Analysis of Genetic Diversity And Population Structure Study In Bread Wheat (*Triticum Aestivum*) Recombinant Inbred Lines And Few Commercial Cultivars Using Simple Sequence Repeats (SSRs) Markers

Chaltu Dugasa Abdisa

A Thesis submitted to the department of Applied Biology,

School of Applied Natural Science

Submitted in Partial Fulfillment of the Requirement for the Degree of Master of Science in Applied Biology (Specialization in Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

January, 2023

Adama, Ethiopia

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Approval Sheet

I/we, the advisors of the thesis entitled "Analysis of Genetic Diversity and Population Structure Study in Bread Wheat (*Triticum aestivum*) recombinant inbred lines and few commercial cultivars Using Simple Sequence Repeats (SSRs) Markers" and developed by

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We, the undersigned, members of the Board of Examiners of the thesis by Chaltu Dugasa have read and evaluated the thesis entitled "Analysis of Genetic Diversity and Population Structure Study in Bread Wheat (*Triticum aestivum*) recombinant inbred lines and few commercial cultivars Using Simple Sequence Repeats (SSRs) 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)

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Declaration

I hereby declare that this MSc. Thesis entitled “Analysis of Genetic Diversity and Population Structure Study of Bread Wheat (*Triticum aestivum*) recombinant inbred lines and few commercial cultivars Using Simple Sequence Repeats (SSRs) 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.

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Recommendation

I/we, the advisor(s) of this thesis, hereby certify that I/we have read the revised version of the thesis entitled "Analysis of Genetic Diversity and Population Structure Study of Bread Wheat (*Triticum aestivum*) recombinant inbred lines and few commercial cultivars Using Simple Sequence Repeats (SSRs) Markers" prepared under my/our guidance by Chaltu Dugasa submitted in partial fulfillment of the requirements for the degree of Master of Science in Applied Biology (Specialization in Biotechnology). Therefore, I/we recommend the submission of revised version of the thesis to the department following the applicable procedures.

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ACKNOWLEDGEMENT

First of all, I would like to thank the Almighty GOD for His mercy in my life. I would like to thank my research main advisor, Dr. Alemneh Mideksa, for his skillful guidance, encouragement up to the accomplishment of this thesis. I would like to express my sincere thanks to my co-advisor, Dr. Kassahun Tesfaye, Director General of Ethiopian Biotechnology Institute (EBTi) for allowing me to work in his Plant Genetics Research Laboratory at College of Natural and Computational Sciences, Addis Ababa University

My special appreciation goes to my co-advisor Dr. Tilahun Mekonnen for his constructive guidance, endless and technical support during the laboratory work, interpretation of the results and review of the thesis. So many people have made meaningful contribution to achieve my goal. I am thankful to all friends who I have met along the way even if your names do not appear here.

I would like to thank Adama Science and Technology University for the support by facilitating condition for continuity of my post graduate study. I would also like to thank Addis Ababa University, Institute of Biotechnology for providing the molecular laboratory facilities to conduct this research.

Last but not least, I would like to pass my heartfelt thanks to my family without their support, encouragement and unconditional love, this work would not be have been possible.

Contents

CONTENTS **PAGE**

Declaration i
Recommendation ii
ACKNOWLEDGEMENT iii
LIST OF TABLES vi
List of Figures and Illustrations vii
List of Acronyms viii
Abstract ix
1. Introduction 1
1.1. General Objective 4
1.2. Specific Objective 4
2. Literature Review 5
2.1. Origin, domestication and distribution of bread wheat 5
2.2. Taxonomy 6
2.3. Ecological Requirement 7
2.4. Bread Wheat's Production Constraints 7
2.5. Importance and Utilization of Wheat 7
2.6. Genetic Diversity of Bread Wheat 8
2.6.1. Molecular Markers for Studying Genetic Diversities 9
3. Materials and Methods 13
3.1. Plant Material 13
3.2. Genomic DNA Extraction 13
3.3. Primer Selection and Optimization 13
3.4. Microsatellite Markers Data Scoring and Analysis 16
4. Results 18
4.1. Genetic Diversity and Population Structure Analysis of Bread Wheat recombinant inbred lines and few commercial cultivars using SSR Markers 18
4.1.1. Microsatellite Markers Based Polymorphism 18
4.1.2. Genetic Variability Within and Among the Populations 21

4.1.3. Genetic Relationships between the Populations 22
4.1.4. Population Genetic Differentiation 23
5. Discussion 29
5.1. Genetic Diversity and Population Structure of Bread Wheat Recombinant Inbred Lines and few Commercial cultivars arevealed by SSR Markers 29
5.1.1. Polymorphism Level of the Microsatellite Markers 29
5.1.2. Population genetic diversity 30
5.1.3. Population genetic structure 32
6. Conclusions and Recommendations 34
6.1. Conclusions 34
6.2. Recommendations 34
7. References 35
8. Appendices 44

LIST OF TABLES

TABLE PAGE

Table 1 . Primers sequence, repeat motif, chromosome number, annealing temperatures and their range of molecular weight detected for the 10 microsatellite loci in 91 bread wheat recombinant inbred lines and few commercial cultivars (*GrainGenes | A Database for Triticeae and Avena*).15
Table 2. Genetic diversity indices across all the recombinant inbred lines and few commercial cultivars using 10 SSR loci 20
Table 3. Allelic patterns and diversity indices[a] across populations averaged over the 10 SSR loci.21
Table 4. Pairwise Nei's genetic distance (below diagonal) and gene flow (Nm) (above diagonal) among the four bread wheat populations 22
Table 5. Population genetic differentiation measured by PhiPT (below the diagonal) between the four bread wheat populations with p-values (above the diagonal). 23
Table 6. AMOVA showing the partitioning of genetic variation within and among populations of Bread Wheat 24
Table 7. Identification of the true number of clusters (K) based on Delta k (ΔK) from structure harvester. 27

List of Figures and Illustrations

Contents **Page**

FIGURE 1PCR products for primer xgwm577 with samples 19 - 42. m = marker (100 bp) DNA ladder). 19

FIGURE 2. Analysis of molecular variation of pie chart for 91 *Triticum aestivum* recombinant inbred lines and few commercial cultivars of four populations 24

FIGURE 3. Unweighted neighbor-joining tree showing relatedness among the 91 bread wheat recombinant inbred lines and few commercial cultivars 25

FIGURE 4.UPGMA dendrogram showing genetic relationships among the four populations of 91 bread wheat recombinant inbred lines and few commercial cultivars based on nei's unbiased genetic distances. 26

FIGURE 5. PCoA of the 91 bread wheat recombinant inbred lines and few commercial cultivars as revealed by 10-microsatellite loci 26

FIGURE 6.Population structure of 91 bread wheat recombinant inbred lines and few commercial cultivars representing four populations of breeding material. 28

List of Acronyms

AMOVA	=	Analysis of molecular variance
EBI	=	Ethiopian biodiversity institute
EIAR	=	Ethiopian institute of agricultural research
He	=	Expected heterozygosity
HRWSN	=	High rainfall wheat screening nursery
Ho	=	Observed heterozygosity
IBWSN	=	International bread wheat screening nursery
ISEPTON	=	International septoria observation nursery
Na	=	Number of alleles
NJ	=	Neighbor joining
PCoA	=	Principal coordinate analysis
PIC	=	Polymorphic information content
RILs		Recombinant inbred lines
STR	=	Short tandem repeat
SSR	=	Simple sequence repeat
UPGMA	=	Unweighted pair-group method using arithmetic averages

Abstract

Wheat is one of the most important cereal crops to sustain food security worldwide. Bread wheat takes the lead in production and economic significance among other species of wheat. Despite many genetic diversity study of bread wheat using varieties of techniques in Ethiopia, there is still a need for continual researching of the species in order to meet the high demand rising with population. One way of exploring potential bread wheat germplasms with wide genetic diversity is engaging different breeding materials in the research. So far to the best extent of available data, genetic diversity of bread wheat RILs has not been done with microsatellite markers in Ethiopia. Therefore, the present study was targeted to investigate the extent of genetic diversity and population structure of 91 bread wheat RILs and few Cultivars using 10 microsatellite markers. Modified CTAB DNA extraction method was used. The DNA of each genotype was extracted from its respective young leaves grown in plastic pot for 21 days in green house. The quality and quantity of the extracted DNA was checked by agarose gel electrophoresis and nanodrop spectrophotometer respectively. The condition for the PCR was initial denaturation held at 94℃ for 4 min, 40 cycles of denaturation for 1 min at 94℃, primer annealing temperature varying for each marker for 1 min, extension at 72℃ for 1:30 min and final extension at 72 ℃ for 10 min. Different software applications were employed to score and analyze the data output. The SSR markers used were highly polymorphic, resulting in 68 alleles. The polymorphic information content of the loci ranged from 0.67 to 0.86 with an overall mean of 0.78. The population showed high gene diversity (0.81), expected heterozygosity (0.81), Shannon's information index (1.61) and percent of polymorphic loci (100%). Analysis of molecular variance (AMOVA) revealed the presence of a genetic differentiation (PhiPT: 0.039) in which 96% of the total genetic variation was accounted by the within populations variation, leaving 4% for the differentiation among populations genetic variation. Population of commercial cultivar to HRWSN showed the highest pairwise genetic differentiation and Nei's genetic distance while the population of ISEPTON to HRWSN resulted in the lowest pairwise genetic differentiation and Nei's genetic distance. Clustering did not sharply grouped the recombinant inbred lines and few commercial cultivars *according to their source of breeding material likely due to the presence of high gene flow (Nm = 6.2). STRUCTURE analysis confirmed the two sub-groups with greater degree of genetic admixture. All the bread wheat populations showed high gene diversity indicating the relevance of the respective breeding material for studies related to bread wheat genetic analysis. In this study HRWSN bread wheat collections showed relatively high gene diversity, indicating that these breeding materials could be potential bread wheat* recombinant inbred lines and few commercial cultivars *sources, and hence future studies and conservation plans shall largely include these breeding materials.*

Key words: Bread wheat; Genetic diversity; SSR marker; Population structure

1. Introduction

Bread wheat (*Triticum aestivum* L.) also called as common wheat (2n=6x=42) is allopolyploid species belonging to the *Triticeae* tribe of grasses (poaceae) family. It is an annual, predominantly autogamous species with its high nutritious content contributing a significant fraction of calorie on daily consumption (Yared Belete *et al*., 2021). Bread wheat is also an important source of protein, dietary fibers, vitamin B, minerals and other phytochemicals in the human diet (Levy and Feldman, 2022).

The south-eastern part of Turkey is considered as the origin of the cultivation of earliest forms of diploid (genome AA) (einkorn) and tetraploid (genome AABB) (emmer) wheat which occurred before 10,000 years ago. About 9000 years ago these cultivation spread to Near East as appearance of hexaploid bread wheat took place for the first time (Shewry, 2009). Hexaploid which is formed through hybridization between tetraploid progenitor and diploid progenitor was able to spread globally in extraordinary manner from its cradle in fertile crescent to new habitats (Levy and Feldman, 2022).

Bread wheat's genome is very large being in approximate about 16000Mbp and it is hexaploid possessing six copies of each of its seven chromosomes, each made of three subgenomes A,B, and D (Bálint *et al*., 2000). *Triticum aestivum* is classified into five subspecies as ssp. spelta, ssp. *macha*, ssp. *aestivum*, ssp. *compactum* and ssp. *sphaerococcum* (Levy and Feldman, 2022).

Wheat surpass all other cereal crops in acreage and production (Uddin *et al*., 2015). The replacement of the highly variable landraces with pure line varieties is the primer reason for the diminished genetic variability within cultivated wheat. Immediate progenitors of wheat (*Triticum* and *Aegilops*) and more distantly related genera (*Secale, Agropyron, Haynaldia, Elymus, Hordeum*, etc.) have been targeted by wheat breeders as supplementary sources to harness beneficial genes (Abdellatif, 2013).

About 5.8 million tons of wheat with mean productivity of 3 tons per hectare (tha^{-1}) is produced annually in Ethiopia. In Ethiopia, wheat accounts for about 17% of total grain production being the third most important cereal crop after teff [*Eragrostis tef (*Zucc.) Trotter] and maize (*Zea*

mays L.). Wheat production prefers altitude range between 1900 and 2700 metres above sea level which is suitable for it. Suitable areas for wheat production in Ethiopia are those with cooler temperatures and high rainfall conditions (Yared Semagn *et al*., 2021)

Being the most common cereal crops along with teff in Ethiopia, wheat has long been consumed as staple food source for its significant carbohydrate and protein content (Kochito Gebremariam *et al*., 2022). Both bread wheat and durum wheat are the main grown wheat species in Ethiopia. Population growth and shift of dietary patterns along with urbanization resulted in increase in the demand of wheat (Hodson *et al*., 2020).

Ethiopia remains as a net importer of wheat despite of being the largest wheat producer in sub-Saharan Africa since 2005 meeting only 70% of demand from domestic production. A national priority is to increase domestic wheat production by employing improved varieties (Hodson *et al*., 2020). Various wheat varieties exists in Ethiopia as the environmental condition for the cultivation of wheat varies being in between 1500-3200 meters above the sea level (Kochito Gebremariam *et al*., 2022).

The most popular molecular markers used in genetic diversity studies are microsatellite markers also called simple sequence repeats (SSR) or short tandem repeats (STR). High efficiency, reproducibility, codominant nature and high degree of polymorphism are the characters of these markers(Mwale *et al*., 2016).

The bread wheat's genome is huge which makes it about 5 times of the human DNA content and about 40 times of rice genome size. Ample scope is offered to use simple sequence repeats as molecular marker to detect differences of individual plants including bread wheat as 80-90% of genome is made of repetitive sequences. Such kind of studies of genetic diversity of individual plants of a particular species is important in crop improvement programs *(Saxena et al*., 2020).

Diseases, caused by both fungi and fungi-like pathogens pose a major threat to wheat production. Yield losses due to diseases can be up to 70% in susceptible varieties (Singh *et al*., 2008). One of the strategies to mitigate the threat from diseases is to identify and utilize diverse sources of durable resistance. While applying molecular analysis to any plant including bread wheat accession, the genetic diversity found could serve as reference for the status of the accession for disease response such as rust (Awale and Sentayew, 2013).

Repeated cultivation of landraces for specific characteristics, narrow adaptation, farmers' varietal selection and the requirement of uniform varieties in industrial seed grain processing are some causes of weakened diversity wheat. Depletion of genetic variation, which is mainly due to implementation of high yielding commercial varieties, necessities the use of genetic resources in wheat breeding, programs (El Baidouri *et al*., 2017).

The study of genetic diversity is a crucial tool in plant breeding as the selection of line with desired traits increases with its genetic diversity extent. Plants with depleted genetic diversity are unable to cope with both biotic and abiotic stresses. Conservation and management of a particular plant species could be facilitated through the study of genetic diversity (El Baidouri *et al*., 2017).

If bread wheat germplasm with wide genetic diversity exists. it means it can withstand a lot of biotic and abiotic stresses (Kochito Gebremariam *et al*., 2022). The presence of wide genetic diversity among bread wheat accessions indicates the capacity of the accessions to hold out against to a great extent of both biotic and abiotic stresses (Kochito Gebremariam *et al*., 2022).

Assessing genetic diversity of bread wheat (*Tritcum aestivum L*.) germplasm is important for plant breeding effort as it grant reduction of genetic vulnerability to biotic and abiotic stresses (Sofalian *et al*., 2008).

Appropriate collection and conservation strategy is assisted on the basis of knowledge of the extent and pattern of genetic diversity within and between populations (accessions). This extends its importance in plant breeding programs. Higher hetrosis, desirable genetic recombination and segregation in progenies are expected to be produced from crosses between genetically diverse parents (Meseret Asmamaw *et al*., 2019).

Despite the importance of knowledge of genetic diversity in bread wheat recombinant inbred lines and few commercial cultivars for future breeding, most of the available information is mostly on basis of agronomic traits (Alemu Dabi *et al*., 2019; Kochito Gebremariam *et al*., 2022). Even the studies of genetic diversity of bread wheat genotypes in Ethiopia by using molecular markers such as SSR, ISSR, and RFLP (Yared Belete *et al*., 2021) and others had not include the genetic diversity of recombinant inbred lines of bread wheat. So this study is aimed

at examining the genetic diversity of recombinant inbred lines of bread wheat along with few commercial cultivars using SSR molecular markers. Therefore, the present study was designed with the following general and specific objectives.

1.1. General Objective

- To investigate the genetic diversity and population structure of *T. aestivum* recombinant inbred lines and few commercial cultivars using simple sequence repeat (SSR) markers.

1.2. Specific Objective

- To assess the extent of genetic diversity of *T. aestivum* recombinant inbred lines and few commercial cultivars using simple sequence repeat (SSR) markers
- To identify the population structure of *T. aestivum* recombinant inbred lines and few commercial cultivars based on simple sequence repeat marker data.
- To determine genetic diversity of *T. aestivum* within population.
- To determine genetic diversity of *T. aestivum* among population.

2. Literature Review

2.1. Origin and distribution of bread wheat

Wheat, a self-pollinating annual plant in the true grass family Gramineae (Poaceae), is the largest cereal crop extensively grown as staple food sources in the world (Gezahegn Fikre *et al*., 2015). The genus *Triticum* occurs as a polyploid series of diploid, tetraploid, and hexaploid species complexes. The tetraploid durum wheat *T. turgidum* L. and the hexaploid bread wheat (common wheat) *T. aestivum* L. are considered important for their cultural and economic value. The economic significance of *T. aestivum* L. is by far greater as it is the most important staple crop in the world (Abdellatif, 2013 and Gezahegn Fikre *et al*., 2015).

The evolution of bread wheat (*Triticum aestivum*) is through polyploidization event taken place twice among *Triticum* species and related species. The first polyploidization is between two diploid proginators that are *Triticum ururtu* (AA genome) and related species-*Aegilops speltoides* (BB genome) to form *Triticum turgidum* ssp. *Diccocoides* (Gezahegn Fikre *et al*., 2015). The modern hexaploid bread wheat (AABBDD) is a result of the second polyploidization which took place 10,000 yrs ago between *Triticum turgidum* spp.*durum* (AABB genome) and *Aegilops tauschii* (DD genome) (El Baidouri *et al*., 2017). The starting point of wheat breeding in Ethiopia dated back to the early 1960's (Meseret Asmamaw *et al*., 2019).

The inception of wheat breeding in Ethiopia dated back to the early 1960's and, as the result of the efforts made hitherto, a number of improved varieties have been developed and released to farmer. The exact origin of common wheat (*Triticum aestivum* ssp. *aestivum*) is still an issue of disagreement among scientists. The creation of the tetraploid species *T. turgidum* ssp. *dicoccoides* (AB) from the hybridization between *T. urartu* (A), as pollinator, and a species related to the Sitopsis section of the *Aegilops* genus (S) as cytoplasm donor is considered as the first step in the development of hexaploid *aestivum* group (ABD) (Levy and Feldman, 2022).

Hexaploid species *T. aestivum* ssp. spelta (ABD) or some other hulled type is resulted from hybridization between *T. turgidum* ssp. dicoccon (AB genome, cytoplasm donor), a descendant of *T. turgidum* ssp. dicoccoides, and Ae. tauschii (D genome, pollinator). *T. aestivum ssp. aestivum* (ABD) along with other naked types may have been raised from this form of ancestral

genome. Its evolution may have involved both human selection and spontaneous hybridization between wild wheat species and their weeds (Bálint *et al*., 2000).

Common wheat possess three basic homoeologous genomes (ABD) which are partially related to each other, even the loss of one chromosome is able to the extent of being compensated by the presence of its homologue. The diploid species of the *Aegilops* genus played an important role in the evolution of the polyploid species of the *Triticum* genus, since the polyploid wheat species probably evolved by means of allopolyploidisation, i.e. through interspecific crosses between *Triticum* and *Aegilops* species, followed by the doubling of the chromosome number (Bálint *et al*., 2000).

In Ethiopia, in terms of total production, wheat ranks third after maize (*Zea mays* L.) and tef (*Eragrostis tef* Zucc.). And it ranks fourth in terms of areas of cultivation being preceded by sorghum (*Sorghum bicolor* L.) as well. Though wheat is widely adopted to grow in diverse environment the mid-altitude (1900 to 2300 m above sea level) and high-altitude (2300 to 2700 m above sea level) regions of the country are considered as the major wheat-producing areas. These regions are characterized with high and reliable rainfall which makes them high potential environment for wheat production. Small-scale farmers produce it mainly under rain fed condition. Ethiopia is ranked first in wheat production in sub-Saharan Africa (SSA) countries being followed by South Africa, Sudan and Kenya. Low adoption of new improved varieties in the country has resulted in lower average productivity of the crop which is 2.4 tons ha^{-1} when compared to that of global (3.4 tons ha^{-1}) (Yared Semagn *et al*., 2021).

2.2. Taxonomy

The global wheat research community has confusion over taxonomic treatment of this species since different classification system mainly Mac Key (1966, 1977) and Dorofeev *et al* (1979) are used by researchers(Systematics and Goncharov, 2015).

The classification of the *Triticum* L. (wheat) genus is as follows: phylum: Angiospermatophyta, class: Monocotyledonopsida, order: Poales (Glumiflorae), family: Poaceae (Gramineae), subfamily: Pooideae, tribe: Triticeae, subtribe: Triticinae, genus: *Triticum* L. Other genera in this subtribe are *Aegilops* L., Amblyopyrum (Jaub. and Spach) Eig, *Secale* L. (rye), *Agropyron Gaertn*. (wheatgrass) and *Haynaldia villosa* (L.) Schur (Bálint *et al*., 2000).

2.3. Ecological Requirement

The sustainability of agricultural production systems is determined by important factor which is climate (Neamatollahi *et al*., 2012; Khaled *et al*., 2015). Wheat (*Triticum aestivum* L.) is a long-day crop of the temperate region and requires relatively low temperatures for satisfactory growth (Mulu Nigus *et al*., 2022). In Ethiopia wheat grows under a variety of environmental conditions, ranging from 1500 to 3200 meters above sea level, permitting for the existence of various wheat varieties (Kochito Gebremariam *et al*., 2022).

2.4. Bread Wheat's Production Constraints

A major threat to wheat production among biotic factors is disease caused by both fungi and fungi-like pathogens. New virulence evolution has been more frequently occurring to cause rust and powdery mildew through migration, mutation, selection, and recombination of virulence genes. Up to 70% of yield losses could result in susceptible varieties (Mondal *et al*., 2016).

In Ethiopia, the most important biotic constraints to wheat production are wheat rust (fungal) diseases including namely stem and stripe rust causing in large scale production losses. A significant and continuing threat is posed to Ethiopia as a new, virulent wheat rust races emerges and in curses. over 100 bread wheat and more than 40 durum wheat varieties has been released by Ethiopian national and regional wheat programs in partnership with international agricultural research centers such as the International Maize and Wheat Improvement Center (CIMMYT) and the International Center for Agriculture Research in the Dry Areas (ICARDA) since 1960 varieties (Hodson *et al*., 2020).

2.5. Importance and Utilization of Wheat

In most parts of the world, common wheat is currently grown as it is among the most important cereal serving as staple food source and one of world's major cereal export earners along maize and rice. One fifth of globally required calorie is provided by this cereal (Mwale *et al*., 2016).

Production of wheat in million hectares of land is significant for the fact that more than 40% of world's commonly consumed food is wheat. People estimated to 95% in the developing countries consume either wheat or maize as main food source (Kefyalew Negisho *et al*., 2021).

Hexaploid bread wheat covers about 95% of cultivated wheat worldwide with the rest remaining being tetraploid durum wheat (Shewry, 2009).

Improving wheat crop by applying intensive activities such as selection of cultivars with desirable attributes may lead to disaster. A reduced genetic diversity over time, increased disease incidences, a decline in crop yield and compromised drought tolerance among many other biotic and abiotic challenges may result (Mwale *et al*., 2016).

Wheat growers could achieve a high improvement rate of desired attributes in the available wheat cultivars without increasing land area for production which is impossible due to population growth, urbanization and competition from other crops (Mwale *et al*., 2016).

2.6. Genetic Diversity of Bread Wheat

The genetic variability of wheat is recognized to be restricted due to several different reasons in comparison to most other crops (Charmet, 2011). The fact that bread wheat is an allohexaploid produced by crosses involving three highly interrelated diploid species, and polyploidization is a restricting force itself for genetic variability. Another reason suggests that the initial genetic variability is restricted by few plants of the ancestral species which were involved in the formation of wheat (Lev-Yadun *et al*., 2000). Since the species is young species, ca.8000 to 10,000 years old, for the species to accumulate mutations or to receive genes or alleles by natural or artificial interspecific cross-breeding processes it had insufficient time (El Baidouri *et al*., 2017).

Several cultivated species including wheat got further restriction in their genetic variability through domestication, centuries of cultivation and modern breeding. Since wheat is several cultivated species, constant selection cycles may have further decreased it variability. The narrow wheat variability might make the cereal not to meet its demands in few decades unless genetic base broadening measures are taken (Venske *et al*., 2019).

Different techniques will need to be applied to broaden the genetic diversity available for wheat breeding including inducing mutation, genetic transformation, genome editing, and introgressions from species of the secondary and tertiary gene pools (Venske *et al*., 2019).

Genetic variation among wheat bread accessions might be used as a confirmation to gene postulation. Assessing the available genetic diversity of wheat is useful for grain yield, adaptation to climate change, disease and insect pest resistance, and nutritional and end-use quality (Nadeem *et al*., 2017).

The establishment of an effective breeding program concerning the intraspecific and interspecific hybridization necessitates the understanding of genetic variation within and between populations (Abdellatif, 2013).

2.6.1. Molecular Markers for Studying Genetic Diversities

DNA/molecular markers are one of the two classifications of genetic markers along with classical markers which include types like morphological, cytological and biochemical markers. These genetic markers are a gene with a known chromosomal location which control specific gene or trait (Nadeem *et al*., 2017). The classical genetic markers might have their own advantages and disadvantages. For instance in the case of morphological markers, they are easy to be used, no requirement of specialized instrument or technique. However it is restricted in number, affected by the stage of growth and other environmental factors. Other classical genetic markers in general are limited in number, show low polymorphism and influenced by external conditions. These reasons are what make the molecular markers preferable.

Several PCR based molecular markers namely random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), sequence-characterized amplified region, inter simple sequence repeats (ISSR), simple sequence repeats (SSR) and single-nucleotide polymorphism (SNP), etc. have been developed and can be successfully used for genetic diversity studies in plants (Nadeem *et al*., 2017).

Evaluation of genetic diversity and determination of inter- and intra-species genetic relationships are possible through the application of molecular marker to recognize polymorphic nucleotide sequences dispersed throughout the genome (Khaled *et al*., 2015).

Molecular markers could be different from one to other types as they exhibit difference in technical principle they have, type of inheritance, extent of reproducibility, amount of polymorphism and in their expense (Khaled *et al*., 2015).

Data generation efficiency and genome area coverage in a study are some of factors which vary among different molecular marker techniques (Mandoulakani *et al*., 2010). The target crop and the issue are stand points for selecting a type of marker tool to be used for study. For instance, random amplified polymorphic DNA (RAPD) markers are known for their simplicity, cost efficiency, fast polymorphism assessment, no prior information of DNA sequences being required and extensive coverage of the intact genome being possible. Molecular markers with low reproducibility will not be selected to be used and other better markers will be prioritized (Nadeem *et al*., 2017).

The different molecular markers that has been used to study genetic diversity in common wheat includes random amplified polymorphic DNA (RAPDs), restriction fragment length polymorphism (RFLPs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSR), sequence-tagged site (STS) and inter simple sequence repeats (ISSRs) *(*Akram *et al*., 2012).

2.6.1.1. Simple Sequence Repeat (SSR)

Microsatellites also referred as simple sequence repeats (SSRs) or STRs are simple sequences that are arranged in tandem repeats of mononucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide motifs. Although mono-(A,T), di-(AT, GA), tri- (AGG) and tetra- (AAAC) nucleotides are the most frequently occurring ones (Koelling *et al*., 2011). Microsatellites are highly rich, polymorphic and randomly disseminated throughout the nuclear, chloroplast and mitochondrial genomes of many species (Feng *et al*., 2016).

Evolutionarily, it has been postulated that SSRs resulted from either single-strand DNA slippage, double-strand DNA recombination or mobility of transposable genetic DNA molecules and nucleotide disparities (Koelling *et al*., 2011). Generally polymorphism is based on the variation in the number of repeats in different recombinant inbred lines and few commercial cultivars (Fry *et al*., 2009). Most of the time these markers are large fragment of non-coding DNA but SSRs have also been committed to be present in protein-coding genes and expressed sequence tags (ESTs) (Feng *et al*., 2016).

Based on the location of SSR in genome, there are nuclear SSR, Cytoplasmic SSR and Mitochondrial SSR. Higher polymorphism levels are provided by cpSSRs with easily genotyping

has made them a very few and desired marker for population genetic studies despite of difficulty in detecting enough sequence variation as mutation rate in chloroplast genome is less. Usually mononucleotide motif which are repeated 8–15 times are present in cpSSRs. Their application in agricultural and basic plant science has been a success (Provan *et al*., 2001).

SSR marker loci are produced with two-primer PCR reactions when set side to side to RAPDs or ISSRs analysis where single primers are used per PCR reaction. One primer anneals to the DNA template in a forward direction and the other primer hybridizes to the template in a reverse orientation. Both primers bind complementary to particular DNA sequences flanking the SSR sequence (Kalia *et al*., 2011).

The basis for development and deployment of PCR based, multiallelic, co-dominant genetic marker system is that plant genome contains large number of simple sequence repeats (Akram *et al*., 2012). SSR markers are regarded as a marker of choice, as they are co-dominant, with high reproducibility and greater genome abundance, and they can be used efficiently in plant mapping studies (Haider,2017). This high polymorphism level is due to the occurrence of various numbers of repeats in microsatellite regions and can be recognized reproducibly easily by PCR (Mei *et al*., 2015).

The capacity of molecular marker to detect genetic differences among individuals of population is what makes it effective tool to be selected. The polymorphism of certain molecular marker is obtained both qualitatively and quantitatively. A molecular marker is said to be polymorphic in terms of qualitative parameters if it has at least two alleles and the most frequent allele has a frequency of up to 99%. While in terms of quantitative polymorphism of a particular marker is measured by various parameters including heterozygosity, polymorphism information content (PIC) and marker index (Serrote *et al*., 2020).

2.6.1.1. Importance of Molecular Markers

The simplest form of marker assisted selection (MAS) is represented by molecular markers when they used to select indirectly for desirable alleles if they are tightly linked to genes of interest (Saxena *et al*., 2020).

The application of molecular markers to probe the level of genetic diversity among different cultivars, within populations, among related species have many applications (Kenzhebayeva *et al*., 2014).

Varietal finger printing for identification and protection, understanding relationships among the units under study, efficiently managing genetic resources, facilitating introgression of chromosomal segments from alien species and even tagging of specific genes are some application of the study of genetic diversity by using molecular markers (Saxena *et al*., 2020).

The naturally highly polymorphic repetitive sequences of a DNA are amplified through polymerase chain reaction by specifically chosen primers which are utilized in SSRs. Several hundred alleles comprised in genetic loci of these repetitive regions differ from each other with respect to length, sequence or both and they are interspersed in tandem arrays ubiquitously (Kenzhebayeva *et al*., 2014; Saxena *et al*., 2020).

Chromosome segments, identification of individuals, studying evolution and orthologous and paralogous relatedness and wildlife conservation are some areas in which SSRs are increasingly being used as genetic markers (Saxena *et al*., 2020).

3. Materials and Methods

3.1. Plant Material

Ninety one bread wheat recombinant inbred lines and few commercial cultivars comprising eighty four recombinant inbred lines and seven commercial bread wheat cultivars were involved in this study. The inbred lines containing 30 IBWSN, 25 HRWSN and 29 ISEPTON were obtained from the International Maize and Wheat Improvement Center, (CIMMYT) while the commercial cultivars were obtained from Holetta Agricultural research centers, Ethiopia (Appendix 1).

Ten seeds of each wheat recombinant inbred lines and few commercial cultivars were planted in plastic pots in greenhouse located at the Addis Ababa University. About 21 days after sowing, seedling leaves were detached and dried with silica gel in plastic bag for about a week and then used for extraction. Genotyping was conducted at Addis Ababa University, Institute of Biotechnology, Plant Genetics Research Laboratory.

3.2. Genomic DNA Extraction

Genomic DNA of each genotype was extracted from their dried young leaves by using modified Cetyl tri methyl ammonium bromide (CTAB) protocol described by Tilahun Mekonnen *et al*., 2017(Appendix 2). The extracted genomic DNA concentration and quality were checked with Nanodrop (NanoDrop™2000/2000c) Spectrophotometer (Termo Scientific™. USA) and on 1% agarose gel at 100 V for 1 h, respectively. Then, each of the extracted genomic DNA was diluted by using nuclease free sterile water into 100 ng μL^{-1} for use in PCR amplification and stored at -20^{o}C until use.

3.3. Primer Selection and Optimization

About 17 simple sequence repeat (SSR) primers referred from previously published wheat scientific literatures were pre-screened for the primers' polymorphism and reproducibility on ten randomly selected bread wheat recombinant inbred lines and few commercial cultivars in

this specific population and then 10 primers which showed reproducible polymorphism were selected for use in PCR(Table 1) *(Simón et al*., 2012).

About 53 simple sequence repeat (SSR) primers previously reported to be polymorphic with other sets of durum wheat genetic materials[12-14] were pre-screened for polymorphism in this specific population and then 12 primers which showed high polymorphism were selected for use in PCR. About 53 simple sequence repeat (SSR) primers previously reported to be polymorphic with other sets of durum wheat genetic materials [12-14] were pre-screened for polymorphism in this specific population and then 12 primers which showed high polymorphism were selected for use in PCR. The gradient PCR (HIMEDIA – Prima-96™ Thermal Cycler 96 wells block) machine was used to optimize the primers' annealing temperatures.

The PCR program for each of the primer pairs involved an initial denaturation at 94 ºC for 4 minutes followed by 40 cycles of denaturation at 94 ºC for 1minute. The optimized annealing temperatures of each of the markers listed in their ranges from 50 ºC to 60 ºC for 1 min (Table 1). Primer extension took at 72 ºC for 1:30 minutes followed by a final extension at 72 ºC for 10 minutes. Then the temperature is held at a temperature of 4 ºC.

The PCR products were separated on 2%agarose gel electrophoresis using 1 X TBE buffer at 100 V for 1:30-2 hr. The gel was stained with ethidium bromide (2 µl), visualized with UV light and subsequently photographed using a BIO-RAD Gel Doc TM EZ Imaging System. A 100 bp DNA ladder was used to estimate the size of the amplified fragments (Table 1).

Table 1. Primers sequence, repeat motif, chromosome number, annealing temperatures and their range of molecular weight detected for the 10 microsatellite loci in 91 bread wheat recombinant inbred lines and few commercial cultivars (*GrainGenes | A Database for Triticeae and Avena*).

Marker	Forward primer (5'-3')	Reverse primer (5'-3')	Repeat Motif	Chromosome Number	[a]Annealing temperature (°C)
Xbarc12	CGACAGAGTGATCACCCAAATATAA	CATCGGTCTAATTGTCAATGTA	(TAA)28	3A	56
Xgwm135	TGTCAACATCGTTTTGAAAAGG	ACACTGTCAACCTGGCAATG	(GA)20	1A	59
Xgwm46	GCACGTGAATGGATTGGAC	TGACCCAATAGTGGTGGTCA	(GA)x2GC(GA)x33	7B	60
Xwmc500	ATAGCATGTTGGAACAGAGCAC	CTTAGATGCAACTCTATGCGGT	(CA)13	3B	51
Xbarc232	CGCATCCAACCATCCCCACCCAACA	CGCAGTAGATCCACCACCCCGCCAGA	(CT)18	5B	62
Xbarc178	GCGTATTAGCAAAACAGAAGTGAG	GCGACTAGTACGAACACCACAAAA	(ATT)17	6B	59
Xgwm577	ATGGCATAATTTGGTGAAATTG	TGTTTCAAGCCCAACTTCTATT	(CA)14(TA)6	7B	55
Xbarc133	AGCGCTCGAAAAGTCAG	GGCAGGTCCAACTCCAG	(CT)24	3B	50

Xgwm335	CGTACTCCACTCCACACGG 3	CGGTCCAAGTGCTACCTTTC 3	(GA)14(GCGT)3	5B	55
Xwmc406	TATGAGGGTCGGATCAATACAA	CGAGTTTACTGCAAACAAATGG	(CA)16	1B	56

3.4. Microsatellite Markers Data Scoring and Analysis

The PyElph software package was used to estimate the sizes of all PCR-amplified SSR regions in relation to the size marker (Pavel and Vasile, 2012). Genetic diversity and population structure were analyzed using the scored marker data. Different statistical software packages were used to calculate the standard indices of genetic diversity.

GenAlex version 6.5 (Peakall and Smouse, 2012) software was used to calculate 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 (%PL) and Principal coordinate analysis (PCoA). Analysis of molecular variance (AMOVA) and the estimation of the variance components were also computed using this software. POPGENE version 1.3.1 (Yeh, 1999) was used to assess population differentiation (Gst) and gene flow (Nm = 1/Fst) −1]/4 across populations. Gene flow (Nm) among populations was estimated using the formula, Gene flow (Nm) calculated as Nm = [(1 / PhiPT) - 1] / 4, PhiPT = the variance among populations/total genetic variations. While locus based diversity indices- major allele frequency (MAF) and polymorphic information content were computed using Power Marker v3.25 software (Liu and Muse, 2005).

Nei's standard genetic distance (Nei and Takezaki, 1983) based Unweighted Pair Group Method with Arithmetic Mean (UPGMA) trees were generated using Powermarker v3.25 (Liu and Muse, 2005). The resulting trees were visualized using MEGA software (Kumar *et al.*, 2018) and Tree View (built in Power Marker v3.25). Significance was tested based on 1000 bootstrap replications (Felsenstein, 1985). This software was also used to compute PIC, heterozygosity (Ho) and gene diversity (expected heterozygosity, He) for each marker as well as the average across markers.

STRUCTURE software ver. 2.3.4 with a Bayesian model-based clustering algorithm was used to determine population structure and admixture patterns (Pritchard *et al.* , 2000). The true number of population cluster (K), a burn-in period of 100,000 was used in each run, and data were collected over 250,000 Markov Chain Monte Carlo (MCMC) replications for K = 1 to K = 10 using 20 iterations for each K.

The optimum K value was predicted following the simulation method of (Evanno *et al.* , 2005) using the web-based STRUCTURE HARVESTER ver. 0.6.92 (Earl, 2012). A bar plot for the optimum K was determined using Clumpak beta version (Kopelman *et al.* , 2015).

4. Results

4.1. Genetic Diversity and Population Structure Analysis of Bread Wheat recombinant inbred lines and few commercial cultivars using SSR Markers

4.1.1. Microsatellite Markers Based Polymorphism

The analysis revealed that all 10 SSR loci were polymorphic and produced 68 alleles with an average of 6.8 alleles per locus among the 91 recombinant inbred lines and few commercial cultivars of bread wheat (Table2). The number of alleles per locus ranged from 4.5 to 9.3 with overall mean value of 6.8. Across the entire populations, the major allele frequencies ranged from 0.17 for locus Xgwm335 to 0.41 for Xgwm135 with an overall mean of 0.26 (Table 2). The highest Na, Ne, I, and Nm were recorded for Xgwm335. Microsatellite loci Xwmc500 and Xgwm335 showed high gene diversity (h = 0.87) while Microsatellite loci Xgwm135 (h = 0.71) resulted in the lowest gene diversity across the entire populations (Table 2).

The Locus Xbarc133 showed the least genetic differentiation (Gst = 0.001) and the highest gene flow (Nm = 11.5) among the populations. The locus XGwm335 showed the highest observed (H_o = 1.00) and expected heterozygosity (H_e =0.83) for each locus whereas the locus X Gwm135 showed the least gene diversity (expected heterozygosity, H_e = 0.63) (Table 2). The highest mean value (0.75) for observed heterozygosity(H_o) was obtained in this experiment (Table 2).The informativeness of individual loci as measured by polymorphic information content ranged from 0.67 (Xgwm135) to 0.86 (Xgmc500) with overall mean of 0.78. From the tested microsatellite loci four of them (Gwm46, Barc178, Barc133 and Gwm335) were insignificant for deviation from Hardy Weinberg equilibrium (P>0.05) (Table 2).

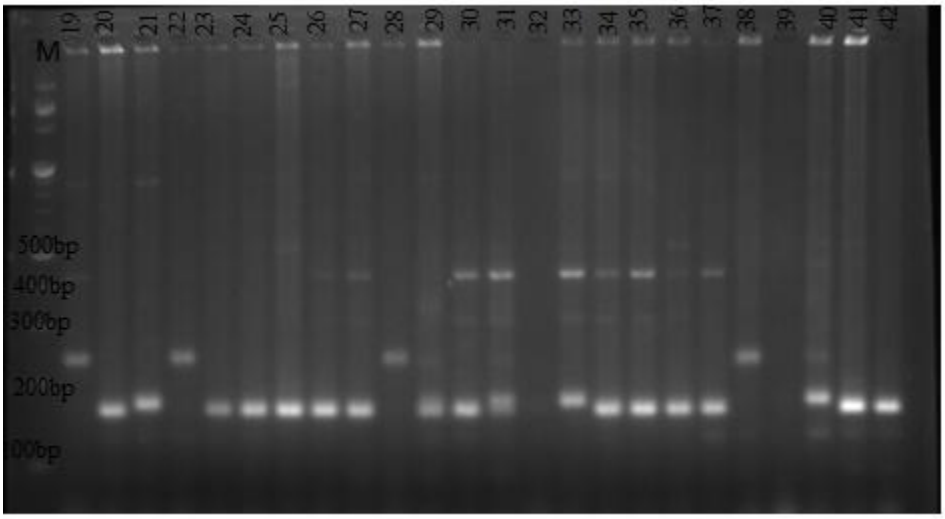

FIGURE 1.P.CR products for primer xgwm577 with samples 19 - 42. m = marker (100 bp) DNA ladder).

Table 2. Genetic diversity indices across all the recombinant inbred lines and few commercial cultivars using 10 SSR loci

SSR Loci	MAF	Na	Ne	I	h	Ho	He	P_{HWE}	Nm	PIC	Fst
Barc12	0.32	8.0	5.0	1.7	0.83	0.32	0.77	0.002	4.0	0.81	0.06
Gwm135	0.41	5.0	2.8	1.2	0.71	0.48	0.63	0.029	2.9	0.67	0.08
Gwm46	0.19	7.5	5.3	1.7	0.85	0.52	0.80	0.188	4.7	0.84	0.02
Wmc500	0.18	7.0	4.9	1.6	0.87	0.49	0.74	0.008	1.2	0.86	0.07
Barc232	0.24	7.0	4.5	1.6	0.83	0.32	0.75	0.022	3.1	0.81	0.04
Barc178	0.20	8.5	5.8	1.8	0.86	0.24	0.81	0.130	5.4	0.84	0.02
Gwm577	0.34	4.5	3.4	1.3	0.74	0.02	0.70	0.002	4.3	0.70	0.03
Barc133	0.25	6.3	4.9	1.6	0.80	0.85	0.79	0.360	11.5	0.77	0.06
Gwm335	0.17	9.3	6.3	1.9	0.87	1.00	0.83	0.069	7.9	0.85	0.03
Wmc406	0.32	4.5	3.6	1.3	0.75	0.05	0.72	0.017	3.7	0.71	0.06
Mean	0.26	6.75	4.6	1.6	0.81	0.43	0.75	0.082	3.6	0.78	0.05

MAF = Major allele frequency, Na = Observed number of alleles, Ne = Effective number of alleles (Kimura and Crow, 1964), I = Shannon's information statistic, h = Nei's gene diversity, H_o = Observed heterozygosity for each locus, H_e = Expected heterozygosity for each locus, Nm = Estimate of the number of migrants (gene flow) from Fst where Nm = [(1/Fst) - 1]/4, Fst = Inbreeding coefficient within subpopulations relative to total, PIC = Polymorphic information content, *P*-value and P_{HWE} for deviation from Hardy Weinberg Equilibrium highly significant at ($p<0.001$), highly significant at ($p<0.01$) and significant at ($p<0.05$)].

4.1.2. Genetic Variability Within and Among the Populations

A summary of the different genetic diversity estimates over all the loci across populations is presented in Table 3.

The overall genetic diversity estimates within the populations showed a mean number of effective alleles of 4.66 with a range of 3.29 to 5.30, a mean number of private alleles (number of alleles unique to a single population) of 0.65 with a range of 0.20 to 1.20, mean Shannon's information index of 1.61 ranging from 1.27 to 1.75, and the mean gene diversity value of 0.75 with a range of 0.67 to 0.79. The population of IBWSN showed the highest Na, Ne, NPA, I, and genetic diversity (Table 3).

The bread wheat recombinant inbred lines and few commercial cultivars from the population HRWSN ranked second in terms of Na, Ne, NPA and I (Table 3). The highest NLCA was observed in ISEPTON population being followed by HRWSN population. The lowest Nei's gene diversity was observed in the populations of Cultivar while recombinant inbred lines and few commercial cultivars from HRWSN showed the highest gene diversity. Sixty percent of the study populations showed a genetic diversity greater than the mean value of 0.75. The percentage of polymorphic loci per population showed 100% polymorphism across all populations (IBWSN, HRWSN, ISEPTON and Commercial cultivar).

Table 3. Allelic patterns and diversity indices[a] across populations averaged over the 10 SSR loci.

Pop	N	Na	Ne	NPA	NLCA	I	Ho	He
IBWSN	30.00	7.80	5.33	1.20	0.40	1.75	0.48	0.78
HRWSN	25.00	7.70	5.30	0.70	0.50	1.74	0.49	0.79
ISEPTON	29.00	7.10	4.91	0.50	0.60	1.68	0.34	0.78
Cultivar	7.00	4.40	3.29	0.20	0.20	1.27	0.40	0.67
Mean	22.75	6.75	4.66	0.65	0.45	1.61	0.43	0.75

[a] Na = Observed number of alleles, Ne = Number of effective alleles, NPA = Number of Private Alleles (i.e., the number of alleles unique to a single population), I = Shannon's information statistic, and NLCA = Number of Locally Common Alleles found in populations. N.B. PPL = 100% across all populations, [b]IBWSN=International Bread Wheat Screening Nursery, HRWSN=High Rainfall Wheat Screening Nursery, ISEPTON=International Septoria Observation Nursery.

4.1.3. Genetic Relationships between the Populations

Table 4 presents pairwise population measures of Nei's genetic distance (below diagonal) and gene flow (Nm) (above diagonal). The pairwise Nei's standard genetic distance of each population from the other populations ranged from 0.18 to 0.49 (Table 4). The lowest genetic distance (0.18) was observed between the bread wheat recombinant inbred lines and few commercial cultivars of ISEPTON and HRWSN population, with the highest gene flow of 11.23. The genetic distance among HRWSN to IBWSN, ISEPTON to IBWSN, Commercial cultivar to IBWSN, and Commercial cultivar to ISEPTON presents a medium value between the lowest and highest value. The highest measure of genetic distance (0.49) with relatively low gene flow (Nm = 2.44) was observed between the populations of Commercial cultivar and HRWSN (Table 4). The second highest genetic distance was observed between the populations ISEPTON, and Commercial cultivar (0.42) with gene flow of 3.53 (Table 4).

Table 4. Pairwise Nei's genetic distance (below diagonal) and gene flow (Nm) (above diagonal) among the four bread wheat populations

Population [a]	IBWSN	HRWSN	ISEPTON	Commercial cultivar
IBWSN	_	9.05	6.78	3.01
HRWSN	0.19	_	11.23	2.44
ISEPTON	0.22	0.18	_	3.53
Commercial cultivar	0.41	0.49	0.42	_

_=Not applicable [a]IBWSN=International Bread Wheat Screening Nursery, HRWSN= High Rainfall Wheat Screening Nursery, ISEPTON=International Septoria Observation Nursery

4.1.4. Population Genetic Differentiation

The pairwise coefficient of genetic differentiation between the populations ranged from 0.02 (between ISEPTON and HRWSN) to 0.09 (between Commercial cultivar and HRWSN) (Table 5). The relatively highest and statistically significant genetic differentiation (PhiPT = 0.09, $p < 0.05$) was observed between populations of Commercial cultivar and HRWSN, implying the relatively lowest gene flow (Nm=2.4) between them. The second highest genetic differentiation of 0.08 was observed between the populations of Commercial cultivar and IBWSN. The genetic differentiation between all the populations was statistically significant ($p < 0.05$) (Table 5).

Table 5. Population genetic differentiation measured by PhiPT (below the diagonal) between the four bread wheat populations with p-values (above the diagonal).

PhiPT/P-values	IBWSN	HRWSN	ISEPTON	Commercial Cultivar
IBWSN	_	0.00	0.00	0.00
HRWSN	0.03	_	0.02	0.00
ISEPTON	0.04	0.02	_	0.00
Commercial cultivar	0.08	0.09	0.07	_

_=Not applicable, IBWSN = International Bread Wheat Screening Nursery, HRWSN = High Rainfall Wheat Screening Nursery, ISEPTON = International Septoria Observation Nursery

4.1.4. Analysis of Molecular Variance

Analysis of molecular variance (AMOVA) revealed that 96% of genetic variation occurred within populations (Table 6, Figure 2). The variability among populations only accounted for 4% of the total genetic diversity. The overall genetic differentiation coefficient among the populations was moderate (PhiPT = 0.04, p = 0.003) with high gene flow (6.2).

Table 6. AMOVA showing the partitioning of genetic variation within and among populations of Bread Wheat

Source of variation	Df	Estimated varience	% variation	MS	SS	[a]Genetic differentiation	[b]P-value
Among population	3	0.47	4%	21.8	65.5	PhiPT =0.04	0.003
Within Population	87	11.7	96%	11.7	1017.9		
Total	90	12.2	100%		1083.5		
Gene flow(Nm)	6.2						

[a] PhiPT = genetic differentiation among population, calculated as = PhiPT = AP / (WP + AP) = AP / TOT, where AP = Est. Var. Among Pops and WP = Est. Var. among Pops, [b] significance based on 1000 permutations, Gene flow (Nm) calculated as Nm = [(1 / PhiPT) - 1] / 4

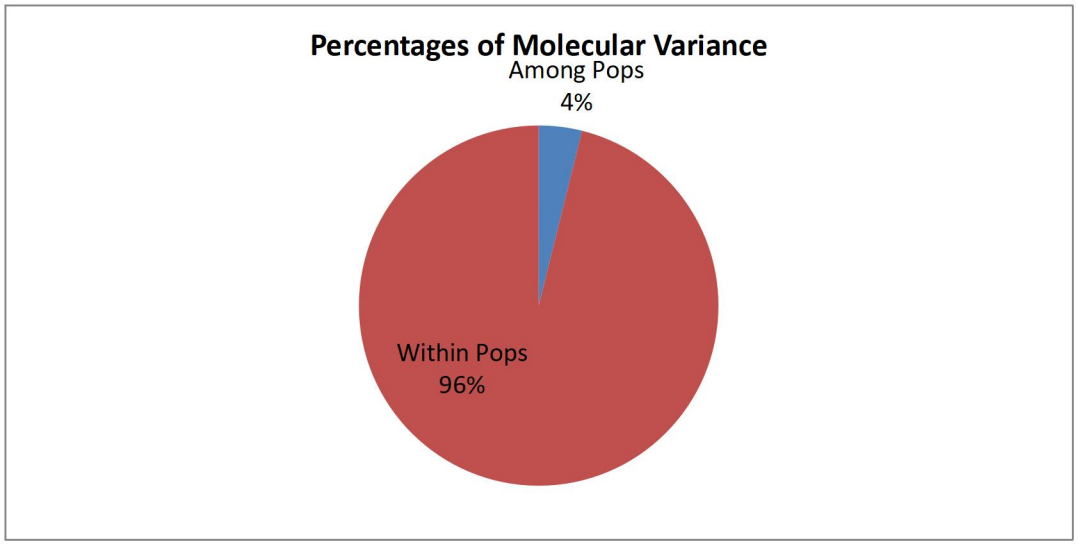

FIGURE 2.Analysis of molecular variation of pie chart for 91 *Triticum aestivum* recombinant inbred lines and few commercial cultivars of four populations

4.1.5. Cluster Analysis, PCoA and Population Structure

The neighborjoining-based clustering of the 91 recombinant inbred lines and few commercial cultivars identified three major clusters (C1, C2, and C3), each of which were further grouped into two sub clusters (i and ii) (Figure 3). Forty seven (51.6%) of the recombinant inbred lines and few commercial cultivars were assigned into C2 which was followed by C1.

The lowest (2) number of isolates was assigned to cluster C3 (Figure 3). None of the major clusters and/or sub-clusters is composed of exclusively recombinant inbred lines and few commercial cultivars from a particular population confirming the existence of considerable intermixing of the recombinant inbred lines and few commercial cultivars. The dendogram generated using the UPGMA grouped the four populations into two major clusters (I and II), where one cluster was composed of three population (IBWSN, HRWSN and ISEPTON), and the other cluster was composed of only one population, that is Commercial cultivar (Figure 4).

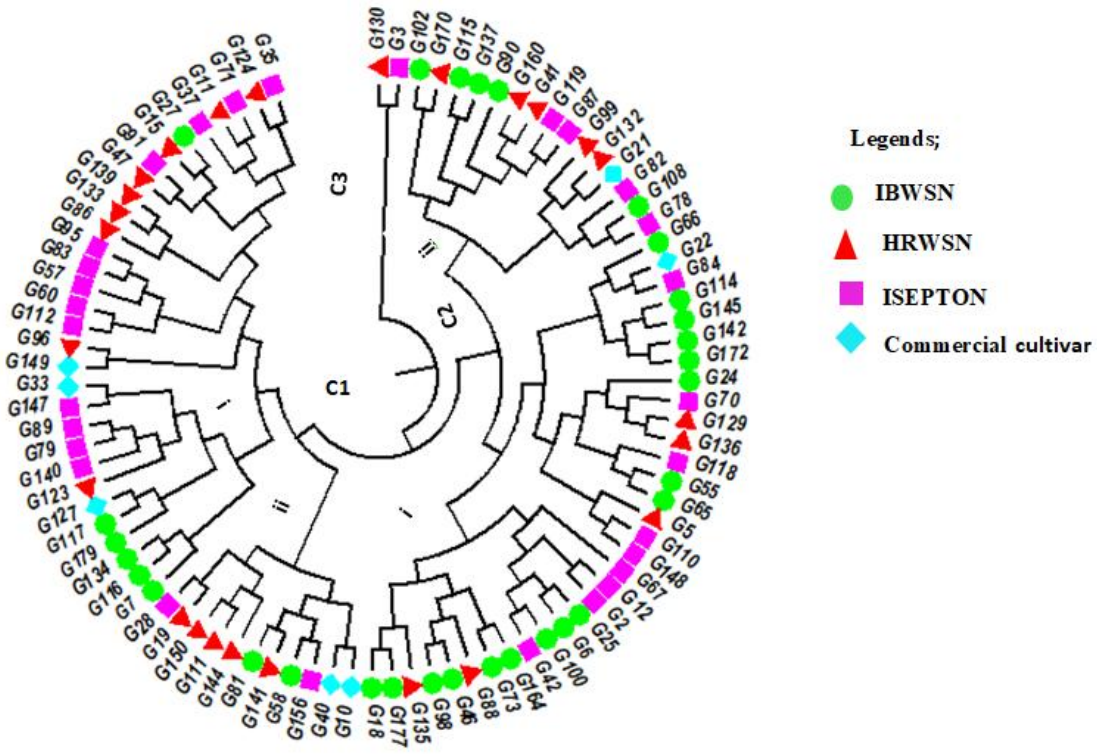

FIGURE 3. UPGMA tree showing relatedness among the 91 bread wheat RILs and few commercial cultivars.

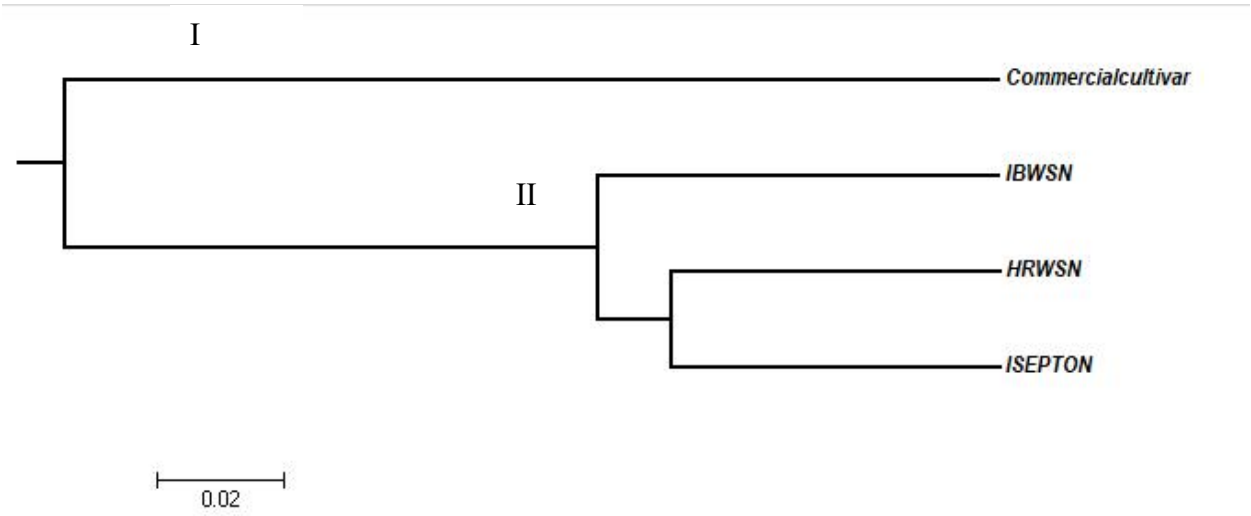

FIGURE 4.UPGMA dendrogram showing genetic relationships among the four populations of 91 bread wheat recombinant inbred lines and few commercial cultivars based on nei's unbiased genetic distances.

The scatter plot obtained from PCoA revealed almost a uniform distribution of the bread wheat recombinant inbred lines and few commercial cultivars of all populations around the center of the two-dimensional coordinate plane with poor population clustering. The first three principal coordinate axes explained 26.44% of the total variations (Figure 5) with each of the dimensions (1, 2 and 3) contributing only 9.87%, 9.32%, and 7.25%, respectively.

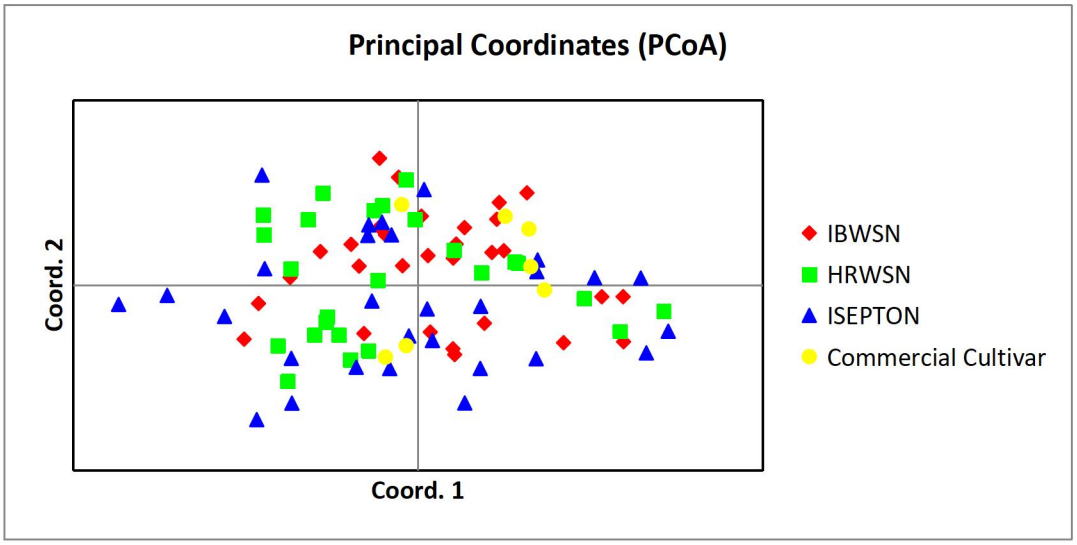

FIGURE 5. PCoA of the 91 bread wheat recombinant inbred lines and few commercial cultivars as revealed by 10-microsatellite loci. Samples coded with the same symbol and color belongs to

the same population. IBWSN=International Bread Wheat Screening Nursery, HRWSN=High Rainfall Wheat Screening Nursery, ISEPTON=International Septoria Observation Nursery

The output of the Structure harvester revealed that the delta K (ΔK) values reached a sharp peak at K = 2 (Figure 6(a), Table 7), confirming that the bread wheat recombinant inbred lines and few commercial cultivars can be clustered into two subpopulations. The Clumpak result (bar plot) detected a greater degree of genetic admixture between the two subpopulations. The result also revealed that each individual bread wheat sample shared alleles from the two sub-populations.

Table 7. Identification of the true number of clusters (K) based on Delta k (ΔK) from structure harvester.

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	20	-3518.775000	0.465522	—	—	—
2	20	-3399.395000	1.083112	119.380000	45.840000	42.322493
3	20	-3325.855000	10.787735	73.540000	30.535000	2.830529
4	20	-3221.780000	2.496650	104.075000	57.610000	23.074917
5	20	-3175.315000	3.620305	46.465000	24.825000	6.857158
6	20	-3104.025000	3.080819	71.290000	60.300000	19.572716
7	20	-3093.035000	9.370237	10.990000	4.400000	0.469572
8	20	-3077.645000	58.710624	15.390000	18.020000	0.306929
9	20	-3080.275000	35.391121	-2.630000	9.640000	0.272385
10	20	-3092.545000	57.376664	-12.270000	—	—

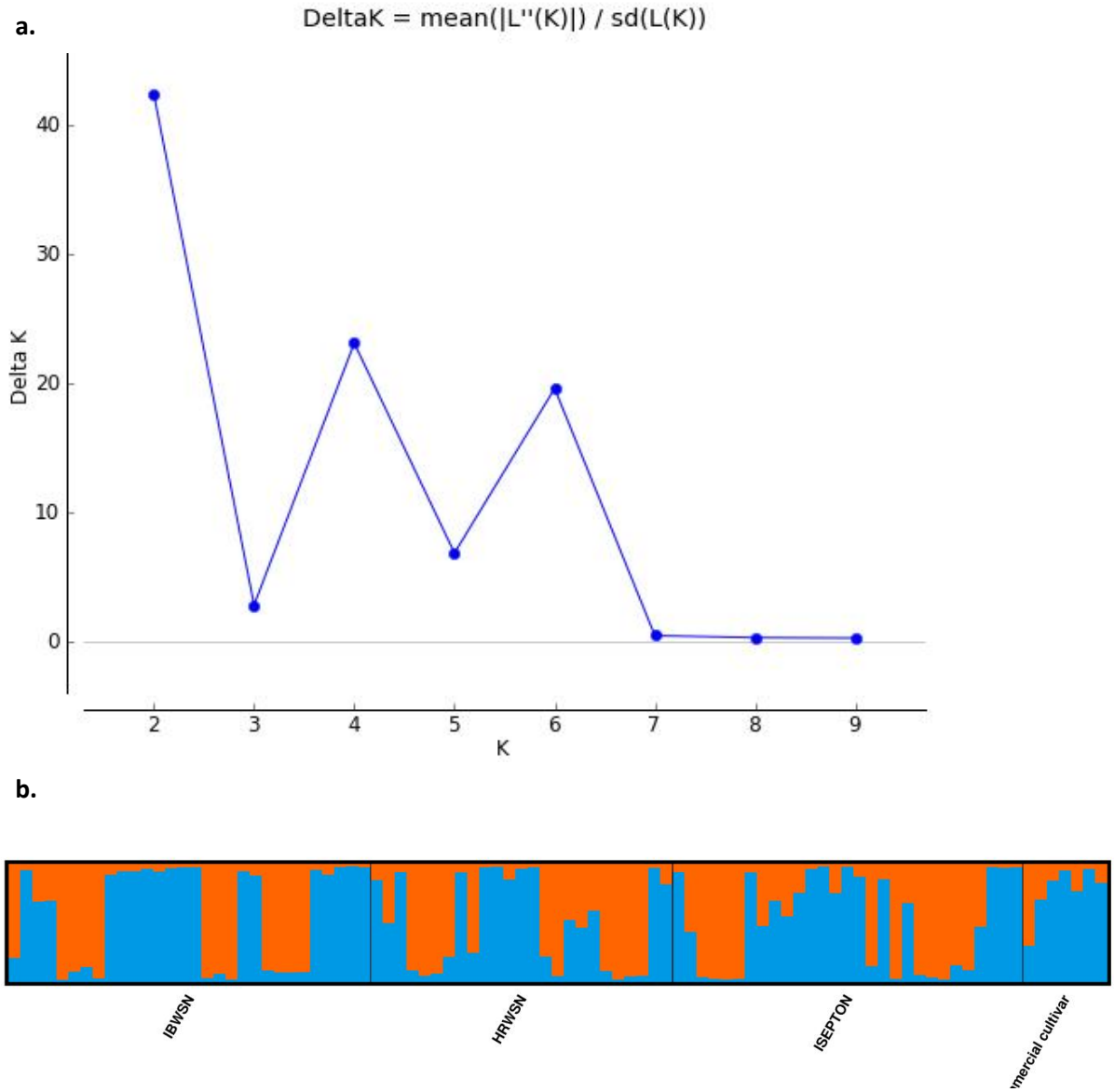

FIGURE 6. Population structure of 91 bread wheat recombinant inbred lines and few commercial cultivars representing four populations of breeding material.(a) Best delta K value estimated using the method of (Evanno *et al.*, 2005); and (b) Estimated population structure for K = 2 according to breeding material. The different (blue, and orange) colors represent genetic groups or sub-populations designated by structure harvester: the x-axis represents individual samples and y-axis represents the proportion of ancestry to each cluster. IBWSN=International Bread Wheat Screening Nursery, HRWSN=High Rainfall Wheat Screening Nursery, ISEPTON=International Septoria Observation Nursery.

5. Discussion

5.1. Genetic Diversity and Population Structure of Bread Wheat recombinant inbred lines and few commercial cultivars as Revealed by SSR Markers

5.1.1. Polymorphism Level of the Microsatellite Markers

Each locus had showed four to nine numbers of alleles per locus with overall mean number of 6.8 per locus. The average number of alleles observed in the present study is similar with previously reported study on durum wheat with different SSR markers by Meseret Asmamaw *et al*. (2019), who described an average of 6 alleles per locus among 141 Ethiopian durum wheat accessions. Ahmed *et al*. (2020) reported a comparable mean value of alleles (7.6) in 105 bread wheat genotypes by using 302 SSR markers. However, the number of alleles reported in the present study is lower than the mean of alleles per locus (18.32) obtained from genotyping of relatively small number of bread wheat genotypes (56) using 30 SSR markers distributed within the genome of the genotypes (Mohi-Ud-Din *et al*., 2022). The diverse nature of recombinant inbred lines and few commercial cultivars used for genotyping and the polymorphism level of the selected SSR markers may serve as a reason for the observed variation.

All the SSR markers in this study were highly polymorphic, confirming their usefulness for genetic analysis. The Polymorphism information content (PIC) ranged from 0.67 (Xgwm135) to 0.86 (Xwmc500), which was comparable to a similar study of wheat collections (Mohi-Ud-Din *et al*., 2022) in which a PIC ranged from 0.434 to 0.932, averaging 0.83 was observed using 25 SSRs across 54 accessions. In similar way Meseret Asmamaw *et al*. (2019) reported PIC value ranging from 0.69 to 0.89, with a mean of 0.76.

The average PIC value of the current study (0.78) was slightly higher than some of the previously reported values on wheat (Ahmed *et al*., 2020; Farhangian-kashani *et al*., 2021;Tsonev *et al*., 2021; Mohi-Ud-Din *et al*., 2022). This could be because of the polymorphic nature of the selected SSR markers utilized for genotyping and the type of the recombinant inbred lines and few commercial cultivars studied. The polymorphism information content (PIC)

value for the primers used in this study, had 10 loci that were considered to be highly informative, since they had a PIC value greater than 0.5.

When the PIC value is > 0.5 the locus is considered to be of high diversity, while if the PIC is < 0.25 the locus is considered to be of low diversity (Ates and Terzi, 2018). The higher PIC values indicated that the primers are useful for molecular characterization and marker assisted breeding.

In the current study, the gene diversity (h) ranged from 0.71 to 0.87 with an overall mean of 0.81. This average gene diversity in this study was greater than the reports of Tsonev *et al* (2021) which is 0.66. The increased genetic diversity in the current study indicates the presence of significant genetic diversity among the studied wheat recombinant inbred lines and few commercial cultivars which in turn provides breeders options to select recombinant inbred lines and few commercial cultivars for crossing and conservation purposes (Awale and Sentayew, 2013).

5.1.2. Population genetic diversity

The presence of high genetic diversity of bread wheat populations in Ethiopian was evidenced by the statistical analysis of population genetic variability across all loci. The four populations had a high average number of observed alleles (6.75), number of effective alleles (4.66), Shannon's information index (1.61), Nei's gene diversity (0.43) and percentage of polymorphic loci (100%).

The gene diversity observed in the current studied populations (0.43) is lower than the diversity value (0.88) reported by Mohi-Ud-Din *et al*., 2022. The modest levels of gene diversity of SSR markers observed in this study was probably due to the presence of modest genetic diversity in these bread wheat recombinant inbred lines and few commercial cultivars that represented different breeding materials.

In the present study, all the four populations showed a highest percentage of polymorphic loci (100%) and the mean number of private alleles was 0.65. A high number of private alleles were resulted in this study, the population IBWSN taking 1.2 values, being followed by HRWSN (0.7), ISEPTON (0.5), and Commercial cultivars (0.2). A lower number of private alleles (NPA) (0.02) was reported by Tilahun Mekonnen *et al*. (2019). The presences of these alleles that are found only in a single population among a broader collection of populations have proven to be

informative for diverse types of population-genetic studies. The informativeness of NPA is that it contributes to indicators of gene flow that is it can be correlated with the mean number of migrants exchanged per generation between populations (Szpiech and Rosenberg, 2011).

Important information which could be employed in crop breeding is provided by private alleles to identify distinctive genetic variability at loci and diversified genotypes (Tomar *et al*.,2021).

The number of private alleles in wheat is estimated to range from 0.3-3.7%, depending on the variety of wheat and the population size of the study (Chen *et al*., 2008). Private alleles are those alleles that are unique to a population or variety, and are not found in the other varieties of wheat. While this number may seem small, each private allele has the potential to have a large impact on phenotypic variation and genetic diversity within a population. Therefore, the presence of private alleles is an important factor to consider when studying wheat genetics (Zauner *et al*. 2019).

It is important to know what a private allele is. According to the study by Zauner et al. (2019), a private allele is an allele that is unique to a sample or population and is not shared with other samples or populations.

In terms of wheat, there have been numerous studies conducted to examine the number of private alleles that are unique to a sample or population and is not shared with other samples or populations, found in wheat. For instance, a study by Zhang *et al*. (2016) examined the private alleles of common wheat in two different regions of China. The study found that there were a total of 8,888 private alleles in the common wheat samples.

In addition, another study by Chen *et al*. (2017) examined the private alleles found in durum wheat in China. This study found that there were a total of 6,236 private alleles in the durum wheat samples. It is important to note that the number of private alleles found in wheat can vary depending on the region and type of wheat.

5.1.3. Population genetic structure

High genetic diversity within populations was observed than among populations through AMOVA. In similar way Busconi *et al*.(2022) reported a higher distribution of genetic variation within species than variation among the population by analysis of molecular variance (AMOVA). AMOVA showed that studied bread wheat recombinant inbred lines and few commercial cultivars had low genetic differentiation, among populations variation accounted for only 4% of the total genetic variation leaving the most for within population variations. Higher proportion of genetic variation within population were reported (Yared Belete *et al*., 2021; Farhangian-kashani *et al*., 2021). Mohi-Ud-Din *et al*. (2022) reported that a relatively higher genetic variation which is 7% among population. Migration and selection by farmers for similar traits could be the reasons for the low genetic differentiation observed between populations (Meseret Asmamaw *et al*., 2019). In this study statistically a significant genetic differentiation is observed along high gene flow. In the same way higher genetic differentiation were reported in previously published papers. Khan *et al*. (2015) reported PhiPT = 0.232 along 23% of genetic variation among population. A moderately high (0.10) mean genetic differentiation estimated by F-statistics at all loci was reported by Meseret Asmamaw *et al*. (2019).

In the present study, the high within-population genetic variability (96%) could be attributed to sexual recombination and spontaneous mutation (Tilahun Mekonnen *et al*., 2019). The low genetic differentiation resulted AMOVA of this study is further confirmed by the high gene flow report. In this study a higher gene flow could be as a result of significant gene exchange among the populations (Kefyalew Negisho *et al*., 2021). In similar way Tomar *et al*.,2021 also reported a high gene flow (Nm=86).

The fact that the individuals of different groups failed to form distinct clusters by PCoA rather mixed up along the axis further confirms the existence of greater genetic variation within populations than among populations (Tilahun Mekonnen *et al*., 2019). This could be due to the presence high genetic materials exchange rate among the various wheat-growing parts of the world.

The 91 recombinant inbred lines and few commercial cultivars obtained from the four different breeding materials were classified into two distinct sub-populations (Delta K = 2) using the structure software. The sub-populations were complementary to the UPGMA tree analysis showing relatedness among the 91 bread wheat recombinant inbred lines and few commercial cultivars. UPGMA dendrogram also showed presence of genetic relationships among the four bread wheat populations and clustered them into three clusters. The structure analysis further substantiated the presence of two sub-populations with significant admixture in the study populations.

6. Conclusions and Recommendations

6.1. Conclusions

The present study was conducted aiming at the overall objective of exploring the genetic diversity and population structure of bread wheat recombinant inbred lines and few commercial cultivars using microsatellite markers. The high average number of alleles per locus (6.8) and PIC value of 0.78 in the present study confirmed the presence of high genetic diversity among bread wheat recombinant inbred lines and few commercial cultivars, indicating the promising possibility to improve desired agronomic traits through breeding. The observed high (96%) within population genetic variation in the studied bread wheat recombinant inbred lines and few commercial cultivars could be due to sexual recombination. The bread wheat populations showed low genetic differentiations (PHiPT= 0.004), likely due to the presence of high gene flow as a result of seed exchange between the breeding materials. Commercial cultivars showed the highest genetic distance with wheat collections HRWSN, implying that these populations could be appropriate for selection of diversified bread wheat recombinant inbred lines and few commercial cultivars for further improvement through breeding. The highest gene diversity was detected in the bread wheat population of HRWSN (h = 0.49), indicating that these breeding material are relevant for genetic diversity study, important genes in bread wheat improvement programs and conservation purposes. Therefore, the present study of the molecular marker technology had generated valuable information for bread wheat improvement through breeding, wise utilization and conservation of the available genetic resource.

6.2. Recommendations

The present piece of work has revealed that HRWSN bread wheat collections showed relatively high gene diversity, indicating that these breeding materials could be potential bread wheat germplasm sources, and hence future studies and conservation plans shall largely include these breeding materials.

7. References

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8. Appendices

Appendix 1. Passport description of the studied bread wheat recombinant inbred lines and few commercial cultivars.

SN	Name	Code	Selection History	Origin
1	EBW172	G172	CMSA08Y00061T-079(1A1RSR26)B-050ZTY-026(1A1RSR26)ZTM-02Y-01B-0Y	MXI12-13\M24ISEPTON\33
2	EBW114	G114	CMSA08Y00378S-050Y-050ZTM-050Y-2BMX-010Y-0B	MXI12-13\M47IBWSN\830
3	EBW145	G145	CMSS08Y00220S-099Y-099M-099Y-11M-0WGY	MXI12-13\M47IBWSN\78
4	G108	EBW108	CMSS08B00391S-099M-099Y-10M-0WGY	MXI12-13\M47IBWSN\496
5	G66	EBW066	CMSS08Y00182S-099Y-099M-099NJ-099NJ-2WGY-0B	MXI12-13\M47IBWSN\69
6	G142	EBW142	CMSS08Y00234S-099Y-099M-099NJ-099NJ-20WGY-0B	MXI12-13\M47IBWSN\95
7	G117	EBW117	CMSS08Y00623T-099TOPM-099Y-099M-099NJ-099NJ-7WGY-0B	MXI12-13\M47IBWSN\217
8	G179	EBW179	CMSS08Y00182S-099Y-099M-099NJ-099NJ-6WGY-0B	MXI12-13\M47IBWSN\72
9	G177	EBW177	CMSS08Y00627T-099TOPM-099Y-	MXI12-

			099M-099NJ-38WGY-0B	13\M47IBWSN\225
	G18	EBW018	CMSS08B00911T-099TOPY-099M-099NJ-12WGY-0B	MXI12-13\M47IBWSN\779
	G81	EBW081	CMSS08Y01116T-099M-099Y-099M-099NJ-099NJ-14WGY-0B	MXI12-13\M47IBWSN\418
	G58	EBW058	CMSS07B00236S-099M-099Y-099M-13WGY-0B	MXI11-12\M46IBWSN\202
	G102	EBW012	CMSS93B00686S-12Y-010M-010Y-010M-7Y-1M-0Y-3SJ-0Y-2PZ-0Y	MXI12-13\M24ISEPTON\102
	G90	EBW090	CMSS07Y01307T-099Y-7M-0Y-3B-0Y	MXI11-12\M46IBWSN\320
	G137	EBW137	CMSS08Y00182S-099Y-099M-099NJ-099NJ-8WGY-0B	MXI12-13\M47IBWSN\73
	G115	EBW115	CMSS08Y00627T-099TOPM-099Y-099M-099NJ-37WGY-0B	MXI12-13\M47IBWSN\224
	G134	EBW134	CMSS08Y00627T-099TOPM-099Y-099M-099NJ-16WGY-0B	MXI12-13\M47IBWSN\222
	G116	EBW116	CMSS08Y00182S-099Y-099M-099NJ-099NJ-9WGY-0B	MXI12-13\M47IBWSN\74
	G7	EBW007	CMSS08Y00704T-099TOPM-099Y-	MXI12-

Appendix I continued …

			099Y-11M-0WGY	13\M47IBWSN\247
	G46	EBW046	CMSS08B00974T-099TOPY-099M-099Y-099B-38WGY-0B	MXI12-13\M47IBWSN\811
20				
	G98	EBW098	CMSA07Y00598S-040ZTY-040ZTM-040Y-5ZTM-010Y-0B	MXI12-13\M24ISEPTON\36
21				
	G73	EBW073	CMSS08Y00627T-099TOPM-099Y-099M-099NJ-6WGY-0B	MXI12-13\M47IBWSN\221
22				
	G164	EBW164	CMSS08Y00235S-099Y-099M-099NJ-1WGY-0B	MXI12-13\M47IBWSN\96
23				
	G27	EBW027	CMSS08Y00869T-099TOPM-099Y-099M-099Y-22M-0WGY	MXI12-13\M47IBWSN\335
24				
	G25	EBW025	CMSS08Y01110T-099M-099Y-099M-099NJ-7WGY-0B	MXI12-13\M47IBWSN\415
25				
	G6	EBW006	CMSS08Y00224S-099Y-099M-099NJ-099NJ-1WGY-0B	MXI12-13\M47IBWSN\81
26				
	G100	EBW100	CMSS08Y00174S-099Y-099M-099NJ-099NJ-17WGY-0B	MXI12-13\M47IBWSN\64
27				
	G24	EBW024	CMSS08B00772T-099TOPY-099M-099Y-21M-0WGY	MXI12-13\M47IBWSN\655
28				
	G55	EBW055	CMSS08Y00627T-099TOPM-099Y-099M-099NJ-5WGY-0B	MXI12-13\M47IBWSN\220
29				

Appendix I continued …

	G65	EBW065	CMSS08Y00871T-099TOPM-099Y-099M-099NJ-099NJ-2WGY-0B	MXI12-13\M47IBWSN\345
30				
	G170	EBW170	CMSS08B00776T-099TOPY-099M-099NJ-099NJ-6RGY-0B	MXI12-13\M25HRWSN\1122
31				
	G129	EBW129	CMSS08B00776T-099TOPY-099M-099NJ-099NJ-14RGY-0B	MXI12-13\M25HRWSN\1123
32				
	G136	EBW136	CMSS08B00919T-099TOPY-099M-099NJ-099NJ-7RGY-0B	MXI12-13\M25HRWSN\1137
33				
	G86	EBW086	CMSA08M00446S-040ZTM-050Y-5ZTM-010Y-0B	MXI12-13\M25HRWSN\1170
34				
	G133	EBW133	CMSS08Y00872T-099TOPM-099Y-099M-099NJ-099NJ-4RGY-0B	MXI12-13\M25HRWSN\1040
35				
	G139	EBW139	CMSA08M00445S-040ZTM-050Y-47ZTM-010Y-0B	MXI12-13\M25HRWSN\1169
36				
	G144	EBW144	CMSS07B00024S-099M-099Y-099M-21RGY-0B	MXI11-12\M24HRWSN\32
37				
	G111	EBW111	CMSS08Y00220S-099Y-099M-099NJ-6RGY-0B	MXI12-13\M25HRWSN\1007
38				
39	G150	EBW150	CMSA08M00124T-050Y-040ZTM-	MXI12-

		050Y-8ZTM-010Y-0B	13\M25HRWSN\1154
G141	EBW141	CMSS08Y00224S-099Y-099M-099NJ-099NJ-4RGY-0B	MXI12-13\M25HRWSN\1008
G19	EBW019	CMSS08B00422S-099M-099NJ-5RGY-0B	MXI12-13\M25HRWSN\1100
G88	EBW088	CMSS08Y00224S-099Y-099M-099NJ-099NJ-16RGY-0B	MXI12-13\M25HRWSN\1011
G135	EBW135	CMSA08Y00556S-050Y-050ZTM-050Y-45BMX-010Y-0B	MXI12-13\M25HRWSN\1150
G132	EBW132	CMSS08Y00351S-099Y-099M-099NJ-099NJ-4RGY-0B	MXI12-13\M25HRWSN\1023

Appendix I continued …

G47	EBW047	CMSA08Y00065T-099B-050Y-050ZTM-050Y-14BMX-010Y-0B	MXI12-13\M25HRWSN\1141
G15	EBW015	CMSA08M00432S-040M-0NJ-10Y-0B	MXI12-13\M25HRWSN\1166
G99	EBW099	CMSS08Y00224S-099Y-099M-099NJ-099NJ-19RGY-0B	MXI12-13\M25HRWSN\1012
G41	EBW041	CMSS08B00381S-099M-099Y-1M-0RGY	MXI12-13\M25HRWSN\1099

	G160	EBW160	CMSS08B00233S-099M-099NJ-099NJ-25RGY-0B	MXI12-13\M25HRWSN\1075
49				
	G96	EBW096	CMSA08Y00469S-050Y-040M-0NJ-21Y-0B	MXI12-13\M25HRWSN\1148
50				
	G11	EBW011	CMSA08M00458S-040ZTM-050Y-50ZTM-010Y-0B	MXI12-13\M25HRWSN\1174
51				
	G124	EBW124	CMSA08M00424S-040ZTM-050Y-13ZTM-010Y-0B	MXI12-13\M25HRWSN\1162
52				
	G123	EBW123	CMSS08B00776T-099TOPY-099M-099NJ-099NJ-21RGY-0B	MXI12-13\M25HRWSN\1124
53				
	G130	EBW130	CMSA07M00443S-040ZTM-040ZTY-47ZTM-010Y-02B-0Y	MXI12-13\M24ISEPTON\51
54				
	G5	EBW005	CMSS08Y00924T-099TOPM-099Y-099M-099NJ-099NJ-10RGY-0B	MXI12-13\M25HRWSN\1053
55				
	G118	EBW118	CMSA07Y00950T-8B-040Y-040ZTM-040Y-10ZTM-010Y-0B	MXI12-13\M24ISEPTON\62
56				
	G70	EBW070	CMSS07B00264S-099M-099NJ-099NJ-8WGY-0B	MXI12-13\M24ISEPTON\88
57				
	G140	EBW140	CMSS07B00869T-099TOPY-099M-099Y-099M-1WGY-0B	MXI12-13\M24ISEPTON\95
58				
59	G89	EBW089	CMSS07B00231S-099M-099Y-	MXI12-

			099M-47WGY-0B	13\M24ISEPTON\86
	G79	EBW079	CMSS07B00614T-099TOPY-099M-099Y-099M-7WGY-0B	MXI12-13\M24ISEPTON\92
60				
	G147	EBW147	CMSA07M00445S-040ZTM-040ZTY-75ZTM-010Y-01B-0Y	MXI12-13\M24ISEPTON\18
61				
	G110	EBW110	CMSS06Y00946T-099TOPM-099Y-099ZTM-099Y-099M-13WGY-0B	MXI12-13\M24ISEPTON\97
62				
	G78	EBW078	CMSS07Y00672T-099TOPM-099Y-099M-099Y-36M-0WGY	MXI12-13\M24ISEPTON\71
63				
	G28	EBW028	CMSS07Y00299S-0B-099Y-099M-099Y-2M-0WGY	MXI12-13\M24ISEPTON\69
64				
	G112	EBW112	CMSA07M00644S-040M-0NJ-0NJ-5Y-0B	MXI12-13\M24ISEPTON\44
65				
	G148	EBW148	CMSA07M00403S-040ZTM-040ZTY-21ZTM-010Y-02B-0Y	MXI12-13\M24ISEPTON\16
66				
	G82	EBW082	CMSA07Y00273S-040ZTY-040ZTM-040Y-30ZTM-010Y-0B	MXI12-13\M24ISEPTON\55
67				
	G119	EBW119	CMSS07Y00704T-099TOPM-099Y-099M-099NJ-099NJ-9WGY-0B	MXI12-13\M24ISEPTON\73
68				
	G57	EBW057	CMSA07M00441S-040ZTM-040ZTY-20ZTM-010Y-01B-0Y	MXI12-13\M24ISEPTON\4
69				

	G87	EBW087	CMSS07B00151S-099M-099Y-099M-7WGY-0B	MXI12-13\M24ISEPTON\83
70				
	G84	EBW084	CMSS06B00001S-0Y-099ZTM-099Y-099M-8WGY-0B	MXI12-13\M24ISEPTON\99
71				
	G83	EBW083	CMSA07M00455S-040M-0NJ-0NJ-2Y-0B	MXI12-13\M24ISEPTON\42
72				
	G95	EBW095	CMSS06Y00706T-099TOPM-099Y-099ZTM-099NJ-099NJ-7WGY-0B	MXI12-13\M24ISEPTON\96
73				
	G91	EBW091	CMSS97M03642T-040Y-020Y-030M-020Y-040M-7Y-3M-0Y	MXI12-13\M24ISEPTON\101
74				

Appendix I continued …

	G60	EBW060	CMSS07Y00346S-0B-099Y-099M-099NJ-099NJ-3WGY-0B	MXI12-13\M24ISEPTON\70
75				
	G37	EBW037	CMSA08Y00045T-024(CRE1)B-050ZTY-067(CRE1)ZTM-03Y-01B-0Y	MXI12-13\M24ISEPTON\32
76				
	G35	EBW035	CMSS07Y00774T-099TOPM-099Y-099M-099NJ-099NJ-6WGY-0B	MXI12-13\M24ISEPTON\74
77				
78	G71	Danda'a	Commercial Cultivar	Danda'a

	G3	EBW003	CMSA07M00022T-032(B=WMC221SR25HET)Y-040ZTM-074(WMC0221SR25HOM+HET)Y-55ZTM-010Y-01B-0Y	MXI12-13\M24ISEPTON\25
79				
	G156	EBW156	CMSA07M00365S-040M-0NJ-0NJ-4Y-0B	MXI12-13\M24ISEPTON\58
80				
	G42	EBW042	CMSS07B00191S-099M-099Y-099M-22WGY-0B	MXI12-13\M24ISEPTON\85
81				
	G12	EBW012	CMSS93B00686S-12Y-010M-010Y-010M-7Y-1M-0Y-3SJ-0Y-2PZ-0Y	MXI12-13\M24ISEPTON\102
82				
	G67	EBW067	CMSS93B00686S-12Y-010M-010Y-010M-7Y-1M-0Y-3SJ-0Y-2PZ-0Y	MXI10-11\C22ISEPTON\102
83				
	G2	EBW002	MXI12-13\M24ISEPTON\56	CMSA07Y00597S-040ZTY-040ZTM-040Y-46ZTM-010Y-0B
84				
	G127	Paven-76	Commercial Cultivar	Paven-76
85				
	G149	Sofomor	Commercial Cultivar	Sofomor
86				
	G40	King-bird	Commercial Cultivar	King-bird
87				
88	G10	Bika	Commercial Cultivar	Bika

Appendix I continued …

89	G33	Huluka	Commercial Cultivar	Huluka
90	G22	Medawolabu	Commercial Cultivar	Medawolabu
91	G21	Alidoro	Commercial Cultivar	Alidoro

Appendix 2. DNA extraction protocol (Modified CTAB method) described by Tilahun Mekonnen *et al*., 2017.

1. Introduction

This protocol describes a modified CTAB method for the isolation of DNA from plant material.

A maximum of 24 samples can be isolated at once using this protocol.

2. Materials

CTAB	-	1 x 700 µl per sample
ß-Mercapto-Ethanol		
Chloroform		
Isopropanol	-	100 %, 4°C
Ethanol	-	100 % and 70% in sterile ddH2O
TE	-	1x, p.a. grade

NH4Ac	-	7.5 M Solution, sterile
NaAc	-	3 M Solution, sterile
Water bath	-	65°C
Centrifuge	-	4°C
Cut 1000 µl tips	-	Sterile

3. Procedure

1. Poor CTAB solution (700 µl per sample) in a 1.5ml-tube and add 0.2 vol % Mercaptoethanol

(Use fume hood!). Mercapto-ethanol 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. Pulverise thoroughly using a Geno grinder machine.

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

6. Centrifuge for 5 minutes at 15000 rpm.

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

8. 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.

9. 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.

10. Shake the samples thoroughly by turning inversing the Eppendorf caps for approximately 5 minutes. (Longer incubation is possible)

11. Centrifuge for 5 min at 15000 rpm.

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

13. Repeat the chloroform extraction (step 9-12) to make sure that all impurities are removed, and then proceed with step 14.

14. 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 2 h at -20°C.

15. Centrifuge 10 min at 15000 rpm.

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

17. Add 200 µl Ethanol 70 % to the pellet. Rinse the inner cap surface by turning the cap.

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

19. 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)

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

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

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

23. Centrifuge 30 min at 15000 rpm. Aspirate fluid carefully.

24. Add 200 µl Ethanol 70%. Rinse the inner cap surface by turning the cap.

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)

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

27. 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 -20^{o}c for further use.