ADDIS ABABA UNIVERSITY

INSTITUTE OF BIOTECHNOLOGY

MSc. Thesis

Genetic Diversity and Population Structure Analysis of *Zymoseptoria tritici* in Ethiopia at Single Field Scale as Revealed by Simple Sequence Repeat Markers

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July, 2021

Addis Ababa, Ethiopia

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MSc. Thesis

A Thesis Submitted to the Institute of Biotechnology, Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

By

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July, 2021

Addis Ababa, Ethiopia

Thesis Approval Sheet

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ACKNOWLEDGMENTS

My deepest and heartfelt gratitude goes to my major advisor Mr. Tilahun Mekonnen at the institute of Biotechnology, Addis Ababa University for his unreserved guidance, support, patience and scientific comments throughout the entire research work. I also thank him for his support for providing different laboratory consumables and facilitating the way to get partial financial support from CYMMY. I would also like to thank my co-advisors; Dr. Bekele Abeyo at CIMMYT- Ethiopia, who gave me partial financial support and advice in all my thesis work and Dr.Teklehaimanot Hailesilassie at the institute of Biotechnology, Addis Ababa University, for his fruitful comments and suggestions to complete this thesis work. I also extend my thanks to Mr. Obsi Desalegn from National Agricultural Biotechnology Research Center (NABRC), for his help to access laboratory reagents and facilities besides to his daily follow-up and critical comments.

I would like to thank the National Agricultural Biotechnology Research Center, EIAR, for providing me study leave, financial support and, permission to use laboratory facilities without restrictions. It is also my pleasure to offer my gratitude to Institute of Biotechnology of Addis Ababa University for giving me the chance to study my MSc. in Biotechnology.

Finally, I appreciate my family for their love and encouragement to pursue my MSc study. Especially I am very grateful to my wife Mrs. Amarech Mhiret for giving me her love and the courage in all difficulties during my thesis work.

The efforts of all other individuals who haven’t been mentioned here because of space but who directly or indirectly put their effort in my thesis work are greatly credited.

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ABBREVIATIONS/ACRONYMS

AMOVA	Analysis of Molecular Variance
CSA	Central Statistics Agency
DArT	Diversity Array Technology
FAO	Food and Agricultural Organization
HARC	Holeta Agricultural Research Center
MAF	Major Allele Frequency
MCMC	Markov Chain Monte Carlo
NABRC	National Agricultural Biotechnology Research Center
Ne	Effective Number of Alleles
PCoA	Principal Coordinates Analysis
PDA	Potato Dextrose Agar
PIC	Polymorphic Information Content
SNNP	Southern Nations, Nationalities and Peoples
SSR	Simple Sequence Repeat
STB	*Septoria Tritici* Blotch
ITS	Internal Transcribed Spacer
YSB	Yeast Extract Sucrose Broth
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UTM	Universal Transverse Mercator Coordinator System

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ABSTRACT

Zymoseptoria tritici the causative agent of *Septoria tritici* blotch (STB), is one of the most economically damaging disease of wheat worldwide. Genetic resistance is a suitable, economical and environmentally safe strategy to control the disease. Knowledge of the genetic structure of the pathogen is vital for designing best management strategy against STB. The present study aimed to investigate the genetic diversity of *Z. tritici* in Ethiopia at a single field scale using simple sequence repeat (SSR) markers. A total of 200 naturally STB infected wheat leaves were collected from 10 plots of unsprayed single field at Holetta Agricultural Research Center. A total of 147 single-spore derived *Z. tritici* isolates were subjected to genetic diversity analysis using ten SSR loci. All the tested loci were polymorphic, and highly informative. Different diversity parameters were executed, and highlighted with the number of alleles, gene diversity (0.82), and polymorphic information content ranged from 5-11, 0.71- 0.88 and 0.67 -0.87 with overall mean of 9 , 0.82, and 0.80, respectively. Hierarchical analysis of molecular variance revealed moderate (PhiPT= 0.13) genetic differentiation where the within population genetic variation accounted for 87% of the total variation 4.18. The variation among population accounted only for 13% of the total genetic variation, likely due to the presence of high (Nm = 3.48) gene flow. The neighbor joining, UPGMA and PCoA failed in sharply grouping the populations into their corresponding sampled plots, confirming the presence of high gene flow. Moreover, the Bayesian-model based structure analysis weakly inferred four sub-groups (K= 4) confirming the weak population structuring with high degree of genetic admixture. Therefore, the study confirmed that *Z. tritici* population shows high genetic variation even in a single field, suggesting need to use integrated disease management strategies to control the disease and also give especial focus for resistance breading to involve pyramiding of several genes that can provide broad spectrum resistance.

***Key words/Phrases**: Septoria tritici* blotch, Microsatellite, Polymorphism, Wheat,

1. INTRODUCTION

Wheat (*Triticum aestivum*) is the second largest globally grown crop next to rice (*Oryza sativa* L.), with ever-increasing area coverage and production (Saintenac *et al*., 2018; Tilahun Mekonnen *et al*., 2020). It is one of the most important strategic and staple food crops, providing 20% of the total daily calories and nearly 35% of protein consumed in the world (Chaves *et al*., 2013). Bread wheat (*Triticum aestivum* L, 2n=6x=42) and durum wheat (*Triticum turgidum* L. 2n=4x=28) are the two most commonly cultivated wheat species. The former alone accounts for 95% of the total global wheat consumption (Randhawa *et al*., 2013).

In Sub-Saharan Africa, Ethiopia is the second largest wheat producer next to South Africa (Tesfaye Letta *et al.,* 2013; Abera Takele *et al*., 2015; Ytagesu Tadesse *et al*., 2018) for use as food, animal feed and income generation. It can grow in a wide range of climatic conditions globally in an altitude ranged from 930 to 3,048 m.a.s.l (Douglas, 2017). It can also grows in the range between 6 and 14 ° N latitudes; and between 35 and 42 ° E longitude and in altitudinal range from 1500 m to 3200 m (Hailu Gebre-Mariam, 1991). In 2019/20 harvesting season, the area covered by wheat and the total annual production in Ethiopia were approximately 1.8 M ha and 5.3 M ton, respectively. Therefore it is the 3rd most cultivated crop next to Teff (*Eragrostis tef*), and Maize (*Zea mays*) (FAOSTAT, 2021), respectively. Oromia, Amhara, SNNPs, and Tigray are the primary wheat growing regions (CSA, 2021). They account for more than 90% of the national wheat production of the country's consumption. Wheat is one of the target crops in the strategic goal of meeting the demand of the country`s food self-sufficiency (CSA, 2021). Although the average yield and total production increased moderately for the last two decades, the national average productivity is relatively low (2.97) t ha^{-1} as compared to the global average of 3.5 t ha^{-1} (FAOSTAT, 2021). The major constraints to wheat production in Ethiopia are

shortage of improved production technologies and agricultural inputs (seeds and fertilizers), biotic stresses like diseases, pests, and abiotic stresses (CSA, 2020; FAO, 2021).

Septoria tritici blotch (STB) caused by the fungal pathogen *Zymoseptoria tritici* (Desm.) synonym (teleomorph, *Mycosphaerella graminicola* (Fuckel) J. Schr¨ot in Cohn previously its asexual stage or anamorph known as, *Septoria tritici* (Quaedvlieg *et al*., 2011). It is one of the most wheat destructive diseases causing 31-54% yield loss globally (Miedaner *et al*., 2006; Dean *et al*., 2012; Fones and Gurr, 2015; Steinberg, 2015; Yosef Gebrehawaryat *et al*., 2017). It is the second important wheat disease and causes 42% yield loss on the sensitive varieties in Ethiopia (Alemayehu Hailu *et al.,* 2015; Ytagesu Tadesse *et al*., 2018). Others also reported wide range of (25- 82%) grain yield loss from Ethiopia in the worst seasons (Getinet Gebeyehu *et al.,* 1990; Mengistu Hulluka *et al*., 1991). The infection begins at the lower leaves where the plant leaves contact with soil and gradually destroys up to the flag leaves, but it can also infect wheat heads (Alemar Said and Temam Hussien, 2016). The pathogen has both primary and secondary sources for its infection. The infection can arise from sexual ascospores produced in the fruiting bodies of the sexual stage (pseudothecia) and also from asexual spores called Pycnidospores. Wheat seeds, wheat volunteers and pycnidiospore-bearing wheat debris are considered to be the primary sources of inoculums (Sanderson and Hampton, 1978; Suffert and Lannou, 2011).

Several disease management strategies have been employed by stakeholders to control STB disease such as fungicide sprays, use of resistant cultivars and different agricultural practices such as late planting, burning previous crop debris, controlling plant density, weeding and use of tall varieties etc. (Eyal, 1999). However, most of STB traditional control mechanisms on field are becoming inefficient due to the fast long distance spread of ascospores in the field (Ytagesu Tadesse *et al*., 2018). Moreover the use of fungicides have been failed from year to year due to

continuous emergence of resistance alleles in the pathogen and it is not also feasible for developing countries because of their high cost and environmental reasons (Torriani *et al*., 2015;Yosef Gebrehawaryat *et al*., 2017).

Host plant genetic resistance is becoming the most suitable, durable, economical and environmentally friendly strategy to control crop diseases including STB (Dean *et al*., 2012; Mejri *et al*., 2018; Tilahun Mekonnen *et al.,* 2019; 2020). Understanding of genetic diversity is very valuable for designing feasible management strategies for prevailing fungal pathogen. McDonald *et al*. (1995) also stated that knowledge of genetic diversity in various strains of pathogenic agents is of great importance in breeding for producing STB resistant cultivars.

So far several DNA markers have been used to explore the genetic diversity of *Z. tritici* population in the world such as RFLP markers (McDonald *et al.*, 1995; Chen and McDonald, 1996; Medini and Hamza, 2008), RAPD markers (Czembor and Arseniuk, 1996), AFLP markers (Schnieder *et al.*, 2001; Kabbage *et al.,* 2009), and also SSR markers were applied even at single field scale in Tunisia (Berraies *et al.,* 2013), North France (Siah *et al.,* 2018). In Ethiopia also different studies have been conducted on this pathogen in the norther, southern, and southeastern and central highlands of the country using SSR markers by (Diriba Guta, 2019; Messele Molla, 2020; Tilahun Mekonnen *et al*., 2020) respectively. SSR markers are preferred for genetic analyses because of their higher rate of polymorphism, informativeness, reproducibility, multiallelic nature, co-dominant inheritance, relative abundance and ease of identification from genomic sequences (Winter and Kahl, 1995; Medini and Hamza, 2008; Gautier *et al.,* 2014). Generally, these studies revealed high genetic diversity, population genetic differentiation and gene flow depending on the sampled geographical areas and/or marker types.

In Ethiopia, despite the various efforts made recently, no considerable investigations were conducted to unfold the fungal genetic structure in small scale, and reports on the genetic structure of the pathogen in more localized level like a single field scale or at single plant or below level is greatly missing. Therefore, the present study was designed with the following objectives.

1.1. Objectives

1.1.1. General objective

The aim of this study was to investigate the genetic diversity of *Zymoseptoria tritici* in Ethiopia at single field scale using simple sequence repeat markers

1.1.2. Specific objectives

- To determine the genetic diversity of *Zymoseptoria tritici* isolates collected from single field using SSR markers.
- To determine population structure of *Zymoseptoria tritici* isolates at single field scale.

2. LITERATURE REVIEW

2.1. Origin and Distribution of *Zymoseptoria tritici*

Zymoseptoria tritici (teleomorph: *Mycosphaerella graminicola*, anamorph: *Septoria tritici*) is an ascomycete fungus, which originated from its relative *Zymoseptoria* species colonizing wild grasses in the Fertile Crescent and it coincides with its host domestication 11,000 years ago in the middle east (Rudd, 2015). The fungal pathogen causes a foliar disease, *Septoria tritici* blotch (STB), on wheat (Fig. 1A.). Even though the pathogen affects both hexaploid bread wheat (*Triticum aestivum L.,* AABBDD, 2n = 42) and tetraploid durum wheat (*T. turgidum L.*, AABB, 2n = 28), the isolates coming from bread wheat do not cause disease on durum wheat and *vice versa* (Ferjaoui *et al*., 2015). In 1842, Desmazieres who is the pioneer investigator of *Z. tritici* has reported the causal agent of STB disease in France, as reviewed in Ponomarenko *et al*. (2011). In addition, *Zymoseptoria pseudo tritici* and *Zymoseptoria ardabiliae* which are close relatives of these fungi were also discovered in Iran during population genetics analysis of fungi collected from wild grasses growing near to wheat (Stukenbrouck *et al*., 2011; Mcdonald *et al*., 2015).

Figure 1. Wheat leaf infected by *Z. tritici* **(A);** HARC main experimental station **(B);** wheat field during sample collection in October, 2019.

Previous reports indicated that, the pathogen have distributed in Europe, United States of America, Australia, Canada, Argentina, Middle East countries alongside of Mediterranean Sea, and Africa including Ethiopia; hence it has become a serious problem for wheat production worldwide (Ponomarenko *et al*., 2011;Yosef Gebrehawaryat *et al*., 2017). The first report on this and other fungal diseases and parasites on plants was released by Castellani (1938) and Ciferri (1939) in Ethiopia. Then, the disease caused by *Z. tritici* has been reported by different researchers since 1930s in different highlands of Ethiopia with high prevalence in central, west, and southwest wheat growing areas (Stewart, 1959; Dagnatchew Yirgu, 1969; Mengistu Hulluka, 1991; Abreham Tadesse, 2008; Abera Takele *et al*., 2015; Alemar Said and Temam Hussien, 2016).

2.2. Life Cycle and Infection Process

Zymoseptoria tritici has sexual and asexual mechanism of reproduction to proceed its life cycle. The sexual (ascospores) are released from pseudothecia, and the asexual pycnidospores are derived from pycnidia (Fig. 2). The former is dispersed with the aid of wind, while the latter is splashed by rain during the wheat growing season to cause infection on wheat leaves at optimal temperatures ranging from 15 to 20 °C with long periods of high humidity (Eyal *et al*., 1987).

The whole infection cycle of *Z. tritici* has three phases: (1) entry of the fungus, 2) colonization of the plant tissue and (3) formation of fruiting bodies (Steinberg, 2015). The first infection is initiated with the germination of either pycnidiospores or ascospores that are both pathogenic.

First, the long-distance dispersing wind-borne ascospores land on the basal host leaves resulting in the initial infection, and a minimum of six hours are required to score a maximum infection and produce mature pycnidospores. These spores are again disseminated by rain splash to upper

leaves of the same or nearby plants. Spore germ tubes are attracted to the stomata, through that they gain entry into the sub-stomatal cavity either directly or indirectly by forming the infection cushion (Duncan and Howard, 2000). They are modified to hyphal growth upon contact with the leaf. Each ascospores and pycnidiospores will germinate to initiate secondary infection when landing on the leaves, and also, they penetrate new plant tissues in favorable conditions (Palmer and Skinner, 2002) (Fig. 2).

The hyphae may grow for many days intercellularly in close contact with the mesophyll cells and little increase in biomass. Once penetration occurs, the pathogen colonizes the mesophyll tissue of the leaves by growing intercellularly but does not produce its feeding structure called haustoria (Steinberg, 2015).The hyphae of the pathogen grows simply on susceptible genotypes, whereas resistant varieties do not allow the establishment of hyphae for the colonization of hosts. The size and the form of chloroplasts have direct effect on the resistance response on hosts. The vulnerable cultivars first response is condensation of chloroplasts and then strong swilling of cells due to accumulation of starch granules that are released for the response of soluble compounds by the fungus. As a result of this, the cells will burst and die. However, the resistant cultivars protect themselves by producing compounds that help for protection of fungal colonization and formation of pycnidium (Kema *et al*., 1996b). The pathogen survives crop-free dry periods primarily as pseudothecia however conjointly in pycnidia on crop stubbles.

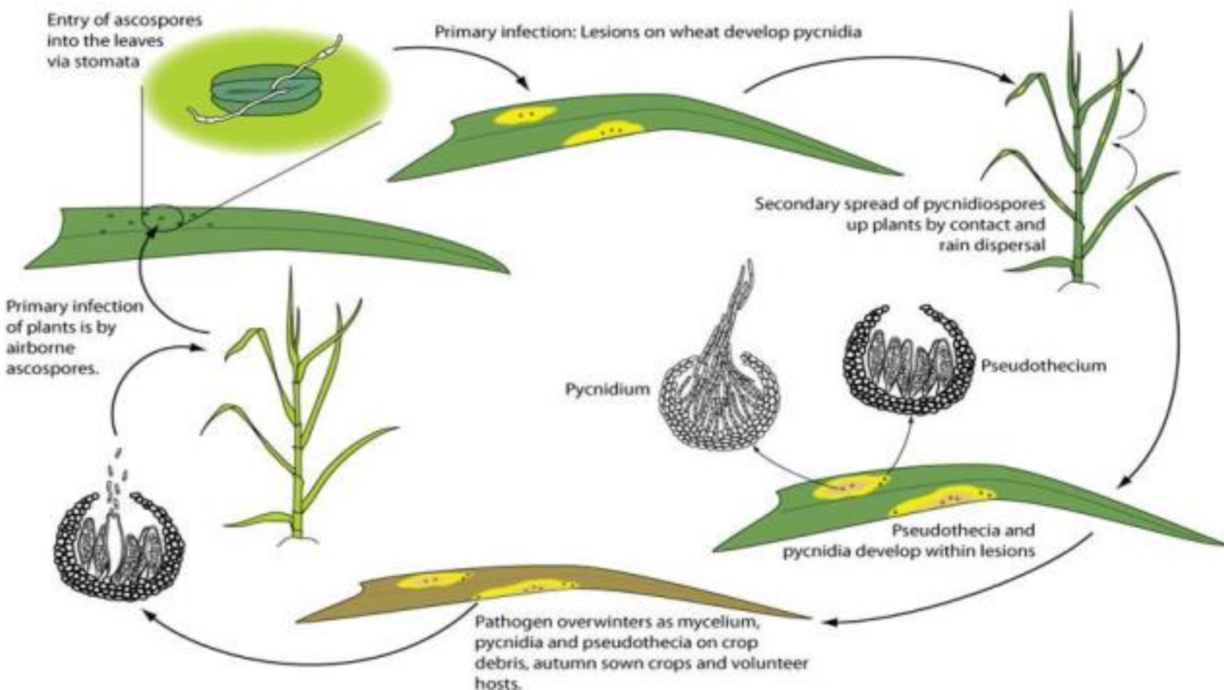


Figure 2. The life cycle of *Z. tritici*. Primary source of infections are wind born sexual ascospores and secondary source of infections are rain splashed asexual pycnidospores. Source (Ponomarenko *et al.,* 2011).

2.3. Taxonomy of *Zymoseptoria tritici*

Zymoseptoria tritici, formerly known as *Mycosphaerella graminicola,* is a species of filamentous fungus and the first class of the *Dothideomycetes* (Testa *et al*., 2015), and its taxonomic status is described below.

Kingdom: *Mycota* (Fungi)

Phylum: *Ascomycota*

Class: *Dothideomycetes*

Subclass: *Dothideomycetidae*

Order: *Capnodiales*

Family: *Mycosphaerellaceae*

Genus: *Zymoseptoria*

Species: *Zymoseptoria triticale*

Source (Ponomarenko *et al*., 2011)

2.4. Genetic Structure of *Zymoseptoria tritici* Populations

Genetic structure refers to the distribution and amount of genetic variation within and among the population in a specified time frame and space. This variability may be created due to the interactions of major evolutionary forces, like mutation, random genetic drift, gene flow, reproduction system, and selection (Abrinbana *et al*., 2010; El- Chartouni *et al*., 2011).

The knowledge of the genetic structure is especially useful for better understanding of the pathogen epidemiology and evolutionary potential, which has direct implications for the development of best management strategies (Siah *et al*., 2018). The reason is the occurrence of high level of genetic variation within a population indicates the rapid genetic evolvement of the pathogen. This allows the pathogen to adapt fungicides or resistant genes found in newly introduced resistant cultivars, and then emerge as potential risk on these target hosts (Mcdonald *et al*., 1995). Therefore, understanding the role of each factor, and the way it influences the emergence of a new adapted pathogen population is important to predict the danger of the pathogen so as to suggest management strategies (McDonald and Linde, 2002). In addition, avoiding use of only major R gene in the host genome is suggested to use the combination of all responsible genes for the control of this disease.

Several studies have been conducted on genetic variation among *Z. tritici* isolates collected from different continents, and variability was reported within the lesions of the pathogen (Linde *et al*., 2002). Except little geographical structures found in the population (El- Chartouni *et al*., 2011), most of the population isolates obtained from different regions globally and even also from the same lesions showed high genetic diversity with low genetic differentiation due to high gene flow (Linde *et al*., 2002; Zhan *et al*., 2003). The high degree of gene flow occurs across large

spatial scales due to air born sexually recombined ascospores carried by different parts of the host (Dalvand *et al*., 2018).

2.4.1. DNA markers for studying *Z. tritici* genetic variability

Historically, genetic structure of *Z. tritici* had been estimated by using phenotypic markers, but it has limitation in determining variability from the randomly collected populations. Since 1990s, neutral DNA based molecular markers that are distributed randomly throughout the genome have been developed from the genome of this fungus to determine population structure, which avoids the biased estimate of genetic diversity (McDonald and Martinez, 1990; Owen *et al*., 1998; Goodwin *et al*., 2007).

The first DNA markers, restriction fragment length polymorphism (RFLP), were developed from *Z. tritici* by McDonald and Martinez (1990). Accordingly, several researches have been done using RFLP markers and high genetic diversity was revealed within *Z. tritici* populations across the world (Boeger *et al*., 1993; Chen and McDonald, 1996; Kabbage *et al*., 2009; Diria *et al*., 2014). Despite that the RFLP markers had high reliability and co-dominance nature, scientists look another way; because these markers are labor-intensive, time-consuming, the polymorphism created by these markers is relatively low, and must be detected by radioisotope.

Due to advances in molecular biology like the innovation of PCR technology; PCR based markers such as random amplified polymorphic DNA (RAPD) was proposed by Williams *et al*. (1990). This technology has been widely used for molecular characterization of different fungal species including *Z. tritici* (Czembor and Arseniuk, 1996; Kema and Silfhou, 1997; Czembor and Arseniuk, 1999; Razavi and Hughes, 2004b), and high genetic variability have been reported within and among *Z. tritici* isolates collected at global level even at a scale of one square meter

area. However, RAPDs are simple, quick and cost effective compared to RFLP; the poor repeatability and reliability of banding profiles, some non-specificity, and their dominant nature limits its application.

The other marker type, amplified fragment length polymorphism (AFLP), was also used effectively in the determination of population structure on *Z. tritici* isolates by many researchers in different regions (Schnieder *et al*., 2001; Kabbage *et al*., 2008; Medini and Hamza, 2008; Kabbage *et al*., 2009; Abrinbana *et al*., 2010; Vagndorf *et al*., 2018). However, they are dominant biallelic and are unable to differentiate dominant homozygous from dominant heterozygous individuals.

The latest PCR based markers are microsatellites such as simple sequence repeat polymorphism (SSR), which consist of randomly repeated, short DNA sequence motifs, and found in both eukaryotes and prokaryotes genome. The number and type of repeat units present in a species are variable between individuals. Thus, primers designed to target specifically to these variations are called SSR markers (Owen *et al*., 1998; Goodwin *et al*., 2007). After the development of species specific diagnostic markers in the internal transcribed spacer (ITS) regions of *Z. tritici* and *Septoria nodorum* by Beck and Ligon (1994), they were effectively used for PCR based detection and genetic differentiation of the pathogen population. The genetic variability and population structure of these fungi including *Z. tritici* were intensively studied worldwide using microsatellite markers (Owen *et al*., 1998; Razavi and Hughes., 2004a; Siah *et al*., 2018; Morais *et al*., 2019; Tilahun Mekonnen *et al*., 2020). These authors reported high genetic variability of *Z. tritici*. Among these, Diriba Guta (2019), Messele Molla (2020), and Tilahun Mekonnen *et al*. (2020) reported the existence of high (95%, 86% and 92%) genetic variability within *Z. tritici* populations collected in different regions of highland Ethiopia such as northern, southern, and,

south eastern and central Ethiopia, respectively. These indicate that the markers were highly informative and best for the determination of the genetic diversity and population structure of the pathogen population.

This is also due to the good features of the SSR markers such as low quantity of the template DNA, co-dominance nature, high accuracy, high reproducibility, and amenable to automation because of their ability to make multiplexed PCR. However, misclassification of heterozygotes may occur when null allele is exhibited due to mutation at the primer binding site of the markers (i.e. if the markers do not amplify at one of the region of a diploid/ or above one ploidy level organism, that individual may have a chance to be consider as heterozygote (Yang *et al*., 2013).

2.5. Septoria tritici Blotch

Septoria is the common name for more than 1000 fungal species, of which more than 100 species are parasitic to cereals and wild grasses (Eyal *et al*., 1987). There are two major *Septoria* diseases that have been considered as main threats for wheat productivity in the temperate regions of the world (Hartmann *et al*., 2017). The first is *Septoria tritici* blotch (STB) which is caused by the *Zymoseptoria tritici* (formerly: *Mycosphaerella graminicola* or *Septoria tritici*). It is *Dothideomycete* hemibiotroph filamentous fungus that has an initial biotrophic and subsequent necrotrophic phase, which is the most wheat destructive disease causing 31-54% yield loss globally (Miedaner *et al*., 2006; Dean *et al*., 2012; Fones and Gurr, 2015; Steinberg, 2015; Yosef Gebrehawaryat *et al*., 2017). In a sever season when *Zymoseptoria tritici* is under favorable growing conditions, the annual yield loss due to *Septoria tritici* blotch (STB) may exceed from 30% to 70% worldwide (Eyal *et al*., 1987 ; Berraies *et al*., 2013). For instance, this disease is one of the current most problematic foliar disease on wheat in France causing a serious yield losses

ranging from 31- 53% during favorable conditions for disease development (Polley and Thomas, 1991; Siah *et al*., 2018). The second type of *Septoria* disease is *Septoria nodorum* blotch which is caused by the fungus *Septoria (Stagonospora) nodorum* (teleomorph: *Leptosphaeria nodorum*).

Similarly, yield loss may reach up to 42% in the susceptible Ethiopian wheat varieties (Alemar Said and Temam Hussien, 2016; Ytagesu Tadesse *et al*., 2018). Currently, it is the second economically destructive disease next to rusts in the main wheat growing regions of Ethiopia.

2.5.1. *Septoria tritici* blotch disease symptoms

The indications of STB disease on wheat is variable according to genotype, cultural practices, and geographical locations, however, the initial disease symptom is mostly noticeable at the beginning of the growing season. A small chlorotic spots on the lower tips of the leaves particularly appears on those in contact with the soil (Ponomarenko *et al*., 2011). After some time, the chlorotic spots can enlarge into irregular lesions with brown-to-reddish brown color (Fig. 3A). As the lesions become oldies and in favorable condition; the lesions at the center becomes bleached with gray or ash-white, with small, dark brown to black spots (Fig. 3C)

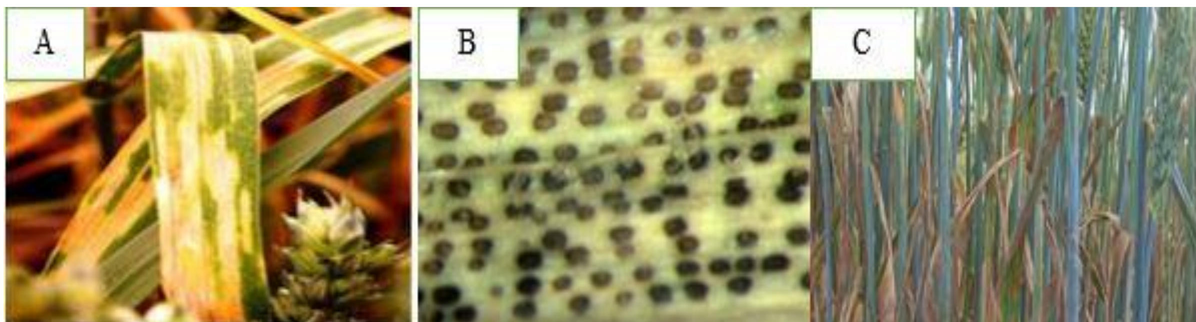


Figure 3. Symptoms of *Septoria tritici* Blotch on wheat leaves. A) Advanced symptoms of *Septoria* on wheat leaves. B) Signs of developed pycnidia in the substomatal cavities of the host within the lesions. C) Symptoms of sever septoria on wheat field. Source (Eyal *et al*., 1987; Halama, 1996; Ponomarenko *et al*., 2011).

The selenderic pycnidiospores are produced from the anamorph *Septoria tritici,* and they are enclosed within a pycnidium. In necrotic injuries, Pycnidia can be developed with colors ranging from light to dark brown. They can be scattered within the lesions of the epidermal and mesophyll tissue on both upper and lower surfaces (Eyal *et al*., 1987). The presence of small dark dots, known as pycnidia in center of lesions is the most valuable character for recognizing the disease symptom of STB (Fig. 3B). The size of pycnidia may be affected by the number of pycnidia on the infected tissue as their number increases their size is decreases while their size varies among different cultivars.

2.5.2. Epidemiology of the disease

Zymoseptoria tritici is considered as one of the most challenging wheat pathogens throughout the world. The primary sources of inoculums which cause STB disease on seedling leaves are infected crop residues and volunteer wheat plants, which usually consist of airborne ascospores. Pycnidiospores survive in pycnidia on infected stubble for several months. The primary lesions due to ascospores will bear evenly dispersed pycnidia that are an asexual structure that allow rapid dispersal of secondary source of inoculum, called conidia (Palmer and Skinner, 2002).

The secondary source of inoculum, conidia for STB can spread readily in the presence of high humidity on leaves, but ascospores are also involved on it. Under sever epidemics, the production of Pycnidia with conidia need roughly 14 to 40 days after infection irrespective of the susceptible plant architecture and stature. However, the infection speed is variable for different cultivars with favorable weather conditions (Eyal *et al*., 1987). Conidia help to spread the disease upwards through the canopy as verified by Ponomarenko *et al*. (2011) who also suggested that

both conidia and ascospores contribute to the epidemic but the asexual cycle seems to dominate during the growing season.

Pycnidiospores survive in pycnidia by concealing themselves in the infected stubble for several months. Ascospores do not originate only from previous season stubbles or volunteer plants, but they are also released from ascocarps produced on infected leaves of the current wheat (Hunter *et al*., 1999). Recent studies show that once the disease established STB, its progress does not merely dependent on splashed spread pycnidospores because it can complete several sexual cycles per season using newly produced wind borne ascospores in growing season (Shaw and Royle, 1993; Kema *et al*., 1996a).

The other two most significant factors are temperature and moisture. Pycnidiospores are viable at a temperature range of 2 to10 °C for several months, and also they shield themselves from radiation and desiccation using slime12 (cirrhus). However, the optimum temperature for the development of *Z. tritici* ranges from15 to 20°C. On the other hand, leaf wetness period and rate of disease development have linear relationship (Magboul *et al*., 1992).

2.5.3. *Septoria tritici* blotch control methods

The STB disease can be controlled by integrated methods but the most common methods that were applied worldwide to combat this disease are application of resistant cultivars, chemicals, cultural practices, and biological methods (Ponomarenko *et al*., 2011).

2.5.3.1. Resistant cultivars

Management of *STB* by planting resistant cultivars is the best approach because it is economical, simple, durable, and environmental friendly. Resistance to *Z. tritici* can be qualitative or

quantitative. According to recent studies, about 21 major (R) resistance genes against STB and close linked to microsatellite markers have been named, mapped, and published. Resistance to STB diseases is derived mostly from the main host species, commonly known as host resistance. Host resistance is divided into two types, all stage resistance (ASR) or seedling resistance (R genes), and adult plant resistance (APR genes). Thus, R genes to STB that are designated as Stb1- Stb18, StbSm3, StbWW, and TmStb1 were identified from Ethiopian Wheat genotypes (Tilahun Mekonnen *et al*., 2019). This number is still not enough to find durable resistant genes against STB, as compared to 73, 89, 61 and 95 mapped resistant genes of wheat against yellow rust, leaf rust, stem rust and powdery mildew, respectively (Ghaffary *et al*., 2011).

The inheritance of these resistance genes in some genotypes is complex which is due to rapid genetic change or change of host range specificity in the pathogen population. For example, Stb1 has remained effective in Indiana for more than 25 years, while Stb4 was effective for less than 2 and 14 years in Oregon and California, respectively. Moreover, majority of them have narrow spectra of host range specificity, some cultivars that are reported as resistant to STB in one region maybe susceptible in another, and hence this limits their use (Ghaffary *et al*., 2011). This may be due to the genetic composition of the local pathogen population, environmental factors during infection, and the relative importance of the sexual stage in the disease cycle (Ponomarenko *et al*., 2011).

Since quantitative resistance genes occur in wheat cultivars, combined use with qualitative resistance genes could be helpful to ensure its stability in susceptible wheat cultivars. Furthermore, use of molecular markers that have been linked to many of the STB genes for marker-assisted selection can also be important to create effective gene combinations in new wheat cultivars.

2.5.3.2. Chemical control methods

Wide ranges of fungicides are designed and are available for the control of wheat diseases including *Septoria tritici* blotch. Protectant fungicides like dithiocarbamates (Maneb, Manzate, Mancozeb, and Zineb) were applied for the control of STB but repeated application at 10-14 day intervals is required for effective management (Eyal *et al*., 1987). As compared to systemic fungicides with curative properties such as benomyl (Benlate), prochloraz (Sportak), triadimefon (Bayleton) and propiconazole (Tilt), these type of fungicides provide shorter time protection (Eyal *et al*., 1987). Thus, combining protectant and systemic fungicides provides better control of the disease.

Even though foliar sprays are the most common type of fungicide treatments prior to applying chemicals, users should consider: 1) early assessment of yield potential and economics of the specific wheat field; 2) disease levels in the specific field; 3) early detection of the diseases and assessment of their progress; 4) weather conditions, and 5) cost of fungicide protection relative to other investments in the crop. In early sown susceptible varieties, a fungicide application at growth stage (GS) 31-32 may be required to suppress the disease and protect emerging leaves.

Therefore, to use chemical control program for *Septoria* diseases effectively through spraying, it should be assisted by an extensive survey of the diseases. Most countries have been applying those chemicals to manage this disease at the seedling stage after gathering the disease history, and other relevant information. For example, triticonazole is sprayed in the region, commonly known as “seed dressing of a fungicide”, where the seedling stage is affected by the pathogen. Hence, strong monitoring and timing of sprays in periods when the pathogen is most likely to be active will yield the greatest economic return.

Since the occurrence of the first fungicide resistance in 2002 by *Z. tritici* as major problem, some chemicals like strobilurin are applying only in areas where resistance have been yet developed. Currently, azoles are being applied to control STB disease while its efficacy has decreased due to mutation in cyp51 gene. Therefore, alternative fungicides with different modes of action help to mitigate the development of *Z. tritici* resistance (Ponomarenko *et al*., 2011).

2.5.3.3. Cultural practices

Since the beginning of agriculture, various cultural practices have been implemented to reduce plant diseases (Howard, 1996). Crop rotation, tillage, fertilizer application, different seeding operations, and disease-free seed are among the oldest approaches that can reduce the incidence and severity of STB. They are economically feasible for the management of *Septoria tritici* blotch disease (Ginkel *et al*., 1999).

Generally, deep plowing of infected crop debris and planting of non - host crops in rotation are important tactics to sanitize the available long distance dispersed ascospores that will initiate new disease cycle in the field. Moreover, burning of stubbles and crop residues are also recommended, however, it has environmental risk factors. Therefore, all stakeholders declined from applying such practices and focused on other alternatives such as use of biological control methods, resistant cultivars, and an integrated management of the above cultural practices.

2.6. Measuring Genetic Variability of a Population

The key tool to estimate the extent of genetic variation within and among the population is the measurement of the existing gene and genotypic frequencies in the target population. The concept of gene diversity was introduced by Nei (1973), and used as a clue to describe the

genetic variability in both sexually and asexually reproducing populations. The gene diversity (H) was also defined by him and considered as the probability of getting two different alleles at a locus when two haploid individuals are sampled randomly from a population. The equation to calculate the Nei's gene diversity is (Eq I).

$$H = 1 - \sum xi^2 \quad => \quad Equation\ - I$$

Where, 'H' or h for (haploid) is the gene diversity of the population, 'Xi' or Pi for (haploid) is the frequency of the ith allele for the population at a particular locus, and $\sum Xi^2$ is the sum of the squared population allele frequencies.

In the co-dominant markers, the maximum (H = 0.5) value of genetic diversity can be revealed when the two allele frequencies occurred equally at a particular locus, in the meantime, the gene diversity could range between 0 and 0.5. But, in multi-locus markers like microsatellites, the maximum value of gene diversity can be revealed as the increase in the number of alleles per locus. On the other hand, the gene diversity will be zero in genetically uniform markers (with no allelic variation at the particular locus).

The distribution of the genetic variability among and within different subpopulations was first proposed by Nei (1973); this variation is partitioned into different components. He also suggested the mathematical representation of genetic differentiation between subpopulations as indicated below (Eq II).

$$Gst = \frac{Ht - Hs}{Ht} => \text{Equation II}$$

Where ‘Ht’ or ht (for haploid) is the total genetic diversity of a population and ‘Hs’ is the average genetic diversity within subpopulations. Gst is the proportion of the total genetic variation found among subpopulations.

The other genetic parameters that are important to estimate intra population genetic variability including major allele frequency (MAF), polymorphic information content (PIC), and inter-population diversity estimators include number of alleles, percent of polymorphic loci (% PPL), effective number of alleles (Ne), Shannon‘s Information Index (I), and unique number of alleles. Furthermore, AMOVA using its fixation indices and population clustering parameters like PCoA, cluster analysis, and population structure analysis have been conducted to estimate the extent of genetic variability in populations (Peakall and Smouse, 2006).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

Two hundred *Z. tritici* infected wheat leaf samples were collected from Holetta Agricultural Research Center (HARC) main experimental field, which is located 29 Km west of the capital city Addis Ababa, Ethiopia (Fig. 4). It is located at 09^0 00'N latitude and 38^0 38 30'E longitude, and at the elevation of 2400 m.a.s.l. The center is a hot spot for *Septoria tritici* blotch disease and serves as national and international wheat germplasm screening center for Septoria by the International Maize and Wheat Improvement Center (CIMMYT). The research center receives an average rainfall of about1144 mm with maximum and minimum annual mean temperatures of 22 °C and 6 °C, respectively. High and long rain coupled with high humidity favors the STB disease development and spreading. The main rainy season extends from June to September, which accounts for 70% of the total annual rainfall. The soil of the center is mainly nitosoil type, which is characterized with average organic matter content of 1.8%, nitrogen 0.17%, pH 4.5 or ranged from 3.95 to 5.25, and phosphorus 4.55 ppm (http://www.eiar.gov.et/holetta). Since the area has enough annual average rain fall, it is one of the suitable bread wheat growing areas of the central highlands of Ethiopia (Ytagesu Tadesse *et al*., 2019).

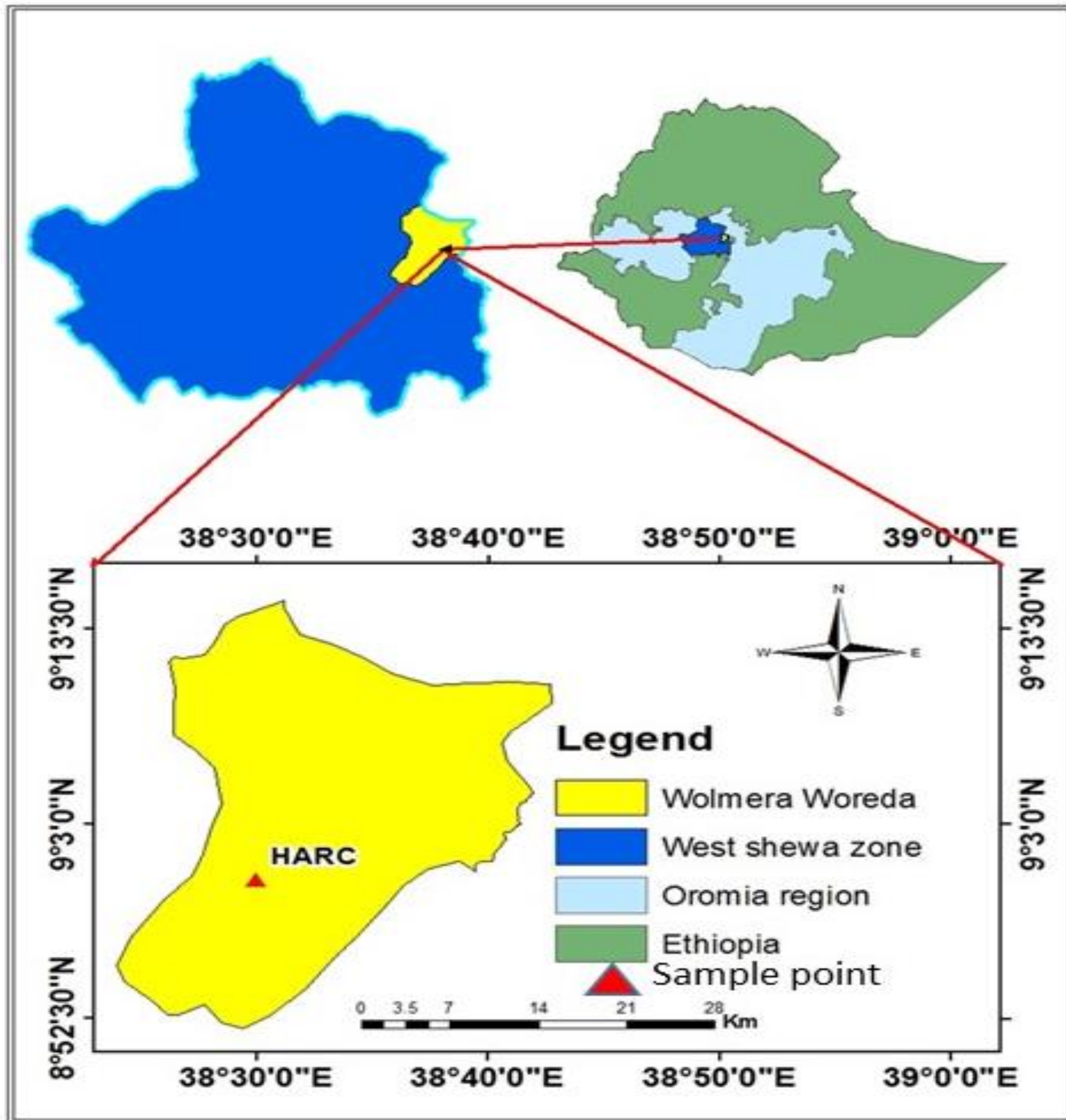


Figure 4. Map of study area: Ethiopia (green), Oromia region (light blue), West Shewa zone (blue), Wolmera woreda (yellow) and HARC (red triangle, exact location of the field where the samples were collected), which is drown by ArcGIS vr. 10.3.1 Software (ESRI, 2015) using the geographical coordinates of the samples collected from HARC wheat single field.

3.2. Sample Collection

STB symptomatic wheat leaves were collected from unsprayed wheat field of 24 m x 100 m in October, 2019. During sample collection, the total area was partitioned in to approximately ten plots; each with 2 m x 100 m by leaving a border area of 2 m x 100 m on both sides as a buffer. From each plot 20 STB infected symptomatic wheat leaves were collected from 20 different plants using hierarchical sampling method. To avoid fungal spore cross contaminations, used scissors were disinfected using 70% ethyl alcohol. The samples were packed in paper bags, and they were labeled with specific code including the location coordinates of each sample.

3.3. *Zymoseptoria tritici* Isolation

The fungal isolation was conducted at the Microbial Biotechnology Laboratory in National Agricultural Biotechnology Research Center, Holetta, Ethiopia according to the isolation method described by Eyal *et al.* (1987) with some modifications. Accordingly, the collected symptomatic wheat leaf samples were cut into about 10 cm length. Then, the prepared leaf samples were placed on sterile Petri dishes with sterile water wetted filter paper. Petri dishes with samples were packed in polyethylene plastic bag, and kept in incubator for 3-4 hours at 24 °C. The incubator was fixed at a high relative humidity to induce oozing of cirrhi having spores from pycnidia. The samples were checked periodically using stereoscopic dissecting microscope under 40X objective for the formation of cloudy ooze on the top of pycnidia.

During transfer to potato dextrose agar (PDA) media, Petri dishes containing the samples and other necessary tools (needles, media plates, stereoscopic microscope, etc.) were prepared and placed in the microbial free laminar air flow cabinet. Observing under the microscope, three oozing drops from each sample were transferred onto 39 g/L PDA medium using flame sterilized

and briefly cooled fine-pointed needle. The medium contained 200 g/l potato, 20 g/l dextrose, and 15 g/l agar supplemented with 250 mg/l chloramphenicol at pH of 5.6. The inoculated plates were then incubated at 24 C° for 10-15 days in the dark until the fungal growth appeared. Pinkish-orange color colonies appeared and were streaked onto fresh PDA medium, and it was kept at the same conditions for the growth of pure isolates. This step was repeated in the same condition to obtain pure isolates. Spore multiplication was carried out by transferring single spore derived colonies into yeast extracts sucrose broth (YSB) medium composed of 1% (w/v) yeast extract powder + 1% (w/v) sucrose using 250 ml flasks. The cultures were maintained on an orbital shaker at 130 rpm for 10 -15 days at 20 C° with a 12/12 hrs day-night cycle (1.103 lux), and then 147 pure isolates were obtained. The multiplied isolates were then centrifuged for 5 minutes at 10,000 rpm to obtain pellets. The pellets were then stored at -80 C° to be used for DNA isolation.

3.4. Genetic profiling of the *Z. tritici* Populations

3.4.1. Genomic DNA Extraction

The DNA isolation was conducted at Molecular Biotechnology Laboratory, NABRC. The extraction was conducted using diversity array technology (DArT) plant DNA extraction protocol with some modifications (appendix.1). A total of 147 single spore derived *Z. tritici* isolates were used for genetic diversity analysis. For genomic DNA extraction, fungal spores were crushed with mortar and pestles using liquid nitrogen. The extracted DNA quality was determined on 1% agarose gel. The DNA concentration and quality was also checked using Nano drop spectrophotometer. The DNA was kept at -20 C° until used for genotyping.

3.4.2. Polymerase chain reaction

Ten SSR pairs of primers (Table 1) were used to assess the genetic diversity of the 147 *Z. tritici* isolates. The PCR reaction was performed in reaction volume of 12.5 µL containing 6.25 µL (OneTaq® 2X Master Mix prepared by *BioLabs* from standard buffer components, Taq DNA polymerase, dNTPs, $MgCl_2$, and stabilizers) mixed, with 1 µL of each of forward and reverse primer, 2µL of gDNA, and 2.25 µL of nuclease free water. The PCR reaction condition was gradient optimized. Except ST1E4, ST1G7, and ST1D7, all primers were effectively worked at the initial denaturation of 94 C° for 2 min, followed by 35 reaction cycles of denaturation at 93 C° for 30 s, optimized annealing temperature of 53 C° for 2 min, and primer extension at 72 C° for 2 min.

The PCR for the remaining three primers (ST1E4, ST1G7, and ST1D7) followed initial denaturation at 94 C° for 3 min followed by 45 cycles of denaturation at 94 C° for 1min, with 58, 65, and 61 C° annealing temperature for 1 min, respectively, and 72 C° of primer extension for 2 min. The final extension for all primers was carried out at 72 C° for 10 min before holding at 4 C°. The PCR reaction was run using BIO-RAD T100 Thermal cycler. The amplified products were fractionated by loading 5 µL PCR product of each sample and 2 µL a mixture of loading dye and gel red on 3 % agarose gel in 1 X TAE buffer at 100 V for 3:00 hrs. The molecular weight of the PCR products was estimated by using both 50 bp and 100 bp DNA ladders. The gels were visualized under UV light and subsequently photographed using BioDoc-It TM Imaging System.

Table1. The ten SSR pair of primer’s sequences, annealing temperatures and the range of expected sizes detected at these microsatellite loci in the *Z. tritici* populations from a single field in Ethiopia.

Locus	Repeat motif	Forward primer (5′ to 3′)	Reverse primers (5′ to 3′)	Annealing temp (°C)	fragment size ranges (bp)	Reference
ST2E4 (MGR 7034)	(GGC)5	GAAGATCAACAGCATGGGCGG	CTCCAGAGGGATCACAAAGGC	58	54-110	Owen *et al.*, 1998
ST1G7 (MGR 7037)	(TG)9	ATGCTGAGAAGTTCGGTGAGG	CGTTCTTCCACCTCCAACACT	65	96-103	>>
ST1D7 (MGR 7039)	(AC)22	TTGAAGTGGCATCCTCCATT	AACTCGGCTGGTGGAACA	61	95-105	>>
tcc-0009	(TCC)8	TCAATTGCCAATAATTCGGG	AGACGAGGCAGTTGGTTGAG	53	161-179	Goodwin *et al.*, 2007
ac-0002	(AC)7	TGAACATCAACCTCACACGC	AGAAGAGGACGACCCACGAG	53	182-206	>>
ggc-0001	(GGC)8	GATACCAAGGTGGCCAAGG	CACGTTGGGAGTGTCGAAG	53	232-256	>>
ag-0009	(AG)10 (GGCA)3	GACTCCATTTACCTGTGGCG	TGTGAAGGACACGCAAAGAG	53	192-200	>>
caa-0005	(CAA)8	AAGAATCCCACCACCCAAAC	’ 5CACACGGCTCCTTTGACAC’	53	263-299	>>
ac-0001	(AC)21	CACCACACCGTCGTTCAAG	CGTAAGTTGGTGGAGATGGG	53	171-227	>>
ag-0003	(AG)15	ACTTGGGGAGGTGTTGTGAG	ACGAATTGTTCATTCCAGCG	53	226-258	>>

3.4.3. Data scoring and analysis

Different software packages were used to compute the different diversity parameters. The fragment size of the products was estimated using PyElph 1.4 software package (Pavel and Vasile, 2012). The scored bands were used to compute the genetic diversity indices. The diversity indices across the entire populations at each locus such as major allele frequency (MAF), number of allele (Na), gene diversity, and polymorphic information content (PIC) were computed using power marker v3.25 software (Liu and Muse, 2005). GenAlEx ver. 6.501 was used to compute gene flow, allelic frequency, genetic distance, Nei's haploid gene diversity, genetic differentiation, percentage of polymorphism, analysis of molecular variance (AMOVA), and principal coordinate analysis (Peakall and Smouse, 2012).

Considering plots as population, population genetic diversity parameters like Na, Ne, and Nei's gene diversity (h) were computed using GenAlEx ver. 6.501. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) based dendrogram was constructed using MEGA7 software (Kumar *et al*., 2016). The Neighbor-Joining based tree was generated using DARwin var.6.0.14 (Perrier and Jacquemoud-Collet, 2006). STRUCTURE software ver.2.3.4 based on Bayesian algorithm was used to determine the population structure in relation to their sampled plots (Pritchard *et al*., 2000). To estimate 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 10 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 and Von Holdt, 2012). A bar plot for the optimum K was determined using Clumpak beta version (Kopelman *et al*., 2015).

4. RESULTS

4.1. Microsatellite Marker Polymorphism

In this study, 10 SSR markers were tested on 147 *Z. tritici* isolates (Table. 2). All of the tested markers were polymorphic, and they produced a total of 1,457 bands with average of 145.7 per locus. Out of the total bands produced, only 105 bands were monomorphic, and the remaining were polymorphic (Fig. 5; Table.2). Among the ten loci, seven markers, ST2E4, ST1G7, ST1D7, ggc-0001, ag-0009, ac-0001, and ag-0003, produced higher number of bands (146) while the remaining three (tcc-0009, ac-0002 and caa-0005) loci gave the lower (145) number of bands (Table.2). The number of polymorphic bands also ranged from 124 (84.93 %) for ac-0001 marker to 142 (97.26 %) for ag-0003, respectively. From the total ten markers, six markers exhibited above the average number of polymorphic markers. The highest (97.26%) percentage of polymorphic bands was recorded for ag-0003 marker followed by tcc-0009 marker (97.24%), and the lowest (84.93%) percentage of the polymorphic bands was recorded for ac-0001 locus (Table. 2).

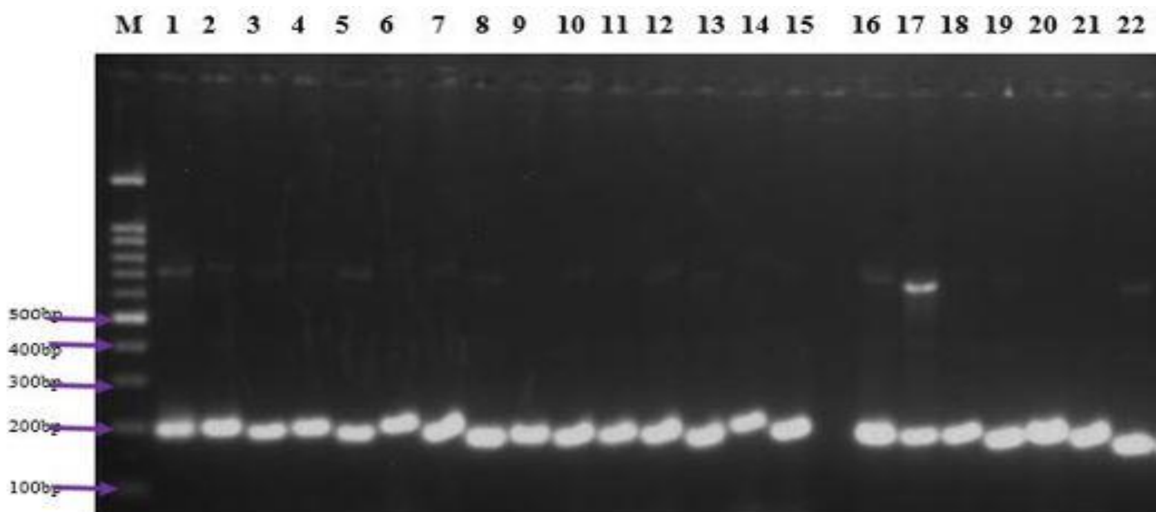


Figure 5. PCR product of Z. *tritici* isolates. **M** is a 100bp DNA ladder and the numbers from 1 to 22 showed fungal isolates that were amplified by marker ac-0001.

Table 2. Number of monomorphic bands, polymorphic bands and the percentage of polymorphic bands generated by 10 SSR markers applied on 147 *Z. tritici* isolates.

Markers	No_ monomorphic bands	No_ polymorphic bands	Total number of bands	Percentage of Polymorphic bands
ST2E4	14	132	146	90.41%
ST1G7	8	138	146	94.52%
ST1D7	7	139	146	95.21%
tcc-0009	4	141	145	97.24%
ac-0002	11	134	145	92.41%
ggc-0001	6	140	146	95.89%
ag-0009	20	126	146	86.30%
caa-0005	9	136	145	93.79%
ac-0001	22	124	146	84.93%
ag-0003	4	142	146	97.26%
Total	**105**	**1352**	**1457**	**92.79%**

The statistical summary of allele frequencies produced by using the 10 SSR markers is given in Table 3. All the markers showed polymorphism, and a total of 90 clearly identified alleles were observed. The observed alleles per locus varied from 5 to 11 with an average of 9 alleles. The highest number of alleles (11) was produced by each five loci: ST1D7, tcc0009, ac0002, ggc0001 and caa0005. Among observed alleles, 18 (22.22%) were scarce (frequency between 0.001 and 0.05). They were resulted by the microsatellite loci ST1D7, tcc0009, ggc0001, ag0009, caa0005 and ac0001 (Table. 3). Most (47.77%) of the observed alleles had the frequencies ranging from 0.05 - 0.1. For each locus, the frequency of the most common allele was less than 0.95 or 0.99, confirming that the markers were highly polymorphic. The alleles with frequencies above 0.1 accounted 30% of the total observed alleles (Table. 3).

Table 3. Summary of the number of alleles with their frequencies

Markers	No_ Alleles with their respective frequencies			Total
	Scarce (0.01-0.05)	(>0.05-0.1)	(above 0.1)	
ST2E4	0	1	5	6
ST1G7	0	2	3	5
ST1D7	4	5	2	11
tcc0009	5	4	2	11
ac0002	0	10	1	11
ggc0001	3	6	2	11
ag0009	1	7	2	10
caa0005	3	5	3	11
ac0001	2	3	4	9
ag0003	0	2	3	5
Total	18	45	27	90
Percentage	**22.22%**	**47.77%**	**30%**	**100%**

The analysis revealed that the major allelic frequency per locus ranged from 0.24 to 0.46 with overall mean of 0.32 (Table. 4**).** The highest frequency (0.46) was recorded by ST1G7 while the lowest (0.24) MAF was shown by ag-0009 (Table. 4). Two markers (ST1G7 and ag-0003) scored the lowest (5) number of alleles while the other five markers (ST1D7, tcc-0009, ac-0002, ggc-0001, and caa-0005) resulted in the highest (11) number of alleles. The highest number of effective allele (5.02), Shannon's information index (1.69), and gene diversity (0.88) were observed for the microsatellite locus ac-0002. Conversely, the lowest records of 2.27, 0.95 and 0.71 for number of effective alleles, Shannon's information index, and gene diversity, respectively were obtained from ST1G7 locus.

The Polymorphic information content (PIC) values of all the tested microsatellite loci were greater than 0.5 with the overall mean value of 0.80 confirming that all the used loci are highly informative and very suitable for diversity analysis. The study revealed that the rate of genetic differentiation at each locus (Gst) ranged from 0.04 (tcc-0009) to 0.25 or 0.26 for ST1G7 with an overall mean of 0.11.

Table 4. Marker informativeness and other genetic diversity statistical parameters for all 10 SSR loci across ten populations of *Z. tritici* at single field level in Ethiopia.

Locus	N	[a]MAF	Na	Ne	I	GD	Gst	PhiPT	*P_value*	Nm	PIC
ST2E4	146	0.33	6	3.19	1.32	0.79	0.09	0.10	0.001	4.40	0.76
ST1G7	146	0.46	5	2.27	0.95	0.71	0.25	0.26	0.001	1.40	0.67
ST1D7	146	0.35	11	2.67	1.14	0.83	0.22	0.24	0.001	1.60	0.81
tcc-0009	145	0.38	11	4.36	1.53	0.80	0.04	0.04	0.004	10.87	0.78
ac-0002	145	0.25	11	5.02	1.69	0.88	0.08	0.09	0.001	5.14	0.87
ggc-0001	146	0.25	11	4.30	1.50	0.88	0.12	0.13	0.001	3.29	0.87
ag-0009	146	0.24	10	3.84	1.43	0.87	0.11	0.12	0.001	3.61	0.86
caa-0005	145	0.28	11	3.97	1.52	0.86	0.10	0.11	0.001	3.98	0.85
ac-0001	146	0.31	9	4.30	1.57	0.84	0.05	0.05	0.002	8.88	0.82
ag-0003	146	0.32	5	3.00	1.21	0.76	0.09	0.10	0.001	4.58	0.72
Mean	**145.70**	**0.32**	**9.00**	**3.69**	**1.39**	**0.82**	**0.11**	**0.12**	**0.001**	**4.78**	**0.80**

[a]MAF = Major allele frequency; N = number of observed samples; Na = number of observed alleles; Ne = Effective number of alleles; I = Shannon's Information index; GD = gene diversity; Gst and PhiPT= Genetic differentiation statistics by locus; Nm = estimate of the number of migrants (gene flow) from GST at Nm = 0.5(1 - Gst)/Gst; p = Differentiation statistics probabilities; and PIC = Polymorphic information content.

4.2. Genetic Variation within and Among Populations

The statistical summary of different genetic diversity parameters over the ten loci across 10 populations is presented in Table 5. The study showed the occurrence of high genetic diversity according to number of alleles, Nei's haploid gene diversity, number of effective alleles, Shannon's information index, number of private alleles, number of locally common alleles, and percentage of polymorphism. Among the studied populations, Plot 3 scored the highest number of alleles (6.00), number of effective alleles (4.06), Shannon's information index (1.48), Nei's haploid gene diversity (0.72), and number of locally common alleles (0.3). On the other side, populations of Plot 6 showed the lowest Shannon's information index (1.24) and Nei's haploid gene diversity (0.62) (Table. 5).

All of the ten populations showed a hundred percent polymorphism across the entire loci confirming the existence of high genetic diversity of *Z. tritici* isolates within the populations. Population of Plot 5 showed the highest number of private alleles (0.20), while most of the populations scored the lowest value with the mean value of 0.04 number of private allele per population (Table. 5).

Table 5. Allelic patterns and diversity indices across ten populations averaged over the 10 SSR loci

[b]Population	Size	[a]Na	Ne	I	h	NPA	NLCA	PPL
Plot1	15	5	3.09	1.26	0.64	0.10	0.10	100%
Plot2	14	5	3.88	1.45	0.72	0.00	0.10	100%
Plot3	15	6	4.06	1.48	0.72	0.00	0.30	100%
Plot4	17	5	3.36	1.34	0.67	0.00	0.10	100%
Plot5	16	5	3.56	1.38	0.69	0.20	0.10	100%
Plot6	14	5	3.32	1.24	0.62	0.00	0.00	100%
Plot7	13	6	3.91	1.42	0.67	0.00	0.00	100%
Plot8	13	5	3.97	1.45	0.70	0.00	0.10	100%
Plot9	16	5	4.03	1.42	0.72	0.00	0.00	100%
Plot10	14	6	3.69	1.44	0.67	0.10	0.00	100%
Gmean	**14.70**	**5.29**	**3.69**	**1.39**	**0.68**	**0.04**	**0.08**	**100%**

[a]Na = Number of Alleles; Ne = Number. of Effective Alleles = 1 / (Sum pi^2); I = Shannon's Information Index = -1* Sum (pi * Ln (pi)); h = Nei's haploid gene diversity = 1 - Sum pi^2; NPA = Number of Private Alleles (i.e. number of alleles unique for a single population); NLCA = Number of Locally Common Alleles (<=25%) and PPL = Percentage of Polymorphic Loci, Where pi = is the frequency of the ith allele for the population & Sum pi^2 is the sum of the squared population allele frequencies.

4.3. Analysis of Molecular Variance (AMOVA)

The hierarchical Analysis of Molecular Variance (AMOVA) without grouping the populations revealed the presence of moderate genetic differentiation (PhiPT: 0.13) between the *Z. tritici* populations with high rate of gene flow (Nm = 3.48) among populations (Table. 6). The analysis also revealed that most (87%) of the total genetic variation (4.18) was accounted by the

variations within populations (Plots) while only 13% of the total genetic variation was attributed to the among population genetic variation (Table. 6).

Table 6. AMOVA summary table

Source of Variations	Degree of freedom	Sum of squares	Mean squares	Estimate of Variance	Percent of variation	[a]Genetic differentiation	[b]*P – values*
Among Populations	9	102.33	11.37	0.53	13%	PhiPT: 0.13	0.001
Within Populations	137	501.18	3.66	3.66	87%	FIS: 0.87	0.000
Total	146	603.52		4.18	100%		
Nm (Haploid)[c]	3.48						

[a]PhiPT = the coefficient of gene differentiation derived from = AP/ (WP+AP), where AP = the variance among population and WP = the variance within population; Fst= F-statistics between populations computed as = WP/ (WP+AP)
[b](**P value* = 0.001 after 999 permutations),
[c]Gene flow {Nm (Haploid)} derived as = [(1 / PhiPT) - 1] / 2

4.4. Measures of Genetic Distance between the Populations

The pair-wise Nei's standard genetic distance (below diagonal) and gene flow (haploid Nm) values above diagonal are presented in Table 7. The pair-wise Nei's genetic distance between populations ranged from 0.08 to 0.97. The highest value (0.97) was recorded for the relationship between plot6 and plot 2 populations, followed by the relationships between plot 10 and plot 4 populations (0.92). Whereas, the lowest record (0.08) of pairwise standard genetic distance was observed from the matrix that was exhibited by the relationship of plot 8 and plot 7 populations. Similarly, the highest (20.27) gene flow was recorded between plot 3 and plot 2 populations,

followed by 19.07 that recorded for the relationship of plot 8 and plot 7 populations. On the other hand, the lowest gene flow (1.93) was observed between plot 6 and plot 1 *Z. tritici* populations at single field level. The second lowest gene flow (Nm = 1.94) was recorded by the relationship of populations between plot 10 and plot 6 (Table. 7).

Table 7. Pair-wise Nei's genetic distance (below diagonal) and gene flow (Nm) (haploid) values (above diagonal) among the ten *Zymoseptoria tritici* populations from a single field in Ethiopia.

populations	Plot1	Plot2	Plot3	Plot4	Plot5	Plot6	Plot7	Plot8	Plot9	Plot10
Plot1	---	3.56	3.38	2.96	2.32	1.93	3.48	2.74	3.38	2.00
Plot2	0.46	---	20.27	2.39	2.67	2.07	3.27	2.99	3.28	3.83
Plot3	0.50	0.09	---	3.69	5.01	2.82	4.08	4.58	3.25	3.29
Plot4	0.50	0.89	0.47	---	8.50	3.18	3.58	7.68	2.60	2.09
Plot5	0.76	0.84	0.36	0.16	---	4.12	4.19	6.76	2.87	2.27
Plot6	0.80	0.97	0.59	0.43	0.33	---	8.81	11.96	4.49	1.94
Plot7	0.42	0.61	0.45	0.43	0.39	0.14	---	19.07	8.91	4.41
Plot8	0.64	0.80	0.44	0.19	0.24	0.10	0.08	---	5.56	3.56
Plot9	0.49	0.71	0.71	0.76	0.74	0.32	0.18	0.34	---	5.23
Plot10	0.91	0.49	0.59	0.92	0.90	0.90	0.36	0.51	0.33	---

The sign represented by (---) is not applicable.

4.5. Cluster Analysis

The Neighbor-Joining tree constructed for 147 *Z. tritici* isolates using the Euclidian distance matrix method over 1000 times replication resulted in four major clusters (C1 to C4) (Fig. 6). This clearly showed none of the major cluster was composed with isolates that come from only a single population. The numbers at the roots of the branches represent the percentage of bootstrap values greater than 56%. Bootstrap values which scored less than 56% were not indicated on the

topology of the tree. The four major clusters: C1, C2, C3, and C4 were composed of 57 (38.77%), 45 (30.61%), 21 (14.29%), and 24 (16.33%) *Z. tritici* isolates, respectively. Each of the major clusters were further sub-grouped into two sub-clusters (I and II). In C1, subclusters I and II were composed of 28 and 29 individual fungal isolates, respectively. Cluster C1 was comprised of individuals from all populations except plot 5 and 6 and the highest percentage (24.56%) of the group members were from plot10 followed by plot2 which contributed 22.81% (Fig. 6). The sub-cluster I and II of the cluster C2 were comprised of 12 and 33 distinct individual fungal isolates, respectively. Members of C2 were from eight populations (plot 1 – plot 5 and plot 7 – plot 9) and the highest (33.33%) group members were from plot 4 followed by plot 5 with 22.22% representatives. The C3 was the smallest cluster having two subclasses I and II; which comprised of 10 and 11 fungal isolates, respectively. This cluster was constructed by six populations (plot 1 and from plot 5 – plot 9), but the highest member (28.57%) were from plot 5, followed by plot 7 and plot 9 which accounted 23.81% of the individuals. The sub clusters, I and II of C4 were comprised of 20 and 4 *Z. tritici* isolates, respectively. This cluster (C4) was constructed by small number of populations (plot 6 – plot 9) and the highest group members (45.83%) were accounted by plot 6 which is followed by populations from plot 8 with 29.16% contribution.

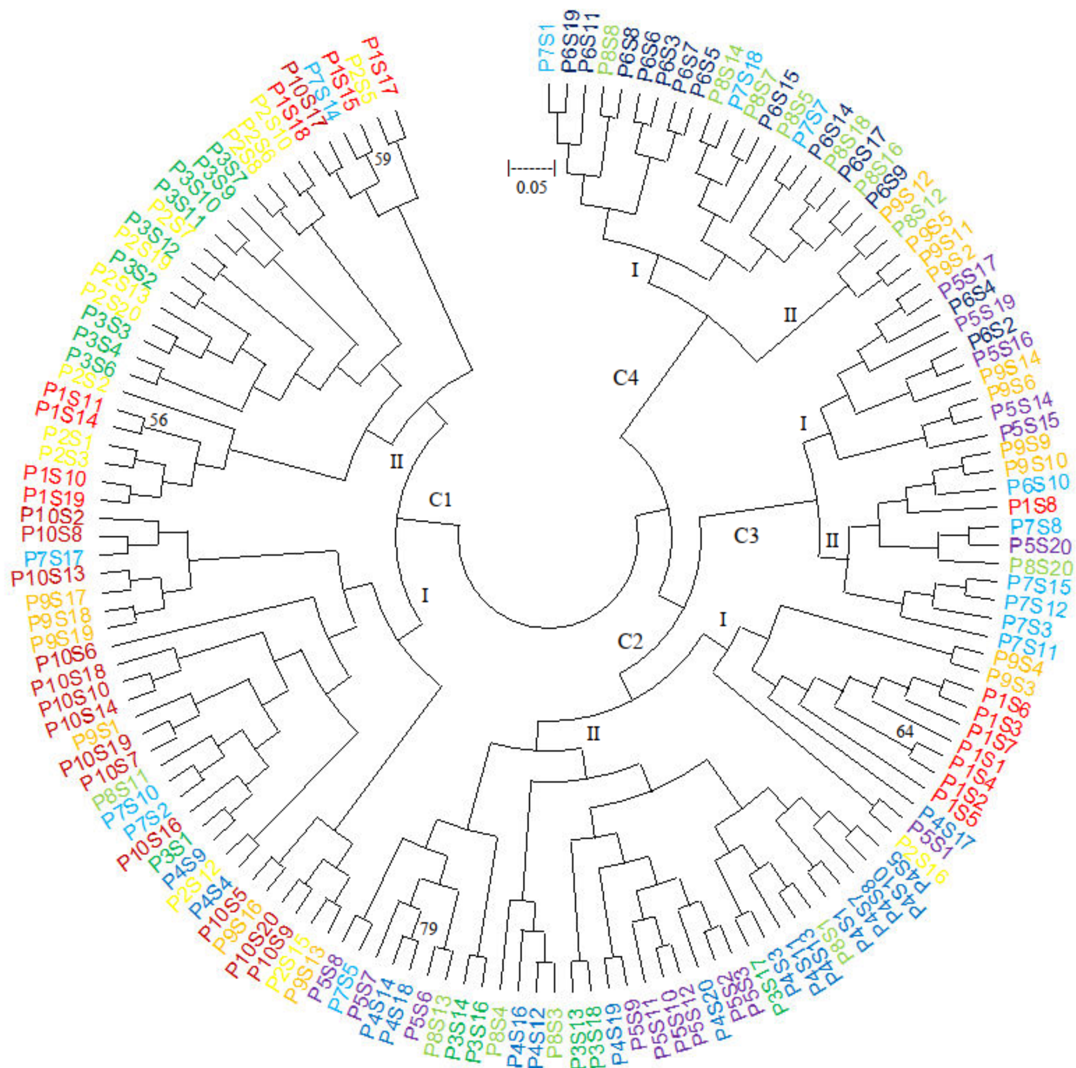


Figure 6. Neighbor-joining tree computed based on Euclidian distance matrix over 1000 replicates for147 individuals of *Z. tritici* isolates collected from 10 plots (populations) at single field studied in Ethiopia. Numbers at the roots of the branches represent percentages of bootstrap values > 56%. Values less than 56% were not indicated. Each individual population was represented by different color: Plot 1 = Red; Plot 2 = Slightly Yellow; Plot 3 = Green; Plot 4 = Deep blue; Plot 5 = slightly blue black; Plot 6 = Blue black; Plot 7 = Cyan; Plot 8 = Lime; Plot 9 = Orange and Plot 10 = Brown. Major clusters were represented from C1 to C4; while I and II were indicated the sub-clusters.

Fig. 7 presents the Unweighted Pair Group Method with Arithmetic mean (UPGMA), based dendrogram generated with ten *Z. tritici* populations. The population cluster analysis weakly grouped the populations into four major clusters (I, II, III and IV), in which the cluster II was

comprised of only a single population (plot1) and cluster III included only two populations (plot 5 and plot 4). Whereas, cluster I and IV were comprised of three (Plot 3, Plot 2, and Plot 10) and four populations (plot 6, plot 7, plot 8 and plot 9), respectively. They were also further partitioned into more sub-clusters.

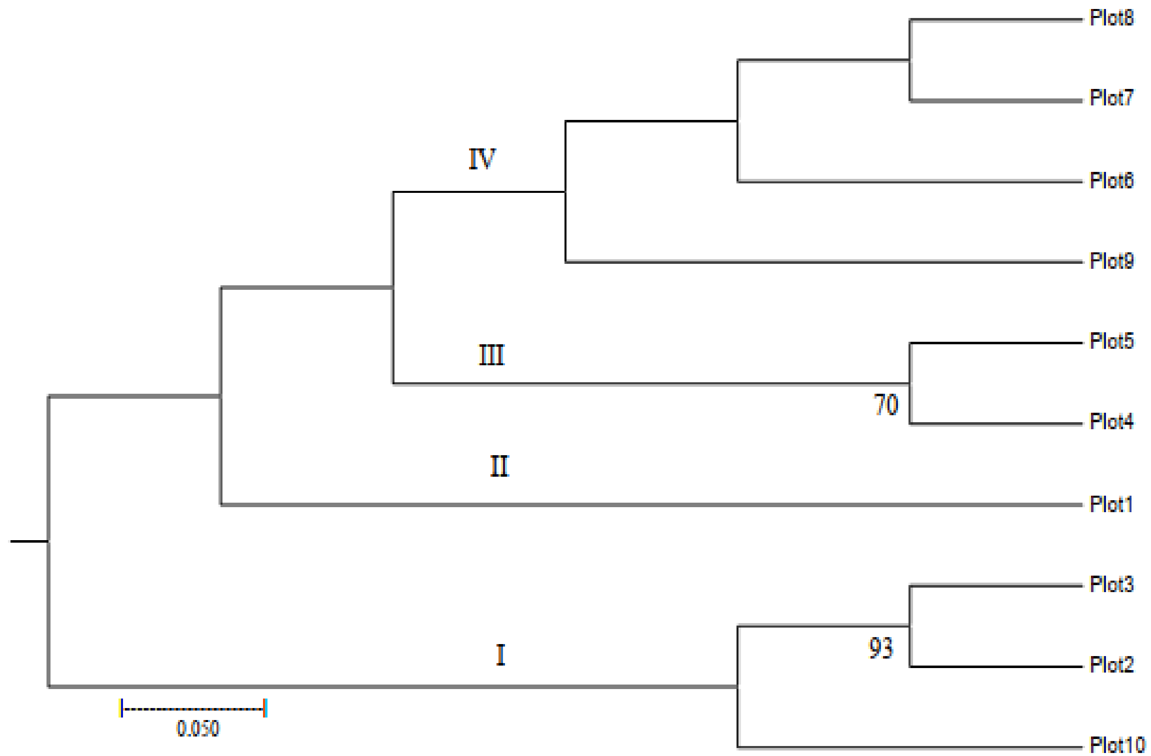


Figure 7. UPGMA dendrogram showing genetic relationships among the 10 populations of *Z. tritici* sampled from single wheat field in Ethiopia based on Nei's genetic distance over 1000 replicates. Numbers at branches represent percentage of bootstrap values, and values less than 60% were not indicated.

4.6. Principal Coordinate Analysis

The Principal Coordinate Analysis (PCoA) was presented in the two dimensional (2D) axes as the scattered plot (Fig. 8). The PCoA was computed considering the first three most informative principal coordinates which accounted about 24.67% of the total genetic variation. The first, second, and third principal coordinates explained about 9.72%, 8.76%, and 6.19%, of the total variation, respectively. None of the isolates collected from the same plot grouped separately on a single coordinate rather they weakly grouped with some admixture of isolates from different plots (Fig. 8). The failure of sharply grouping of isolates distinctly based on their corresponding sampled plots, confirming the existence of poor clustering of the *Z. tritici* isolates, rather than high admixture of the samples across the study field. This is directly complimenting to the result of NJ cluster analysis.

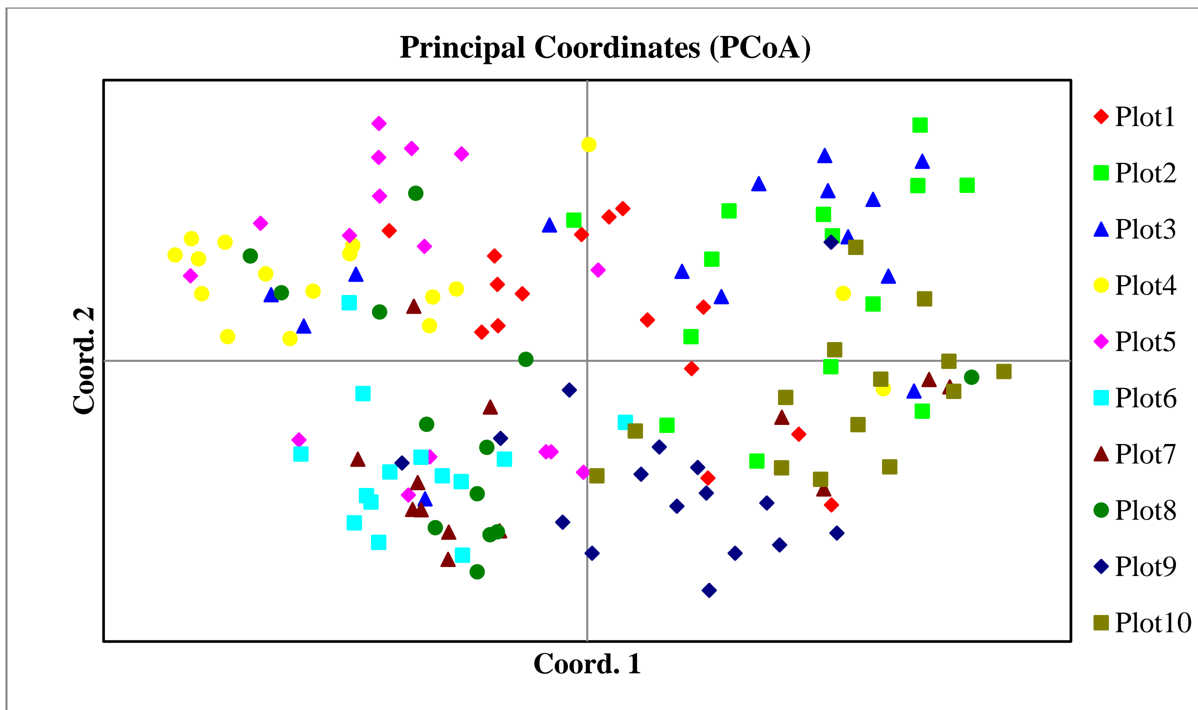
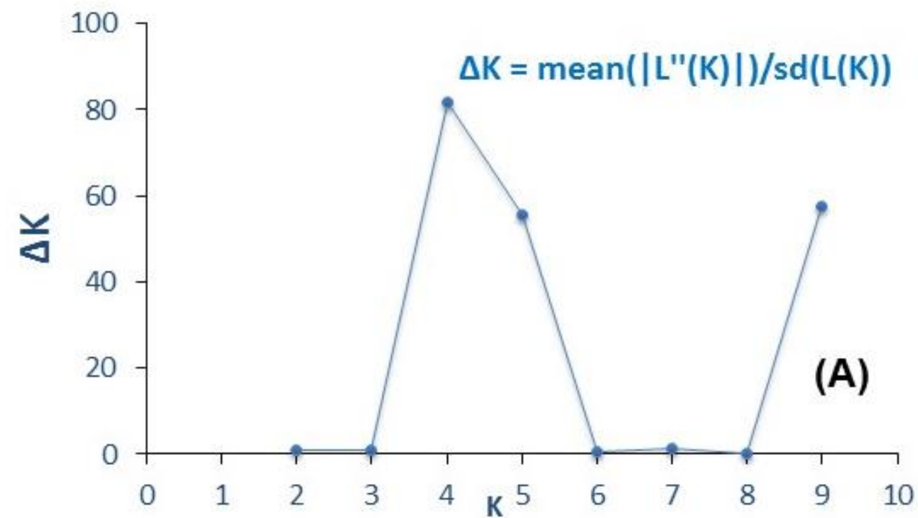


Figure 8. Principal Coordinate Analysis (PCoA) of the 147 *Z. tritici* isolates amplified by 10-SSR loci presented in the 2D plane. Individuals represented by the same symbol and color were belonged to the same population.

4.7. Genetic Structure of *Z. tritici* Populations Collected at Single Field

The Bayesian-model based grouping of the 147 *Z. tritici* isolates into different population, and determining their population structure using STRUCTURE ver. 2.3.4 software (Fig. 9). The structure harvester detected the predicted peaks (K = 4) is the most likely number of clusters that corresponds to the number of groups created by the populations (Fig. 9A). Based on the output of the web based structure harvester (K = 4), individual isolates were partitioned into four predicted populations (Fig. 9B). Using the K - value, the Clumpak result confirmed the presence of greater degree of admixture. It also confirmed that there was no clear plot based structuring of the populations.



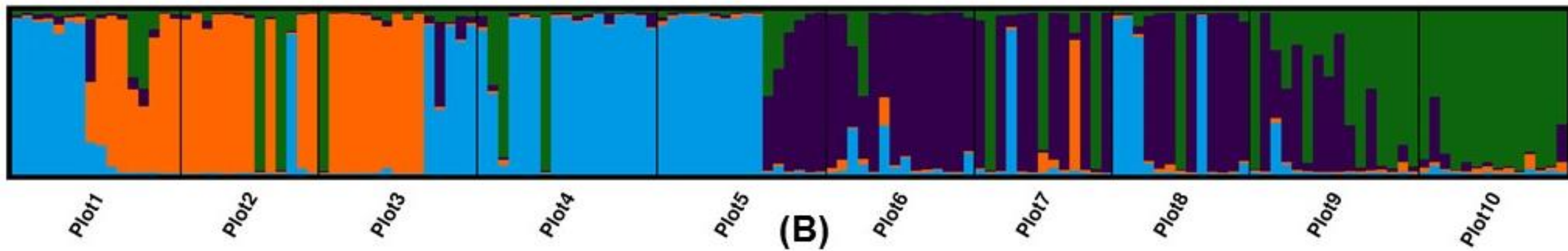


Figure 9. Population structure of 147 *Z. tritici* isolates represent ten populations in Ethiopia. (A) Estimated population structure from Bayesian cluster inference, at K = 4 according to Plots, where each group of isolates were derived. The four clusters were represented by different colors (K1 = blue; K2 = orange; K3 = blue black and K4 = green). Individuals inferred in the same ancestor or had the same subpopulations were designated by the same color: the x-axis represents individual isolates and y-axis represents the proportion of ancestry to each cluster. (B) Delta K value calculated using Evano *et al*. (2005) method from Ln P (D); function of the number of clusters K range (1-10).

5. DISCUSSION

A total of 147 single spore derived *Z. tritici* isolates were profiled using 10 microsatellite markers. As expected, each of the tested primer pairs amplified a single copy of an allele per each *Z. tritici* isolate, confirming that each individual fungal isolate was haploid. All the used microsatellite markers were polymorphic, and resulted a total of 1457 bands. Out of the total bands produced, 1357 (92.79%) were polymorphic, and this result is significantly higher as compared to the results reported by Diriba Guta (2019) who reported polymorphic bands of 142 (58.26%) out of 242 total bands which produced from 24 *Z. tritici* isolates collected from the northern regions of Ethiopia using nine SSR markers. This could be due to the sample size difference and the HARC isolates were varied due to sexual recombination from this hot spot field. Similar, with some lower percentage (92.71%) of polymorphic bands out of 439 total bands reported by (Messele Molla, 2020) from 51 *Z. tritici* isolates collected from Southern Ethiopia using nine SSR markers. This similarity in the percentage of polymorphic bands could be due to the markers type and number and the environment of SNNPs region is also favorable for the population evolvement of Z. tritici next to HARC.

The present study revealed that some of the primers failed to produce an amplicon on the loci of one to two individuals in the populations likely due to the occurrence of mutation at the primer binding site on the genome of these individuals (Owen *et al*., 1998; El Chartouni *et al*., 2011; Berraies *et al*., 2013).

The recorded 90 alleles across all isolates and the number of alleles per locus ranged from five to eleven with an overall mean of 9 alleles was indicated the genetic diversity of Ethiopian *Z. tritici* isolates is higher as compared to Owen *et al*. (1998) and Berreiaes *et al*. (2013) who reported 2

and 4 alleles per loci from 12 and 45 *Z. tritici* isolates of UK and Tunisia, using SSR markers, respectively. This might be due to their use of smaller sample size and marker number. However, the number of alleles per locus observed in the present study was substantially lower than the report of Siah *et al.* (2018) in north France, who detected 3 to 23 alleles per locus with overall mean of 9.5 alleles per locus. This could be the higher *Z. tritici* population adaptation in France than in Ethiopia, because Z. tritici first found in France by Desmazieres in 1842 (Ponomarenko *et al*., 2011)

In this result, the frequency of the common allele was less than 0.95 or 0.99; which confirms that the used microsatellite markers were polymorphic; and the probability of finding the true distribution of allelic frequency in the given sample size was within the permissible error bound. The markers polymorphism rate is supported by the level of major allelic frequency that ranged from 0.24 (for ag-0009) to 0.46 for ST1G7. This is lower than the average number of major allelic frequency reported by Tilahun Mekonnen *et al*. (2020) who obtained an average MAF of 0.59 using 14 SSR loci across 182 *Z. tritici* isolates collected from Southeastern and central highlands of Ethiopia, likely due to our focus only on a single very hot spot area than the analysis from pool of various locations.

In the present study, most marker parameters showed significantly higher average number of alleles (Na = 9.00), effective number of alleles (Ne = 3.69), shanonn's index (I = 1.39), gene flow (Nm = 4.78), polymorphic information content (PIC= 0.80), and gene diversity (GD = 0.82) values than the coresponding diversity paramenter reported by Tilahun Mekonnen *et al*. (2020). This could be the higher allele shoaring comes from the mutated region during sexual recombination favored by the shorter distance in HARC than the sample locations across Southeastern and central highlands of Ethiopia. This agrees with the report of Cordo *et al*. (2006)

and Berreiaes *et al*. (2013) who conducted at single field scale in Argentina and Tunisia, respectively.

The observed high range of PIC (0.67 - 0.87) per locus indicates informativeness of the used microsatellite markers, and hence a good tool for estimation of the genetic diversity of the *Z. tritici* populations. The average gene diversity per locus observed in the present study (0.82) was higher than the previous studies of gene diversity ranged from 0.440 - 0.484 which was reported by Razavi and Hughes (2004a); Berreiaes *et al*. (2013); Siah *et al*. (2018).

In this study, the population parameter analysis across all the tested loci revealed existence of high genetic diversity in the *Z. tritici* populations obtained from ten plots at single filed level. This is supported by the higher range of genetic diversity indices including the number of alleles (5- 6), effective number of allele (3.09- 4.03), Shannon's Information index (1.24 -1.48), number of private alleles (0.0 - 0.2), Nei's haploid gene diversity (0.62 - 0.72), number of locally common alleles (0.0 - 0.3), and 100% of polymorphic loci. Similar, but mostly lower values of these parameters were reported from different parts of the world. In line with this, EL-Chartouni *et al*. (2011) reported lower range of Nei's haploid gene diversity (0.45 to 0.68) and number of unique alleles (2.32 to 3.51) in *Z. tritici* populations of north France as compared to the present results. The Nei's haploid gene diversity and number of alleles within population in the present study were significantly higher than the level reported by Siah *et al*. (2018) (h = 0.40 - 0.47 and Na = 4.12 - 4.87) in north France and Boukef *et al*. (2012) (h=0.44 - 0.53 and Na = 4.45 – 5.45) in Tunisia. Similarly, Dalvand *et al*. (2018) reported lower number of alleles (1 - 1.98), effective number of alleles (1 - 1.58), Shannon's Information index (0.0-0.51) and percent of polymorphic loci (0 - 97.8%) for *Z. tritici* populations in Iran. This could be Ethiopia is considered as center of diversity and site of Durum wheat (*T. turgidum L*) (Vavilov, 1951) and HARC have been

served as a screening site for STB resistant that leads the pathogen could be evolve do to long time exposure to fungicide and resistant cultivars this scenario is agree with the report of Medini and Hamza (2008).

Moreover, the level of genetic diversity parameters within populations observed in the present study were higher than the level reported by Tilahun Mekonnen *et al*. (2020) who described the number of effective alleles of 1.66-2.47, Shannon's information index of 0.55-1, Nei's haploid gene diversity of 0.34- 0.58, number of private alleles of 0.0-0.14, and 79 -100 percentage of polymorphic loci . The discrepancy with the present finding could be likely due to their focus at larger area: *Z. tritici* populations of Southeastern and Central highlands of Ethiopia than our present single location data.

Similar to Tilahun Mekonen *et al.* (2020) our study confirmed high genetic diversity at single field level, suggesting the need to design and implement integrated disease management to control STB disease and lower the yield loss due to the disease. Among studied plot level *Z. tritici* populations, those from Plot3 gave the highest record on number of alleles, number of effective alleles, Shannon's information index, Nei's haploid gene diversity, number of locally common alleles, and percentage of polymorphism. The observed high genetic diversity for *Z. tritici* populations of Holetta, indicates that the center is a hot spot for *Z. tritici* which makes it an ideal place for germplasm screening for STB resistance, and studies on host pathogen interaction this is agree with the report of Abera Takele *et al*. (2015) who obtained high (99.8%) of mean STB incidence in West Showa zone. The observed very few number of unique alleles indicates that each population did not evolve independently to create and maintain unique alleles at the population level.

The Analysis of Molecular Variance (AMOVA) confirmed the presence of statistically significant ($p > 0.001$) and moderate genetic differentiation (PhiPT= 0.13) among *Z. tritici* populations whereas the variation within populations accounted 87% of the total variation (4.18). A relatively similar lower rate of among population variation (12%- 17%) were reported in previous studies by Linde *et al*. (2002) in USA and Razavi and Hughes (2004a) for Switzerland, Israel, and Canada in *Z. tritici* populations, respectively. Moreover, Siah *et al*. (2018) also reported existence of higher within populations genetic variation (99%) in *Z. tritici* populations of north France populations. This low degree of genetic differentiation and percentage of variation among populations could be attributed due to high gene flow across plots as a result of the spread of ascospores by wind, rain splash, and/or mechanically by human during weeding which provide potential to distribute the spores uniformly throughout the field (Consolo *et al*., 2009; Dalvand *et al*., 2018). Conversely, the high degree of within populations genetic variability indicates that the population may come from genetically heterozygous sources (sexual ascospores) as a result of several cycles of sexual recombination in a single season (Razavi and Hughes, 2004a; Berraiaes *et al*., 2013). These ascospores can create new combination of virulence genes during sexual recombination and spontaneous mutation in the population, which gives chance for the pathogen to destroy the resistant hosts using single resistance genes (Razavi and Hughes, 2004a; Berraiaes *et al*., 2013).

The result of hierarchical clustering, PCoA principal coordinate, and population structure analysis of the 147 *Z. tritici* isolates revealed the presence of low genetic differentiation possibly due to the high gene flow between populations. On the other hand high genetic diversity within the population was observed, which could be due to the wind born ascospores that infected most of the plots and favors variation by recombination due to the occurrence of sexual stage of the

pathogen in this wheat field this scenario is agree with the previous reports by Hamada *et al*. (2008); Boukef *et al*. (2012). Thus, the genetic background of the studded *Z. tritici* populations didn't affect them to group samples based on their sites of sampling (Berreiaes *et al*., 2013; Tilahun Mekonnen *et al.,* 2020). Likewise, UPGMA based population clustering based on Nei's genetic distance also weakly grouped the populations to their corresponding sampled plots, but the clustering pattern is weak enough to support the concept of "isolation by distance". Similar weak population differentiation, clustering, and population structure were reported for *Z. tritici* populations by (Linde *et al*., 2002; Siah *et al*., 2018; Boukef *et al*., 2012) in Israel, France, and Tunisia, respectively. Conversely, strong population stratification were reported for *Z. tritici* populations in France (EL- Chartouni *et al*., 2011), and Iran (Abrinbana *et al*., 2010).

6. CONCLUSION

Septoria tritici blotch caused by *Z. tritici* is critical problem to wheat cultivation in Ethiopia. Identification and deployment of host resistant varieties through genetic resistance with the help of breeding can find environmentally friendly and durable control of the disease. Knowledge of the pathogen genetic structure is crucial for designing and implementing appropriate disease management strategies. The present study profiled the genetic structure of 147 *Z. tritici* isolates recovered from single field (Holetta agricultural research center) using 10 polymorphic microsatellite markers. The study revealed that all the used loci were highly informative, and thus successfully uncovered the genetic diversity of the *Z. tritici* populations. The *Z. tritici* populations showed high genetic diversity, where 87% of the total variation (4.18) was accounted by the within population variation. In general, the Cluster analysis, PCoA, and population structure analysis exhibited weakly grouping of samples and unstructured population to their corresponding sampled plots. This could be due to the high gene flow (Nm = 3.48) confirms, that is not strong enough to full fil the concept of "isolation by distance". The generated information is valuable for wheat breeders and pathologists to control genetically diverse population at the study field in particular and in the country at large.

7. RECOMMENDATIONS

Considering the observed high genetic diversity of *Z. tritici* isolates collected at HARC single wheat field we suggest a gene pyramiding resistance breeding strategy to control the broad genetic base of the pathogen. We also recommend future studies on the pathogen dynamics across the country including HARC. We advise future studies on the potential of pathogenicity of *Z. tritici* isolates recovered from HARC. Further study on genetic structure *Z. tritici* at single plat even from different lesions on the same leaf is recommended to understand its population evolution with respect to environmental conditions and associated with different hosts. Finally, we suggest HARC, is an ideal location for germplasm screening against STB.

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9. APPENDICES

Appendix 1: Summary information about the ten populations of Z. tritici isolates and the collection site

Range of isolate's code(s)	population[a]	The plots' geographical positions (UPM)[b]		Elevation range (m)
		Latitude range	Longitude range	
P1S1 up to P1S19	Plot1	9° 3' 27.8"'N - 9° 3' 28.1"'N	38° 30' 10.5"'E - 38° 30' 10.8"'E	2400
P2S1 up to P2S20	Plot2	9° 3' 27.8"'N - 9° 3' 28.2"'N	38° 30' 9.8"'E - 38° 30' 9.9"'E	>>
P3S1 up to P3S18	Plot3	9° 3' 27.9"'N - 9° 3' 28.6"'N	38° 30' 9.6"'E - 38° 30' 9.7"'E	>>
P4S1 up to P4S20	Plot4	9° 3' 28.0"'N - 9° 3' 28.2"'N	38° 30' 9.4"'E - 38° 30' 9.5"'E	>>
P5S1 up to P5S20	Plot5	9° 3' 28.3"'N - 9° 3' 28.5"'N	38° 30' 9.1"'E - 38° 30' 9.3"'E	>>
P6S2 up to P6S19	Plot6	9° 3' 28.6"'N - 9° 3' 28.9"'N	38° 30' 8.8"'E - 38° 30' 9.0"'E	>>
P7S1 up to P7S18	Plot7	9° 3' 29.0"'N - 9° 3' 29.1"'N	38° 30' 8.6"'E - 38° 30' 8.7"'E	>>
P8S1 up to P8S20	Plot8	9° 3' 29.3"'N - 9° 3' 29.7"'N	38° 30' 8.5"'E - 38° 30' 8.6"'E	>>
P9S1 up to P9S19	Plot9	9° 3' 29.8"'N - 9° 3' 30.2"'N	38° 30' 8.3"'E - 38° 30' 8.5"'E	>>
P10S2 up to P10S20	Plot10	9° 3' 30.4"'N - 9° 3' 30.6"'N	38° 30' 8.2"'E - 38° 30' 8.4"'E	>>

[a]Plot1 to Plot10 are locations from the sampled single field and considered as population
[b]UTM = Universal Transverse Mercator Coordinator System

Appendix 2: Pair-wise Population Matrix of Nei's standard Genetic Identity (bellow diagonal) of *Z. tritici* isolates.

Populations	Plot1	Plot2	Plot3	Plot4	Plot5	Plot6	Plot7	Plot8	Plot9	Plot10
Plot1	1.00									
Plot2	0.53	1.00								
Plot3	0.51	0.74	1.00							
Plot4	0.53	0.35	0.53	1.00						
Plot5	0.40	0.36	0.58	0.73	1.00					
Plot6	0.39	0.32	0.47	0.57	0.62	1.00				
Plot7	0.56	0.44	0.52	0.55	0.57	0.74	1.00			
Plot8	0.44	0.36	0.52	0.69	0.65	0.76	0.76	1.00		
Plot9	0.52	0.40	0.40	0.40	0.40	0.62	0.69	0.58	1.00	
Plot10	0.34	0.50	0.46	0.34	0.34	0.35	0.58	0.49	0.60	1.00

Appendix 3: Plant DNA Extraction Protocol for DArT

BUFFER STOCK SOLUTIONS

EXTRACTION BUFFER STOCK

To make 500 ml:

- 0.35 M sorbitol
- 0.1 M TrisHCl pH 8.0
- 5 mM EDTA pH 8.0

- 31.9 g sorbitol
- 50 ml 1M TrisHCl pH 8.0
- 5 ml 0.5 M EDTA pH 8.0
- fill up to 500 ml MiliQ H2O

LYSIS BUFFER STOCK

To make 500 ml:

- ✓ 0.2 M Tris HCl pH 8.0
- ✓ 0.05 M EDTA pH 8.0
- ✓ 2M NaCl
- ✓ 2% CTAB

- ✓ 100 ml 1M Tri HCl pH 8.0
- ✓ 50 ml 0.5 M EDTA pH 8.0
- ✓ 200 ml 5 M NaCl
- ✓ 10 g CTAB
- ✓ fill up to 500 ml with MilliQ H20

SARCOSYL STOCK 5% (w/v)

FRESH BUFFER WORKING SOLUTION*:

0.5 % (w/v) sodiumdisulfite (= sodium metabisulfite)

2 % (w/v) PVP-40 (K29-32) Sigma

dissolve in required volume of extraction buffer stock; add same volume of lysis buffer stock and 0.4 volume

of extraction (=lysis) buffer stock of sarcosyl stock.

For example to make 120 ml:

Add 0.6 g sodiumdisulfite (= sodium metabisulfite) and 2.4 g PVP-40 (K29-32) to 50 ml extraction buffer stock and dissolve; add 50 ml lysis buffer stock and 20 ml sarcosyl stock

For example to make 30 ml:

Add 0.15 g sodiumdisulfite (= sodium metabisulfite) and 0.6 g PVP-40 (K29-32) to 12.5 ml extraction buffer stock and dissolve; add 12.5 ml lysis buffer stock and 5 ml sarcosyl stock

*This buffer may settle into two layers on standing. Heat to 65ºC and shake immediately before adding to extraction tubes.

PROTOCOL

For 2 ml Eppendorf tubes:

● aliquot 1 ml of freshly prepared preheated to 65ºC, well mixed "fresh buffer solution" and place tubes to the 65ºC incubator or water bath, (3, 4 days old "fresh buffer solution" works fine),

● grind required amount (same across all samples) of plant material in mortar and pestle under liquid nitrogen to fine powder,

● suspend powder in 1 ml "fresh buffer solution" kept at 65ºC (make sure there are no clumps, vortex if necessary),

● incubate at 65ºC for 1 h (can extend for another 30 min), invert tubes in every 20 minutes or incubate with gentle shaking,

● cool down for 5 min and add 1 ml of chloroform : isoamyl alcohol (24 : 1) mixture,

● mix well for 30 min,

● spin 20 min, 10000 x g, RT,

● transfer water phase to fresh tube, add same volume of ice cold isopropanol and invert tube ~ 10 times, nucleic acids should become visible,

● spin 30 min, 10000 x g, RT,

● discard supernatant, wash pellet with 2 ml 70 % EtOH,

● discard Et OH, dry pellet and dissolve in 250 µl of 1 x TE (10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0),

● check DNA quality and quantity on 0.8 % agarose gel. (If RNA quantity is several fold less than DNA, RNase treatment is not necessary for DArT applications).