on the STRUCTURE based stratification. The smaller numbers of variance explained by the second and consecutive PCs indicated that only few PCs couldn't encapsulate the existing genetic variance in Ethiopian *H. vulgare*.

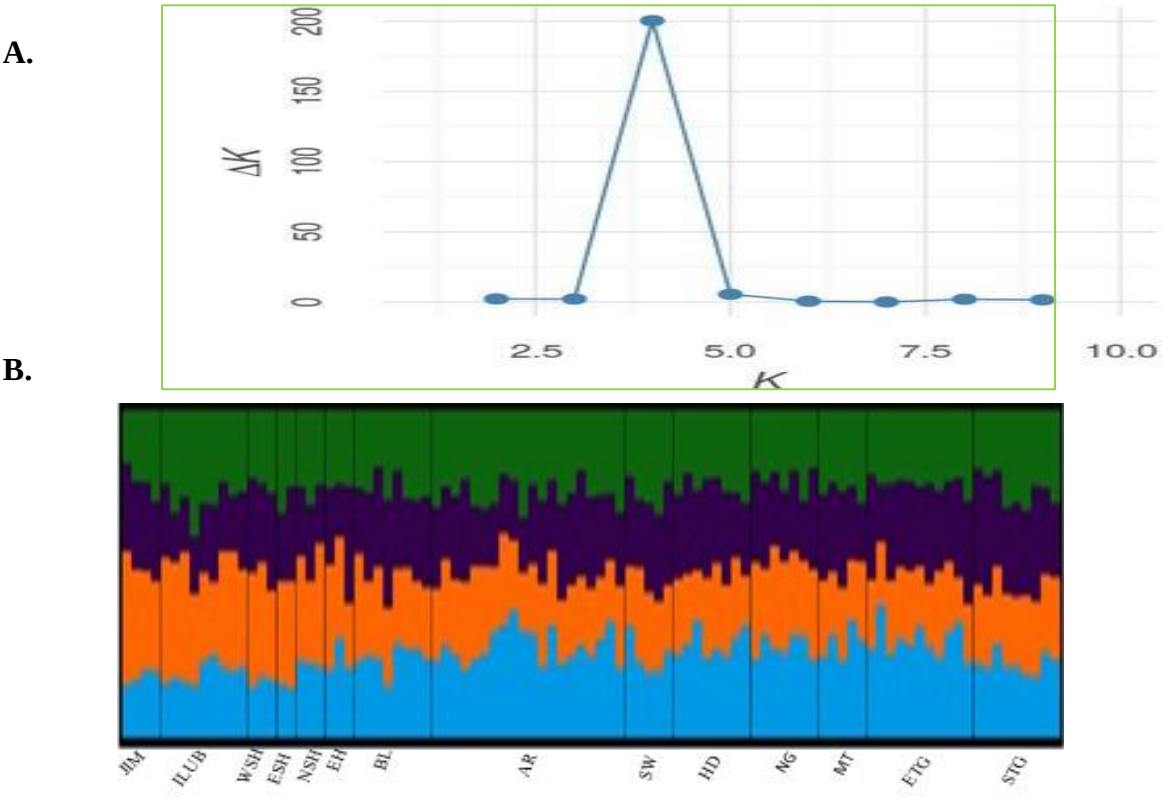

Figure 10 : Population structure analysis for 14 populations representing 97 H. Vulgare germplasm in Ethiopia.

A best estimated delta K value and B estimated population structure for K = 4. The blue, deep orange, green and pink colures represent genetic groups designated by Structure Harvester and each number represents fourteen populations.

5. DISCUSSIONS

The genetic diversity analysis of H. vulgare germplasm is the greatest technique to evaluate genetic diversity and population structure in order to manage crop genetic resources more effectively and improve breeding programs. Such genetic diversity analysis was essential for plant breeders to perform strategic integration and target selection while preserving important economic traits of individual crops. Such genetic diversity analysis was essential for plant breeders to perform strategic integration and target selection while preserving important economic traits of individual crops (Yirgu et al., 2023).

In this study, the highest gene diversity (GD = 0.373) was revealed in barley genotypes (Table 6). This high gene diversity may be a natural crossing due to cultivating mixed genotypes in a field. The genetic diversity for the tested markers showed the smallest (0.119) and the highest values (0.373) for UBC 811 and UBC 809 respectively. The average gene diversity was 0.193, which is less than that Iraqi barley accessions reported by Yirgu et al.,(2023), using SNP, indicated genetic diversity value ranged from 0.32–0.96, with an average value of 0.77 per SNP marker with a mean PIC of 0.74. This high genetic distance indicated, Ethiopia is a center of origin and diversity of many cultivated crops and their wild relatives including barley (Shimelis Tesfaye and Berhanu Sime, 2022). The expected heterozygosity was higher (He = 0.219) in the Arsi, indicating the existence of high genetic variability between the genotypes (Table 6). This may be due to outcrossing in barley. In this work, the average observed number of alleles was greater (Na = 0.753) than the average expected heterozygosity (He=0.098), showing highly related among the barley genotypes. Higher MAF 1.00 with an average value of 0.929 was found in barley genotypes (Table 6); indicating further valuable genes can be exploited from those genotypes. The lower MAF obtained could be due to the smaller number of genotypes studied from the regions. Hence, the MAF value observed in the present study was greater than Yirgu et al., (2023), genetic diversity study for 105 barley genotypes by SNP Markers. This could be due to the region from which genotypes were collected. The average percent of polymorphism per population revealed in the present study 63% (Table 10) was considerably higher than that reported by Yirgu *et al*., (2023) which was 52.85%. This implies that Ethiopian H. *Vulgare* populations exhibit higher genetic diversity.

In this research, the polymorphic information content of the used ISSR loci was found across

populations ranged from 0.127 to 0.263, with an overall mean of 0.178. The moderate PIC observed in most of the ISSR primers could be attributed to the diverse nature of the barley germplasm and informativeness of the ISSR markers used. The ISSR markers used are highly informative, and have been found to be useful genetic tools for barley genetic structure analysis. Moreover, the PIC indicating that all the used markers are informative less to moderate and suitable to assess genetic diversity and phylogenetic relationship analysis among *H. Vulgare* germplasm in Ethiopia. According to Atsbeha et al., (2023), marker with PIC values below (0.25) is somewhat informative, a marker with PIC values between 0.25 and 0.5 is considered as moderate informative, and those greater than 0.5 is said to be highly informative. Thus, the other author (Chesnokov & Artemyeva, 2015), also reported that the maximal value of PIC for dominant markers is 0.5. However, for the markers with multiple alleles and equal distribution in the population, the PIC values are much higher than 0.674. Based on our findings, PIC values were ranged from 0.127 to 0.263. Thus, PIC shows less and moderate informative.

The present study showed the highest genetic distance (0.245) was found between genotypes derived from Eastern Tigray and West Shewa (Table 8), which indicated that the barley genotypes had a high genetic difference. This result was too much higher than the result reported by Yirgu et al., (2023), which shows highest genetic distance of (0.08). Similarly other researcher Allel et al., (2017), showed that the average gene diversity (Ht) over all loci was (0.258). This could be types of primers used and the restricted gene flow and the influence of eco-geographical differences on the existence of high genetic distance. On the other hand, the least genetic distance (0.036) was recorded between Ilubabora and Jima (Table 7). The lower genetic distance among collection regions probably has high levels of the farmer to farmer seed exchange and gene flow across regions. The results indicated genotypes collected from the close geographical location showed high genetic admixture.

The genetic identity ranged from 0.784 to 0.964 (Table 9). The least genetic identity was observed between genotypes collected from East Shewa and Southern Tigray. However, the highest genetic identity (0.9641) was from Jima and Ilubabora and Bale with Arsi, (Table 8). This is possibly due to natural selection for shared genetic components being the key force in determining the high genetic identity between the geo- graphical origins or the lower genetic distance among collection regions probably has high levels of the farmer to farmer seed exchange and gene flow across regions.

Similarly, Wang et al., (2009), reported genetic similarity coefficient was high between the two barley regions (Tibet and the Middle East), being 0.947 and 0.982 for ISSR and SSR respectively, while the observed genetic distance was low, being 0.055 and 0.018 for ISSR and SSR respectively.

As it was reported by (Tiegist Dejene, 2013), the AMOVA result indicated that geographic region accounted for 7.84% of the total variance followed by the variation among accessions within regions (27.77%), while the highest variation was attributed to the within accessions category (64.39%) with degree of population differentiation among accessions within geographical regions (FST = 0.356) which was considered as great genetic differentiation. This finding supports the assertion that geographic regions are not the basis of the genetic variation observed in these barley populations. However, in this study, AMOVA revealed that *H. vulgare* has also great genetic differentiation (Fst = (0.367) with relatively low among populations (37%) variation which was higher within the population (63%). This indicates that even a single population could be a source of breeding materials due to the genetic variability that exists within each population.

In this study, high level of gene flow (Nm=2.193) confirmed the presence of high genetic exchange or gene flow between the populations of *H. Vulgare* germplasm. This result is consistent with earlier studies and it was somewhat similar. The difference could be due to the types of markers, the size of the population, the number of germplasms used and reproducibility of the markers. For instance, (Tiegist & Le´on, 2010), reported that (Nm=2.947) and also suggested as lowest and non-significant population differentiation was observed among Arsi and Bale (Fst=0.028), Arsi and Harerghe (Fst=0.040), Gamo Gofa and Gonder (FST=0.037), Gamo Gofa and Shewa (0.016) and Gojam and Hararghe (0.023) which indicated these regions were more similar. But in this current finding, lowest and non-significant population differentiation was only observed among Arsi with Bale (Fst=0.047), and Ilubbabora with Jimma (Fst=0.037). According to Yirgu *et al.,* (2023), moderate genetic differentiation was recorded between the Tigray and SNNP (0.117) followed by Tigray and Amhara (0.089) and Tigray and Oromia (0.088). However, the current study detected the higher genetic differentiation (0.245) observed between eastern Tigray and West Shewa following Southern Tigray and East Shewa (0.243), south Wollo and Metekel (0.240) with West Shewa.

This revealed the barley genotypes obtained from those regions shows the existence of the

highest genetic variations. Allel et al., (2017), also reported high level of population differentiation (GST = 0.257), which was still blow of the total genetic differentiation (0.367) obtained in this current study. Similar finding reported by Dido *et al*. (2022), the populations of Metekel and Hadiya have less private alleles. Metekel exhibits the least genetic differentiation among the population under investigation in this study as well.

The PCoA showed that the genotypes of the fourteen populations not tended to form grouping based on their geographic regions. While, some individuals were intermixed from one to another population, likely due to the presence of gene flow among *H. vulgare* populations. UPGMA based clustering of the fourteen populations resulted in four major clusters, revealing the presence of moderate population differentiation as a result of the presence of gene flow among population.

Population genetic structure analysis also confirmed that delta K reached its maximum (a sharp peak at K=4), indicating that the populations of *H. vulgare* studied could be grouped into four main populations with greater degree of genetic admixture. The analysis revealed that many populations are possessed genetic background (alleles) from each sub-population confirming the presence of admixture due to the nature of the primers used the origin, distribution and pollination patterns of *H. vulgare* germplasms and gene flow among the populations.

6. CONCLUSIONS AND RECOMMENDATION

6.1. CONCLUSIONS

H. vulgare is one of the most important cereal crops that are widely used in industrial applications. Knowledge of the genetic diversity of this crop plants is very important for its improvement through selection and also conservation purposes. The markers used were highly polymorphic and informative to describe the genetic diversity and population structure. In this current study, the ISSR data generated using this molecular tool provides useful information that can be utilized in breeding and genetic research in barley. Based on ISSR data, the barley genotypes were genetically divergent.

The average total of gene diversity (Ht) was ranged from 0.118 to 0.260 with average mean 0.1968. The highest polymorphism information content (PIC) of 0.263, with mean value of major allelic frequency (MAF) was 0.928, and this revealed a high genetic variation in barley genotypes. The genetic differentiation also showed the existence of variations, ranging from 0.019 to 0.402, indicating moderate to strong genetic differentiation between barley populations (Table 6).

The AMOVA indicated high genetic variations within the populations (63%) than among population (37%). This high diversity could be the foundation for developing and generating desirable new barley varieties with superior grain yield potential and wide adaptability, enhanced with abiotic and biotic resistant traits.

In this study ISSR markers were observed to be an appropriate molecular marker for generating the detail intraspecific genetic diversity data to evaluate the extent and distribution of genetic diversity within and among *H. vulgare* germplasm. Hence, assessment of genetic diversity is vital to know the extent and patterns of genetic diversity in the available genetic resources to exploit the genetic potential of the crop in breeding, its wise use and also conservation measures. In this regard, the high average mean of effective number alleles (1.323) and mean value of PIC (0.178) in the present study confirmed the presence of high genetic diversity among selected major barley growing region of Ethiopian and thus, there is high possibility to improve desired agronomic traits through breeding. Determining the level of variation within and among barley populations is an essential step towards conserving genetic resources and developing future

strategies for plant improvement (Dido *et al*., 2022).

In summary, the populations from Jimma and Arsi, Southern and Eastern Tigray demonstrated high gene diversity among the studied populations based on all parameters analyzed, indicating that these locations could be potential sites for *H. vulgare* genetic diversity and should be targeted for traits improvement through breeding and conservation. Conversely, the Metekel population had the lowest genetic diversity, indicating that special attention should be paid to balancing the genetic resources of such beneficial genes across different agro-ecologies and genetic backgrounds. The results show Tigray was the highly differentiated region, while Arsi and Bale, geographically closer regions, had the least genetic differentiation observed.

Therefore, in addition to baseline data for further research, the current study offered helpful guidance for *H. vulgare* improvement through selection and conservation. The populations were firmly classified into four clusters by PCoA-based clustering, UPGMA analysis, and Structure Harvester analysis, confirming high genetic intermixing and high gene flow among populations.

6.2. RECOMMENDATION

This study is not exhaustive in terms of sample size and area coverage. Hence, more survey and sample collection has to be carried out so as to cover other major barley growing region of Ethiopia where probably *H. Vulgare* occurs. Thus, similar further studies should be carried out on other populations from the remaining parts of the country using a greater number of primers to generate a nationwide picture of the diversity of the crop. Moreover, due attention should be given to Jimma population since it is highly diversified.

Analysis with codominant markers system, like microsatellites, needs to be conducted to better understand and estimate the gene flow, the size of a population and levels of inbreeding.

The present study only assessed the genetic structure of 97 *H. vulgare* germplasm is not enough and it is too few to estimate the genetic variation of the crop. In order to cover the entire region and obtain a better understanding of the genetic makeup of barley nationwide, future research should concentrate on comprehensive data collection and analysis through the surveying of a greater number of accessions and high-density markers that contain large sets of *H. vulgare* genotypes. This will also aid in the development of the most productive cultivars.

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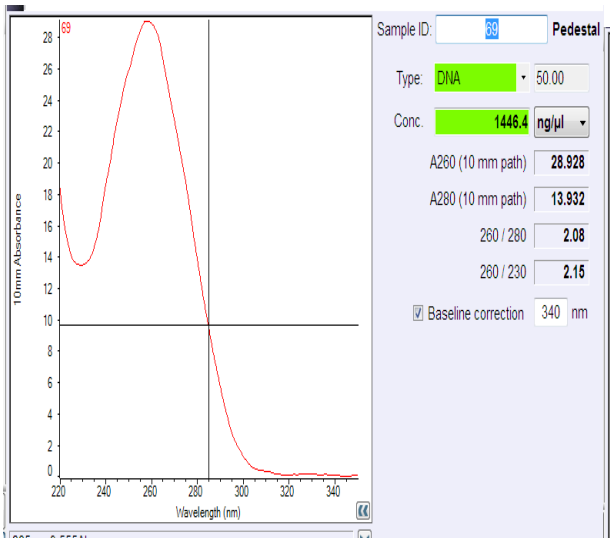
8. APPENDICES

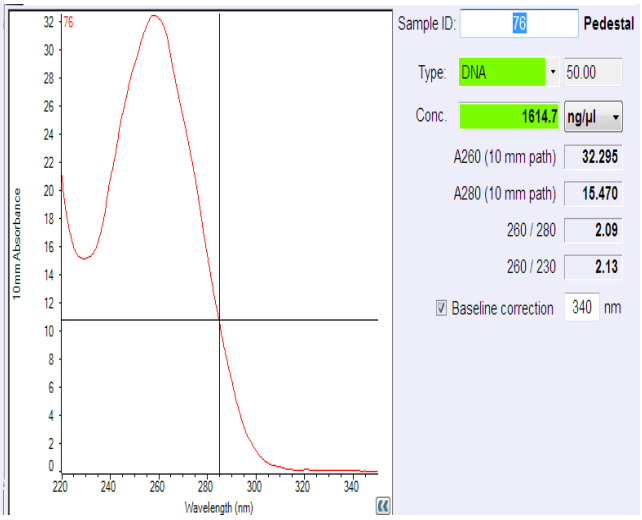
Appendices 1: Representative pictures to give further illustration of all practical works beginning from sample collection to the end of laboratory session

Appendices 2: Quantification and qualification of genomic DNA using Nano drop

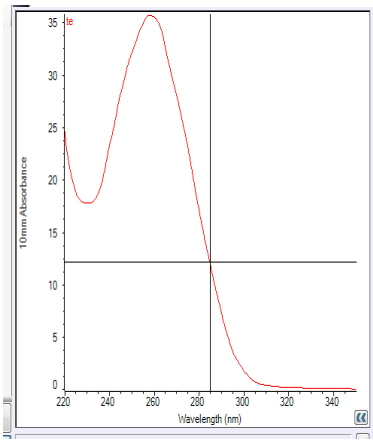
A



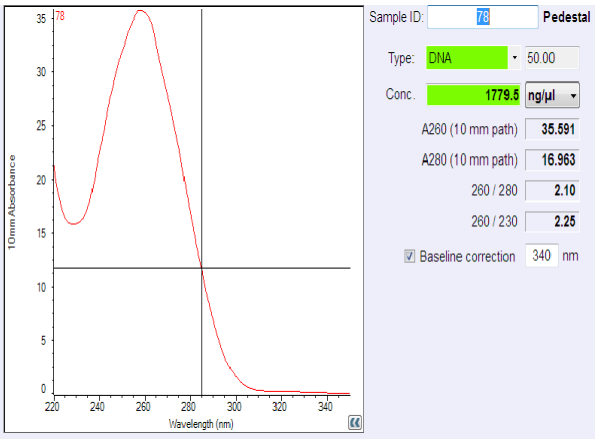
B



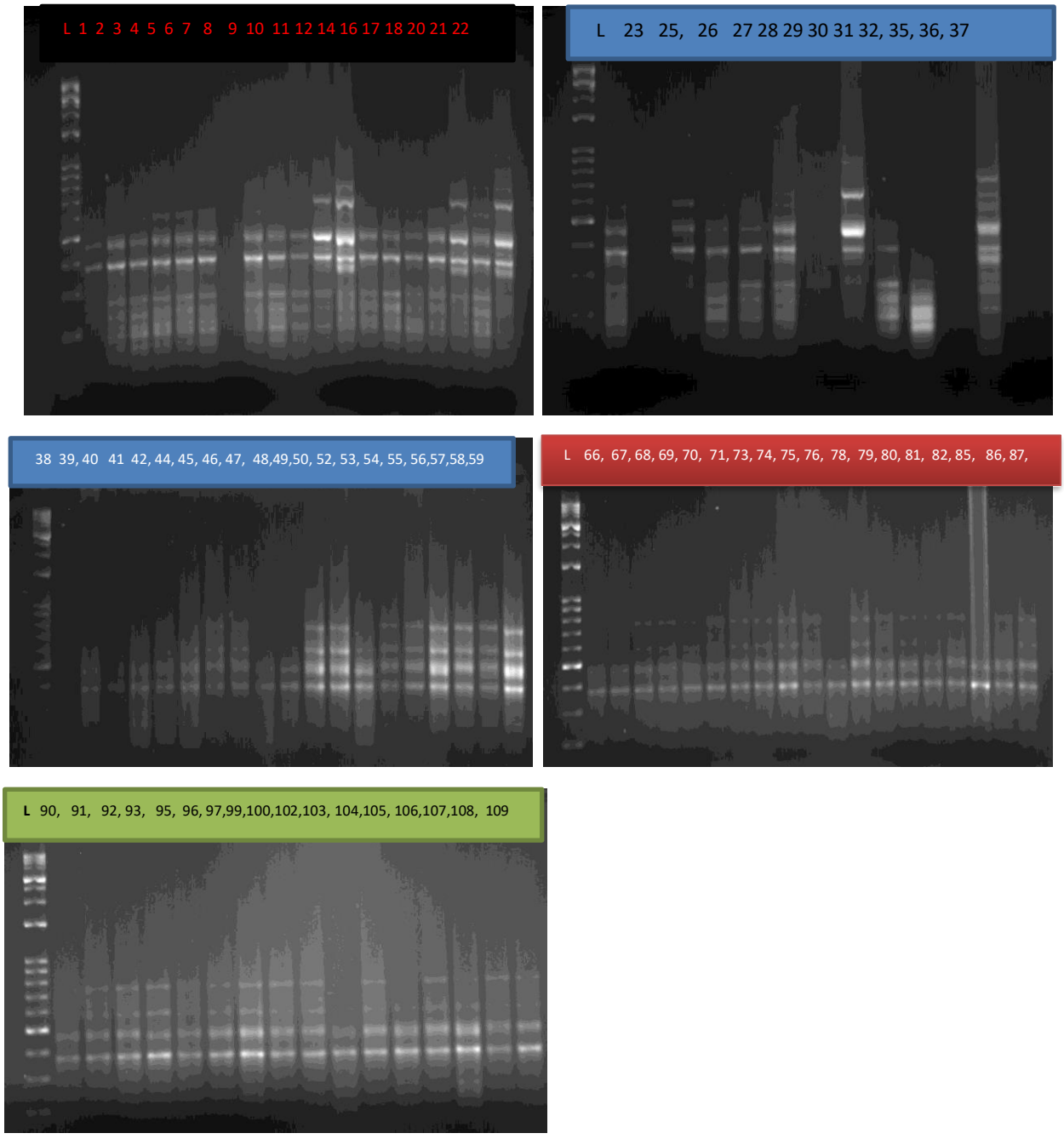
C



B



Appendices 3: ISSR profiles of *Hordeum Vulgare* genotypes generated for primer UBC809



Appendices 4: List of *Hordeum vulgare* samples used in this study

No	Acc. No	Acc.	Region	Zone	Altitude	Longitude	Latitude
1.	1	234917	Oromia	Jimma	1620	08-17-00-N	35-33-00-E
2.	9	234925	Oromia	Jimma	1620	08-17-00-N	35-33-00-E
3.	12	234938	Oromia	Jimma	1620	08-17-00-N	-08-27-00-N
4.	100	232409	Oromia	Jimma	2000	08-17-00-N	35-33-00-E
5.	2	234918	Oromia	Ilubabora	1965	35-33-00-E	08-27-00-N
6.	3	234919	Oromia	Ilubabora	1920	35-33-00-E	08-17-00-N
7.	4	234920	Oromia	Ilubabora	1880	36-22-00-E	08-27-00-N
8.	5	234921	Oromia	Ilubabora	1880	36-22-00-E	08-27-00-N
9.	6	234922	Oromia	Ilubabora	1840	36-22-00-E	08-33-00-N
10.	7	234923	Oromia	Ilubabora	1840	35-43-00-E	08-20-00-N
11.	8	234924	Oromia	Ilubabora	1750	35-51-00-E	08-21-00-N
12.	10	234927	Oromia	Ilubabora	1965	36-21-00-E	08-21-00-N
13.	11	234935	Oromia	Ilubabora	1965	36-20-00-E	08-38-00-N
14.	14	220539	Oromia	West Shewa	1920	39-17-00-E	09-18-00N
15.	15	220540	Oromia	West Shewa	1910	39-17-00-E	09-18-00N
16.	16	220541	Oromia	West Shewa	1900	39-17-00-E	09-18-00N
17.	17	220546	Oromia	East Shewa	1670	39-17-00-E	09-18-00-N
18.	18	220547	Oromia	East Shewa	1860	39-17-00-E	09-18-00-N
19.	19	220553	Oromia	North Shewa	3190	39-17-00-E	09-18-00-N
20.	20	220554	Oromia	North Shewa	3340	39-17-00-E	09-11-00-N
21.	23	220557	Oromia	North Shewa	3340	39-10-00-E	09-11-00N
22.	21	220555	Oromia	East Hararghe	1920	41-37-00-E	09-19-00N
23.	22	220556	Oromia	East Hararghe	1600	41-37-00-E	09-19-00N
24.	99	232362	Oromia	East Hararghe	2020	41-37-00-E	09-19-00-N
25.	25	230612	Oromia	Bale	2040	40-22-00-E	07-01-00N
26.	26	230616	Oromia	Bale	1680	40-29-00-E	07-00-00N
27.	27	230624	Oromia	Bale	1800	40-42-00-E	07-08-00N
28.	28	230626	Oromia	Bale	1780	40-42-00-E	07-09-00N
29.	29	230631	Oromia	Bale	1750	40-43-00-E	07-07-00N
30.	30	230632	Oromia	Bale	1950	40-44-00-E	07-06-00N
31.	31	230634	Oromia	Bale	2250	39-58-00-E	07-10-00N
32.	32	230635	Oromia	Bale	2240	39-57-00-E	07-10-00N
33.	35	220690	Oromia	Arsi	2940	39-27-00-E	07-35-00N
34.	36	220691	Oromia	Arsi	2940	39-27-00-E	07-35-00N

35.	37	220692	Oromia	Arsi	2750	39-28-00-E	07-36-00N
36.	38	220693	Oromia	Arsi	2750	39-28-00-E	07-36-00N
37.	39	220694	Oromia	Arsi	2750	39-28-00-E	07-36-00N
38.	40	220695	Oromia	Arsi	2750	39-28-00-E	07-36-00N
39.	41	220696	Oromia	Arsi	2750	39-28-00-E	07-36-00N
40.	42	220697	Oromia	Arsi	2340	39-30-00-E	07-38-00N
41.	44	232220	Oromia	Arsi	2360	39-37-00-E	08-28-00N
42.	45	232222	Oromia	Arsi	2750	39-37-00-E	08-26-00N
43.	46	232223	Oromia	Arsi	2760	39-35-00-E	08-26-00N
44.	47	232225	Oromia	Arsi	2550	39-41-00-E	08-25-00-N
45.	48	232226	Oromia	Arsi	2250	39-58-00-E	07-10-00-N
46.	49	232227	Oromia	Arsi	2510	39-43-00-E	08-24-00N
47.	50	232228	Oromia	Arsi	2550	39-41-00-E	08-25-00N
48.	92	220709	Oromia	Arsi	2250	39-22-00-E	07-10-00-N
49.	93	220710	Oromia	Arsi	2240	39-27-00-E	07-33-00-N
50.	95	231222	Oromia	Arsi	3150	39-27-00-E	07-35-00-N
51.	96	231223	Oromia	Arsi	2940	39-28-00-E	07-36-00-N
52.	97	231224	Oromia	Arsi	2940	39-30-00-E	07-38-00-N
53.	52	232808	Amara	South Wollo	3500	39-24-00-E	10-51-00-N
54.	53	232809	Amara	South Wollo	2280	39-25-00-E	10-50-00-N
55.	54	232810	Amara	South Wollo	2450	39-39-00-E	10-58-00-N
56.	55	232811	Amara	South Wollo	2300	39-39-00-E	10-58-00-N
57.	56	232812	Amara	South Wollo	2530	39-39-00-E	10-58-00-N
58.	57	232813	Amara	South Wollo	2840	39-39-00-E	11-00-00-N
59.	58	232815	Amara	South Wollo	2380	39-39-00-E	11-03-00-N
60.	59	232816	Amara	South Wollo	2300	39-39-00-E	11-05-00-N
61.	60	232817	SNNP	Hadiya	2580	37-44-00-E	07-16-00-N
62.	61	232818	SNNP	Hadiya	2330	07-15-00-N	37-53-00-E
63.	63	232820	SNNP	Hadiya	2720	07-39-00-N	37-52-00-E
64.	64	232821	SNNP	Hadiya	2720	07-39-00-N	37-52-00-E
65.	65	232822	SNNP	Hadiya	2800	37-52-00-E	07-41-00-N
66.	66	232823	SNNP	Hadiya	2720	37-51-00-E	07-39-00-N
67.	67	232824	SNNP	Hadiya	2410	37-31-00-E	07-36-00-N
68.	68	232825	Amara	North Gondar	2510	37-42-00-E	12-44-00-N
69.	69	232826	Amara	North Gondar	1810	37-52-00-E	12-50-0-N
70.	70	232827	Amara	North Gondar	2720	37-52-00-E	13-07-00-N
71.	71	232828	Amara	North Gondar	2720	37-40-00-E	13-07-00-N
72.	72	232829	Amara	North Gondar	1750	37-52-00-E	12-47-00-N
73.	73	232830	B-Gumuz	Metekel	2560	35-55-00-E	11-15-00-N

74.	74	232831	B- Gumuz	Metekel	2300	35-50-00-E	10-30-00-N
75.	75	232832	B-Gumuz	Metekel	2200	35-50-00-E	10-30-00-N
76.	76	232833	B-Gumuz	Metekel	2200	35-50-00-E	10-30-00-N
77.	78	232835	B- Gumuz	Metekel	2700	35-50-00-E	10-30-00-N
78.	80	232837	Tigray	Eastern Tigray	1748	35-50-00-E	39-26-00-E
79.	81	232838	Tigray	Eastern Tigray	1650	39-26-00-E	14-16-00-N
80.	82	232839	Tigray	Eastern Tigray	2200	39-28-00-E	14-18-00-N
81.	83	232840	Tigray	Eastern Tigray	1650	39-21-00-E	14-18-00-N
82.	85	232870	Tigray	Eastern Tigray	1955	39-28-00-E	14-18-00-N
83.	86	232871	Tigray	Eastern Tigray	1860	39-31-00-E	14-36-00-N
84.	87	232872	Tigray	Eastern Tigray	1912	39-31-00-E	13-26-00-N
85.	88	232894	Tigray	Eastern Tigray	1770	39-28-00-E	13-29-0-N
86.	89	232895	Tigray	Eastern Tigray	1880	39-28-00-E	14-29-00-N
87.	90	232896	Tigray	Eastern Tigray	1980	39-28-00-E	14-29-00-N
88.	91	232897	Tigray	Eastern Tigray	1738	39-22-00-E	13-27-00-N
89.	79	232836	Tigray	southern Tigray	1726	39-38-29-E	13-31-00-N
90.	102	232941	Tigray	Southern Tigray	1930	38-04-00-E	13-31-00-N
91.	103	232942	Tigray	Southern Tigray	2190	38-45-00-E	13-26-00-N
92.	104	232943	Tigray	Southern Tigray	1880	39-30-00-E	13-29-00-N
93.	105	232944	Tigray	Southern Tigray	1600	39-31-00-E	13-27-00-N
94.	106	232945	Tigray	Southern Tigray	1781	39-28-00-E	13-31-00-N
95.	107	232946	Tigray	Southern Tigray	1660	39-28-00-E	13-31-00-N
96.	108	232947	Tigray	Southern Tigray	1628	39-28-00-E	13-31-00-N
97.	109	232948	Tigray	Southern Tigray	1892	39-31-00-E	13-26-00-N

Appendices 5: A Modified CTAB protocol for plant DNA extraction

1. Materials and Chemicals used in this study

Geno grinder	Agarose
Metal beads	Water (sterile)
65°C water bath	Chloroform: Iso Amyl Alcohol (24:1)
Microfuge tubes	75 % Ethanol (ice cold)
Tips	Absolute Ethanol (ice cold)
Microfuge	NaAc
Refrigerator	ß-Mercapto-Ethanol

2. Protocol

1. Pour CTAB solution (700 µl per sample) in a 1.5ml-tube and add 0.2 vol % Mercaptoethanol (Use fume hood!). Mercaptoethanol is stored at 4°C.
2. Aliquot CTAB in 1.5 ml Eppendorf-caps and warm in water bath up to 65°C.
3. Weigh in 100 mg fresh leave material (50mg dry material) per sample. Pulverized thoroughly using a Geno-grinder machine.
4. Add 700 µl of pre-warmed CTAB solution to the powdered sample (open the caps carefully), dissolve the powder and incubate the sample for 30 minutes at 65°C.
5. Centrifuge for 5 minutes at 15000 rpm.
6. Transfer the supernatant (only clear liquid) in a new of 2ml Eppendorf tube Use blue pipette tips which are cut.
7. Add new CTAB solution (700 µl) to the tissue pellet and stir slightly with a new 1000 µl pipette tip, incubate 30 min at 65°C.Step 6 and 7 are repeated. The same is carried out for a third extraction. Each fraction proceeds with step 9 and is treated separately.
8. Add 600 µl Chloroform to the cap with supernatant and shake carefully a few times upside down. This chloroform step should be carried out immediately.
9. Shake the samples thoroughly by turning inversing the Eppendorf caps for approximately 5 minutes. (Longer incubation is possible)
10. Centrifuge for 5 min at 15000 rpm.
11. Transfer the supernatant (only clear liquid) in a new 1.5ml Eppendorf tube. Use blue pipette tips which are cut. Work carefully; do not transfer suspended matter (normally the

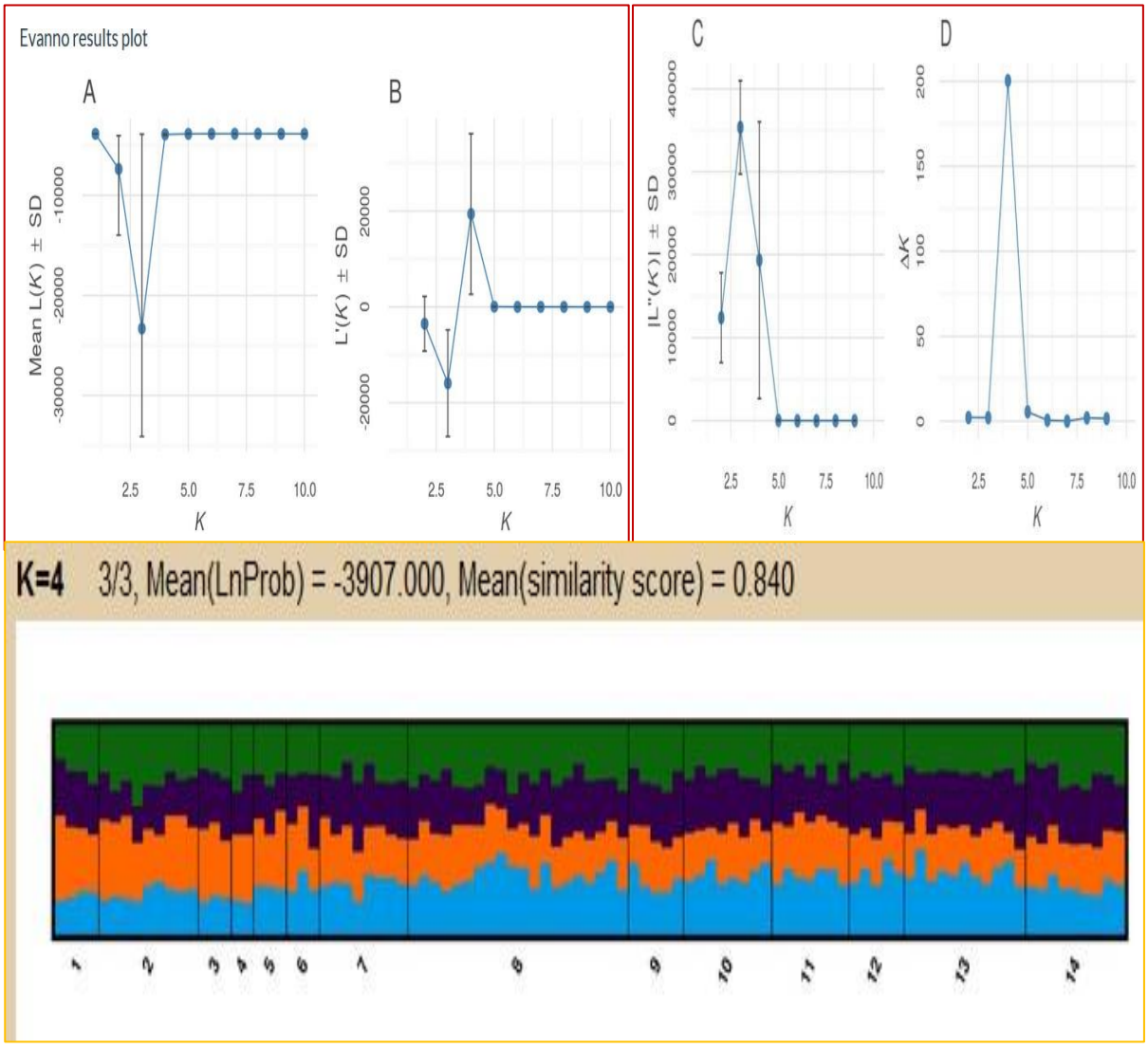
Chloroform Is covered by a thin layer of fine sediment material). Chloroform has to be disposed of in a special waste bottle.

12. Repeat the chloroform extraction (step 9-12) to make sure that all impurities are removed, and then proceed with step
13. Add cooled Iso-propanol (4°C), approximately 2/3 of the solution volume. Shake carefully by inversing the Eppendorf cap. In most cases DNA becomes visible as white threads. Freeze for more than 2h at -20°C.
14. Centrifuge 10 min at 15000 rpm.
15. Aspirate liquid using yellow tips (without touching pellet!). If pellet is solid enough the larger part of the liquid may be poured out. (Alternatively add TE).
16. Add 200 µl Ethanol 70 % to the pellet. Rinse the inner cap surface by turning the cap.
17. Centrifuge for 10 min at 15000 rpm in a cooled centrifuge.
18. Aspirate Ethanol using yellow tips. Dry the DNA-pellet at room temperature. (Usually 15 min are sufficient; after drying no liquid drops are to be seen)
19. Dissolve pellet in 100 µl TE (1x, p.a. grade) and store at 4°C.
20. Add cool Ethanol 100 % (double of the solution volume). Mix carefully. Freeze for more than 2 hr at -20°C.
21. Centrifuge 30 min at 15000 rpm. Aspirate fluid carefully.
22. Add 200 µl Ethanol 70%. Rinse the inner cap surface by turning the cap.
23. Centrifuge 10 min. at 15000 rpm. Aspirate liquid and dry pellet at room temperature.
24. Dissolve the pellet in 100 µl TE (1x, p.a. grade)

Repeat steps 21 to 24 with 3 M Na-AC-solution (4°C, half the volume) then proceed with Step 27.

25. Centrifuge 10 min. at 15000 rpm. Aspirate liquid and dry pellet at room temperature. Dissolve the pellet in 100 µl TE (1x, p.a. grade) and store at -20oc for further use.

Appendices 6: Output of the Evanno method results



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