ADDIS ABABA UNIVERSITY

INSTITUTE OF BIOTECHNOLOGY

Genetic Diversity and Population Structure of *Zymoseptoria tritici* Populations of Southern Ethiopia Using SSR Markers

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Addis Ababa, Ethiopia

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By

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List of Abbreviations

AMOVA	Analysis of molecular Variance
CTAB	Cetyl trimethylammonium bromide
CSA	Central Statistical Agency
DArT	Diversity Array Technology
ITS-rDNA	Internal transcribed spacer ribosomal DNA
PCoA	Principal coordinates Analysis
PDA	Potato dextrose agar
SDS	Septoria Disease Severity
SLB	Septoria Leaf Blotch
SPL	Scientific Phytopathological Laboratory
SSR	Simple Sequence Repeats
STB	*Septoria tritici* Blotch
UPGMA	Unweight Pair Group Methods with Arithmetic average
YSB	Yeast extracts Sucrose Broth

Genetic Diversity and Population Structure of *Zymoseptoria tritici* Populations of Southern Ethiopia Using SSR Markers

ABSTRACT:

Septoria tritici blotch caused by the fungal pathogen *Zymoseptoria tritici* is considered to be one the major threats to wheat production in Ethiopia and elsewhere in the world. Understanding the genetic structure of the pathogen has great importance in designing and implementing suitable management strategies. Therefore, the present study was targeted to explore the genetic structure of 51 *Z. tritici* isolates collected from four wheat producing zones of South and Southwesten parts of Ethiopia using nine microsatellite marker systems. Different diversity indices were computed. A *Z. tritici* specific diagnostic marker that targets the ITS rDNA had amplified an expected fragment size of 345bp in all the tested isolates. All the markers were found to be highly polymorphic and thus useful tools to depict the genetic structure of the pathogen populations. The analysis revealed a high degree of genetic diversity within populations with number of alleles, gene diversity and polymorphic information content per locus ranging from 9 - 14, 0.80 - 0.88 and 0.70 - 0.87, respectively. Analysis of molecular variance (AMOVA) confirmed a moderate (0.14) genetic differentiation where 86% of the total genetic variability (3.93) resides within populations, and only 14% was due to among populations' difference. Dendrogram produced UPGMA and PCoA also showed a moderate population clustering in which the populations were not distinctly clustered according to their areas of sampling due to presence of high gene flow. Moreover, Bayesian model-based population structure analysis weakly clustered the population into five (ΔK) sub-groups with high level of genetic admixture. Among the considered populations, populations of Kembata -Tembaro zone and Hadiya zone showed relatively higher genetic variability, and thus can be considered as STB hot spots for further studies on the dynamics of the pathogen and germplasm screening and host-pathogen interactions. Therefore, the present study had generated useful baseline information for wheat breeders and pathologists for designing and implementing durable and effective STB control strategies.

Key words/phrases: Genetic structure, Microsatellites, Wheat

1. INTRODUCTION

Wheat is the third most widely produced cereal crop in the world next to maize (*Zea masys* L.) and rice (*Oryza sativa* L.). It is the fourth most important staple crop in Ethiopia after Tef (*Eragrostis tef*), Maize (*Zea mays*) and Sorghum (*Sorghum bicolor*) (Letta *et al*., 2013). According to Tesfaye Letta *et al*., (2013), next to South Africa, Ethiopia is the second largest wheat producer in Sub- Saharan Africa. Both bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum*ssp. *durum* L.) are widely cultivated in Ethiopia for multiple purposes including food, feed and income generation (Dixon *et al*., 2006). In 2017, about 1.7 M ha of land was covered with wheat and the national annual production and productivity were 4.8million metric tonnes and 2.8 t /ha, respectively (FAOSTAT, 2018). The potential wheat growing regions of the country includes Oromiya, Amhara, Tigray and Southern Nations Nationalities and Peoples region (SNNPR). In spite of its larger production coverage and multiple uses, the average productivity of wheat in Ethiopia is 2. 8 t/ha; which is by below the global average of 3.27 t/ha (Alemar Said and Temam Hussien, 2016).

Septoria tritici blotch (STB) caused by the ascomycete *Mycosphaerella graminicola* (asexual stage: *Zymoseptoria tritici*) is the major wheat devastating fungal disease next to rust in Ethiopia (Abera Takele *et al*., 2015; Tilahun Mekonnen *et al*., 2019; 2020) and elsewhere in the world (Eyal *et al.,* 1985). Under favorable growing conditions with high relative humidity (85%) and optimal temperature (22 °C), STB could decrease yield by 30 to 70% (Eyal *et al*., 1987). The disease is mainly a foliage disease and the primary infection may arise from airborne or rain splashed asexual pycnidospores and sexual ascospores from infested crop debris (Shaner, 1981; Gilchrist and Dubin, 2002). Changes in farming practices (higher sowing densities and nitrogen

fertilization), mono cropping system and limited number of cultivars contributed significantly to the increase of the disease (Berraies *et al*., 2013). In Ethiopia up to 82% wheat yield loss has been reported (Mengistu Huluka *et al*., 1991; Abreham Tadesse, 2008). STB is becoming serious problem in southern and southwestern part of Ethiopia including Haddiya, Kambata, Silte and Southwest Shewa (Alemar Said and Temam Hussien, 2016). Integrated disease management strategies including genetic resistance (resistant varieties), crop rotation, appropriate fertilizer and fungicide applications, proper seeding rates and dates would be alternatives to control STB disease (Berraies *et al*., 2013). Genetic resistance remains the first line of defense against this foliar disease, especially in developing countries for resource poor farmers, and is the most environmentally friendly and profitable strategy for farmers (Teklay Abebe *et al*., 2015). However, in Ethiopia most of the high-yielding wheat cultivars grown today are susceptible to STB. All commercial and candidate wheat varieties are affected by the disease at varying intensity (Alemar Said and Temam Hussien, 2016). This calls for searching for new source of resistance to the diseases. In this regard, knowledge of the genetic diversity of the pathogen could have direct implications for development of effective and durable disease management strategies (Schnieder *et al*., 2001)

So far, different molecular marker systems have been used to explore the genetic structure of *Z. tritici* populations including Sequence Characterized Amplified Region (SCAR)**,** simple sequence repeats (SSRs)**,** restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs) and Rapid amplified polymorphic DNAs (RAPDs) (Schnieder *et al***.** 2001). Simple sequence repeats (SSRs) also called microsatellites are considered to be the marker of choice for assessing genetic diversity of population as they are

co-dominant, easy to use, highly polymorphic, multiallelic, locus specific, and highly reproducible **(**Czembor and Arseniuk, 1999).

In breeding for disease resistance cultivars, knowledge of pathogen's genetic diversity has a great importance (Sebei and Harrabi, 2008). However, information on the pathogen's geographic distribution and genetic diversity in Ethiopia is limited. By considering the greater efficiency and speed of SSR markers, this research aimed to use them to study genetic diversity of *Z. tritici* from southern part of Ethiopia.

In Ethiopia, Tilahun Mekonnen *et al.* (2020) used SSR markers to explore the genetic diversity of *Z. tritici* populations of central highlands and south eastern parts of Ethiopia and confirmed the existence of higher genetic diversity among the pathogen populations. However, information on the genetic structure of the pathogen populations of Southern Ethiopia is lacking. Therefore, the present study was designed with the following general and specific objectives.

1.1 Objectives

1.1.1 General objectives

To assess the molecular diversity of *Zymoseptoria tritici populations* collected from southern part of Ethiopia to generate useful baseline information for the development of durable Septoria resistant cultivars.

1.1.2 Specific objectives

- To identify *Z. tritici* isolates collected from southern parts of Ethiopia using race specific markers.
- To investigate the genetic diversity of *Z. tritici* populations of Southern Ethiopia using SSR markers.
- To identify the population structure of *Z. tritici* populations of southern Ethiopia.

2. LITERATURE REVIEW

2.1 Origin and Distribution of *Zymoseptoria tritici*

Mycosphaerella graminicola is the name of the sexual stage (teleomorph) of the wheat pathogen *Zymoseptoria tritici or* it is the asexual anamorph *Septoria tritici*. The pathogen was first described as the causal agent of STB in 1842 by Desmazieres. *Z. tritici* is thought to be originated from closely related *Zymoseptoria* species colonizing wild grasses in the Fertile Crescent regions of the Middle East, where wheat domestication began 10,000 years ago (McDonald *et al*., 1993, Stukenbrock *et al*., 2010).

Nowadays, *Z. tritici* is a worldwide problem (Fig. 1) affecting wheat-production in Europe, Australia, Canada, the United States Argentina, Iran, United States, Netherlands, Russia, New Zealand, Australia Tunisia, and East Africa including Ethiopia (Ponomarenko *et al*., 2011). In the early1960s, *Z. tritici* was not perceived as an economically significant pathogen on wheat. However, with the introduction of dwarf wheat varieties, this pathogen has become a problem causing considerable grain yield loss of 30 to 40%, especially during growing seasons with significant rainfall (Palmer and Skinner, 2002). Several reports indicated the occurrence of *Septoria tritici* blotch wheat disease in Ethiopia (Stewart and Dagnachew, 1967; SPL, 1975; SPL, 1978; Eshetu Bekele, 1985; Sebei and Harrabi, 2008; Tilahun Mekonnen *et al.*, 2020).

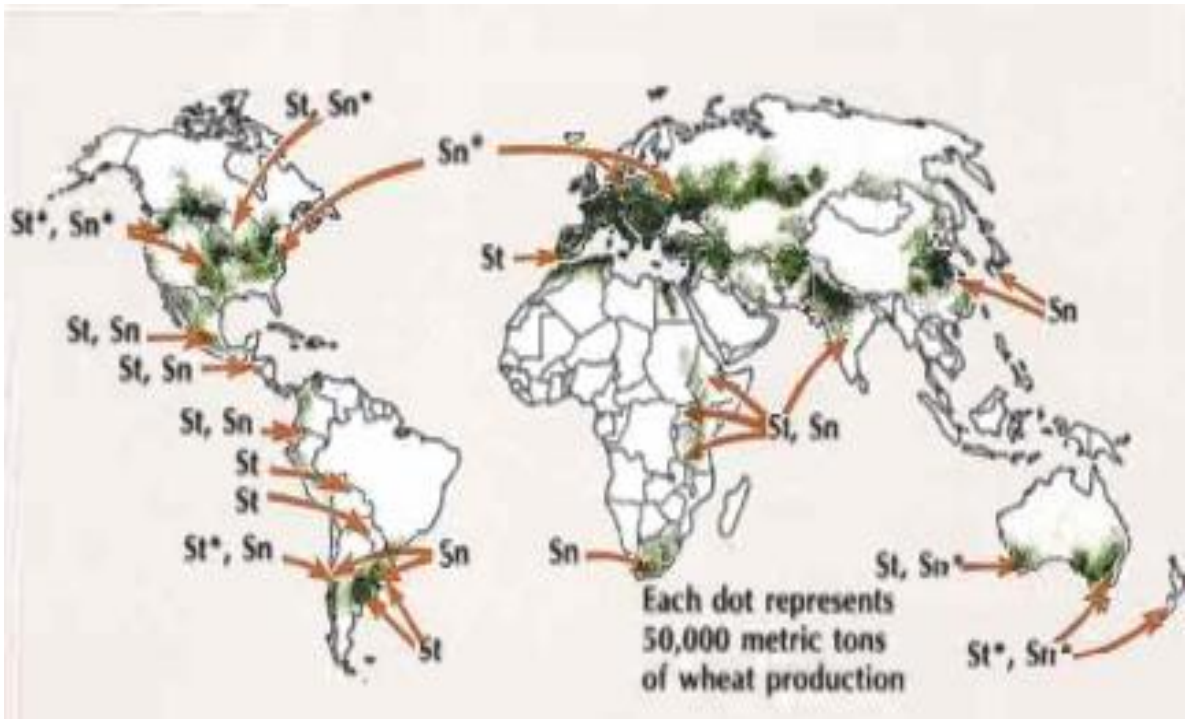


Figure 1: Global distribution of Septoria spp.: *Zymoseptoria tritici* or *Septoria tritici* (St) and *Septoria nodorum* (Sn). An asterisk indicates locations where the sexual state (pseudothecia and ascospores) have been reported.Source Eyal *et al*. (1987).

2.2 Taxonomy and Biology of *Zymoseptoria tritici*

Zymoseptoria tritici species is grouped to the kingdom Fungi, Phylum *Ascomycota*, Class *Dothideomycetes* (Testa *et al*., 2015), order *Capnodiales*, family *Mycosphaerellaceae* and Genus *Zymoseptoria* (Ponomarenko *et al*., 2011).

The pathogen shows considerable morphological diversity. Steinberg, (2015) described the different growth forms of the pathogen including the single-celled yeasts, multi-cellular and tip growing hyphae and asexual and sexual spores. The vegetative growth forms of *Z. tritici* fall into all three categories. 1) The "yeast like"stage also called macropycnidiospore is the most common cell type, grown under laboratory conditions the individual cells within this multicellular

structure are ~1.5–3.5 µm wide and can be up to ~40–100 µm long. 2) Micropycnidiospores which are small (~1 µm wide, 5–10 µm long) and unicellular structures (Eyal *et al*., 1987) that are formed by lateral budding from hyphae or macropycnidiospores. Neither macro- nor micropycnidiospores, are formed in asexual fruiting bodies (pycnidia) and dispersed by rain splash (Steinberg, 2015). 3) Hyphae, a germinated form of macropycnidospores consisting of very elongated cells that extends by polar tip growth. This morphogenic transition can be triggered in liquid culture upon nutrient deprivation and following an increase in temperature (Wiese, 1987).

2.3 Genetics of *Zymoseptoria tritici*

Z. tritici full genome sequence was completed and thus used as model for fundamental genetic studies of plant-pathogenic fungi (Goodwin *et al*., 2011). It is a haploid plant-pathogenic fungus (Wittenberg *et al*., 2009) carrying 21 chromosomes. Eight of the chromosomes could be lost with no visible effect on the fungus and thus are dispensable (Goodwin *et al*., 2011). These dispensable chromosomes range from 0.39 to 0.77 Mb and are not required for saprophytic growth and hence are accessory chromosomes (AC). They also contain high number of repetitive elements (Dhillon *et al*., 2014) and show a faster rate of evolutionary change (Stukenbrock *et al*., 2010). However, their biological role remained unclear. The existing assumption is that the dispensable chromosomes may contribute to rapid adaptation to changing environments (Wittenberg *et al*., 2009) or participate in development of fungicide resistance (Torriani *et al*., 2009) and appear to have originated by ancient horizontal gene transfer from an unknown donor. *Z. tritici* chromosomes have an odd size range, varying from 0.39 to 6.09 Mb (Wittenberg *et al*., 2009). The length of the genome of the reference isolate IPO323 is 39.7 Mb (Wittenberg *et al*., 2009; Goodwin *et al*., 2011). At least half of the genome of the reference is contained within the

six largest assembled sequences, with the sixth largest sequence having a length of 2.67 Mb. A surprising feature of its genome compared to other sequenced plant pathogens is that it contained very few genes for enzymes that break down plant cell walls, which is more similar to endophytes than to pathogens (Goodwin *et al*., 2009).

2.4 *Zymoseptoria tritici* Infection Process and Epidemiology

The infection cycle of *Z. tritici* involves (1) entry of the fungus, 2) colonization of the plant tissue and (3) formation of fruiting bodies (Steinberg, 2015). The infection process begins with the landing of either pycnidiospores or ascospores from stubbles and crop debries of the previous harvesting season on to newly growing wheat leaf tissue (Fig. 2). Pycnidiospores are asexual spores released from a fungal fruiting body called pycnidia. While ascospores are sexual spores resulted from the fusion of two strains with opposite mating type. They are produced in a structure called fungal fruiting structure known as pseudothecia or perithecia. Within 12-24 hours after infection, the spores germinate to produce a vegetative growth structure called hayphae.

In 2-4 days post infection, the hayphae enters to the leaf tissue through the stomatal opening and starts to colonize fill the substomatal cavity that will be followed by invasion of the mesophyll tissue, a stage is called colonization. After 5- 9 days of post infection, pre-pycnidia are formed in the colonized and substomatal cavities (Pre-pycnidia formation). During this colonization stages there is no symptom and the leaves look healthy (Duncan and Howard 2000).

Depending on wheat genotype-fungal isolate combination and the weather conditions, the asymptomatic ‘‘latent phase (also named biotrophic phase) can be extended up to 6 to 36 days (Shipton *et al*., 1971). After 10 -12 days post infection, chlorotic lesions will appear which will be followed by necrotic development (Duncan and Howard, 2000; Kema *et al*., 1996a). The necrotrophic phase is characterized by disintegration of host tissues as a consequence of extensive programmed plant cell death (Kema *et al.,* 1996a). Feeding on the nutrient released from dried host tissue, the fungus proliferates rapidly and the pycnidium gets matured. By water splash and /or contact the maturated pycnidospores will be released out from pycnidia and will be spread into new plants to initiate secondary infection (Fig. 2) (Steinberg, 2015). A combination of wind and rain provides the most favorable conditions for spread of both asexual and sexual spores for the STB disease development within and between paddocks and cause disease epidemics in wheat growing areas (Raman and Milgate, 2012). The pathogen escapes the overwintering season on crop debris as pycnidia and pseudothecia, off-season grown cultivars and voluntary hosts from which it begins a new infection cycle during the main cropping seasons (Fig. 2).

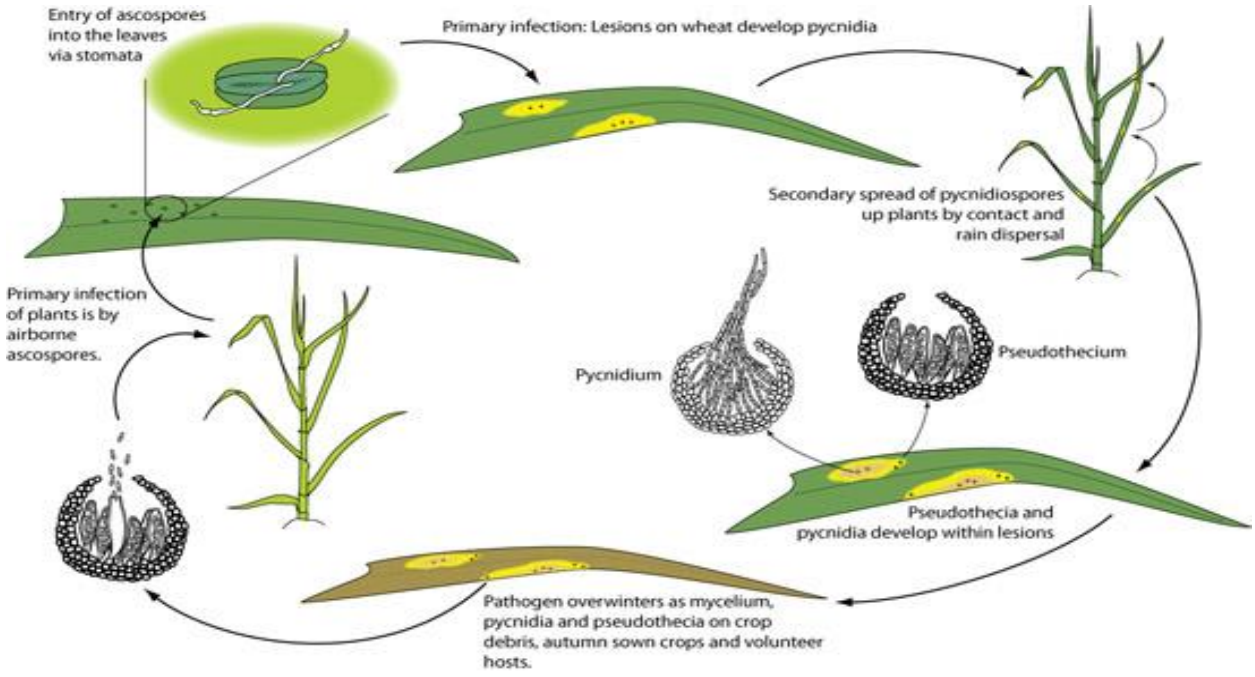


Figure 2: *Septoria tritici* blotch disease cycle. Source: Ponomarenko *et al*. (2011)

2.5 *Septoria tritici* Blotch Disease Symptom

STB symptoms vary according to cultivar, cultural practices, and geographic location. The initial symptoms of STB are small chlorotic spots on the leaves that appear almost immediately after seedling (Ponomarenko, *et al.*, 2011). Although the disease can affect the plant at any developmental stage, the initial lesions start as small yellow flecks usually on lower leaves that are in contact with the soil. Gradually, the flecks expand into irregular brown-to reddish coloured lesions with 1.6-3.2 mm wide by 6.35-19 mm long margin. The lesions get old; the center becomes bleached with gray or ash-white centers, with small, dark brown to black specks. These black dots at the center are the fungal fruiting body called pycnidia (Fig.3).

The disease can be distinguished from other foliar diseases by the presences of these small black fruiting bodies within the lesions (Eyal, 1987). The diseases development depends on the wheat genotypes, environmental conditions including moisture, temperature (optimum temperature

range is 15.6 to 21 degree Celsius), and humidity (Váry *et al*., 2015). The diseases development could be favored by high humidity, higher atmospheric CO_2, frequent rain and cool wet weather.

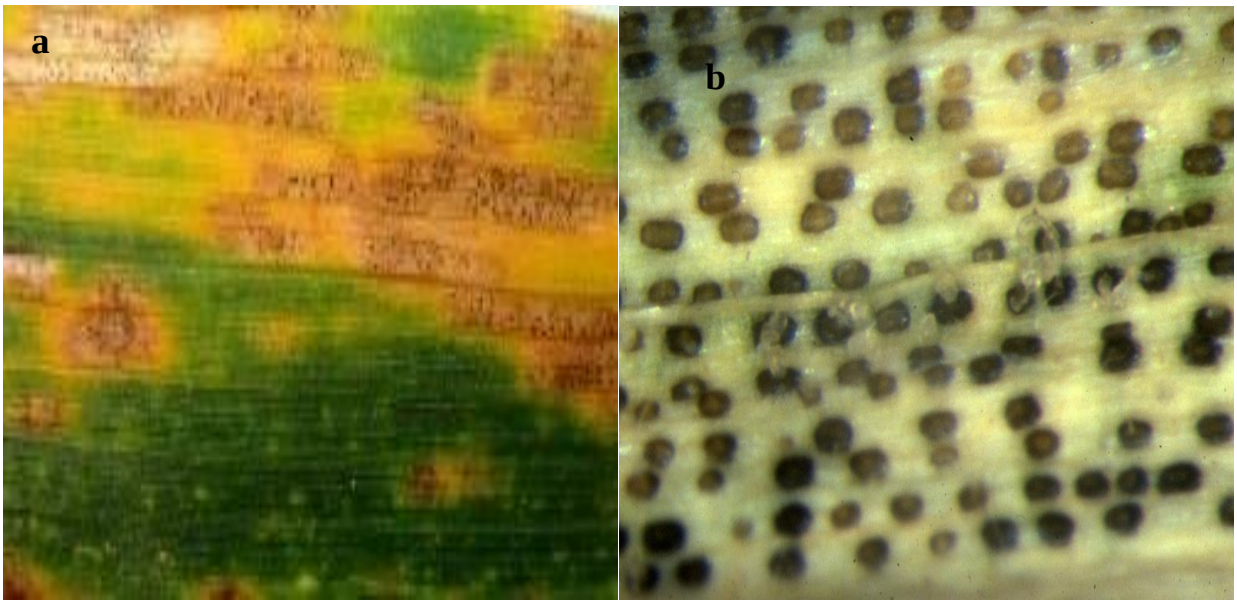


Figure 3: Symptoms of *Septoria tritici* blotch on wheat leaf. a**)** Typical *Septoria tritici* blotch symptoms produced on young wheat leaves. b) The pycnidia develop in the substomatal cavities of the host within the lesions.

2.6 Impact of *Septoria tritici* Blotch Disease on Wheat Production

Septoria tritici blotch (STB) is a worldwide problem causing considerable wheat production losses in different parts of the world including Europe, Mediterranean area, Africa, Americas, and in Australia (Fones and Gurr, 2015). STB infections occur during all stages of plant development; however, infection on the flag leaves can cause the most severe losses by reducing grain weight. Under conducive environmental conditions, yield losses as a result of this disease can range from 30 to 70% (Eyal *et al*., 1987). In Europe, annual losses from STB are estimated to be $400 million USDdollars, and similarly loss estimates for the United States are more than $275 million dollars per year. STB affects wheat yield by causing premature death of wheat

leaves and hampering photosynthesis. In Ethiopia, most of the high-yielding wheat cultivars grown today are STB susceptible and there are no varieties that are fully resistant (Teklay Abebe *et al*, 2015). This disease is widely distributed over all wheat growing areas of the country (Tilahun Mekonnen *et al.*, 2020), and the yield loss as a result of this disease reaches up to 82% at hot spot areas and susceptible varieties (Abreham Tadesse, 2008).

2.7 *Septoria tritici* Blotch Control Methods

2.7.1 Chemical control method

Chemical spraying and planting fungicide treated seeds are some of the method to control Septoria severity in wheat. However, it is important to correctly identify *Septoria tritici* blotch before spraying with a fungicide as nutritional disorders such as aluminium toxicity or zinc deficiency can be confused with *Septoria tritici* blotch. It is important to consider the appropriate growth stage in order to apply the fungicides. 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. Once the flag leaf has fully emerged, at GS39, another fungicide application may be required to protect the upper canopy (Figueroa *et al*., 2018). However, reports revealed that the high input of fungicides in combating this disease has led to a high percentage of fungal strains showing resistance to fungicides like resistance strobilurin (Qo inhibitor, QoI azoles, triazole and succinate dehydrogenase inhibitors (SDHIs) (Cools and Fraaije, 2013).

Moreover, the use of fungicides to control STB is expensive, inaccessible and unaffordable by resource poor small holder farmers, and environmentally not safe (Kosina *et al*., 2007). Reports reveled that approximately 70% of the estimated volume of fungicide used on cereals in Europe is used to control STB.

2.7.2 Cultural method

Cultural management can reduce the incidence and severity of STB. Crop rotation, appropriate fertilizer, proper seeding rates and planting dates would be an alternative control to STB disease (Berraies *et al*., 2013). Rotation to non- hosts and sanitation achieved by deep plowing of crops debris can decrease the amount of inoculums available to initiate anew disease cycle. Late early planting of early flowering and susceptible varieties destroying stubble by grazing or cultivation are some of the cultural methods to reduce STB severity. This is not, however, feasible in light soil areas where stubble must be kept to prevent erosion (Holloway, 2014).

2.7.3 Resistance cultivars

Planting of resistant cultivar is considered to be the most economical, durable and safe strategy to control crop diseases including STB. Genetic resistance to *Z. tritici* can be qualitative or quantitative. The inheritance of the resistance to STB is complex and challenging to track because STB rapidly changes its host-specificity, leading to overcoming host disease resistance. So far, 21 genes were identified to confer resistance to STB and were designated Stb1 to Stb18, StbSm3, StbWW, and TmStb1. When compared to yellow rust, leaf rust, stem rust and powdery mildew with 73, 89, 61 and 95 mapped résistance genes, respectively and this number is limited. Moreover, the majority of these genes have spectra of specificity towards *Z.tritici* Nevertheless, 10s of quantitative trait loci (QTL) for STB resistance have been detected in a number of mapping populations reporting an extraordinary diversity, and complexity of the genetic basis of STB resistance (Stewart *et al*., 2015). Understanding the genetic bases of the pathogen population has great importance for adopting and/or developing durable and effective management strategies like the use of STB resistant wheat cultivars (Fones and Gurr, 2015).

2.8 Genetic Diversity of *Zymoseptoria tritici*

Consideration of the genetic variation of *Z. tritici* population is essential to understand their virulence patterns on the different cultivars. *Z. tritici* population around the world show wider ranges of genetic variations which could be attributed to variation in regular recombination, different migration patterns, and presence and importance of the sexual form. Sexual reproduction creates large numbers of genetically diverse isolates. Populations in this fungus are in genetic equilibrium as well as in drift migration equilibrium attributed to a high rate of sexual recombination (Simon *et al*., 2012). Moreover, the pathogen shows high migration rate that can result in high gene flow.

Due to high gene flow, remarkably little geographical structure was found on the global scale, with an average of 92% of global pathogen diversity found within a wheat field and 84% of global diversity found within a one square meter area of any wheat field. By comparison, only 3% of global genetic diversity was distributed among continents. Molecular analysis revealed that *Z. tritici* populations show high gene flow within and between populations (Chen and McDonald, 1996). A high degree of gene flow occurs across large spatial scales due to air dispersal of the sexually recombined ascospores that emerge from infected plants during the growing season and from infected straw and other crop debris between growing seasons (Zhan *et al*., 2002). Similarly, Dalvand *et al., (*2018) described the three ways in which gene flow could happen in *Z. tritici* : formation of a sexual reproduction cycle, presence of intermediate hosts such as some grasses that can play the role of a green bridge, and movement of infected seeds and straw from one place to another by people.

2.9 DNA markers used for *Zymoseptoria tritici* genetic analysis

Genetic structure of *Z. tritici* populations have been studied over the last decade around the world. Many molecular studies revealed the presence of a high level of genetic variability within *Z. tritici* populations (Zhan *et al*., 2003). Molecular markers are commonly used to characterize populations of fungal plant pathogens and are increasingly most commonly used for identification and diagnostic purposes of *Z. tritici* populations.

Different marker systems have been enveloped and widely used to explore the molecular diversity and population structure of *Z. tritici* populations including restriction fragment length polymorphisms (RFLP) (McDonald *et al.* 1995; Chen and McDonald 1996), random amplified polymorphic DNA (RAPD) (Czembor and Arseniuk 1999; Razavi and Hoghes**,** 2004), amplified fragment length polymorphisms (AFLP) (Schnieder *et al.*, 2001; Kabbage *et al.*, 2008; Medini and Hamza, 2008 Abrinbana *et al.*, 2010), and simple-sequence repeats (SSR) or microsatellite (Owen *et al*., 1998; El Chartouni *et al*., 2011; Berraies *et al*., 2013; Boukef *et al*., 2012; Samia *et al.*, 2013; Siah *et al.*, 2018;Tilahun Mekonnen *et al.*, 2020). These studies confirmed that *Z. tritici* populations exhibit higher genetic variation in which the within population genetic variation accounts for most of the overall genetic differences (Kabbage *et al.*, 2008; Medini and Hamza, 2008; Dalvand1*et al.*, 2018; Tilahun Mekonnen *et al.*, 2020); which could be due to sexual reproduction biology of the pathogen.

Among these marker systems, SSRs are the marker of choice for fungal plant pathogens genetic structure analysis because of their higher rate of polymorphism, informativeness, reproducibility, multi-allelic nature, co-dominant inheritance, relative abundance and ease of identification from genomic sequences (Tautz et *al*., 1989; Winter and Kahl, 1995; Medini and Hamza, 2008;

Gautier *et al*., 2014). The high levels of polymorphisms observed in SSR markers and the relative ease of detection of these polymorphisms by PCR amplification has led to the wide applications of SSRs as genetic markers.

The genetic polymorphism among microsatellite loci is the result of the difference in the number of repeat units among study samples. The polymorphism can be detected by using pairs of primers that target the conserved sequence flanking the microsatellite region and amplify the variable region or locus (Mohan *et al*., 1997; Govindaraj *et al*., 2015). However, any change in the SSR flanking regions' sequence can result in failure of amplification (null alleles), as the primers will fail to bind the flanking region to amplify the region between. Although, SSR marker is widely used in many fields of biology that span from forensic DNA studies to genome mapping, paternity testing, population genetics and biological resources conservation **(**Luikart *et al*., 2003; Berraies *et al*., 2012; Peixoto *et al*., 2014), its development requires extensive knowledge of DNA sequences, and also sometimes underestimates the genetic structure in diversity analysis (Matsuoka *et al*., 2002; Govindaraj *et al*., 2015).

2.10 Measuring Molecular Variability of a Population

Various diversity parameters are used to determine the extent of genetic variability of a population. Nei (1973) gene diversity (H) defined as the probability of obtaining two different alleles at a locus when two haploid individuals are sampled randomly from a population is one of the parameters used to measure population diversity in both sexually and asexually reproducing populations. Mathematically gene diversity can be calculated by:

$$H = 1- \Sigma Xi^2$$

Where: H is the gene diversity of the population and Xi is the frequency of different alleles at a particular locus. The minimum value of gene diversity is 0 and it is the locus is monomorphic (only one allele per locus). In dominant markers the value of gene diversity ranges from 0 to 0.5 and H= 0.5 when the frequencies of the two allele in particular locus are equal. For multi-allelic markers like microsatellites, the gene diversity values can increase with increasing the number of alleles per locus. It becomes 0.67, 0.75 and 0.8 for multi-allelic markers with 3, 4 or 5 alleles per locus, respectively. The other important genetic parameter to be considered in assessing the gene diversity is determining how the variability is distributed among and within the different subpopulations. Nei (1973) proposed a method for partitioning the genetic diversity of a population into different components. He suggested that genetic differentiation between subpopulations can be estimated as:

$GST = (H_T - Hs) / H_T$

Where: GST = the proportion of the total genetic variation accounted for by variation among sub populations. H_T = the total gene diversity of the population, and Hs = the average gene diversity within subpopulations. GST value will be small, when the amount of gene diversity within subpopulations is higher than among populations, while it becomes large when the amount of gene diversity among subpopulations is high, but low within subpopulations.

It is also important to consider the intra-population and inter- population genetic diversity parameters. The intra-population diversity estimators include the number of alleles, polymorphism or rate of polymorphism, major allele frequency (MAF), polymorphic information content (PIC), proportion of polymorphic loci (% PPL), average number of alleles per locus, effective number of alleles (Ae), Shannon's Information Index (I), and estimate of the deviation

from Hardy-Weinberg equilibrium (HWE),and etc. While the inter-population genetic diversity measuring parameters include inter-population differentiation for several loci (G_{ST}), Fst (Wright) state, analysis of molecular variance (AMOVA) etc. Population differentiation tests: Wrights fixation index, determination of the dissimilarity coefficient (genetic distance), cluster analysis and inferring the pattern of population structure (Berraies *et al.*, 2013)

3. MATERIALS AND METHODS

3.1 Description of the Study Area

The *Z. tritici* isolates used in this study were isolated from STB symptomatic wheat leaf samples collected from naturally infected wheat fields of three zones (Kembata- tembaro, Hadiya, Silite) of the SNNPR state, and one bordering zone *i.e* Southwest Shewa zones from Oromia in 2018 main cropping season (Fig. 4). The zones were selected based on their wheat production potentials in Southern part of Ethiopia. From each zone, Woreda /District and Kebels (the lowest local administration) were subsequently selected based on their wheat production potential and road accessibility.

Table 1 Description of areas from where samples of *Zymoseptoria tritici* were collected.

Study area	Longitude	Latitude	Altitude (m.a.s.l.)	Temperature (ºC)
Kembata- tembaro,	37º.34”- 38 º	7º.10” –7º.50"	501-3000	126-27.5
Hadiya	37º23'14" - 38º52'13"	7º3'19" - 7º56'1"	1500-3000	10-35
Silite	38° 16' 60"	7° 58' 0"	2317	10-35
Southwest-Shewa	37º05´-38º 46´	8º16-90´ 56"	1600-3576	10-35

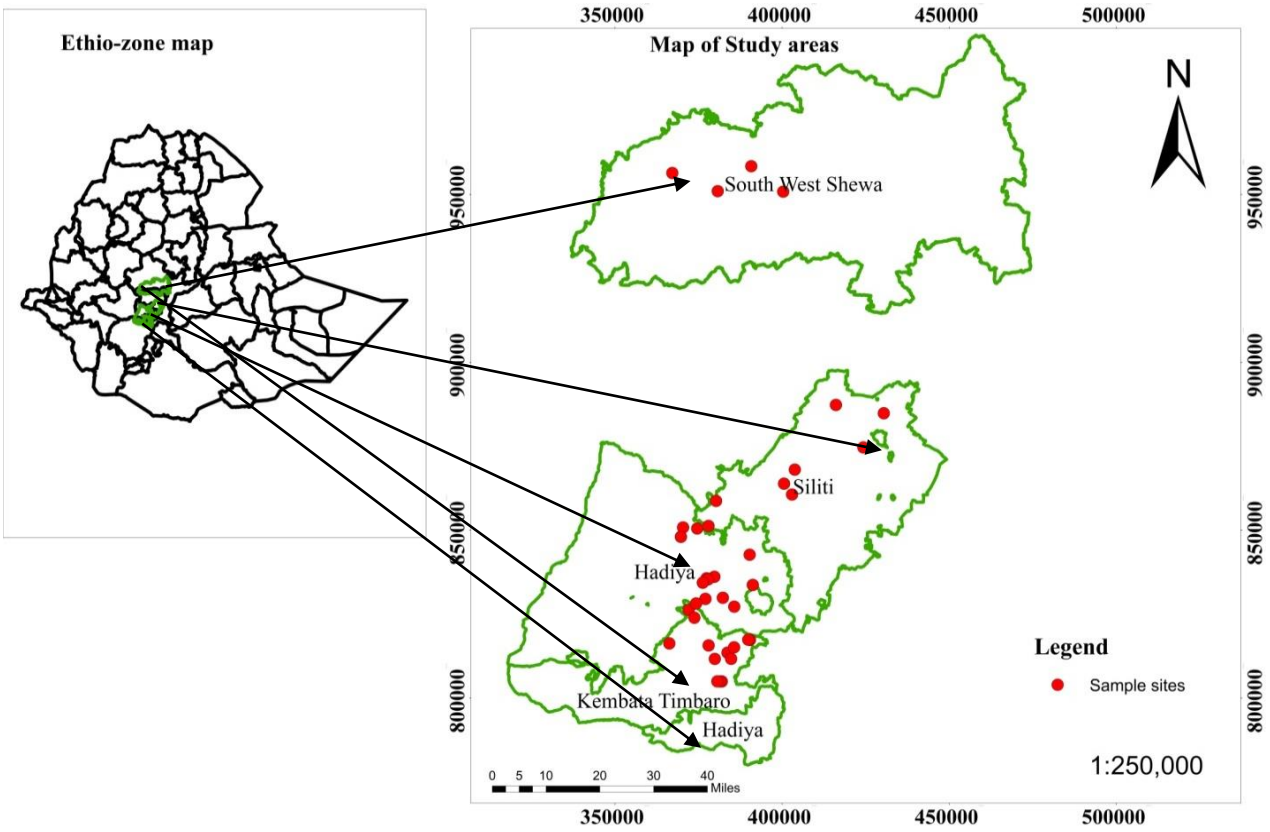


Figure 4: Map of Ethiopia (on the left) with the four zones (on the right) representing the four *Zymoseptoria tritici* populations. The map was constructed using geographic coordinates and elevation data gathered from each collection site using the global positioning system (GPS).

3.2 Sample Collection

STB symptomatic wheat leaf samples were collected from naturally infected wheat fields (Fig. 5) from the four zones at the beginning of October in 2018 main cropping season following the main roads and accessible routes at 5-10 km intervals based on vehicle odometers. Hundred samples were collected at medium milk and early dough growth stages (GS) as described by

Zadoks et al. (1974). We have visited about 120 naturally grown wheat fields and 0.83 average samples were collected from each field.

During collection, green leaf samples in the wheat field naturally infected with STB with black spots (pycnidia) on the necrosis area were collected. Scissors were swiped with 70 % ethanol prior to the next sample collection to avoid cross contamination. Collected samples were placed in paper envelopes, and followed by recording of collection date, sample code, latitude, longitude, altitude, and disease severity score. The samples were left to dry at room temperature for a week, and then were placed in zipped plastic bag and stored at 5 °C until isolation begins.

3.3 Diagnostic and Disease Assessment in the Field

Diagnosis of the disease on wheat is based on the observation of the typical symptom caused by *Z. tritici*. The STB was identified by chlorotic spots and light tan lesions with small blackings (Ponomarenko *et al.*, 2011). Disease severity was scored based on double digit scale (00-99) where the first digit (0-9) indicates the necrotic leaf area on the four uppermost infected leaves of 10 - 20 plants and the second 0-9 digit represent the blotch development up the plant height (for instance 5 if the disease reached at the middle (50%) of the plant height, 8 for flag leaf and 9 for spike), and the second digit stands for disease severity as a percentage but in terms of 0-9 (1=10%, 2=20% … and 9=90%). Depending on the size of the wheat field three to five stops were made in an "X" pattern and average result was taken to describe the disease severity of the field (Eyal *et al.*, 1987)

3.4 Isolation of *Septoria tritici* Pathogen

Isolation of *Zymoseptoria tritici* fungal spore and following activities were conducted at National Agricultural Biotechnology Research Center (NABRC), Holeta, 29 Km west of the capital city- Addis Ababa. The isolation was carried out as described by Eyal *et al.* (1987) with some modifications. During isolation a necrotic wheat leaf with a pycnidium was placed on wet filter paper in Petri dish (Fig. 5a) at room temperature (20-25 °C) for four hours. Under high humidity ooze that contains pycnidiospores were emerged from the opening of the pycnidium (ostiole) and formed a drop (cirrhus) on top of the dark pycnidium. Observing under stereoscopic dissecting microscope (Fig. 5b), mono-pycnidial oozing drops were transferred onto potato dextrose agar plate (PDA) containing 250 mg/l chloramphenicol succinate using flame sterilized needle. Inoculated Petri plates were kept at 24 °C for 10 days until fungal growth was observed. Developed pinkish-orange colony was streaked on a new PDA plates without antibiotics and kept at the same conditions.

Developed mono-spore derived colonies (Fig. 5c) were transferred to yeast-sucrose broth (YSB) (1% sucrose, 1% yeast extract) and incubated at room temperature on orbital shaker (180 rpm) for two weeks for spore multiplication (Fig. 5e). Spore pellets were recovered (Fig. 5f) by centrifugation at 10, 000 rpm for 5 minutes and stored at -80 °C until used for DNA extraction. For long term storage fungal isolates were preserved in yeast extract (4g/l), malt extract (4g/l), and sucrose (4 g/l), supplemented with 30% glycerol and stored at - 80 °C

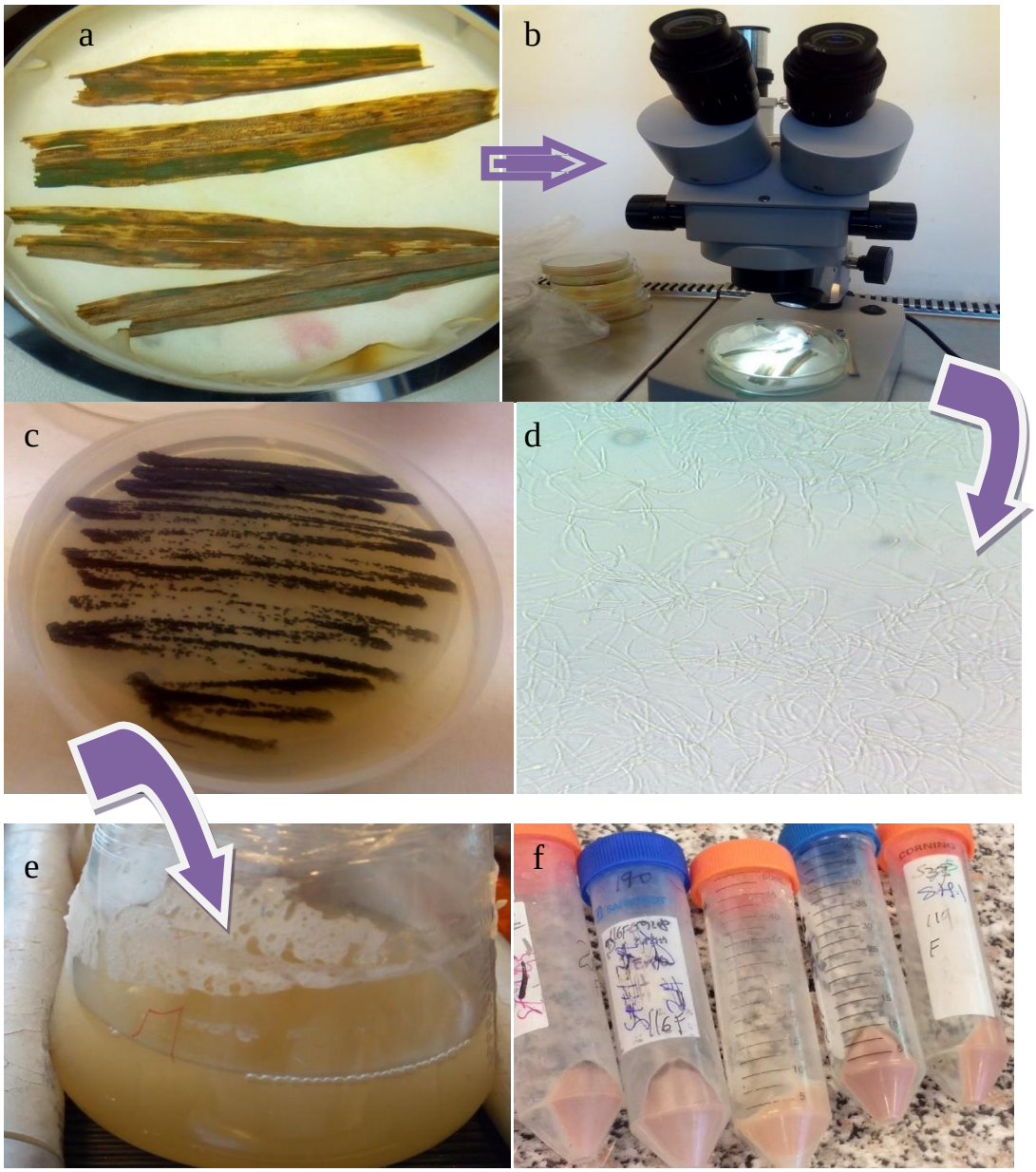


Figure 5: *Z.ymoseptoria tritici* isolation procedure. a)10 cm long STB symptomatic leaves on samples on wet filter paper in Petri dish b) transferring oozing drops to PDA observing using stereoscopic dissecting microscope under sterile cabinet, c) Pure *Z. tritici* culture on PDA ready for multiplication, d) *Z. tritici* spores under 10x magnification objectives, e) Spore multiplication in YSB, and f) Spore pellets recovered through centrifugation.

3.5 Assessing the Genetic Diversity of *Zymoseptoria tritici* Isolates

3.5.1 Genomic DNA isolation

Fungal genomic DNA isolation was carried out using diversity array technology (DArT) with minor modification (Appendex 2). The DNA was quantitatively and qualitatively checked using a Nano drop spectrophotometer (ND-1000) and gel- electrophoresis. DNA concentration was adjusted to50 ng/µl using sterile nuclease free water and stored at -20˚C for further use.

3.5.2 Molecular detection of *Zymoseptoria tritici*

All 51 isolates were confirmed by running polymerase chain reaction (PCR) using a race specific diagnostic markers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') as forward and JB446 (5'CGAGGCTGGAGTGGTGT-3') as reverse primer (Beck and Ligon 1995). The PCR reaction was performed in a volume of 12.5 µl containing 6.25 µl (2x Taq plus master mixes), 2µl Nuclease free water, 1µl of each of the forward (ITS1) and reverse (JB446) primers and 2.5 µl of 50 ng template DNA. Amplification conditions were set as an initial denaturation at 94 ℃ for 3 min followed by 35 cycles with 1 min denaturation at 94 ℃, 1 min annealing at 60 ℃, primer extension at 72 C for 2 min followed by final extension step of 10 min at 72 ℃. The PCR amplified products were fractionated in 3% agarose gel electrophoresis by loading 5µl of each of the PCR product mixed with 2 µl of loading dye with 1x gel red using 1x TAE buffer at 100V for three hours. Amplicon fragment size was estimated using 100 bp DNA molecular ladders. The gel was visualized and also photographed using gel-documentation system (BioDoc-It^{TM} imaging system).

3.5.3 SSR marker based genotyping

For molecular diversity and population structure analysis, the confirmed *Z. tritici* isolates were genotyped using nine published single locus microsatellite primers as described by (Owun *et al.* 1998 and Berraies *et al.* 2013) (Table 1).

Table 2: SSR markers and their respective primers used for genotyping of *Z. tritici* isolates.

Marker	Repeat Motif	Annealing temperature (°C)	Expected Size (bp)	Forward	Reverse
ST1E7 (MGR 7038)	(CGG)5	58	85-91	5'GATCTCGAGCAGGGCGGAAGT3'	5'TCACACGCTGGTCTGTGAATC3'
ST2E4 (MGR 7034)	(GGC)5	58	75	5'GAAGATCAACAGCATGGGCGG3'	5'CTCCAGAGGGATCACAAAGGC3'
ST1A4 (MGR 7032)	(CCG)7	58	98-116	5'GGTTCGATGGAGAGATTT3'	5'TCACCTCCTCATCGCAGA3'
ST1D7 (MGR 7039)	(AC)22	61	85-105	5'TTGAAGTGGCATCCTCCATT3'	5'AACTCGGCTGGTGGAACA3'
ST2C10 (MGR 7036)	(AGCGG)4	58	75-87	5'AGGCGAGAAACTTGCTTGCAG3	5'AATGAACGTCCCATGGACGTG3'
AG-0003	(AG)15	52	230-258	5'ACT TGG GGA GGT GTTGTA AG 3'	5' ACT TGG GGA GGT GTT GTA AG 3'
AC-0001	(AC)7	52	171-200	5'CACCACCAC GTGGTTGAA G 3'	5'CGTAAG TTC GTG GAG ATG GG 3'
AG-0009	(AG)7	52	192-200	5' C GAC TGC ATTTACTTGTGG CG 3'	5'TGTGAAGG CAC GCAAAG AG 3'
AC-0002	(AC)7	52	172-210	5' TGA ACA TCA ACC TCA CAC GC 3'	5'ACAAGA GGA CGACCCACGAC 3'

PCR was performed in a total volume of 12.5 µl containing 6.25 µl master mix, 2 µl DNA tamplate, 1µl of each of the forward and reverse primers, 0.25 µl DMSO and 2 µl nuclease free water. PCR amplification was conducted on a Biometra thermocycler program as follows: 3 min at 94°C for initial denaturation followed by 40 cycles of denaturation at 94°C for 1min, annealing at 52 – 65 °C for 1 min, and extension at 72 °C for 2 min. The final extension was adjusted to at 72 °C for 10 min. PCR products were resolved on 3% agrose gels electrophoresis using 1× TAE buffer at 100 V for 3 h. The gel was stained with gel red and visualized under UV light and subsequently photographed. To estimate the amplicon size A 50 Bp and 100 Bp DNA ladder were used.

3. 5.4 Data scoring and statistical analysis

The PCR amplified SSR regions fragment size on gel were estimated using PyElph 1.4 software package (Pavel and Vasile, 2012). Genetic diversity analyses were carried out on the basis of the scored bands. Only clearly resolved and unambiguous bands were scored for each primer and sample. For each locus, amplified fragments with different molecular sizes were considered as different alleles. Appropriate statistical software packages were employed to compute the standard indices of genetic diversity. Locus based diversity indices including major allele frequency (MAF), the number of allele (Na), and gene diversity(GD) and Polymorphic information contents (PIC) were computed using Powermarker v3.25 software (Liu and Muse, 2005). Effective number of alleles (Ne) and Shannon's Information index (I) were determined using POPGENE version 1.31(Yeh and Yang, 1999). Allelic frequency and population genetic structure and differentiation among and within isolates based on their geographic origin and the analysis of molecular variance was performed using GenAlEx ver. 6.503 software (White and Peakall, 2015).

The patterns of variation among individual samples and highlight the resolving power of ordination was examined by PCoA. The weighted neighbor joining based clustering and relative position of genotypes on principal coordinate axis (PCoA) was computed by GenAlex version 6.503 (White and Peakall, 2015).

Genetic dissimilarity (GD) between isolates was calculated according to the formula of Nei (1973). The UPGMA (unweighted pair-group method with arithmetic averages) clustering method was used to obtain the dendrogram using DARwin var. 6.0.14 (Perrier and Jacquemoud-Collet, 2006).

Population structure and admixture patterns were determined using STRUCTURE software ver. 2.3.4 based on Bayesian algorithm (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 200,000 Markov Chain Monte Carlo (MCMC) replications for K = 1 to K = 9 using 20 iterations for each K. The most likely number of K was determined based on Evanno correction (Evanno *et al.*, 2005) method using the web-based STRUCTURE HARVESTER ver. 0.6.92 (Earl and Von Holdt, 2012). Bar plot for the optimum K was determined using Clumpak beta version (Kopelman *et al.*, 2015).

4. RESULTS

4.1 Molecular Based Identification of *Zymoseptoria tritici*

Conserved regions of the ribosomal DNA (ITS-derived primers) were used to amplify specific fragments from the isolates for the detection of *Z. tritici* species. Diagnostic analysis using a pair of primers (ITS and JB446) revealed that all the 51 tested *Z. tritci* isolates resulted in positive unambiguous amplification of the expected fragment size of about 345 bp. This confirms that all the morphologically identified study materials are *Zymoseptoria tritici* and considering their molecular diversity is thus important.

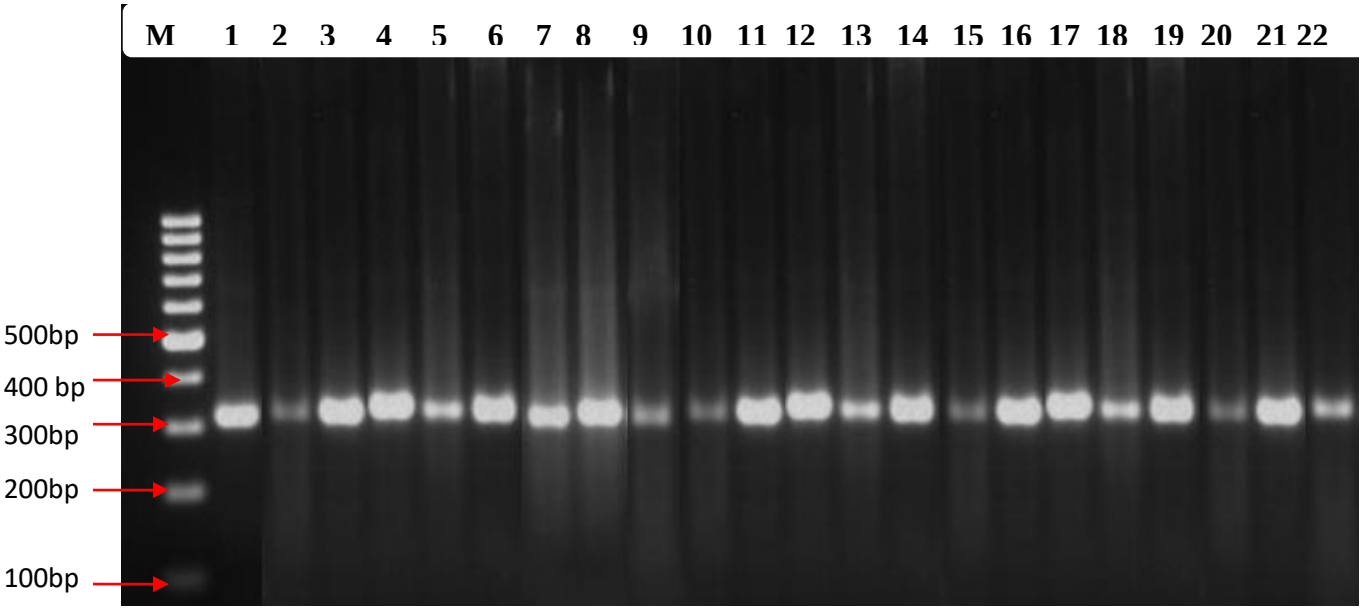


Figure 6: PCR products for molecular diagnostics of *Zymoseptoria tritici*. M=molecular marker, number 1 up to 22 *Z. tritici* isolated samples.

4.2. Molecular Diversity Analysis

4.2.1 Microsatellite markers level of polymorphism

The analysis revealed that all the nine loci were found to be polymorphic and produced a total of 439 bands with an average of 48.77 bands per locus (Table 4). The highest number (51) of bands per locus was recorded for AG-0003 marker, out of which 48 (94.11%) were polymorphic. The highest (95.65%) percentage of polymorphic bands was produced by the locus ST1E7, followed by AG-003 and then AC-002 which resulted in 94.11 % and 94 % polymorphic bands, respectively. Five (55.56 %) of the considered SSR marker resulted in above average percentage of polymorphic bands. The lowest (90%) percentages of polymorphic bands were exhibited by two SSR loci (ST2E4 and AC-0001) (Table 4).

Table 3: Number of polymorphic bands, monomorphic bands and percentage of polymorphic bands produced by 9 SSR markers tested on 51 *Z. tritici* isolates.

Primers	Total no of bands	No Monomorphic bands	No of polymorphic bands	Percentage of polymorphic bands
ST1E7 (MGR 7038)	46	2	44	95.65
ST2E4 (MGR 7034)	50	5	45	90.00
ST1A4 (MGR 7032)	49	4	45	91.83
ST1D7 (MGR 7039)	49	3	46	93.87
ST2C10 (MGR 7036)	45	3	42	93.33
AG-0003	51	3	48	94.11
AC-0001	50	5	45	90.00
AG-0009	49	4	45	91.83
AC-0002	50	3	47	94.00

Total	439	32	407	92.71

The study revealed that the number of alleles per locus varied from 9 to 14 with an average of 12 alleles per locus. The highest number of alleles (14) was resulted from two loci (ST2E4 and AG-0009). The analysis showed that 57% of the alleles were scarce (frequency between 0.01 and 0.05). The frequency of 15 (14%) alleles was between 0.05 and 0.1, while 31 (29%) alleles had a frequency of 0.1-1.00 (Table 4).

Table 4: Summary of the number of alleles with their respective frequencies

Markers	Number of alleles with their respective frequencies			Total
	Scarce(0.01-0.05)	0.05-0.1	0.1 and above	
ST1E7 (MGR 7038)	6	4	1	11
ST2E4 (MGR 7034)	10	1	3	14
ST1A4 (MGR 7032)	5	3	3	11
ST1D7 (MGR 7039)	10	0	2	12
ST2C10 (MGR 7036)	4	3	4	11
Ag-0003	7	2	4	13
AC-0001	7	0	5	12
AG-0009	8	2	4	14
AC-0002	4	0	5	9
Total	61	15	31	107
Percentage	57%	14%	29%	100%

The analysis showed that the major allele frequency per locus ranged from 0.18 (AG-0009) to 0.43 (ST1A4) with overall mean of 0.29. The gene diversity (GD) ranged from 0.73 to 0.88 with an average of 0.82. The highest gene diversity (0.88), polymorphic information content (0.87), number of alleles (14), effective number of alleles (4.605) and Shannon's Information Index

(1.61) were recorded for the microsatellite locusAG-0009 (Table 5).On the other hand, the highest major allele frequency (0.43) and the lowest gene diversity (0.73), polymorphic information content (0.70) and Shannon's information index (0.96) were observed for primer ST1D7 (Table 5). The PIC values of all the SSR loci were found to be high (>0.5) confirming their high informativeness (Table 5).

Table 5: Informativeness and levels of different diversity indices of 9 SSR markers across the entire *Z. tritici* populations.

Marker	N	MAF	GD	PIC	Na	Ne	I
ST1E7 (MGR 7038)	51	0.29	0.85	0.84	11	3.34	1.17
ST2E4 (MGR 7034)	51	0.37	0.79	0.77	14	2.98	1.221
ST1A4 (MGR 7032)	51	0.29	0.81	0.78	11	3.41	1.31
ST1D7 (MGR 7039)	51	0.43	0.73	0.70	12	2.21	0.96
ST2C10 (MGR 7036)	51	0.29	0.83	0.81	11	3.33	1.33
AG-0003	51	0.28	0.85	0.83	13	4.27	1.51
AC-0001	51	0.20	0.85	0.83	12	3.93	1.42
AG-0009	51	0.18	0.88	0.87	14	4.61	1.61
AC-0002	51	0.26	0.81	0.78	9	3.11	1.20
Mean	51	0.29	0.82	0.80	12	3.47	1.30

MAF = Major allele frequency, N= Number of samples, NA= Number of alleles, Ne = Effective number of alleles, GD= Gene diversity and PIC= polymorphic information content.

4.2.2 Genetic variability within and among the populations

Summary of the different genetic diversity indices over the entire SSR loci for the four populations is presented in Table 5. The analysis showed the existence of high diversity among the four populations of *Z. tritici* with regard to number of alleles, effective number of alleles, genetic diversity, private allelic richness, Shannon's information index and percentage of polymorphic loci (Table 5). Among the studied populations, populations of Kembata- Tembaro scored greater values of number of alleles (6.78), effective number of alleles (4.15), genetic diversity (0.73) Shannon's information index (1.57) and private allelic richness (3.22) (Table 6). While isolates of Southwest Shewa showed lowest number of alleles (3.22), effective number of alleles (2.60) genetic diversity (0.57) and Shannon's information index (1.00) (Table 5). The analysis confirmed that all the studied populations showed 100% percentage of polymorphism. Based on the number of locally common alleles (Freq. >= 5 %) which is found in 50% or fewer populations, populations of Hadiya showed the highest value (2.11).

Table 6: Summary of different population diversity indices averaged over the 9 loci for each population.

Population	N	Na	Ne	H	Arp	I	Na Freq. >= 5%	No. LComm Alleles (<=50%)
Kembata - Tembaro zone	21	6.78	4.15	0.73	3.22	1.57	4.11	1.22
Hadiya zone	17	6.67	4.38	0.73	2.11	1.63	6.67	2.11
Silite zone	7	3.33	2.73	0.57	0.67	1.01	3.33	0.67
South west Shewa zone	6	3.22	2.60	0.57	1.00	1.00	3.22	0.44

Mean	12.75	5.00	3.47	0.65	1.00	1.30	4.33	1.11

N= Number of samples, Na= Number of alleles (allelic richness), Ne = Effective number of alleles, h = genetic diversity, Arp = Private allelic, I = Shannon's Information Index, PPL= Percentage of polymorphic loci, No. LComm Alleles (50%): Number of Locally Common Alleles (frequency! 5%) found in 50% or fewer population.

4.2.3 Analysis of molecular variance (AMOVA)

There was moderate degree of genetic differentiations among the tested *Zymoseptoria tritici* populations as revealed by Analysis of Molecular Variance (AMOVA) (PhiPT = 0.14; p = 0.001). The analysis showed that among populations genetic variation accounted for only 14% of the overall (3.93) genetic variations allocating the 86% for the within populations molecular diversity (Table 6). The analysis also confirmed the presence of considerable (3.14) gene flow (Nm) or gene migration (3.14) among the studied populations (Table 6).

Table 7: Analysis of molecular variance of 51 isolates of *Z. tritici* genotypes grouped in to population based on their geographical location

Source of variation	DF	Sum square	Mean square	Est. variance	Percent variance	Genetic differentiation(PhiPT)	P value
Among population	3	29.101	9.700	0.54	14%	0.14	0.001
Within population	47	159.527	3.394	3.39	86%	0.14	0.001
Total	50	188.627		3.93	100%		

4.2.4 Measures of Genetic identity and Genetic distance between the populations

Genetic grouping of populations based on Nei's genetic diversity showed that the pair wise genetic distance between the populations ranged from 0.36 to 0.82 and the maximum genetic

distance (0.82) was observed between population from Kembata-Tembaro zone and population from Southwest Shewa zone (Table 7). The lowest genetic distance (0.36) was observed between populations of Silte and Southwest Shewa zone. On the other hand, Nei's genetic identity between the populations ranged from 0.43 to1.00 (Table 7).

Table 8: Pair- wise Population Matrix of Nei Genetic Distance

Kembata-Tembaro	Hadiya	Silte	Southwest-Shewa	
0.000				Kembata-Tembaro
0.68	0.000			Hadiya
0.54	0.85	0.000		Silte
0.82	0.48	0.36	0.000	Southwest-Shewa

4.2.5 Cluster analysis

Cluster analysis of the 51 *Z. tritici* isolates revealed three major clusters (C1, C2 and C3,) with different hierarchical sub-groups. Most of the individuals (34 or 66.6 %) were assigned to C1. The lowest (3) number of individuals were assigned to sub-group C3 (Fig. 7). None of the clusters consisted of individuals of single population exclusively. The first cluster constituted isolates from Kembata- Temibaro (61.8 %), Silte (17.6 %), Hadiya (8.8 %) and Southwest Shewa (11.8). The second cluster consisted of isolates from Hadya (71.4 %), Southwest Shewa (21.4 %) and Silte (7.1 %). Whereas the third cluster comprised of isolates of Hadiya zone (Fig. 7).

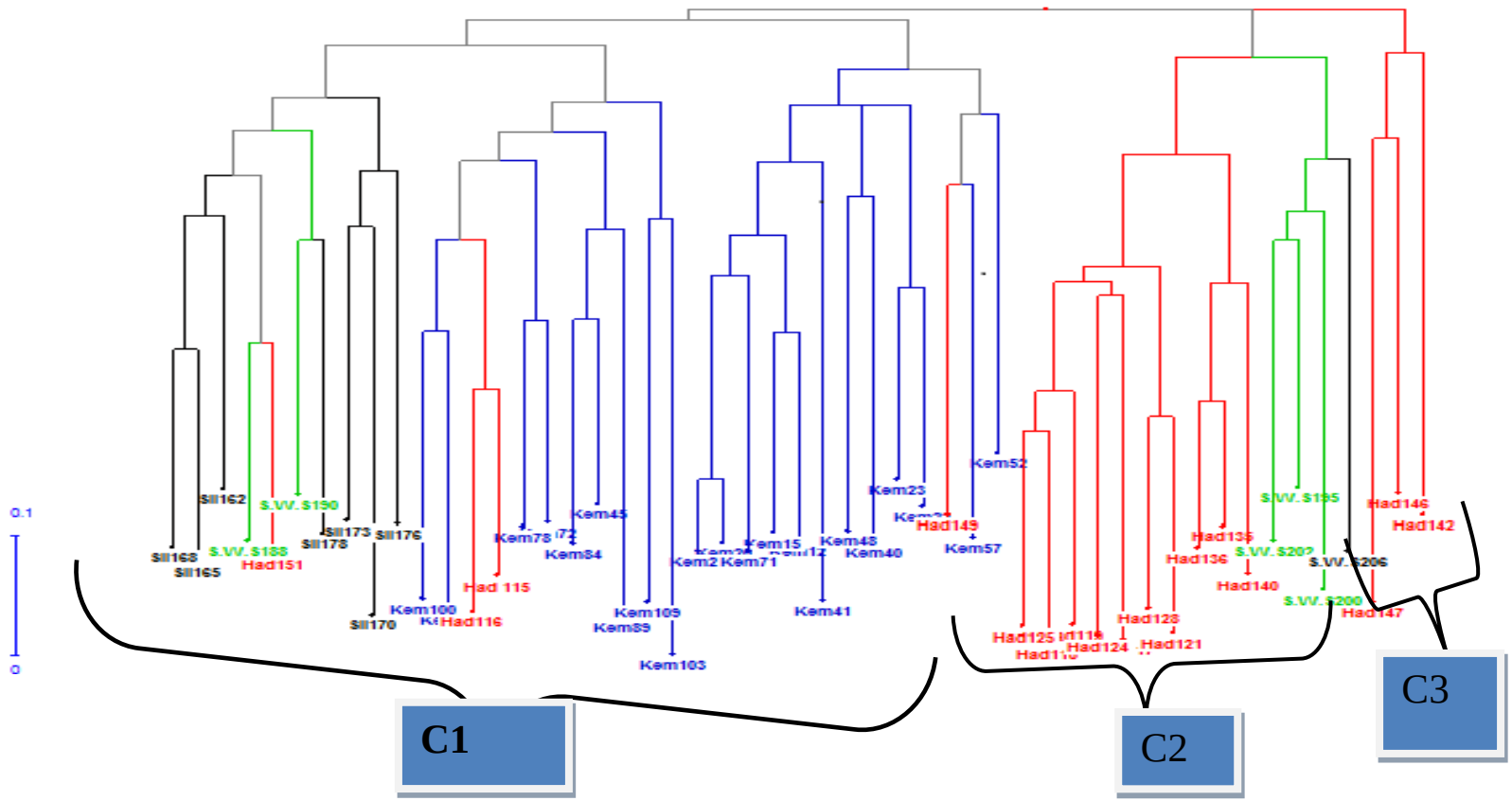


Figure 7: Neighbor-joining tree for 51 isolates of *Z. tritic*i based on Jaccard coefficient of similarity with 1000 replication as revealed by 9 SSR molecular markers. Kem stands for isolates from Kembata-Tembaro zone, Had refers to Hadiya isolates, S.W.S stands for South West Shewa collections and Sil stands for Silte zone collections.

4.2.6 Principal coordinate analysis

The principal coordinate analysis (PCoA) also confirmed the presence of poor population structure in the pathogen. It showed that the first three coordinates accounted for about 37.75 % of the genetic variation. The first, second, and third principal coordinates explained about 15.46 %, 12.27 % and 10.02 % of the gross variation, respectively. The PCoA analysis in the two-dimensional plot displayed in Fig. 8 showed that isolates from different collection sites were clustered together. It is not cluster the isolates distinctly based on their geographical areas of sampling (Fig. 8) complementing the result of NJ cluster analysis.

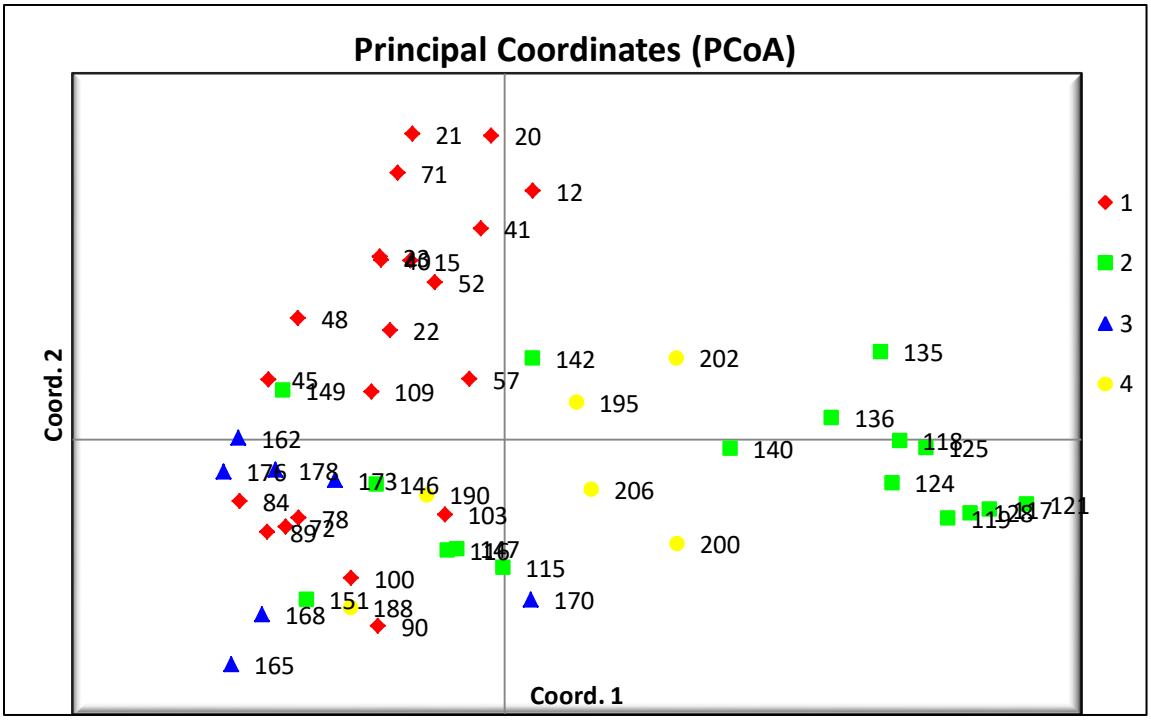
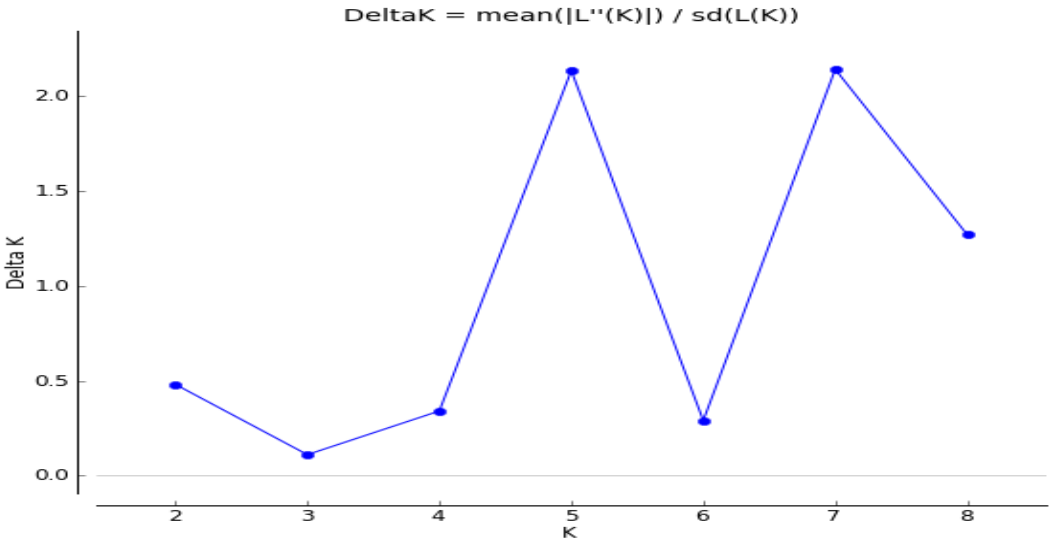


Figure 8: Two dimensional plot of PCoA analysis of 51 Z. tritici isolates and four Population (1= Kembata- Tembaro, 2 = Hadiya, 3 = Silte and 4 = Southwest Shewa populations) based on 9 SSR markers. Samples coded with the same symbol and colors belong to the same population.

4.2.7 Population structure and admixture pattern

Population structure analysis was carried out using STRUCTURE v 2.3.4 software based on Bayesian phylogenic method. The Structure harvester detected two picks (both at K = 5 and K=7 (Fig. 9A). In such cases the most likely number of genetic clusters corresponds to the smallest value of K that captures the major structure in the data (Pritchard *et al.*, 2000). Hence, the model detected the presence of five (K = 5) subpopulations. Based on this value, Clumpak result (bar plot) showed wide admixtures and hence there was no clear geographic origin-based on structuring of the populations (Fig. 9B).

A



B

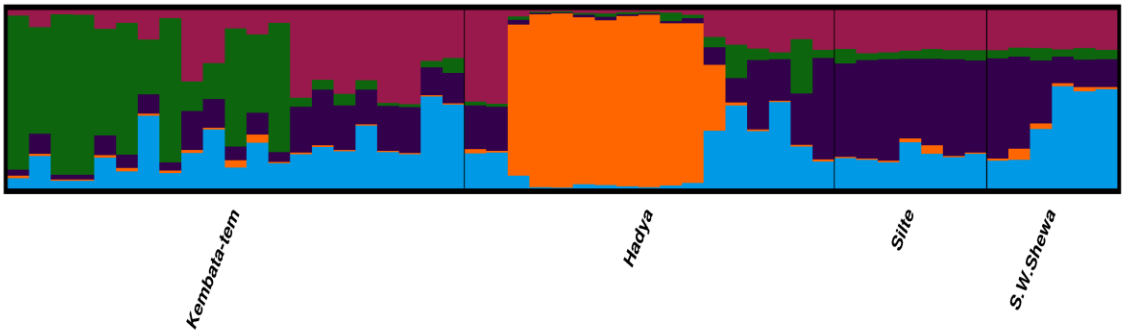


Figure 9: Inferred population structure of 51 *Z. tritici* isolates A) number of population subgroups based on Evano *et al*. (2005) and B) Estimated population structure for K = 5 according to geographical locations. The different colures represent genetic groups or sub-populations designated by Structure Harvester: the x-axis represents individual samples and y-axis represents the proportion of ancestry to each cluster.

5. DISCUSSION

In the present study a diagnostic molecular marker specific to *Zymoseptorai tritici* was used for identification of the pathogen. The primer pair (ITS1 and JB446) target the ITS of ribosomal DNA region and amplified about 345 bp long products in all the 51 tested isolates indicating that all of them were likely to be the target pathogen. Similarly, Beck and Ligon (1995) used same primer pairs to differentiate Z. *tritici* isolates from other fungal species such as *S. nodorum*, *S. glycines, S. passerinii, P. herpotrichchoides, P.aestiva, C. cereal,* and *D. sorokiniana.* Moreover Diriba Guta *et al.* (2019) used the diagnostic markers to identify *Z. tritici* of Northern Ethiopia. The Authors also used similar diagnostic markers to determine *Z. tritici* isolates of Northern Ethiopia. The study confirmed that *Z. tritici* is a widespread and critical problem of wheat production in SNNP regional state of Ethiopia as well.

Analysis of the molecular diversity showed that the *Z. tritici* populations sampled from naturally infected wheat fields had high level of genetic diversity. In total, 439 bands were detected of which 92.71 % where polymorphic. This figure is significantly higher than Kabbage *et al*. (2008) who reported 75% - 81% polymorphic bands at different population scales. This is because of primary source of inoculum which was due to airborne ascospores that would be dispersed uniformly within the field and long year's exposure of the pathogen populations to diverse resistance genes in the host materials.

Per locus, wide range of number of alleles (9 -14), gene diversity (0.73 - 0.88) and polymorphic information content (0.70 -0.87) were recorded indicating that all the microsatellite loci were highly informative, and useful genetic tools to disclose the genetic structure of the pathogen populations.

The PIC values provide discriminating power of a marker by taking into account not only the number of alleles at a locus but also relative frequencies of these alleles. Lower PIC values might be result of closely related genotypes and vice versa. Marker loci with an average number of alleles running at equal frequencies will have the highest PIC value. Markers polymorphism with > 0.5are considered as highly informative, between 0.5 and 0.25 moderate and those with values below 0.25 are low informative (Smith, *et al.*, 1997). In the current studies PIC value close to 1 suggests the presence of more alleles indicating polymorphism in that population which showed more uniform distribution of polymorphism bands among the isolates.

The average gene diversity (0.82) and number of alleles (12) per locus observed in the present study were significantly higher than the level reported by Berraies *et al.* (2013) who described a mean gene diversity and number of alleles of 0.49 and 2.5 alleles, respectively using 45 isolates of *Z. tritici* in Tunisia. The probable reason may be the number of SSR markers used in the present study is higher than they used and the genotype difference due to different in terms of locations of isolates collection may create the variation. Gene diversity is the probability that two randomly chosen alleles from the population are different. The difference between the highest (0.88) and lowest (0.73) gene diversity indicates the presence of variability among 51 *Z. tritici* isolates.

The observed gene diversity in the present study was also by far greater than the report of Owen *et al.* (1998) and Razavi and Hughes (2004b) who found an average gene diversity of 0.49 and 0.44 for 12 UK isolates and for 90 Canadian isolates, respectively. Moreover, the mean number of alleles (12) per locus we reported is significantly higher than the level reported by Medini and Hamza (2008) and Siah *et al.* (2018) who found an average of 2.5 and 4.2 alleles per locus, respectively. Whereas, the PIC (0.70 -0.80) detected in the present study equivalent to the report

of by El-Wahsh *et al*., (2016) who described PIC values ranging from 0.51 - 0.83 using 16 SSR markers.

The higher genetic diversity of the pathogen could be due to sexual reproductive biology or sexual recombination and spontaneous mutation. Moreover, geographical conditions, especially relative humidity in the air, and agricultural practices such as mono-cropping in places including Kembata-Tembaro and the Hadiya zones allow the annual incidence of the disease. This, by itself, can be effective in expanding, displacing, and creating diversity in the causal agent of the disease and can influence diversity of the pathogen (Berraies *et al.* 2013).

Moreover, population genetic variability analysis across the entire loci revealed higher genetic variability among the tested *Z. tritici* populations. This can be explained by higher mean number of alleles (Na =5.0), gene diversity (0.65), effective number of alleles (Ne = 3.47, Shannon's information index (I = 1.3) and 100% percentage of polymorphism. Similarly, Dalvand *et al.* (2018) reported a mean number of alleles of 2.0, Nei's genetic diversity of 0.35, Shannon's information index of 0.53, and percentage of polymorphic loci value of 65%. Similarly, high gene diversity values of 0.31 to 0.70 were reported for *Z. tritici* populations from USA (Gurung *et al*., 2011), Northern France (El Chartouni *et al*., 2011; Siah *et al*., 2018), and Tunisia (Boukef *et al*., 2011). The studied *Z. tritici* population showed higher gene diversity, and this could be attributed to sexual recombination, and long year's exposure of the pathogen populations to diverse resistance genes in the host materials which might have resulted in the pathogen evaluation (Siah *et al*., 2018)

Among tested populations, those of Kembata -Tembaro and Hadiya populations had more mean number of alleles (Na) and Shannon's information index (I) than those of Silte and Southwest Shewa populations. These differences could be attributed to the lower population sizes incorporated in the latter two populations.

Moreover, the populations showed a wide range of private alleles ranging from 0.67 (Kembata-Tembaro) - 3.22 (Silte populations), indicating that the populations possessed unique alleles and a certain level of independent evolution that allowed the maintenance of the private alleles at a population level. The private alleles reported in the present study were relatively lower than the private alleles values of 12, 10, and 11 reported by (Kabbage *et al.*, 2008). This might be because of the presence of high gene flow between in the current study areas.

Analysis of molecular variance (AMOVA) revealed that the populations showed statistically moderate (0.14) genetic differentiation rate where most (86%) of the total genetic variation (3.93) was accounted by the within populations genetic variability, leaving only 14% for among populations. Similarly, in Canada Razavi and Hughes (2004) reported a within and among population genetic variability of 88% and 12%, respectively. In another study conducted by Linde *et al.*, (2002) on samples from various countries including Switzerland and the United States, it was found that 77% was related to intra-population and 23% to inter-population diversity. Sexual recombination could be the major source of intra-population genetic variability (Siah *et al*., 2018). The lower inter-populations genetic variation could be due to the presence of strong gene flow (NM =3.14) among the study population through long distance movement of spores, seed exchange through marketing among neighboring zones and also transfer of STB infected seeds or straws from place to place by people (Schnieder *et al***.,** 2001; Consolo *et al***.,** 2009; Abrinbana *et al***.,** 2010).

The presence of low population differentiation rate and higher gene flow was evidenced by dendrogram results, principal coordinate analysis (PCoA) and population structure analysis in which the populations didn't sharply clustered based on their geographical areas of sampling. Population structure analysis confirmed that the populations were composed of five sub-populations with higher degrees of admixture. Similarly, unstructured population was reported for *Z.tritici* populations of Tunisia (Boukef *et al*., 2012). To the contrary, El Chartouni *et al*. (2011) and Siah *et al.* (2018) in northern France and Abrinbana *et al*. (2010) in Iran reported a 5 - 6 distinct clustering of the *Z. tritici* populations based on geographical origin of the samples.

6. CONCLUSION

In the present study, a total of 51 *Z. tritici* isolates from five zones of South and southwestern parts of Ethiopia were profiled using molecular tools. The diagnostic marker that target the ITS region of rDNA amplified an expected fragment size from all the tested isolates. All the used markers were found to be highly informative with PIC information contents ranging from 0.7 - 0.87) and gene diversity of 0.73- 0.88, confirming that they are useful genetic tools to depict the molecular diversity and population structure of the pathogen populations.

The high within population's gene diversities (0.57 to 0.73) in all the populations indicates that areas included in the present study are very appropriate for multi-location germplasm screening, the pathogen dynamics over time and also host-pathogen interactions. Among the tested populations, those from Kembata-Tembaro and Hadiya showed relatively higher gene diversity and these could be suitable locations if site selection for resistance evaluation is required. Moreover, the Z. *tritici* populations didn't genetically cluster according to their geographical origin due to the presence of high gene flow, and they share genetic background from five sup populations with greater degrees of admixture.

7. RECOMMENDATION

The effect of genetic diversity in pathogenicity must not be ignored because many loci are associated with pathogenicity genes. As it is potential wheat production problem, attention must be given to genetic resistance, because of its durability and environmental suitability for resource poor Ethiopian farmers. It is the preferred and sustainable method to manage various crop diseases including STB, and broad spectrum resistance breading strategies that involve gene pyramiding and quantitative resistances are suggested for effective control of the disease.

Further sampling and pathogen population analyses are needed to assess samples from different wheat growing regions. We also would like to recommend further scanning of the pathogen populations' genetic diversity using more robust genomic tools. More over Kembata-tembaro and Hadiya zones are suitable sites if resistance evaluation is required.

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9. APPENDICES

Appendix 1. : Collection sites of wheat leaf samples infected with SLB (*Z. tritici*) in southern part of Ethiopia.

Dis	Code	Region	Zone	Woreda	Kebele	Longitude	Latitude	Altitude	score
SLBSLB	12	SNNP	Kenbata- Tembaro	Angacha	Anbercho wasera	037°50'624"	07° 18'335"	2422	53
SLBSLB	15	SNNP	Kenbata- Tembaro	Angacha	Bucha	037°53' 41"	07°22'41"	2128	51
SLBSLB	20	SNNP	Kenbata- Tembaro	Angacha	Anbercho wasera	037°50'608"	07° 18' 337"	2440	46
SLBSLB	21	SNNP	Kenbata- Tembaro	Angacha	Kelema	037°53' 009"	07°22' 492"	2076	16
SLBSLB	22	SNNP	Kenbata- Tembaro	Angacha	Garba fandide	037°49'086"	07°18' 626"	2506	13
SLBSLB	23	SNNP	Kenbata- Tembaro	Angacha	Bondena	037°50' 99"	07°19' 628"	2394	25
SLBSLB	40	SNNP	Kenbata- Tembaro	Giyota garba	Giyota garba	037°56'46"	07°21'31"	2214	22
SLBSLB	41	SNNP	Kenbata- Tembaro	Giyota garba	Funto-01	037°54'42"	07°20'30"	2243	24
SLBSLB	45	SNNP	Kenbata- Tembaro	Damboya	Gerinba	037°54'201"	07°20'031"	2227	52
SLBSLB	48	SNNP	Kenbata- Tembaro	Damboya	Demboya 01	037°58' 171"	07°20' 748"	2255	53
SLBSLB	52	SNNP	kenbata tembaro	Damboya	Geyota gerba	037°56'110"	07°21'083"	2215	45

SLBS LB	57	SNNP	Kenbata- Tembaro	Damboya	Geyota gerba	037°56'45"	07°21'24"	2220	43
Dis	Code	Region	Zone	Woreda	Kebele	Longitude	Latitude	Altitude	score
SLBS LB	71	SNNP SNNP SBNP	Kenbata- Tembaro	Damboya	Geyota gerba	037°55'51"	07°16'52"	2098	75
SLBS LB	72	SNNP SNNP	Kenbata- Tembaro	Kacha bira	Hobichaka	037°44'719"	07°15'923"	2335	65
SLBS LB	78	SNNP SNNP	Kenbata- Tembaro	Kacha bira	Hobichaka	037°44'442"	07°15' 729"	2341	60
SLBS LB	84	SNNP SNNP	Kenbata- Tembaro	Durame	Teza agara	037°55'7"	07°16'52"	2292	65
SLBS LB	89	SNNP SNNP	Kenbata- Tembaro	Durame	Teza agara	037°55'32"	07°16'52"	2098	57
SLBS LB	90	SNNP SNNP	Kenbata- Tembaro	Doyogena	Hawara arara	037°47'456"	07°22'707"	2486	66
SLBS LB	100	SNNP SNNP	Kenbata- Tembaro	Doyogena	Awera arara	037°47'18"	07°22'59"	2451	72
SLBS LB	103	SNNP SNNP	Kenbata- Tembaro	Doyogena	Murasa yeramo	037°55'7"	07°16'52"	2292	71
SLBS LB	109	SNNP SNNP	kenbata tembaro	Doyogena	Anchasadicho	037°47'650"	07°20'537"	2591	65
SLBS LB	115	SNNP SNNP	Kenbata- Tembaro	Doyogena	Anchasadicho	037°47'650"	07°20'537"	2591	66
SLBS LB	116	SNNP SNNP	Hadiya	Limu	Ambicho gode	037°53'20"	07°33'31"	2267	33

SLBS LB	117	SNNP SNNP	Hadiya	Misha	Hage	037°51'315"	07°37' 316"	2356	32
Dis	Code	Region	Zone	Woreda	Kebele	Longitude	Latitude	Altitude	score
SLBS LB	118	SNNP SNNP	Hadiya	Misha	Abushura	037°50'290"	07°39'422"	2525	45
SLBS LB	119	SNNP SNNP	Hadiya	Misha	Morsito	037°49'29"	07°41'44"	2594	46
SLBS LB	121	SNNP SNNP	Hadiya	Misha	Morsito	037°49'8"	07°40'13"	2737	36
SLBS LB	124	SNNP SNNP	Hadiya	Misha	Morsito	037°49'167"	07°41' 034"	2607	37
SLBS LB	125	SNNP SNNP	Hadiya	Limo	Morsito	037°49'9"	07°40'13"	2729	40
SLBS LB	128	SNNP SNNP	Hadiya	Limo	Kidogsa	037°51'894"	07°35'831"	2325	40
SLBS LB	135	SNNP SNNP	Hadiya	Limo	Masbra	037°51'560"	07° 36'812"	2327	25
SLBS LB	136	SNNP SNNP	Hadiya	Limo	Ambicho gode	037°53'20"	07°33'31"	2267	35
SLBS LB	140	SNNP SNNP	Hadiya	Limo	Tachignaambichu	037°53'34"	07°33'20"	2248	74
SLBS LB	142	SNNP SNNP	Hadiya	Limo	Ambichu gode	037°52'43"	07°32'49"	2232	73
SLBS LB	146	SNNP SNNP	Hadiya	Limo	Ambichu gode	037°52'44"	07°32'50"	2284	72
SLBS LB	147	SNNP SNNP	Hadiya	Limo	Shurmo	037°53'281"	07°38'591"	2392	70

SLBS LB	149	SNNP SNNP	Hadiya	Limo	Lemo	037°52' 159"	07°33' 322"	2300	40
Dis	Code	Region	Zone	Woreda	Kebele	Longitude	Latitude	Altitude	score
SLBS LB	151	SNNP SNNP	Hadiya	Limo	Dijo demale	037°53' 221"	07°40'780"	2487	50
SLBS LB	162	SNNP SNNP	Silte	MirabAzernet Berbere	Dijo demale	037°54' 819"	07°45'367"	2598	40
SLBS LB	165	SNNP SNNP	Silte	Lera	Duna	037°55'39"	07'45'19"	2644	30
SLBS LB	168	SNNP SNNP	Silte	Lera	Duna	037°53'37"	07'45'16"	2660	25
SLBS LB	170	SNNP SNNP	Silte	Lera	Duna	037°53'37"	07'41'59"	2651	65
SLBS LB	173	SNNP SNNP	Silte	MirabAzernet Berbere	Duna	037°55' 732"	07°45'126"	2651	75
SLBS LB	176	SNNP SNNP	Silte	Wulbareg	Datte wezir	038°09' 787"	07°53' 436"	2369	67
SLBS LB	178	SNNP SNNP	Silte	Hulberg	Menzofeten	038°03' 948"	07°47'465"	2236	65
SLBS LB	188	oromia	Southwest shewa	Amaya	Gulti bulla	037° 47' 806"	08° 40' 912"	2352	31
SLBS LB	190	oromia	Southwest shewa	Amaya	Direoraji	037° 45' 599"	08° 36' 624"	2145	11
SLBS LB	195	oromia	Southwest shewa	Amaya	Direaroji	037°46' 94"	08° 39' 173"	2195	15
SLBS LB	200	oromia	Southwest shewa	Woliso	Adamigotu	038° 01' 899"	08°01' 043"	2302	27
SLBS LB	202	oromia	Southwest shewa	Woliso	Adamigotu	038° 01' 799"	08°01' 036"	2315	32

SLBS LB	206	oromia	Southwest shewa	Woliso	Adamigotu	038° 01' 279"	08° 36' 364"	2262	78

Appendix 2: Plant DNA Extraction Protocol for DArT

BUFFER STOCK SOLUTIONS

EXTRACTION BUFFER

STOCK

	To make 500 ml
0.35 M sorbitol	31.9 g sorbitol
0.1 M TrisHCl pH 8.0	50 ml 1M TrisHCl pH 8.0
5 mM EDTA 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	100 ml 1M Tri HCl pH 8.0
0.05 M EDTA pH 8.0	50 ml 0.5 M EDTA pH 8.0
2M NaCl	200 ml 5 M NaCl
2% CTAB	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.