MOLECULAR CHARACTERIZATION OF WHEAT LEAF BLOTCH PATHOGEN (*ZYMOSEPTORIA TRITICI)* ISOLATES FROM NORTHERN ETHIOPIA USING SSR MARKERS

MSc. THESIS

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MOLECULAR CHARACTERIZATION OF WHEAT LEAF BLOTCH PATHOGEN (*ZYMOSEPTORIA TRITICI)* ISOLATES FROM NORTHERN ETHIOPIA USING SSR MARKERS

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ABBREVIATIONS

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

ABSTRACT

Molecular Characterization of Wheat Leaf Blotch Pathogen (*Zymoseptoria tritici)* isolates from Northern Ethiopia using SSR Markers
Diriba Guta, Teklehaimanot Hailesilassie, Tilahun Mekonnen, Kindie Tesfaye and Tadessa Daba

Septoria leaf *blotch, caused by* Zymoseptoria tritici*, is one of the most devastating diseases of wheat. Understanding its genetic diversity is paramount importance in designing effective and sustainable management strategies to control the disease. In Ethiopia, studies on the pathogen identification and genetic diversity are greatly missing. Therefore, the present study was designed to detect and determine the genetic diversity and population structure of* Z. tritici *isolates collected from northern Ethiopia: Amhara and Tigray regional States using Simple Sequence Repeat (SSR) markers. In this study, a total of 24 single spore derived* Z. tritici *isolates were subjected to a pair of* Z. tritici *specific marker (ITS1 and Primer JB446) to confirm the isolates, and nine Simple Sequence Repeat markers to assess their genetic diversity and population structure. The PCR based detection using a pair of primers that target the internal transcribed spacer sequences of rDNA resulted in a positive amplification size of 345 base pair (bp) confirming that all the isolates are* Z. tritici*. The molecular study revealed a total of 242 bands, out of which 58.3% were found to be polymorphic. All loci across the entire populations were found to be highly (100%) polymorphic and informative with Polymorphic information contents (PIC) ranging from 0.95 (ST1A2) to 0.60 (ST1G7), confirming the power of the markers to be used as molecular tools to determine the extent of genetic diversity and population structure of the pathogen. The genetic diversity across the entire populations ranged from 0.96 (ST1A2) to 0.63 (ST1G70.87) with overall mean of 0.87. Moreover, Analysis of molecular variance (AMOVA) revealed that 95% (3.989) of the total genetic variation (4.19) was accounted within populations, leaving only 5% (0.2) for among populations. The smaller Fixation Index value (0.047) observed in the study indicates the presence of lower population differentiation as a result of higher gene flow (Nm =4.73) between the* Z. tritici *populations of Amhara and Tigray area. The unweight pair group with methods arithmetic average (UPGMA) based cluster, Principal components of Analysis (PCoA) and Structure analysis poorly grouped the individuals into distinct clusters confirming the presence of population admixture due to the long distance movement of the sexual ascospores. Hence, it is possible to conclude that the present study has successfully identified Z. tritici isolates of Northern Ethiopia and disclosed their genetic diversity and population structure using highly informative molecular marker system. The observed higher genetic diversity in both populations indicates that both regions are hot spot for the pathogen study and also can serve as site for germplasm evaluation. The information is very relevant for wheat breeders and pathologists to design and implement integrated management strategies to control the disease and hence to contribute to increased and stable wheat production and productivity in the regions and in Ethiopia at large*.

Key words: Identification, Genetic diversity, Population structure, and Simple sequence repeats

1. INTRODUCTION

Wheat is the world's third most important food crop next to maize (*Zea masys* L.) and rice (*Oryza sativa* L.) (Green *et al*., 2012; Endale and Getaneh, 2015; Tewodros *et al*., 2016). The most common cultivated species are *Triticum aestivum* L. (common or bread wheat) and *Triticum turgidum var. durum* L. (durum /pasta wheat), and the former accounts for 95% of the total global wheat consumption (Randhawa *et al*., 2013). In 2016, 220 M ha of the world was cultivated with wheat, and the global production and average productivity were 735.3 million tons and 3.3 tons/ha, respectively (FAOSTAT, 2018).

Wheat is the most important stable food crop in Ethiopia and ranks fourth after Teff (*Eragrostis tef*), Maize (*Zea mays*) and Sorghum (*Sorghum bicolor*) in area coverage and third after Maize and Teff in total production (CSA, 2017). The crop is cultivated for various purposes including for food (bread, biscuits, pasta, macaroni, "dabokolo", "genfo", and "kinche"), animal feed and income generation (Abera *et al*., 2015; Fanos and Gurr, 2015; Tewodros *et al*., 2016). The wheat production zones in Ethiopia lie between 6° and 16° North, and 35° and 42° East, at altitudes ranging from 1500 to 3000 m.a.s.l. (Endale and Getaneh, 2015). Oromia, Amhara, Tigray, and Southern Nations, Nationalities and Peoples (SNNP) regional states are considered to be the primary wheat growing areas of the country accounting for more than 90% of national wheat production. In 2015, about 1.7 million ha of land of the country was covered with wheat with annual production of 4.5 million metric tons (Bezabeh and Eyob, 2015).

However, in spite of its significant contributions to food and nutritional security, the national average wheat productivity in Ethiopia is about 2. 37 t/ ha; far below the global average of 3.27 t/ha (FAO, 2018). The traditional production system, biotic and abiotic stresses are the major limiting factors of wheat production in Ethiopia. Among the biotic factors, diseases play

significant role in yield reduction. In Ethiopia, wheat is susceptible to more than 30 types of diseases which highly affect its yield (Hailu and Woldeab, 2015; Tewodros *et al*., 2016).

Currently, the fungal disease *Septoria tritici* blotch (STB) caused by the ascomycete *Zymoseptoria tritici (*formerly *Mycosphaerella graminicola* or *Septoria tritici*) (Steinberg, 2015) is among the major bottlenecks to wheat production across the world (Ginkel *et al*., 1999; Thomas *et al*., 2013) including Ethiopia (Hailu and Woldeab, 2015). Both durum wheat (*Triticum turgidum* sub. *durum* Desf.) and bread wheat (*Triticum aestivum* L.) are affected by *Z. tritici* (Ferjaoui *et al*. 2015).

Septoria leaf blotch is principally a foliage disease and the primary infection may begin with airborne or rain splashed asexual pycnidospores and sexual ascospores from infested crop debris (Shaner, 1981; Gilchrist and Dubin, 2002). The disease development depends on favorable conditions such as frequent rain and moderate temperature, traditional agricultural practices, availability of inoculum and presence of susceptible cultivars (Ferjaoui *et al*., 2015). Moisture is required for all stages of infection: germination, penetration, development of the mycelium within the plant tissue, and subsequent pycnidial formation (Ferjaoui *et al*., 2015; Tewodros *et al*., 2016).

Zymoseptoria tritici is a worldwide wheat pathogen (Ginkel *et al*, 1999) which causes 20 - 50% yield loss (Thomas *et al*. 2013). In Ethiopia, 25% to 82% wheat production losses have been reported due to *Septoria tritici* (Bekele *et al*., 2011). Currently, it is among the top two or three most economically damaging diseases of this crop in the Tigray region (Teklay *et al.,* 2015). The use of resistant cultivars is currently seen as the best, economical and environmentally friendly

method to control crop diseases including *Septoria* (Eyal *et al.*, 1987; Eyal, 1999; Ponomarenko *et al*., 2011; Thomas *et al*., 2013).

In breeding for disease resistance, knowledge of the pathogen distribution and its genetic diversity and population structure has paramount importance (Sebei and Harrabi, 2008). Medini and Hamza (2008) revealed that understanding the genetic variability of the pathogen's populations represents a prerequisite for the development of a rational strategy of resistant gene deployment. So far different marker systems have been used to disclose the genetic diversity of *Z. tritici* populations in different parts of the world. McDonald *et al.* (1995) and Chen and McDonald (1996) used RFLP markers to study field populations of *M. graminicola* in USA. Similarly, Medini and Hamza (2008) used AFLP to examine the genetic diversity of Z. *tritici* populations of Tunizia, Algeria and Canada. Among marker systems, simple sequence repeats (SSRs) also called microsatellite markers have become markers of choice for genetic analysis because of their higher rate of polymorphism, informativeness, reproducibility, multi-allelic nature, co-dominant inheritance, relative abundance *etc.* (Winter and kahl, 1995; Angelique *et al*., 2014).

However, the application of molecular tools to assess the genetic diversity of *Z. tritici* in Ethiopia is greatly missing. So far limited efforts have been done to isolate, detect and characterize *Z. tritici* populations collected from northern Ethiopia. Therefore, the present study was initiated with the following general and specific objectives.

1.1. General objective:

- To detect and assess the extent of genetic diversity and population structure of *Z. tritici* populations collected from northern Ethiopia using SSR markers.

1.1.1. Specific objectives:

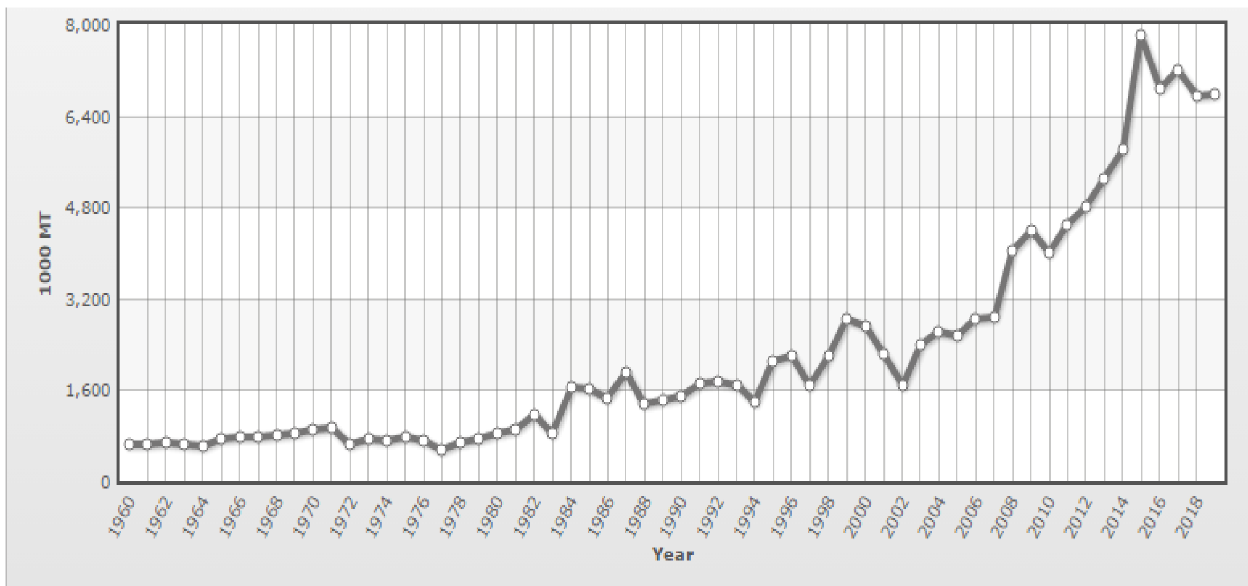
- ✓ To identify *Zymoseptoria tritici* isolates collected from northern parts of Ethiopia using race specific markers.
- ✓ To determine the molecular diversity of *Zymoseptoria tritici* populations of Amhara and Tigray regional states using SSR marker system.
- ✓ To determine the pattern of population structure in *Z. tritici* isolates of Northern Ethiopia

2. LITERATURE REVIEW

2.1. Wheat cultivation in Ethiopia

Wheat is an important cereal crop in Ethiopia that is widely cultivated in a wide range of altitude of mid and highland regions. It is produced exclusively under rain fed conditions (Endale and Getaneh, 2015; Tewodros *et al*., 2016). Wheat is one of the most important small cereal crops and Ethiopia is the second largest producer of wheat in sub-Saharan Africa, following South Africa. The crop is also one of the most important cereal crops being cultivated in the mid and high land areas of Tigray and Amhara regions (Gebru and Abay, 2013).

Many cereal crop are most commonly cultivated, out of these wheat is the most important stable food crop in Ethiopia and ranks fourth after Teff (*Eragrostis tef*), Maize (*Zea mays*) and Sorghum (*Sorghum bicolor*) in area coverage and third after Maize and Teff in total production (CSA, 2017). Production level of the wheat is in averagely increasing from year to year, maximum production were recorded at 2015 year and summarize of production amounts were show on graph 1 below.



Graph 1: Wheat production distribution in Ethiopia, source: CSA, (2019)

However, the national average wheat productivity in Ethiopia is about 2.37 t/ha; far below the global average of 3.27 t/ha (FAO, 2018). The traditional production system, biotic and abiotic stresses are the major limiting factors of wheat production in Ethiopia. Among the biotic factors, diseases play significant role in yield reduction (Hailu and Woldeab, 2015; Tewodros *et al*., 2016).

Wheat is susceptible to many diseases, 30 diseases have been reported on wheat in Ethiopia (Hailu and Woldeab, 2015; Tewodros *et al*., 2016). The most commonly affect wheat production and productivity were included rusts (*Puccinia* spp.), Septoria leaf blotches (*Septoria tritici*), Fusarium head blight (*Fusarium graminearum*), smut (*Ustilago tritici*) and powedery mildew (*Erysiphe graminis* f.sp.tritici). Production of the crop constrained by several infection diseases including rust and *Septoria leaf* blotch diseases which are the major bottle neck of wheat production in Ethiopia (Hailu and Woldeab, 2015) and in Tigray region (Teklay *et al.,* 2015).

Rust (*Puccinia* spp.): Rusts are the most important diseases of wheat worldwide included to Ethiopia (Hailu *et al*., 2015). Considered the major diseases of wheat since no other wheat disease could result in greater loss over large area in a given year. Rusts can cause up to 60 percent of yield loss for leaf or stripe (yellow) rust and 100 percent loss for stem rust (Hailu *et al*., 2015). The highland of Ethiopia including; Oromia, Amhara and Tigray regions were considered as a hot spot for the development of stem rust diversity (Bezabeh and Eyob, 2015; Hailu *et al*., 2015). Stem rust causes blister-like lesions on leaves, leaf sheaths, and stems. Infection of glumes and awns is also possible. The reddish-brown spores of the fungus cause considerable tearing as they burst through the outer layers of the plant tissues. To the best management of these pathogens using a genetic resistance and foliar fungicides is best (Wolf *et al*. 2011).

Fusarium head blight (*Fusarium graminearum*): Fusarium head blight (FHB), caused by the fungal plant pathogen *Fusarium graminearum* (*Gibberella zeae)*, is a devastating disease of wheat and barley (Erena and Steffenson, 2015). Diseased spikelets exhibit symptoms of premature bleaching shortly after infection. The fungus produces a mycotoxin known as deoxynivalenol that poses a significant threat to the health of domestic animals and humans (David *et al*., 2010). Symptoms of Fusarium head blight include tan or light brown lesions encompassing one or more spikelets. Some diseased spikelets may have a dark brown discoloration at the base and an orange fungal mass along the lower portion of the glume. Grain from plants infected by Fusarium head blight is often shriveled and has a white chalky appearance. Some kernels may have a pink discoloration (Wolf *et al*. 2011; Dweba *et al*., 2017). Disease forecasting models may help to optimize FHB management by targeting fungicide, bio-control applications and avoid the most susceptible varieties (David *et al*., 2010).

Powedery mildew (*Erysiphe graminis* f.sp.tritici): Powdery mildews are a group of fungal diseases caused by obligate biotrophic species (species that derive nutrients from living tissue). Their distribution is world-wide including to Ethiopia and they infect most staple crops (Negassa, 1985; Ge *et al*. 2016). Powdery mildew causes white lesions on leaves and leaf sheaths. Glumes and awns also can be infected when disease is severe. Fungal growth is largely limited to outer plant surfaces and can be easily wiped away by rubbing a finger across affected areas. Mature lesions may have dark, reproductive structures mixed with the white, cottony growth of the fungus. Management: Genetic resistance, foliar fungicides (Wolf *et al*. 2011).

2.2. Origin and distribution of *Zymoseptoria tritici*

Zymoseptoria tritici is an Ascomycete fungus that is thought to have originated from closely related *Zymoseptoria* species colonizing wild grasses in the Fertile Crescent. It is a fungus that

has been a pathogen of wheat since the domestication of the crop10,000–12,000 years ago (Stukenbrock *et al*., 2010; 2012; Sanchez-Vallet *et al.* 2017). The genus is widespread, and estimated to contain 1072 species (McDonald *et al*., 2011). This fungus is a causal agent of *Septoria tritici* blotch (STB), the main leaf disease of wheat in temperate regions and a major threat for wheat production globally (Sanchez-Vallet *et al.* 2017).

In Ethiopia, although no or limited studies have been done to identify and characterize the pathogen, the diseases is most widely distributed and it is becoming serious problem to wheat production in major wheat growing areas of the country (Abera *et al* 2015). It is pathogenic on both Hexaploid bread wheat (*Triticum aestivum* L., AABBDD, 2*n* = 42) and Tetraploid durum wheat [*T. turgidum* L. (Thell.) ssp. *durum* L., AABB, 2*n* = 28] (Ferjaoui *et al*. 2015).

Septoria diseases are important in warm and humid wheat growing areas of the world (Fig.1) especially in the coastal areas of the Mediterranean, South America, highlands of East Africa, Australia and Western Europe (Rajaram and Dubin, 1977; Saari and Wilcoxson, 1974). There are two major Septoria diseases that cause problems in wheat in the world. These are *Septoria tritici* blotch (syn. *septoria leaf* blotch, *Speckled leaf* blotch of wheat) caused by the fungus *Zymoseptoria tritici,* formerly called *Septoria tritici* (sexual state: *Mycosphaerella graminicola)* and *Septoria nodorum* blotch (syn. Septoria glume blotch of wheat) caused by the fungus *Septoria nodorum* (sexual state: *Leptosphaeria nadorum)* (Fig.1). The occurrence of *Septoria* diseases in Ethiopia has been recorded by a number of reports (Stewart and Dagnachew, 1967; SPL, 1975; SPL, 1978; Eshetu Bekele, 1985; Sebei and Harrabi, 2008). Many of the studies conducted so far indicated that *Septoria tritici* blotch is more common type of *Septoria* disease in Ethiopia. Hailu and Woldeab (2015) and Takele *et al.* (2015) have reported that *Septoria* leaf

blotch is one of the major bottleneck of wheat production in West and south west Shewa zones of Ethiopia.

Similarly, Abebe *et al.* (2017) reported that STB of wheat is critical problem to wheat production in the major wheat-growing areas of the country. Despite the significant effect of the disease in limiting wheat production and productivity, less or no efforts were made to characterize the causative pathogen or its interaction with the host. The available literatures are limited to field surveys or field evaluations of the wheat germplasms either in a particular region or zone. Thus, more research work is required on the pathogen side (collection, isolation characterization and assessing its genetic diversity), the host and their interaction to generate valuable information that helps to develop durable *Septoria* resistant and high yielding wheat cultivars that lead to increased wheat production and productivity in Ethiopia.

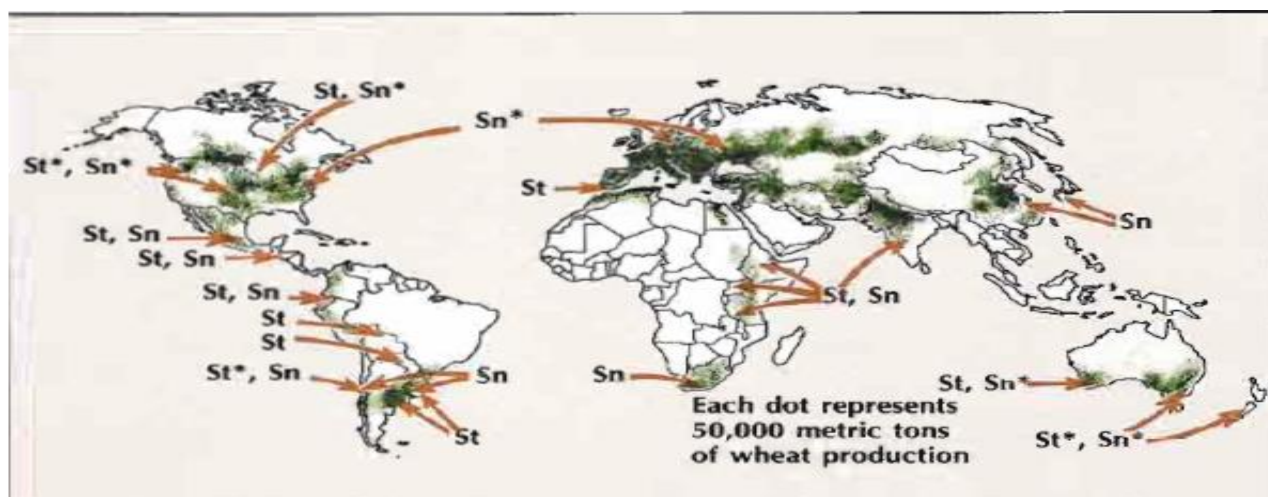


Figure 1: World distribution of *Septoria* spp. on wheat. *Z. tritici or Septoria tritici* (St) and *Septoria nodorum* (Sn) are used to designate the pathogens in all locations. An asterisk indicates locations where the sexual state (pseudothecia and ascospores) has been reported. (Source: Ginkel *et al*, 1987).

2.3. Genetics of *Zymoseptoria tritici*

Z. tritici represents an interesting model for fundamental genetic studies of plant-pathogenic fungi. Goodwin *et al*. (2011) have reported a fully sequenced genome of *Z. tritici* and it was the

first completely sequenced genome of a filamentous fungus. It is haploid plant-pathogenic fungus (Wittenberg *et al*., 2009). The sequenced finished genome contains 21 chromosomes; eight of the chromosomes could be lost with no visible effect on the fungus and thus are dispensable (Goodwin *et al*., 2011). The dispensable chromosomes range from 0.39 to 0.77 Mb and are not required for saprophytic growth and hence are accessory chromosomes (AC). These chromosomes are dynamic in field and progeny isolates, are different from the core genome in gene and repeat content, and appear to have originated by ancient horizontal gene transfer from an unknown donor. *Z. tritici* chromosomes have an extraordinary size range, varying from 0.39 to 6.09 Mb (Wittenberg *et al*., 2009; Goodwin *et al*., 2011).

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. Its chromosomes have been designated into core chromosomes (CCs) and accessory chromosomes (ACs), numbered 1–13 and 14–21, respectively (Goodwin *et al*. 2011; Thomas *et al*., 2018). A surprising feature of the *Z. tritici* 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*., 2011; Wittenberg *et al*., 2009).

2.4. Taxonomy

Zymoseptoria tritici formerly called as *Mycosphaerella graminicola* or *Septoria tritici (*Vallet *et al., 2015*) is a species of filamentous fungus that belongs to kingdom Mycota (Fungi) Phylum Ascomycota, Class Dothideomycetes (Testa *et al.,* 2015) in the family Mycosphaerellaceae, Genus: *Zymoseptoria, and the* species, *Zymoseptoria tritici.* The classification *Zymoseptoria tritici* is summarized below.

Kingdom:	Fungi
Phylum:	Ascomycota
Class:	Dothideomycetes
Subclass:	Dothideomycetidae
Order:	Capnodiales
Family:	Mycosphaerellaceae
Genus:	*Zymoseptoria*
Species:	*Zymoseptoria triticale*

Source: (Testa *et al.,* 2015)

2.5. Life cycle of *Z. tritici*

Zymoseptoria tritici blotch is propagated by both asexual and sexual reproduction methods. Asexual propagation of the pathogen takes place by simple division of cells and this propagation is called pycnidiospores. Asexual propagation is slight, elongated, hyaline, and enclosed within a pycnidium. This stage of *Zymoseptoria tritici* was first identified on wheat by Desmazières (1842) as quoted in Sanchez-Vallet *et al.* (2017).

A sexual mating system of *Zymoseptoria tritici* requires two compatible partners of opposite mating types to come together to produce the sexual spores. Sexual fruiting bodies are called pseudothecia. This sexual stage was identified 130 years later by Sanderson in New Zealand (Medini and Hamza, 2008; Sanchez-Vallet *et al.,* 2017). After sexual mating or asexual mating spores land on a leaf, they germinate and grow as filamentous hyphae that enter the host through stomata, other natural openings or wounds (Sanchez-Vallet *et al.*, 2017). After penetration the fungus grows very slowly and does not produce haustoria or other visible feeding structures, while the plant remains symptomless for 8–11 days (Kema *et al.*, 1996; Duncan and Howard, 2000; Keon *et al.*, 2007). Coincident with the onset of necrotic symptoms, fungal growth spikes and pycnidia and pycnidiospores begin to develop. After the host tissue is dead, saprophytic

growth begins and the fungus forms sexual fruiting structures (pseudothecia) which appear 25–30 days after the initial infection.

Vallet *et al.* (2015) stated that many aspects of the biology of *Z. tritici* and its interaction with wheat remain unclear. The pathogen has evolved diverse lifestyles to infect and obtain nutrients from the host plant. The early infection stage is called biotrophic phase. This is during which the pathogen grows asymptomatically in the host tissue that will be followed by a necrotrophic phase during which host cell death is induced. As *Z. tritici* involves both biotrophic and necrotrophic phase, it is considered to be hemibiotrophic pathogen (Ziming *et al*., 2017; Xin *et al*., 2018).

With regard to dissemination, *Z. tritici* can survive for several years in the form of vegetative strands (mycelium) and pycnidia in wheat residues. The sexual stage is very essential in the disease cycles. The sexual ascospore enables the pathogen to disperse to the next year by staying on crop residues (María *et al*., 2012). Sexual spores (ascospores) from pseudothecia and asexual spores (pycnidiospores) from pycnidia are released and dispersed by wind or rain splash during the wheat-growing season and can initiate infections under favorable environmental conditions (Fig.2; Kidane *et al*. 2017). A combination of wind and rain provides the most favorable conditions for spread of this disease within crops. *Z. tritici* requires more than 24 hours of wetness and is most destructive between 10 and 20°C. In Argentina, the relative abundance of *M. graminicola* ascospores becomes higher during the two growing seasons (June to December) (María *et al*., 2012). Similarly, in Ethiopia STB disease severity becomes higher during wheat growing seasons June to November and the disease distribution is strongly influenced by

temperature and other environmental conditions (Tewodros *et al* 2016).

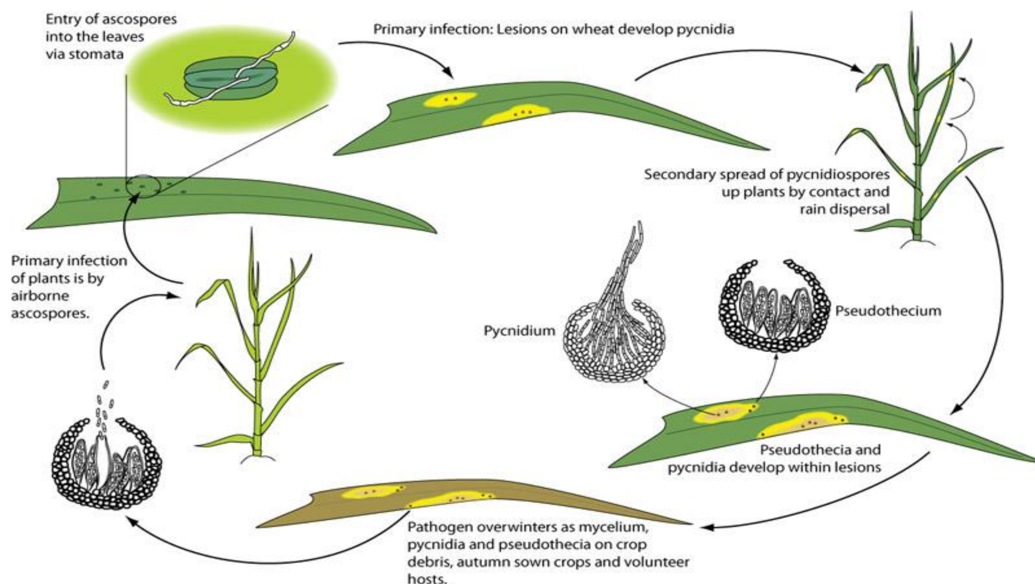


Figure 2: Life cycle of *Z. tritici* (Source: Kidane et al., 2017)

2.6. Symptom of *Septoria tritici* blotch

Symptoms of *Septoria tritici* blotch are usually detected on the lower leaves and gradually progresses to the flag leaf and also attacks the leaf sheaths (Wolf; 2008). In wet years, the *Zymoseptoria tritici* blotch fungus can move onto the heads and cause brown lesions on the glumes and awns known as glume blotch (Wolf, 2008; Ponomarenko *et al.,* 2011).

Although this pathogen affects the plants at any stage of development, the spread of *Septoria tritici* blotch usually decreases as temperatures increase. As a result, *Septoria tritici* blotch won't be more common on lower leaves of plants early in the growing season than on upper leaves (Wolf, 2008; Ponomarenko *et al.,* 2011). Reports show that STB has inverse relation to plant height; very tall wheat varieties are less affected than dwarf wheat varieties (Thomas *et al*., 2013; Xin *at el*., 2018).

The initial symptoms are yellowish or chlorotic flecks on leaves, especially those in contact with the soil. These flecks enlarge into irregular lesions, brown-to-reddish brown in color. As the lesions age, the centers become somewhat bleached with gray or ash-white centers, with small, dark brown to black specks. These are pycnidia or spore producing bodies of the fungus. The presence of small, black pycnidia in lesions is the most reliable in-field character for identifying the disease (Fig.3; Ponomarenko *et al.,* 2011).

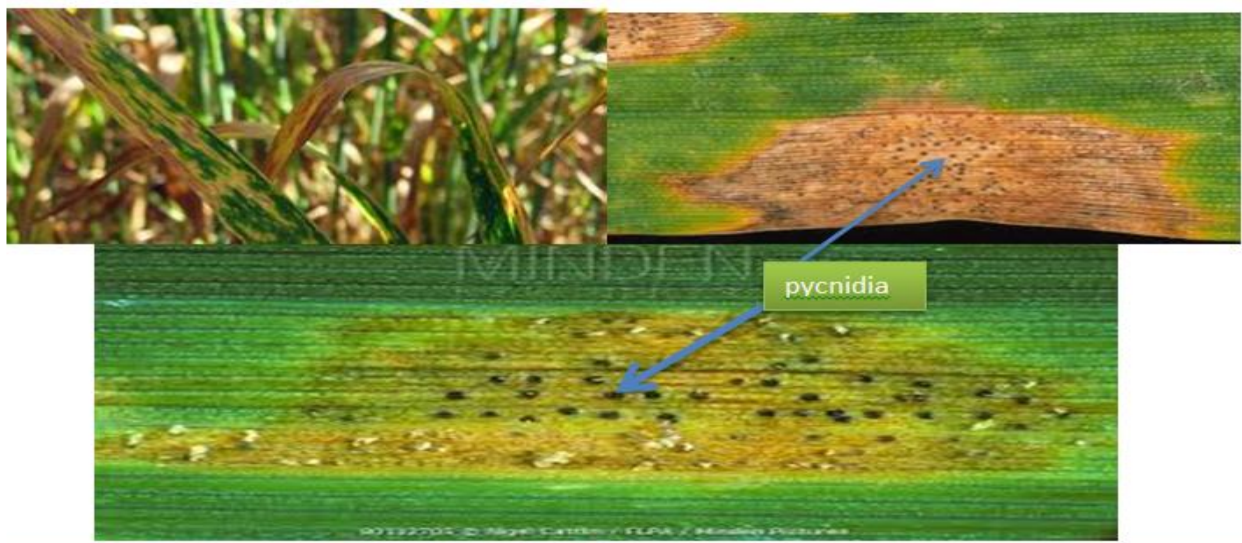


Figure 3: Symptoms of ***Septoria tritici blotch*** on wheat leaf collected from the field. Pychnidia are represented by small black dots lesion on leaf area. **Source: Ponomarenko *et al.* (*2011*).**

2.7. The prevailing weather for *Septoria tritici* blotch development

The impacts of *Septoria tritici* blotch on wheat crops are dependent on the climate change scenarios. Whilst changes in rainfall and temperature would influence STB spread and severity depending on the agro-ecology under evaluation, higher atmospheric levels of CO_2 may boost the development of the disease (Ferjaoui *et al*., 2015; Kidane *et al*., 2017). Similarly, Fanos and Gurr (2015) and Tewodros *et al*. (2016) stated that STB rainfall and crop moisture play a key part in disease dispersal and spore survival. The other important environmental factor which affects the speed of disease development is temperature. During wet and windy seasons the

spread of the fungus becomes rapid, even the dispersion from the lower leaves to the upper leaves becomes high. Whereas, dry weather not only prevents infections, but also slows down lesions development and spore production on hosts of pathogen (Fanos and Gurr, 2015; Tewodros *et al.*, 2016). Hence, it is important to deduce that STB development in wheat is associated with frequent rain and moderate temperatures, cultural practices, availability of inoculum and presence of susceptible cultivars. In Tunisia, the average yield losses increase with weather conditions even 35% up to 60% (Ferjaoui *et al*. 2015; Sameh *et al*. 2013).

According to Suffert *at el*. (2018), ecology critically affects the sexual reproduction cycle of *Zymoseptoria tritici*. The ecological mechanisms include: competition for resources between the two modes of reproduction, and competitive disequilibrium between the two parental isolates, due to differential interaction dynamics with the host. Disease severity of 30 to 45% was maximizing offspring (ascospores) number, and its eco-evolutionary consequences are considered by environmental effect. Cultural practices are very effective in the *Zymoseptoria tritici* distribution, for example, early sowed wheat in the UK were more affected than late planted crops. Sowing of wheat in autumn, around September, increased the challenge of *Zymoseptoria tritici* (Fanos and Gurr, 2015).

2.8. Impact of *Z. tritici* on wheat production

Understanding of the interaction between the fungus and its host constrains are very essential, because we can optimize STB disease control strategies (Fanos and Gurr, 2015). STB is globally distributed, difficult to control because populations contain extremely high levels of genetic variability (McDonald *et al*., 2015). The disease is important in warm and humid wheat growing areas of the world especially in the coastal areas of the Mediterranean, South America, highlands

of East Africa, Australia and Western Europe (Saari and Wilcoxson, 1974; Rajaram and Dubin, 1977).

Globally, wheat yield losses of 30 – 54% (Eyal *et al.,* 1987), >60% (Shipton *et al.,* 1971) have been attributed to *Septoria* diseases (Eyal *et al.,* 1987) with the diseases affecting yield by causing reduced tiller, poor seed set, poor grain fill or shriveled kernels, death of leaves, spikes or the entire plant (van Ginkel *et al*., 1999; Simón *et al*., 2002; Goodwin, 2007; Ponomarenko *et al*., 2011). The cost incurred due to STB in wheat is high. For instance, the three European states known with their higher wheat production France (26%), Germany (17%) and UK (8.5%) register higher loss due to STB, losses due to these usage per annum of €800 to 2400 m (Fanos and Gurr, 2015). In Ethiopia, although it needs detailed studies, *Septoria* leaf blotch was found to be among the most wheat destructive disease in the major wheat growing areas of the country (Abera *et al*., 2015). Hailu and Woldeab (2015) studies revealed that *Septoria* leaf blotch prevalence, incidence and severity around the south west and west shewa are becoming very high problem of wheat production in Ethiopia are show in Table 1 below.

Table 1: Distribution of SLB of wheat in South West and West Shewa zones

Zone	Woreda	Altitude (m.a.sl)	*Septoria leaf* blotch		
			Prevalence (%)	Incidence (%)	Severity (%)
South west Shewa	Ameya	1896-2006	100	87	45
	Bacho	2161-2293	100	80	76
	Dawo	2148-2306	100	82	76
	Seden Sodo	2292-2413	100	83	54
	Wolisso	1967-2415	100	100	76
	Wenchi	2100-2817	100	78	77
West Shewa	Chelia	2383-2870	100	90	65
	Dendi	2227-2497	100	64	84
	Toke kutaye	2247-2413	100	100	54
	Ambo	2073-2679	100	70	35
	Grand mean	1967-2817	100	83	66

Source: Hailu and Woldeab, (2015)

According to Teklay *et al.* (2015), most of the high-yielding wheat cultivars grown in Ethiopia are susceptible to *Septoria tritici* blotch and none of them have been found to be fully resistant. Large numbers of the wheat genotypes (80.9%) grown in West and Southwestern parts of Ethiopia are identified to be vulnerable to *Septoria tritici* blotch and hence categorized within the susceptible to highly susceptible groups (Teklay *et al.,* 2015). Although it needs detailed assessment on yield loss, it is important to state that *Septoria tritici* blotch is critical problem to wheat production in Ethiopia.

2.9. *Zymoseptoria* leaf blotch management

A better understanding of *Septoria tritici* blotch biology, the molecular mechanisms and infection process is crucial to design novel and effective approaches for STB management. To manage *Septoria tritici* blotch, an integrated approach that incorporates variety selection, cultural practice, crop rotation, bio-control and fungicides are the most effective way (Ponomarenko *et al* 2011; David and Pierce, 2016).

Resistant Cultivars: Planting of resistant cultivars is the most economical and environmental friendly approach for managing STB disease. As compared to others, developing resistant cultivars is found to be a very simple approach to manage the disease. Qualitative or quantitative genes were available for resistance to *M. graminicola*. So far, 18 loci associated with *Septoria* resistance genes were identified and incorporated into wheat breeding programs, providing some quantitative resistance to the disease (Fanos and Gurr, 2015). For example, the *AvrStb6* is a virulence effector that facilitates fungal infection in plants lacking the *Stb6* R-gene, but is recognized and activates plant defenses in plants expressing *Stb6* R-gene. However, the effector and virulence functions of the *AvrStb6* protein were not identified (Ziming *et al*., 2017).

In the field, some *Stb* genes have been quite durable while others have failed due to rapid genetic change in the pathogen population where it has even remained effective for more than 25 years (Ponomarenko *et al*., 2011). Reports indicate the resistivity of gene depends on the environment *i.e.,* wheat cultivars reported as resistant in one region have been found to be susceptible in another. This may be connected to the genetic composition of the local pathogen population, which can be affected by cultivars grown, the suitability of the environment for infection, and the relative importance of the sexual stage in the disease cycle (Ponomarenko *et al*., 2011).

Cultural practices: Following *Septoria* outbreak farmers do not sow wheat into infected stubble and avoid early sowing as a high number of ascospores are released early in the season. Moreover, destroying stubble by grazing or cultivation of other crops will reduce the number of spores available to infect the new season's crop. In the absence of resistant wheat varieties, it is a good Z. *tritici* control practices. Such practices will have more effect if undertaken on a locality basis. However, the practice is not practicable in light soil areas where stubble must be retained to prevent erosion (Ponomarenko *et al* 2011; David and Pierce, 2016).

Crop rotation: This is a practice where the wheat field is planted by another crop in the next year. A one-year rotation out of wheat is generally effective to provide a disease break. However, the fungus may survive for over 18 months on stubble during very dry seasons (Ponomarenko *et al* 2011).

Biological Control: Biological controls are currently not available in the commercial production. Fungi belonging to *Trichoderma* spp. have been used previously as bio-control agents to protect wheat plants against leaf spot diseases in Argentina. A collection of *Bacillus megaterium* bacteria spp. originating from the wheat rhizospheres and leaves, barley, oat chaff, and grains

have been screened for their ability to inhibit STB. This bacterium consistently retarded STB development by up to 80% in small-scale field trials (Ponomarenko *et al* 2011). Moreover, Samara *et al.* (2018) have reported lipopeptides from *Bacillus subtilis* (mycosubtilin, M; surfactin, S; fengycin, F) and two mixtures (M + S and M + S + F), and reported that M + S containing lipopeptides are the best protectants and have been found to reduce *Septoria* severity up to 82%.

Chemical control: Some seed-treated with fungicides can suppress early infection and should be used in areas where STB is known to occur. Where necessary, effective foliar fungicide sprays are available. However, it is important to correctly identify *Septoria tritici* blotch before spraying with a fungicide as nutritional disorders such as Aluminum toxicity or Zinc deficiency can be confused with *Septoria tritici* blotch (David and Pierce, 2016).

Nowadays, STB control is becoming the main problem because; due to the repeated emergence of resistance alleles in the pathogen and some fungicides retain high monetary and environmental costs. For all these reasons, breeding for host plant resistance is an appealing perspective to achieve an economical, durable, and environmentally friendly control of STB in wheat fields (Raman and Milgate, 2012; Bruce and Christopher, 2016; Yosef *et al*. 2017).

With regard to the timing of fungicide application, the very best practice to control the disease is early spraying of the fungicide before disease appears. However, if lesions begin to show on the leaves, STB has taken hold and fungicide application will be of limited utility (Ziming *et al.,* 2017). Azoles and Succinate Dehydrogenase Inhibitors (SDHIs) are the main fungicides available for *Z. tritici*.

2.10. Resistance breeding

Septoria tritici is highly diversified because it has high levels of sexual reproduction, large population sizes and long-distance dispersal of the disease. This makes control of STB using genetic resistance more challenging. Breeding for qualitative resistance is not sustainable because it can be broken down relatively quickly due to rapid evolutionary changes. The most sustainable control measures are likely to involve an integrative approach that combines quantitative resistance, multi-target fungicides and agronomical practices that limit the survival of *Zymoseptoria tritici* between growing seasons (Sanchez-Vallet *et al.,* 2017; Thomas *et al*., 2013).

Reports from Tunisia on the management of the *Z. tritici* indicate that application of fungicide could be very important but it has its own limitations because of increased production costs, possible side effects on human and animal health and harm to the environment. As a result, using the host plant resistance has been always regarded as the cheapest, most practical and the least environment damaging control methods for wheat production (Gharbi *et al*., 2000).

Breeding for resistance to STB can benefit greatly from the long history of breeding crops to control other diseases. To aid this, knowledge about genetic diversity in order to identify genes for resistance to *Zymoseptoria tritici* is very important (Fanos and Gurr, 2015). These traits which confer qualitative resistance are controlled by genes which control large fractions of genetic variation. For example, *Stb6* has been shown to control a gene-for-gene relationship. Quantitative resistance is generally controlled by genes with small-to-moderate effects on STB such as *Stb6 and Stb16q* and these are present in several sources (Thomas *et al*. 2013).

Another fundamental requirement for breeding for STB-resistance are genetic diversity for resistance in wheat germ-plasm and a field trial site at which STB epidemics occur regularly and effective selection can be conducted for resistance combined with other desirable traits (Brown e*t al*., 2015).

2.11. Genetic structure of *Zymoseptoria tritici*

Genetic structure refers to the distribution and amount of genetic variation within and among populations. Understanding the genetic structure of a population is useful for development of control strategies. McDonald *et al*. (1995) have stated that pathogen populations that have high levels of genetic variability are more likely to adapt to resistant cultivars than populations with less genetic variability.

Understanding the genetic variation of *M. graminicola* is very important for designing effective management strategies for the deployment of resistant genes. It helps to determine which genotypes predominate within a geographic area (Simon *et al*., 2012; Fanos and Gurr, 2015). In principle, if a population has a high level of pathogenic variation within local populations, such populations would have the potential to adapt rapidly to resistance genes. In such case the use of single major resistance genes will not be effective for controlling the disease (Goodwin *et al*. 1992).

Variation between individuals in a population or between populations in a species, derived from genes and/or environmental effects, can be easily evaluated through the use of a variety of markers. Mostly phenotypic markers such as pathogen physiological reaction and virulence spectrum analysis on a set of wheat differential lines carrying different resistant genes have been used to characterize the genetic structure of pathogen populations. In addition to these phenotypic markers like pycnidia density, total necrotic leaf area with or without pycnidia, latent

period and disease progress have been used to determine genetic variability in pathogen populations. These traits are scored visually, either by eye or by computer aided image analysis (Brown *et al*., 2015). Phenotypic markers, for instance virulence analysis, represent small fraction of the pathogen and are also influenced by the interaction with the host plant and also by the environmental conditions. This implies that morphological markers are less powerful to explain the true genetic variability in the pathogen populations.

The second type of marker used for genetic analysis of populations is the protein or isozymes markers (Scandalios 1969; Brown, 1978; Hamrick *et al.,* 1979). Although protein markers circumvent the effects of environment, they have the drawbacks of a limitation in the number of detectable isozymes as well as tissue and developmental stage specificity. Hence, protein markers provide limited insight into the true genetic diversity of the pathogen population. These all drawbacks necessitated the development and use of DNA marker systems.

DNA marker systems, which were introduced to genetic analysis in the 1980s, have many advantages over the traditional morphological and protein markers that are used in genetic and ecological analyses of plant populations: firstly, an unlimited number of DNA markers can be generated; secondly, DNA marker profiles are not affected by the environment, and, thirdly DNA markers, unlike isozyme markers, are not constrained by tissue or developmental stage specificity (Park *et al*., 2009).

2.11. Molecular tools to study genetic diversity of *Z. tritici*

Advance in molecular biology has come up with the development of different DNA markers useful for genetic analysis of plant pathogen populations. The first generation of DNA marker systems is restriction fragment length polymorphisms (RFLP). The technique involves restriction

digestion of organism genomic DNA followed by southern blotting which also involves hybridization of the DNA fragments with known sequence of DNA (probes). The possible source of fragment size polymorphism could be point mutations in restriction enzyme recognition sites or chromosomal mutations like insertions, deletions, inversions, and translocations. The advantages of RFLPs include: detecting unlimited number of loci and being codominant, robust, and reliable and results are transferable across populations. However, RFLPs are highly expensive, time consuming, labor intensive, larger amount of DNA is required and limited polymorphism especially in closely related lines (Govindaraj *et al*., 2015; Dubcovsky *et al*., 2001).

The second generations of DNA markers for genetic analysis were those derived from PCR polymerase chain reaction (PCR) (Mullis *et al.,* 1986). Single nucleotide polymorphisms (SNPs) and microarrays are considered to be the third generation of molecular markers system (Park *et al.,* 2009). Of the many PCR-marker techniques that have been developed, RAPD, AFLP and SSR are the major systems, with the other systems being modifications of these three (Park *et al.,* 2009). Rapid amplified polymorphic DNAs (RAPDs) were the first of the PCR-based markers. The advantages of RAPDs include being quick and simple and inexpensive and the facts that multiple loci from a single primer are possible and a small amount of DNA is required.

However, the results from RAPDs may not be reproduced in different laboratories and can only detect the dominant traits of interest (Govindaraj *et al*., 2015). Tingey and del Tufo (1993) stated that RAPD has been a marker of choice in studying fungal systematics because of its potential for detecting polymorphism among individuals at various taxonomic levels including species, subspecies, strains and isolates. In line with this Czembor and Arseniuk (1996) used RAPD

marker to determine the genetic similarity of three species, *Septoria tritici, Stagonospora nodorum* and *Stagonospora avenae* f ssp. *triticea.*

The other DNA marker system widely used for fungal plant pathogen study is amplified fragment length polymorphism (AFLPs) that combines both PCR and RFLP (Mohan *et al*., 1997). The method involves restriction digestion of genomic DNA with two different restriction enzymes and then ligating the fragments with specific adaptor sequences. PCR amplification will be carried out using pair of primers having complementary sequence with the adaptor sequence. The PCR products will be separated by gel electrophoresis and hence fragment size polymorphism can be analyzed. Mehdi *et al.* (2008) used AFLP to evaluate the genetic structure of Kansas populations of *M. graminicola* at different spatial scales (micro-plot, macro-plot, and statewide) and noticed that the genetic identities among populations were >98%.

The study also revealed that 98% of the genetic variability of the populations occurred within populations with a corresponding migration rate of 16 to 23 individuals per generation. Similarly, Schnieder *et al.* (2001) studied the population structure and genotypic diversity of *Mycosphaerella graminicola* from six natural field populations in Germany using AFLP marker system. The study revealed presence of a high degree of genotypic diversity of the *M. graminicola* population with lower genetic differentiation (F_{ST} =0.04) in which 96% of the total genetic variations was accounted by the within populations genetic variation.

Another most popular PCR based marker system widely used for genetic analysis of the populations of *Z. tritici* are microsatellites (Mohan *et al*., 1997) also known as simple sequence repeats (SSRs) markers. They are short tandem repeats of two, three, or four nucleotides length (di-, tri-, and tetra-nucleotide repeats) widely dispersed in the genome and common in

eukaryotes (Tautz, 1989; Hamada *et al*., 1982), including those of fungi (Rosewich and McDonald, 1994). Polymorphism among microsatellite loci is mainly due to variation in the number of units. Variation is detected using pairs of primers that target the conserved sequence flanking the microsatellite region and amplify the variable locus (Mohan *et al*., 1997; Govindaraj *et al*., 2015). However, a change in the sequence of the flanking regions can result in null alleles, because the primers will not be able to bind and amplify the microsatellite region. Microsatellites markers are evenly distributed throughout the genome, easily automated, highly polymorphic, are high reproducible and have good analytic resolution than the other marker systems.

In line with this, Powell *et al*. (1996b) compared four genetic marker systems including RFLP, AFLP, RAPD and SSR in an analysis of soybean germplasm and found that SSR markers showed the highest expected heterozygosity (0.60). Likewise, Teulat *et al*. (2000) used AFLP and SSR marker to assess the genetic diversity in 14 coconut *(Cocos nucifera)* populations and confirmed that SSR markers had a greater ability to separate closely related individuals within a population. The SSR genetic tools are easy-to-use, accurate, repeatable, economical, and faster technical strategy can provide useful genetic information for evolutionary inferences concerning *Z. tritici* populations (Angelique *et al*., 2014; Medini and Hamza, 2008).

However, the development of microsatellites requires extensive knowledge of DNA sequences, and sometimes they underestimate genetic structure measurements (Matsuoka *et al*., 2002; Govindaraj *et al*., 2015). Owen *et al*. (1998) isolated and characterized nine single-locus microsatellite markers in *M graminicola.* They also developed specific primers for the flanking regions of these loci. They used these primers to study genetic diversity among 12 isolates of the pathogen collected from a single field at Long Ashton Research Station, England. The number of alleles ranged from two to four with a genetic diversity value between 0.278 and 0.736. Also

Samia *et al.* (2013) used microsatellite markers to assess the genetic diversity of *M. graminicola* at a micro geographical scale in Tunisia, and observed higher genetic diversity that ranged from 0.403 to 0.555 with an average of 0.484. Thus, it can be safely concluded that SSR genetic tools are easy-to-use, accurate, repeatable, economical, and faster the capacity to provide useful genetic information for evolutionary inferences concerning *Z. tritici* populations (Angelique *et al.*, 2014; Medini and Hamza, 2008).

2.12. Measuring molecular variability of a population

The genetic variability of a population can be estimated by measuring gene and genotypic frequencies. Nei (1973) introduced the concept of gene diversity to describe genetic variability in both sexually and asexually reproducing populations. Gene diversity (H) is defined as the probability of obtaining two different alleles at a locus when two haploid individuals are sampled randomly from a population. This 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 when the locus is monomorphic (only one allele per locus). In dominant markers, the value of gene diversity range from 0 to 0.5 and H= 0.5 when the frequencies of the two allele in particular locus are equal. However, in multi-allelic markers like microsatellites, the maximum value for gene diversity will increase with increasing the number of alleles per locus. For instance, if the number of alleles per locus becomes 3, 4 and 5, the gene diversity will become 0.67, 0.75 and 0.8, 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: $G_{ST} = (H_T - H_S) / H_T$

Where, H_T is the total gene diversity of the population, Hs is the average gene diversity within subpopulations and GST is the proportion of the total genetic variation accounted for by variation among sub populations. If the amount of gene diversity within subpopulations is high, but among subpopulations is low, the G_{ST} value will be small and vice versa (Nei, 1973).

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, richness of allelic variants (A), average number of alleles per locus, effective number of alleles (Ae), average expected heterozygosity (He; Nei's genetic diversity), observed, heterozygosity (Ho), Shannon's Information Index (I), and estimate of the deviation from Hardy-Weinberg equilibrium (HWE), rarified allelic richness (Ar), and private rarified allelic richness (Ap).

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.

3. MATERIALS AND METHODS

3.1. Description of study area

The study was carried out at Amhara and Tigray regional states of Ethiopia (Fig.4) collected the samples follow the main road. Both of which represent the high lands of major wheat production areas of Northern Ethiopia with high rainfall (1000-2200mm) and expected to be the suitable environment (hot spot) of the disease (Teklay *et al.* 2015). The regions were located at 14°50’0”N and 41°40’0”E and about 1500-3000 m.a.s.l. The annually average minimum and maximum temperatures are 10°C and 25°C, respectively. The dominant soil type is Vertisols and Vertic Cambisols and slightly acidic. It belongs to the sub humid agro climatic regions.

3.2. Study Area

The study was carried out on *Zymoseptoria tritici* pathogen isolated from *Septoria leaf* blotch infected samples collected from Amhara and Tigray Regional States in northern parts of Ethiopia. The leaf samples were collected in 2017 main cropping seasons. During collection, first zones and then districts known for their wheat production were randomly selected. In this study a total of seven zones (three from Amhara: West Gojjam, North Shewa and North Gondar and four from Tigray: Western Tigray, Central Tigray, Eastern Tigray and South Tigray) were included (Fig. 4).

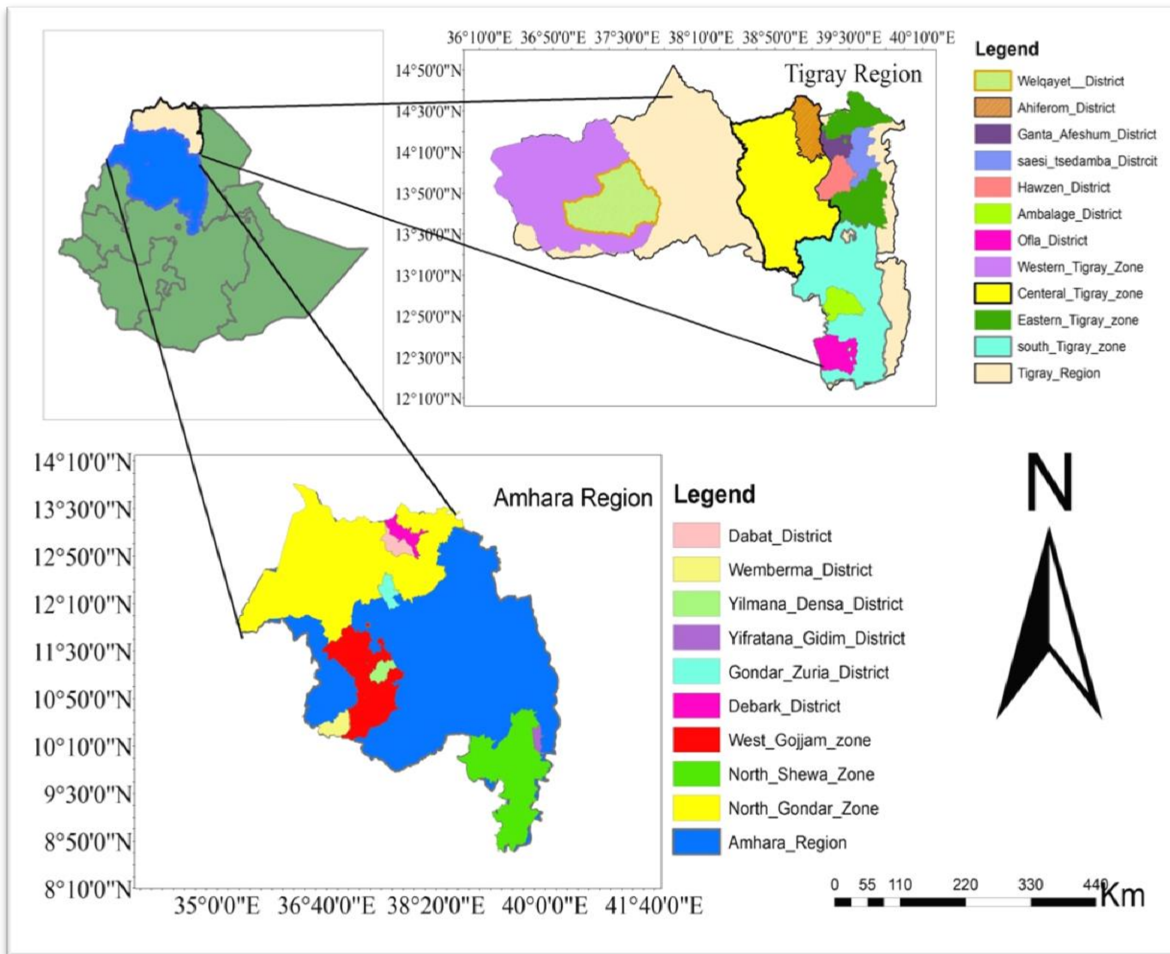


Figure 4: Location of areas in Amhara and Tigray Regional States, northern Ethiopia, from where samples of *Zymoseptoria tritici* were collected.

3.3. Sample collection

The collection was conducted following the main roads and accessible routes in each selected district when the crop growth stage (GS) was on average between the medium milk (60 GS) and early dough (75 GS) stages according to Zadoks *et al*. (1974). During collection, green leaf samples in the field naturally infected with SLB with black spots on the necrosis area were collected using scissors (Fig. 5). To avoid cross contamination, collection items like scissors were swiped with 70% ethanol before proceeding to the next sample collection.

Collected samples were placed in paper envelopes. Totally, 73 samples were collected and these were subjective for study, but the isolated were only 24 samples (Table 2). Each collection envelope was labeled with the following information: collection date, sample code, latitude, longitude, altitude, and disease severity score. After air drying at room temperature, each samples with its envelop was placed in zipped plastic bag and stored at 5°C for 4-5 months.

Figure 5: Collection of *Zymoseptoria tritici* infected wheat leaf samples in the wheat field

3.4. Survey of *Septoria tritici* blotch Infection

During sample collection, the wheat fields were assessed for Septoria disease severity. Depending on the size of the wheat field, three to five stops were made in an "X" pattern (Eyal and Brown, 1976). At each point, Septoria disease severity (SDS) was estimated visually by considering percent of necrotic leaf area on the four uppermost infected leaves of 10 - 20 plants using double digit 00-99 scoring scale (Saari and Prescott, 1975), where the first digit (0-9) represents 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%).

Table 2: Collection sites of wheat leaf samples infected with SLB (*Mycosphaerella graminicola)* in Ethiopia.

Dis	Code	Region	Zone	Woreda	Kebele	Altitude	Latitude	Longitude	00-99
SLB	S1	Tigray	Southern Tigray	Ofla	Hayalo	120 28. 253’	0390 31.309’	2341.778	11
SLB	S2	Tigray	Southern Tigray	Ambalage	Ayiba	120 52. 596’	0390 33. 242’	2779.471	75
SLB	S3	Tigray	Southern Tigray	Ambalage	Adei sheou	120 58.104’	0390 31.612	2571.902	32
SLB	S4	Tigray	Eastern Tigray	Hawzen	Belada	130 49. 261’	0390 36. 254’	2024.482	21
SLB	S5	Tigray	Eastern Tigray	Saesi Tsadamba	Siniketa	140 00. 175’	0390 35. 589’	2420.112	42
SLB	S6	Tigray	Eastern Tigray	GantaAfeshum	Adikineyi	140 15. 250’	039o 20. 401’	2348.789	11
SLB	S7	Tigray	Central Tigray	Ahiferom	Sefeo	140 28. 357’	0390 14. 538’	2162.251	32
SLB	S8	Tigray	Central Tigray	Ahiferom	Inticho Town	140 19. 045’	0390 11. 392’	2166.518	11
SLB	S9	Tigray	Western Tigray	Welqayet	Maycheho	140 19. 045’	0390 11. 392’	2897.429	11
SLB	S10	Tigray	Western Tigray	Welqayet	Maycheho	140 19. 045’	0390 11. 392’	2897.429	11
SLB	S11	Amhara	North Gondar	Debark	Debir	130 10. 716’	0370 53. 722’	2897.429	54
SLB	S12	Amhara	North Gondar	Debark	Debir	130 10. 716’	0370 53. 722’	2897.429	54
SLB	S13	Amhara	North Gondar	Debark	Miga iyesus	130 07. 050’	0370 52. 513’	2767.889	54
SLB	S14	Amhara	North Gondar	Debat	Weken zuria	130 03. 109’	0370 49. 294’	2710.891	64
SLB	S15	Amhara	North Gondar	Debat	Weken zuria	130 03. 109’	0370 49. 294’	2710.891	64
SLB	S16	Amhara	North Gondar	Debat	Weken zuria	130 03. 109’	0370 49. 294’	2710.891	64
SLB	S17	Amhara	North Gondar	Debat	Debat Zurya	120 59. 413’	0370 46. 316’	2629.814	74
SLB	S18	Amhara	North Gondar	Debat	Carbita	120 53. 501’	0370 43. 971’	2723.998	53
SLB	S19	Amhara	North Gondar	Gondor Zuriya	Sihor	120 39. 959’	0370 29. 779’	2380.488	64
SLB	S20	Amhara	North Shewa	Yifratana Gidim	Ber Gibi	120 24. 664’	0370 31. 208’	1923.288	22
SLB	S21	Amhara	Western Gojam	Yilmana Dansa	Adet Zuria	110 17.315’	0370 28. 709’	2210.714	87
SLB	S22	Amhara	Western Gojam	Yilmana Dansa	Adet Zuria	110 17.315’	0370 28. 709’	2210.714	87
SLB	S23	Amhara	Western Gojam	Yilmana Dansa	Adet Zuria	110 17.315’	0370 28. 709’	2210.714	87
SLB	S24	Amhara	Western Gojam	Wemberma	Sebader Abo and Kelo	100 39. 252’	0360 57. 305’	2130.552	86

3.5. Isolation of *Zymoseptoria tritici (Mycosphaerella graminicola)*

The isolation was carried out at Microbial Biotechnology Laboratory, National Agricultural Biotechnology Research Center, Holeta, Ethiopia in 2018. Isolation was conducted following the procedure of Eyal *et al*. (1987) with some modifications. Fig.6 shows the steps followed for *Zymoseptoria* isolation from infected wheat leaf samples. Accordingly, collected leaves with pycnidia were cut into about 10 cm length and placed on sterile filter paper in Petri plates wetted with distilled water. The Petri dishes with specimen were placed in polyethylene plastic bag and then incubated for 3-4 h. at 24°C.

The samples were periodically checked under stereoscopic dissecting microscope (x 40 objective) for the formation of cloudy ooze on the top of pycnidia. When the oozing drops were found to be ready to transfer, the Petri dishes containing the samples and other necessary tools (needles, media plates, stereoscopic microscope, etc.) were placed in the microbial free laminar air flow cabinet. Observing under the stereoscopic microscope, oozing drops were quickly transferred onto potato dextrose agar (PDA) supplemented with 250 mg/l chloramphenicol (ant-biotic for control bacteria developments) using flame sterilized and briefly cooled fine-pointed needle. Inoculated Petri plates were kept at 24°C for 10-20 days in the dark until fungal growth was observed.

Developed pinkish-orange colonies were streaked on new PDA supplemented with chloramphenicol, and then kept at the same conditions for growth. After 10-20days old streaked colonies were developed in to black colors. Then after, single colony was transferred into liquid medium (YSB) composed of 1% (w/v) yeast extract powder + 1% (w/v) sucrose for further spore propagation and for use as inoculums and stored as a stock culture or to isolate genomic DNA.

Cultures were maintained on orbital shaker at 130 rpm for two to three weeks for spore multiplication and a total of 24 isolates were prepared.

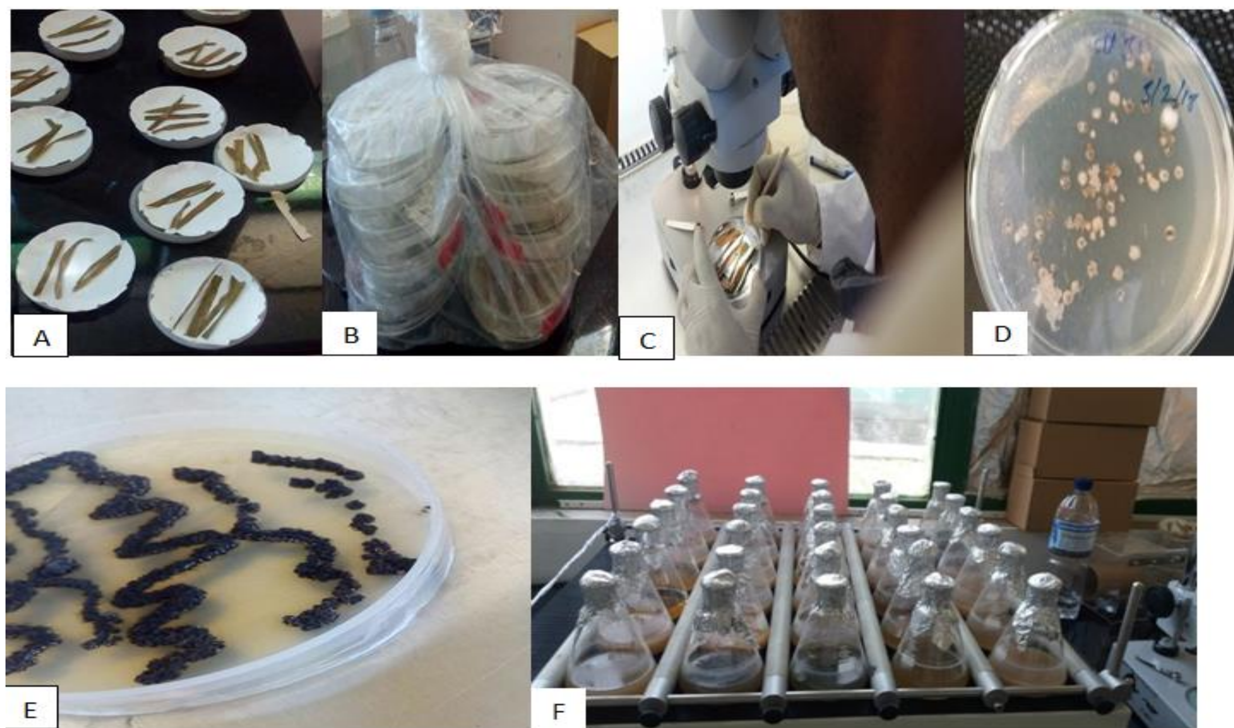


Figure 6: *Zymoseptoria tritici* isolation procedure from SLB infected wheat leaf samples. A) Putting samples on wet filter paper in Petri dish, B) Petri dish with leaf samples incubated at 24°C for 3 to 4 hrs., C) Transferring oozes to PDA using stereoscopic dissecting microscope under sterile cabinet, D) a 5 – 7 days old *Z. tritici* colonies grown from transferred mono-pycnidial oozes E) Pure *Z. tritici* obtained after sub-culturing on PDA developed to black colors, and F) *Zymoseptoria* cultures multiplication in liquid medium (YSB) to be used for DNA isolation and culture preservation.

3.6. Determining the genetic diversity of *Z. tritici* populations

3.6.1. Genomic DNA extraction

Isolates grown in YSB (1% yeast extract + 1% sucrose broth) for two to three weeks were centrifuged at 10,000 rpm for five minute to collect the pellet of spore. The fungal genomic DNA extraction was carried out from spores using plant DNA extraction Protocol described in Diversity Array Technology (DArT) with some modifications (Appendix 1). Extracted DNA quality was checked by loading 5µl DNA + 2 µl of 6xloading dye with gel red on gel electrophoresis at 1% agarose gel and 100 V for 40 minutes (Fig. 7A). The concentration was

checked using Nano Drop Spectrophotometer (ND-8000, 8-sample Spectrophotometer) (Fig. 7B). High molecular weight gDNA was stored at -20°C until use for genotyping.

Figure 7**:** Quality and concentration test of genomic DNA: A) on 1% agarose gel electrophoresis and B) with Nano Drop Spectrophometry.

3.6.2. Molecular based identification of *Zymoseptoria tritici*

Hence, the diagnostic markers were used for PCR based detection or identification of the isolates. The PCR reaction was performed in a volume of 25µl containing 12.50µl (2x Taq plus master mixes), 5.50µl Nuclease free water, 2µl of each of the forward (ITS1) and reverse (JB446) primers and 50 ng template DNA.

The PCR reaction was run in BIO-RAD T100 Thermal cycler. The reaction program involved an initial denaturation at 94°C for 3 minutes followed by 35 cycles with 1minute denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute, and 2 minutes primer extension at 72°C, that was followed by final extension step at 72°C for 10 munities. The PCR amplified products were fractionated in 3% agarose gel electrophoresis by loading 10 µl of each of the PCR product mixed with 2 µl of gel red using 1x TAE buffer at 100V for three hours. The product was visualized under gel documentation system (BioDoc-It^{TM} imaging system) and

subsequently photographed. A 50 bp size marker was used to estimate the size of the amplified products.

3.6.3. Genotyping of *Z. tritici* isolates using SSR markers

The study involved a total of 24 isolates (14 from Amhara and 10 from Tigray regional states) which were grouped into two populations based on their geographical location of collection.

Table 3: SSR markers and their respective primers used for genotyping of *Z. tritici* isolates

Marker	Repeat Motif	Annealing temperature (°C)	Expected Size (bp)	Forward	Reverse
ST1A2 (MGR 7031)	(GGC)7/ (GGT)2	65	67-76	5’CTCTCTCCCGTGCTGTGTTT3’	R-5’CAGACCACCTGCACAGCAT3’
ST1E3 (MGR 7035)	(CGG)5	65	67-70	5’GTTCCGCCGGTCGAAGTCG3’	R-5’GCCAAGGCACTGCTGCTCC3’
ST1E7 (MGR 7038)	(CGG)5	58	85-91	5’GATCTCGAGCAGGGCGGAAGT3’	5’TCACACGCTGGTCTGTGAATC3’
ST1G7 (MGR 7037)	(TG)9	65	90-96	5’ATGCTGAGAAGTTCGGTGAGG3’	5’CGTTCTTCCACCTCCAACACT3’
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’	R-5’AACTCGGCTGGTGGAACA3’
ST1B3 (MGR 7033)	(CGG)8	58		5’CGCGCACTAGTAGACGCTCT3’	5’TCTACCTTAATCCTCACCGCC3’
ST2C10 (MGR 7036)	(AGCGG)4	58	75-87	5’AGGCGAGAAACTTGCTTGCAG3	5’AATGAACGTCCCATGGACGTG3’

3.6.4. Polymerase chain reaction (PCR) Program

The PCR amplification was carried out in 25 µl reaction mixture composed of 2.5 µl 10×PCR buffer, 2.5 µl $MgCl_2$, 1µl of 10 mM dNTPs, 1 µl of 400 nM each of forward and reverse primers, 0.5 µl Taq DNA polymerase (5 Units), 1 µl template DNA (50 ng/ µl) and 15.5 µl nuclease free water using a thermal cycler (PeQ START). The PCR amplification condition was programmed to initial denaturation at 94°C for 3 min followed by 45 cycle of denaturation at 94 ^{o}C for 1minute, optimized annealing temperature of 65 or 58°C for 1minute (Table 3), primer extension at 72°C for 2 minute which was followed by final extension at 72°C for 10 minutes and holding temperature at 4°C forever. PCR product was fractionated in 3% agarose gel electrophoresis

using 1× TBE buffer at 100V for 3 h. The gel was stained with gel red and visualized under UV light and subsequently photographed. A 50 bp DNA ladder was used to determine the amplification size (Fig. 8).

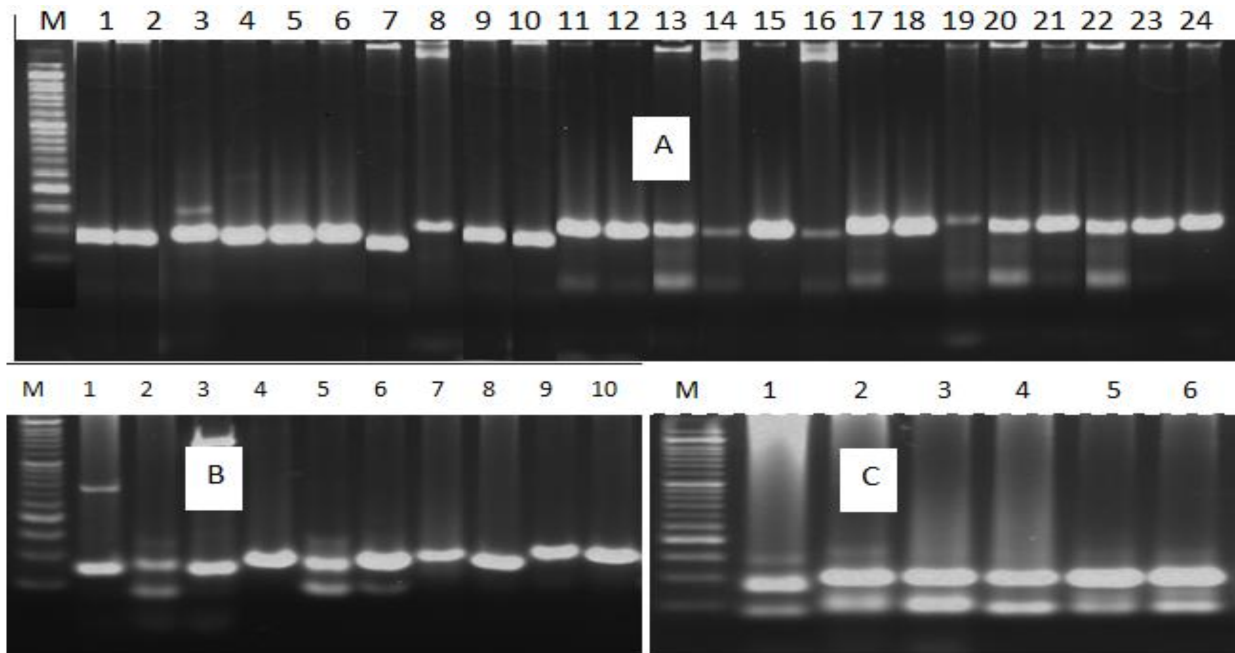


Figure 8: Sample of gel electrophoretic pattern of SSR markers generated from *Z. tritici* isolates collected from Amhara and Tigray regional states of Ethiopia using pairs of SSR primers: A) ST1B3F, B) ST1A4F, and C) ST1G7. Where M is a 50bp size marker and the numbers in lanes1-24 indicates the individual isolates.

3.7. Data scoring and analysis

All the PCR amplified SSR regions fragment size on gel were estimated using PyElph 1.4 software package. Genetic diversity analyses were carried out on the basis of the scored bands. Different 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; Polymorphic information contents (PIC) and heterozygosity were computed using Power marker v3.25 software (Liu and Muse, 2005). Effective number of alleles, Shannon's Information index (I) and Gene flow (Nm) were determined using POPGENE

version 1.31(Yeh and Yang, 1999). Allelic frequency, observed heterozygosity (Ho), expected heterozygosity (He), fixation index (F) and estimate of the deviation from Hardy-Weinberg equilibrium (HWE) over the entire populations were computed with GenAlEx ver. 6.501 software (White and peakall, 2015). The same software package was used to compute population differentiation test: Wrights fixation index (FST) and pairwise FST at 1000 bootstraps. Moreover, rarified allelic richness (Ar) and private rarified allelic richness (Arp) were computed using HP-Rare 1.1 software (Kalinowski, 2005).

Analysis of molecular variance (AMOVA) and estimate of the variance components were conducted using Arlequin ver. 3.5.2.2 (Excoffier and Lischer, 2010). To examine the genetic relationship between the different isolates, a genetic dissimilarity matrix was calculated using Jaccard's formula and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) based Neighbor-Joining tree and hieratical clustering (dendrogram) were generated 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 = 10 using 20 iterations for each K. The optimum K value was predicted following the simulation method of Evanno *et al.* (2005) using the web-based STRUCTURE HARVESTER ver. 0.6.92 (Dent and Bridgett, 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*

In the current study, the PCR based detection of the *Z. tritici* isolates using pairs of diagnostic markers that target the internal transcribed spacer (ITS) sequences within the ribosomal RNA genes (ribosomal DNA) resulted in positive amplification in all the isolates (Fig. 9). The pairs of primers (ITS and JB445) resulted in band size of about 345 bp in all the 24 isolates (Fig. 9). All the band sizes produced were in the expected region and specific to *Z. tritici*. Using the same primer, Beck and Ligon (1995) observed a fragment size of 345 bp in all *Septoria tritici* (*Z. tritici*) while no amplification was detected in non-target fungal species.

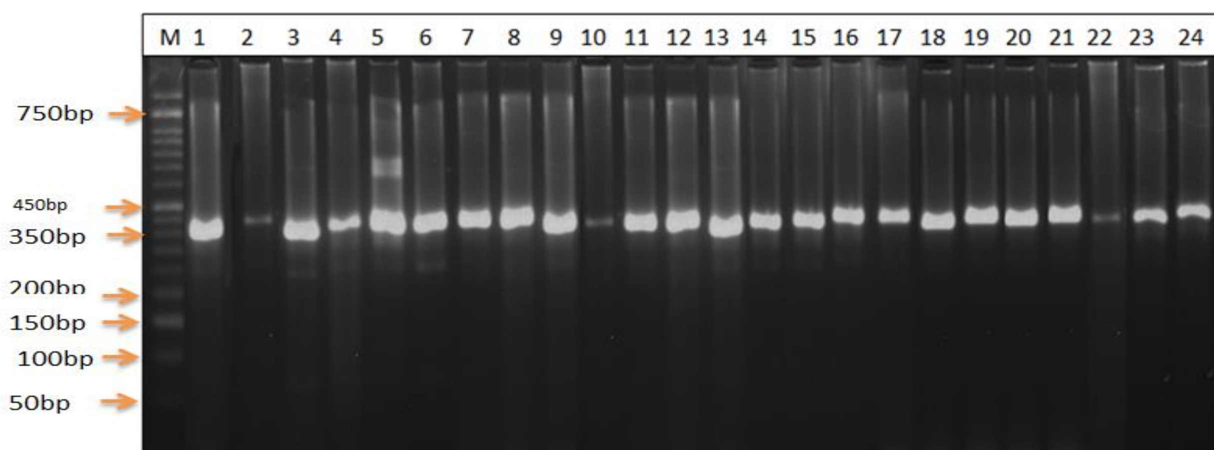


Figure 9: PCR products for molecular diagnostics of *Zymoseptoria tritici*. M=molecular marker, number 1 up to 24 *Z. tritici* isolated samples

4.2. Genetic diversity analysis

4.2.1. Microsatellite markers level of polymorphism

The isolates that were confirmed to be *Z. tritici* with the diagnostic marker were further assessed for their genetic diversity using nine primer pairs (Table 3). The result revealed that the number of DNA fragments detected varied with the marker and the isolate used (Table 4). All the 9 loci were found to be polymorphic and produced a total of 242 bands with an average of 26.9 bands per locus (Table 4). The highest number (43) of bands per locus was recorded for ST1A2 marker,

out of which 32 (74.42%) were polymorphic. ST1A4 resulted in the highest percentage of polymorphic bands (79.17%), whereas ST1G7 gave the smallest percentage (29.17%) of polymorphic bands (Table 4). The percentage of polymorphic bands produced by the other microsatellite markers were 40.74% (ST1E3), 58.33 % (ST1E3), 53.85% (ST2E4), 66.67% (ST1D7), 60.00 % (ST1B3), and 52.00 (ST2C10) (Table 4).

Table 4: Number of polymorphic bands, monomorphic bands and percentage of polymorphic bands produced by each primer, when tested on isolates of *Z. tritici*

Primers	Total no of bands	No Monomorphic bands	No of polymorphic bands	Percentage of polymorphic bands
ST1A2	43	11	32	74.42
ST1E3	27	16	11	40.74
ST1E7	24	10	14	58.33
ST1G7	24	17	7	29.17
ST2E4	26	12	14	53.85
ST1A4	24	5	19	79.17
ST1D7	24	8	16	66.67
ST1B3	25	10	15	60.00
ST2C10	25	12	13	52.00
TOTAL	242	101	141	58.26

The study resulted in a total of 133 alleles (average 14.80 alleles per locus), out of which 82 (61.7%) were scarce (frequency between 0.01 and 0.05) (Table 5). The frequency of 25 (18.78%) alleles was between 0.05 and 0.1, whereas 26 alleles (19.55%) had a frequency of 0.1 or higher (Table 5).

Table 5: Summary of the number of alleles with their respective frequencies

Markers	Number of alleles with frequency			
	Scarce (0.01 - 0.05)	0.05 - 0.1	0.1 or higher	Total
ST1A2	26	3	0	29
ST1E3	4	3	4	11
ST1E7	7	4	3	14
ST1G7	3	2	2	7
ST2E4	10	0	4	14
ST1A4	13	4	1	18
ST1D7	7	3	4	14
ST1B3	6	4	4	14
ST2C10	6	2	4	12
TOTAL	82	25	26	133
Percentage	61.65	18.80	19.55	

The analysis showed that the major allele frequency within population ranged from 0.58 (ST1A2) to 0.08 (ST1G7) with mean frequency of 0.21 per locus (Table 6). The highest gene diversity (0.96), allelic richness (7.86), private allelic richness (6.64), polymorphic information content (0.95), number of alleles (29), effective number of alleles (23.04) and Shannon's Information Index (3.25) were recorded for the microsatellite marker ST1A2 (Table 6).

On the other hand, the highest major allele frequency (0.58) and the lowest gene diversity (0.63), allelic richness (4.98), polymorphic information content (0.60), number of alleles (7.00), effective number of alleles (2.67), and Shannon's information index (1.39) were observed for ST1G7 (Table 6). The study also revealed that all the markers were highly informative with the PIC ranging from 0.60 (ST1G7) to 0.95 (ST1A2) (Table 6). The highest gene flow (9.364), observed heterozygosity (0.931), expected heterozygosity (0.764) and the lowest fixation index (0.181) were observed for ST1A2 (Table 6). With regard to test for the Hardy Weinberg equilibrium, 8 (88.89%) of the markers showed highly significant ($p<0.0001$) deviation from HWE. Only one marker (ST1A2) showed non-significant ($p=0.284$) deviation from HWE (Table 6).

Table 6: Informativeness and levels of different diversity indices of the SSR loci across populations of *M. graminicola* collected from Amhara and Tigray regions.

Marker	N	MAF	GD	Ar	Arp	PIC	Na	Ne	I	Fst	Nm	Ho	He	P_{HWE} [a]	F
ST1A2	24.00	0.08	0.96	7.86	6.64	0.95	29.00	23.04	3.25	0.026	9.364	0.764	0.931	0.284	0.181
ST1E3	24.00	0.25	0.86	6.20	3.69	0.84	11.00	7.11	2.14	0.072	3.241	0.150	0.808	0.000***	0.826
ST1E7	24.00	0.13	0.91	5.47	4.18	0.91	14.00	11.52	2.53	0.067	3.460	0.000	0.849	0.000***	1.000
ST1G7	24.00	0.58	0.63	4.98	4.05	0.60	7.00	2.67	1.39	0.227	0.853	0.000	0.522	0.000***	1.000
ST2E4	24.00	0.23	0.87	5.47	3.33	0.86	14.00	7.57	2.3	0.042	5.718	0.071	0.831	0.000***	0.915
ST1A4	24.00	0.13	0.93	7.11	5.55	0.93	18.00	15.16	2.81	0.039	6.204	0.000	0.899	0.000***	1.000
ST1D7	24.00	0.17	0.91	5.64	4.31	0.90	14.00	11.08	2.52	0.054	4.365	0.000	0.859	0.000***	1.000
ST1B3	24.00	0.13	0.91	6.50	4.57	0.91	14.00	11.41	2.52	0.047	5.067	0.050	0.871	0.000***	0.943
ST2C10	24.00	0.17	0.88	5.66	3.59	0.87	12.00	8.29	2.26	0.056	4.252	0.050	0.831	0.000***	0.939
Mean	24.00	0.21	0.87	6.10	4.43	0.86	14.80	10.872	2.413	0.070	4.725	0.121	0.822		0.867

Where MAF = Major allele frequency, N= Number of isolates, NA= Number of alleles, Ne = Effective number of alleles, GD= Gene diversity, Ar = Allelic richness, Arp = Private allelic richness, Ho = Observed heterozygosity, He = Expected heterozygosity, Fst = Inbreeding coefficient within subpopulations relative to total (genetic differentiation among subpopulations), Nm = Gene flow estimated from Fst= 0.25(1- Fst)/Fst, PIC = Polymorphic information content, I = Shannon's Information Index, F = Fixation Index, P_{HWE} [a] = *P*-value for deviation from Hardy Weinberg equilibrium, ns = not significant, * = $P < 0.0001$ and hence highly significant.

4.2.2. Genetic variability within and among the populations

Summary of the different genetic diversity indices over the nine markers for the two populations are presented in Table 7. The comparative analysis showed that there is no much difference among the two populations of *Z. tritici* with regard to genetic diversity indices including similar number of alleles, effective number of alleles, private allelic richness, Shannon's information index, observed heterozygosity, expected heterozygosity, percentage of polymorphic loci and fixation index (Table 7). Comparatively, the populations of Amhara scored greater values in number of alleles (9.57), effective number of alleles (7.62), private allelic richness (6.22), and fixation index (0.87) (Table 7).Whereas, expected heterozygosity in Tigray population is higher than the population of Amhara and also from the mean. However, both populations showed same value in observed heterozygosity (0.12) and percentage of polymorphic loci (100) (Table 7).

Table 7: Summary of different population diversity indices averaged over the 9 loci for each population

Population	N	Na	Ne	Arp	I	Ho	He	PPL	F
Tigray	10	8.56	7.30	5.22	2.03	0.12	0.85	100	0.86
Amhara	14	9.57	7.62	6.22	1.98	0.12	0.79	100	0.87
Mean	12	9.06	7.46	5.72	2.00	0.13	0.82	100	0.87

N= Number of samples, Na= Number of alleles, Ne = Effective number of alleles, Arp = Private allelic, I = Shannon's Information Index, Ho = Observed heterozygosity, He = Expected heterozygosity, PPL= Percentage of polymorphic loci, F = Fixation Index.

4.2.3. Measure of population differentiation and gene flow

The analysis revealed that the greatest (82%) genetic variations in *Z. tritici* was due to individuals within population (Table 8), which could be resulted due to the heterozygosity of the individuals within each population. Among populations and within individuals variations accounted for 5% and 13% of the total genetic variations, respectively. The overall Wright's *F*

statistic also known as the fixation index value, used as a measure of population differentiation was very small (Fst= 0.047 which is < 0.05) (Table 8).

Table 8: Analysis of molecular variance of 24 isolations of *Z. tritici* genotypes grouped in to population based on their geographical location

Source of Variation	DF	Sum of Square	Mean Square	Est. Variance	% Variance		Fixation index	*P* value
Among Population	1	12.019	12.019	0.196	5%	Fst	0.047	0.001
Among individuals (within population)	22	163.564	7.435	3.447	82%	Fis	0.864	0.001
Within individuals	24	13.000	0.542	0.542	13%	Fit	0.871	0.001
Total	47	188.583		4.185	100%			

4.2.4. Measures of Genetic identity and Genetic distance between the populations and the individuals

The analysis showed that the genetic distance between the populations was 1.0, whereas, the Nei's genetic identity between the populations was 0.4. The pairwise genetic distance between samples ranged from 0.0 to 0.5 and the highest genetic distance of 0.5 was observed between sample 10 (From Tigray) and sample 16 (from Amhara) (Table 9). The next higher genetic distance value of 0.34 was observed between sample 17 and 20 which were from Amhara regional state.

Table 9: Dissimilarity matrix among 24 *Z. tritici* isolates using Darwin

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
2	0.06																						
3	0.11	0.05																					
4	0.06	0.08	0.05																				
5	0.05	0.07	0.04	0.33																			
6	0.05	0.07	0.03	0.23	0.22																		
7	0.00	0.02	0.00	0.00	0.00	0.00																	
8	0.06	0.22	0.05	0.08	0.07	0.07	0.02																
9	0.04	0.08	0.02	0.06	0.05	0.04	0.13	0.08															
10	0.02	0.07	0.01	0.05	0.04	0.03	0.07	0.07	0.13														
11	0.03	0.08	0.02	0.05	0.04	0.04	0.12	0.08	0.33	0.12													
12	0.01	0.06	0.00	0.04	0.03	0.02	0.06	0.06	0.12	0.22	0.11												
13	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.07	0.17	0.06	0.22											
14	0.04	0.09	0.03	0.07	0.06	0.05	0.14	0.09	0.22	0.14	0.22	0.12	0.07										
15	0.05	0.10	0.04	0.07	0.06	0.06	0.09	0.10	0.15	0.18	0.15	0.17	0.12	0.16									
16	0.04	0.09	0.02	0.06	0.05	0.04	0.08	0.09	0.14	0.50	0.14	0.23	0.18	0.15	0.20								
17	0.01	0.06	0.00	0.04	0.03	0.02	0.06	0.06	0.12	0.17	0.11	0.15	0.10	0.13	0.17	0.18							
18	0.00	0.05	0.00	0.02	0.01	0.00	0.04	0.05	0.10	0.30	0.10	0.19	0.14	0.11	0.16	0.31	0.14						
19	0.04	0.09	0.03	0.06	0.05	0.04	0.08	0.09	0.14	0.17	0.14	0.16	0.11	0.15	0.33	0.18	0.16	0.14					
20	0.02	0.07	0.01	0.05	0.03	0.03	0.07	0.07	0.13	0.18	0.12	0.16	0.11	0.13	0.18	0.19	0.34	0.15	0.17				
21	0.02	0.07	0.01	0.04	0.03	0.02	0.06	0.07	0.12	0.17	0.12	0.16	0.11	0.13	0.18	0.18	0.20	0.14	0.16	0.21			
22	0.02	0.07	0.01	0.04	0.03	0.03	0.06	0.07	0.12	0.17	0.12	0.16	0.11	0.13	0.18	0.19	0.20	0.14	0.17	0.21	0.33		
23	0.00	0.05	0.00	0.02	0.01	0.00	0.04	0.05	0.10	0.15	0.10	0.14	0.09	0.11	0.15	0.16	0.33	0.12	0.14	0.32	0.18	0.18	
24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.10	0.04	0.08	0.03	0.06	0.10	0.11	0.15	0.07	0.09	0.16	0.13	0.13	0.13

4.2.5. Cluster analysis and population structure

These analyses resulted in three clusters (C1, C2 and C3) (Fig. 10). Both C1 and C2 were further grouped into two sub-clusters designated as i and ii (Fig. 10). The first two major clusters (C1 and C2) are composed of individuals from the two regional states. The third cluster is composed of only two samples from Amhara population. Although there could be considerable intermixing, sub-clustering resulted in the individuals to be grouped according to their geographical regions of collection (Fig. 10). Sub-clustering of the major cluster C1 gave two groups (one composed of isolates from Amhara regional state only and the second group made of isolates from both regions). Similarly, sub-clustering of C2 resulted in a group composed of isolates from Tigray regional state and a second group made of isolates from the two populations (Fig. 10).

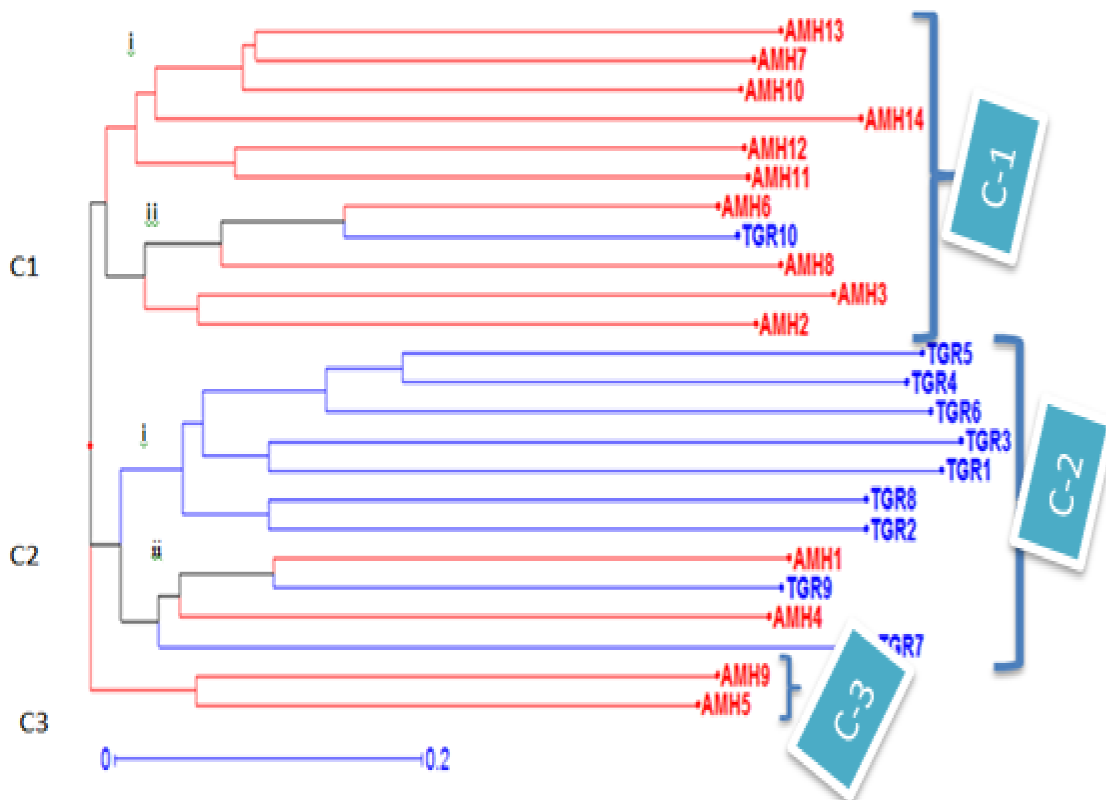


Figure 10: UPGMA dendrogram for 24 *Z. tritici* isolates based on the Jaccard's coefficient as revealed using 9 microsatellite markers. The name Amh1-14 stands for isolates collected from Amhara regional state, and TGR1-10 shows isolates from Tigray.

The principal coordinate analysis (PCoA) also confirmed the presence of poor population structure in the pathogen populations and roughly clustered the isolates into three clusters (C1, C2 and C3) (Fig. 11). The first three principal ordinate axes explained 24.27 % of the total variation (Fig. 11). The long standing issue in population genetics is the identification of genetically homogeneous groups of individuals. In this regard, a recent Bayesian algorithm implemented in the software STRUCTURE allows the identification of such groups. The algorithm allows estimating the true number of clusters (K) in a sample of individuals under study. The analysis revealed that maximum ΔK (which is good indicator of the true number of clusters) becomes peak both at K = 4 and K= 9 (Fig 12A). In such cases it should be aimed for the smallest value of K that captures the major structure in the data (Pritchard *et al.*, 2000). Accordingly, K = 4 is found to be the most likely number of clusters to assign the 24 *Z. tritici* isolates. Based on this value, Clumpak result (bar plot) showed wide admixtures and hence there was no clear geographic origin-based structuring of populations (Fig.12B).

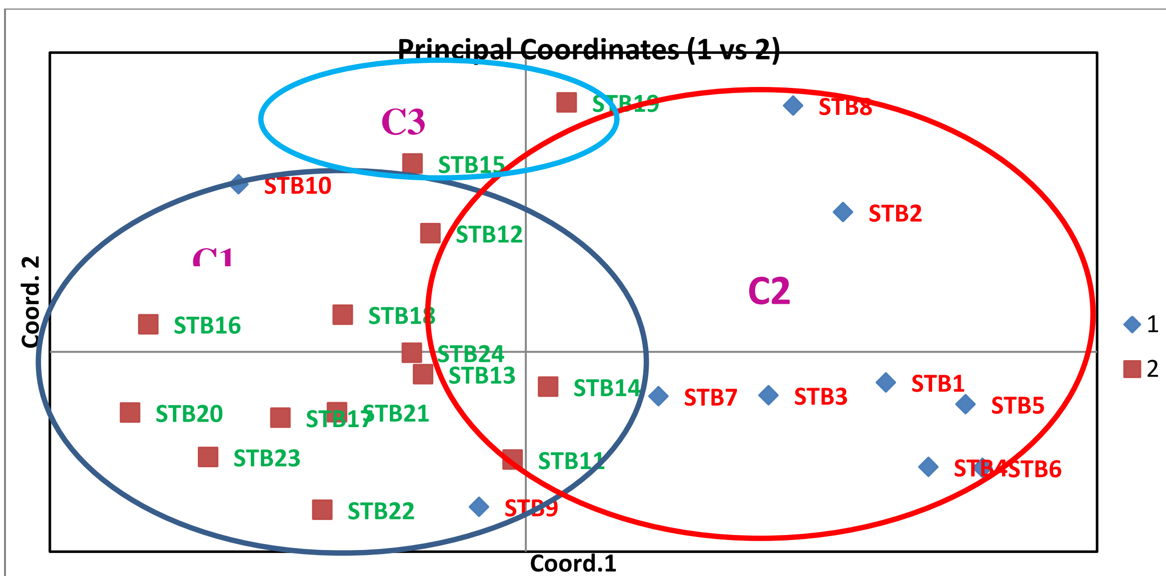


Figure 11: Principal coordinates analysis (PCoA) bi-plot showing the clustering pattern of 24 *Z. tritici* isolates from the two populations (1= Tigray and 2= Amhara populations). Samples coded with the same symbol and colors belong to the same population. Note: The percentages of variation explained by the first 3 axes (1, 2 and 3) are 14.1, 9.6, and 8.9%, respectively.

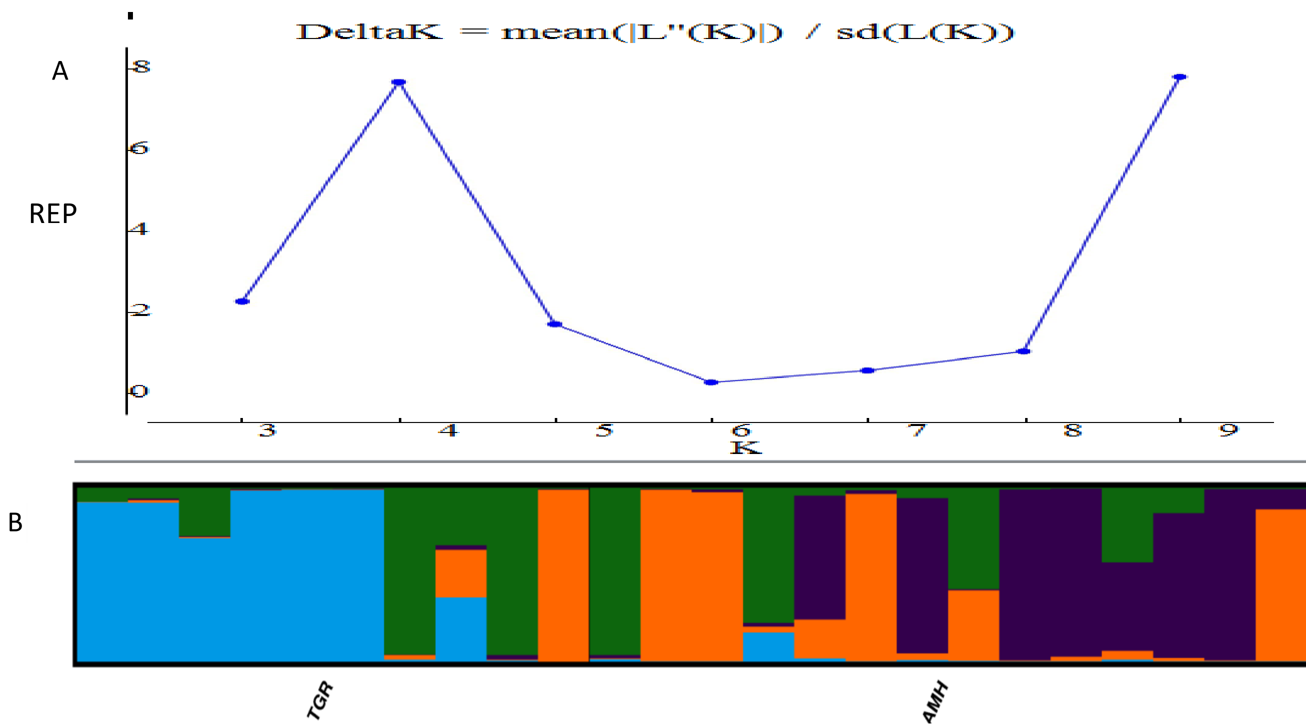


Figure 12: Inferred population structure of 24 *Zymoseptoria tritici* isolates: A) Δ K value estimated using Evano *et al* (2005) method (highest peaks at K=4 and K= 9), (and B) Bayesian model-based estimation of population structure (K = 4) where each color represent a different cluster, and the length of the colored segment shows the genotype's estimated proportion of membership in that cluster as calculated by STRUCTURE. The population names are indicated below the Figure.

5. DISCUSSION

Septoria tritici blotch (STB) of wheat caused by the fungal pathogen *Z. tritici* occurs in all wheat-growing areas world-wide, with an increasing economic impact over the last decades (Eyal ,1999). Currently, the fungus represents a major economic concern for global wheat production causing 20-50% yield loss (Thomas *et al*. 2013). Survey studies revealed that *Septoria tritici* blotch is becoming a serious threat to wheat production in Ethiopia (Hailu and Woldeab, 2015). Presently, the disease incidence and severity is increasing in major wheat growing areas of the country. Hence, correct identification, knowledge of the pathogen genetic diversity and population structure has paramount importance in designing integrated diseases management strategies. Hence, the present work reports the molecular based identification, genetic diversity analysis and population structure of *Z. tritici* populations of Northern Ethiopia.

Accurate identification of the pathogen is a basis for precise prediction of disease and also essential for precise timing in fungicide applications to get the best control effects (Fraaije *et al*., 1999). Complementing the traditional microscopic and culture based techniques, in recent years molecular techniques are widely used for early and precise detection and quantification of plant pathogens (Miller and Martin, 1988). Detection using specific primers basically involves the amplification, using specific primers, of a specific region of the genome that contains phylogenetic information (Liew *et al.,* 1998). Regions of the ribosomal RNA genes (rDNA) from the nucleus more specifically the ITS regions have great potential as targets in molecular-based assays for the characterization and identification of fungi (Iwen *et al.,* 2002).

In the present study, the use of the *Z. tritici* specific diagnostic markers (ITS1 and JB446) successfully amplified a 345 bp fragment (specific to *Z. tritici* species) from all the 24 isolates. As the primer pairs are species specific and can result in the same amplification size in *Z. tritici,*

it confirms that our isolates were possibly *Z. tritici*. Similarly, using the same primer pairs, Beck and Ligon (1995) have observed a fragment size of 345 bp in all *Septoria tritici* species (*Z. tritici*), while no amplification was detected in non-target fungal species. Hence, it is possible to reason that the present studies successfully identified *Z. tritici* isolates collected from Northern parts of Ethiopia. So far limited effort has been made to isolates and characterizes *Z. tritici* pathogen of wheat in Ethiopia and probably this could be the first report in the study area. Hence, further sequence based identification is important to confirm the present finding.

Microsatellite or SSR markers are a 1-6 base long repeats that are scattered throughout the genome and preferably used for genetic analysis due to their abundance, highly polymorphism, reproducibility, co-dominant inheritance (Park *et al.,*2009) and simple assays using agarose gel electrophoresis (Singh *et al.,* 2010). In the present study, the average polymorphic bands per locus for the entire population is 58% which is similar to the report of Medini and Hamza (2008) who observed 57% polymorphic bands from 42 isolates of *M. graminicola*, collected from Tunisia, Algeria, and western Canada.

The mean number of alleles (14.80) and gene diversity (0.87) per locus (Table 5) reveled by the current study are indicated to high diversity within the population. Samia *et al.* (2013) reported, using similar SSR markers, they described average of 2.5 alleles and 0.485 genetic diversity per locus for 45 isolates of *M. graminicola* in Tunisia. But, our result indicated to the highest genetic diversity within the population per locus. The present average genetic diversity (0.87) is also significantly higher as the previous investigations on one single field derived average genetic diversity of 0.49 and 0.441 for 12 UK isolates (Owen *et al.,* 1998) and for 90 Canadian isolates (Razavi and Hughes, 2004 b), respectively. The same authors also explained that the high genetic

diversity in *M. graminicola* could be due to genetic recombination during the sexual life cycle of the pathogen.

Hence, the high percent of polymorphism, number of alleles and gene diversity coupled with higher value of polymorphism information content (0.86) observed in the present study indicates the great potential and in-formativeness of the markers for use in genetic analysis of *M. graminicola* to deliver relevant information that helps to design suitable and sustainable management strategies to control the problem. The study revealed that except for ST1A2 (p = 0.284), 88.89 % of the loci showed significant difference between observed and expected heterozygosity in which all of them showed excess heterozygosity that led to a significant departure from HWE across populations. Such excess heterozygosity is expected in sexually reproducing organisms that can maintain their heterozygosity through sexual recombination, or if other factors such as natural selection pressure.

Moreover, the study revealed that the mean Nei's genetic diversity (0.87), Shannon's information index (2.413) and expected heterozygosity (0.82) were very high confirming the presence of higher genetic vibration within the populations. In fact, no clones were obtained and each isolate showed a unique haplotype. Both populations were genetically highly diverse and showed almost similar genetic diversity parameters including genetic diversity, expected heterozygosity, percent of polymorphic loci, and fixation index.

The high genetic diversity of the pathogen might have originated due to the presence of various source of resistance in the host plant that forced the pathogen to evolve to overcome host resistance (Zhan *et al*., 2002). In line with this Medini and Hamza (2008) stated that the exclusive and widespread use of major resistance genes to control *Septoria leaf* blotch is likely to

lead to the rapid emergence of new virulent strains in the pathogen's population. Sexual recombination is considered to be the major source of genetic variation in the pathogen population (Zhan *et al.*, 1998) which provides optimum conditions for the emergence of new virulence and/or fungicide resistance alleles. Most efficient and sustainable management strategy involves the deployment of moderately resistant cultivars (Gharbi *et al.*, 2000).

Another fundamental measure of genetic diversity is allelic richness (the number of alleles in a sample). The average allelic richness observed in the current study is very high (6.10) indicating higher evolutionary potential of the pathogen population. The study also indicated that both populations experience higher private alleles: Tigray (5.22) and Amhara (6.22) confirming a certain level of independent evolution of their gene pools that allowed maintenance of private alleles at a population level.

Analysis of Molecular Variance (AMOVA) revealed that *Z. tritici* has very low genetic differentiation among populations, it accounted only 0.196 or 5% of the total genetic variations (4.185). The high proportion (95 %) of within population genetic variations could be attributed to sexual recombination, spontaneous mutation and possibly as yet undescribed teleomorph ascospores of *M. graminicola* (Samia *et al*., 2013). Similar results were reported by previous investigations, using 75 *Z. tritici* isolates of Iran, and Dalvand *et al.* (2018) reported an intra-population and inter-population genetic diversities of 69% and 31%, respectively.

In a study carried on *M. graminicola* populations of Tunisia, Algeria, and Canada, Medini and Hamza (2008) have reported that the within population genetic diversity (Hs=0.315) accounted for 87.5% of the total genetic diversity (HT = 0.360). The study also revealed the presence of lower genetic differentiation among populations of *Z. tritici* ($F_{ST=}$ 0.045). Basically, fixation

index ranges from 0 (indicating no differentiation between the overall population and its subpopulations) to a theoretical maximum of 1; and it can be considered as small (0 to 0.05), moderate (0.05 to 0.15), large (0.15 to 0.25) or very high (>0.25). The smaller genetic differentiation could be due to high gene flow (Nm = 4.725) through long miles movement of the ascospores by wind, germplasm exchange through marketing etc.

The UPGMA based clustering confirmed the existence of weak genetic differentiation in *Z. tritici* populations indicating the genetic composition of the pathogen population is weakly correlated with their geographical isolation. This could be due to high within-population diversity (95%), and significant gene flow (Nm=4.73) which could have prevented genetic isolation and differentiation of putative geographically separated populations (Schnieder *et al.,* 2001). The sexual ascospores have the potential for long distance travel (Shaw and Royle, 1989) and thus, could have contributed to the genetic exchange between spatially distant populations (Schnieder *et al.,* 2001). Populations genetic structure analysis also revealed the presence of weak sub-structuring (K=4) of the two populations of *Z. tritici* with high potential of admixture. Both populations possessed alleles from the four clusters confirming the presence of gene flow between the populations that resulted in less population differentiation.

6. CONCLUSION

The species specific diagnostic marker based identification confirmed that all the isolates were found to be *Z. tritici*. All the markers were highly polymorphic (100%) and informative (PIC) to describe the genetic diversity and population structure of the pathogen. The study also revealed existence of high genetic diversity in the *Z. tritici* isolates of Northern Ethiopia, where the major (95%) of the genetic variation is due to the within population genetic differences. The study also showed the presence of high and relatively similar genetic variation between Tigray and Amhara *Z. tritici* populations. It also implies that both regions can serve as potential hot spot for wheat germplasm screening and other resistance evaluation experiments against the pathogen. UPGMA based cluster analysis showed the existence of weak genetic differentiation in the *Z. tritici* populations because of the high genetic exchange or gene flow between geographically distant populations. Therefore, it is possible to conclude that the present study has successfully identified *Z. tritici* isolates of Northern Ethiopia and disclosed their genetic diversity and population structure. The study also revealed that the pathogen has broad genetic base and thus can change its virulence spectrum that can result in frequent resistance breakdown on the host side. Such base line information is very relevant for wheat breeders and pathologists to design and implement integrated management strategies to control or minimize yield loss due to *Septoria tritici* blotch which ultimately contributes to an increased and stable wheat production and productivity to address current food security challenges.

RECOMMENDATION

- Because of the limited financial resources for sample collection the present study was delimited to two regions: Amhara and Tigray regional states. Thus, I strongly recommend similar studies to be carried on other major wheat growing areas of the country.
- The study involved diagnostic marker based identification and microsatellite based analysis of the pathogen genetic diversity. Thus, further sequence based identification of genetic diversity analyses would be more informative.
- The study revealed that the pathogen has high genetic variability and broad gene pool that provides it the capacity to overcome newly introduced genes that lead to fast break down of resistance. Hence, integrated disease management strategies shall be applied to control or minimize yield loss caused by *Septoria tritici* blotch of wheat.
- As the disease is economically very important in the northern parts of Ethiopia, the pathogen physiological race and virulence spectrum analysis and durable resistance breeding shall be targeted to boost wheat production and productivity of the regions.

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APPENDIXCES

Appendix 1: Plant DNA Extraction Protocol for Diversity Array Technology (DArT)

BUFFER STOCK SOLUTIONS EXTRACTION

EXTRACTION BUFFER STOCK to make 500 ml:

- ✓ 0.35 M sorbitol 31.9 g sorbitol
- ✓ M TrisHCl pH 8.0 50 ml 1M TrisHCl pH 8.0 h
- ✓ 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) sodium di-sulfite (= sodium meta-bisulfite)
- ✓ 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 of working solution:

- Add 0.6 g sodium di-sulfite (= sodium metabisulfite)

- 2.4 g PVP-40 (K29-32)
- Add 50 ml extraction buffer stock and dissolve;
- Add 50 ml lysis buffer stock and
- Sarcosyl stock

For example to make 30 ml:

- Add 0.15 g sodiumdisulfite (= sodium metabisulfite)
- 0.6 g PVP-40 (K29-32)
- 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 1.5 ml Sarsted tubes:

- ➢ Single spore derived colony multiplied in liquid media on orbital shaker at 130 rpm were collected
- ➢ Centrifuge at 10000rpm for 5 min and discard the supernatant
- ➢ The pellets were collected and sink into liquid nitrogen to dried the pellets
- ➢ Aliquot 6 ml of freshly prepared preheated to 65ºC well mixed “fresh buffer solution” and place tubes to the 65ºC incubator or water bath
- ➢ Grind required amount (same across all samples) of fungi spore in mortar and pestle under liquid nitrogen to fine powder

- Suspend powder in 6 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 6 ml of chloroform : isoamyl alcohol (24 : 1) mixture and mix well for 30 min
- Spin 20 min for 10000X RTM
- Transfer water phase to fresh tube, add same volume of ice cold isopropanol and invert tube ~ 10 times, nucleic acids should become visible
- Spin 10000 RTM for 30 min
- Discard supernatant and wash pellet with 1 ml 70 % EtOH
- Discard EtOH, dry pellet and dissolve in 100 µl of nuclease free water
- Check DNA quality and quantity on 1% agarose gel

Appendix 2: Numbers of allele in each locus

Locus	Allele	Tigr	Amh	Locus	Allele	Tigr	Amh	Locus	Allele	Tigr	Amh	Locus	Allele	Tigr	Amh	Locus	Allele	Tigr	Amh
primer1	52	0.100	0.000	primer2	60	0.100	0.000	primer4	66	0.000	0.143	primer6	90	0.100	0.000		170	0.100	0.000
	56	0.050	0.000		63	0.150	0.071		67	0.200	0.857		100	0.100	0.000		174	0.100	0.000
	59	0.050	0.000		65	0.100	0.071		77	0.100	0.000		101	0.100	0.000		178	0.000	0.071
	60	0.100	0.000		67	0.200	0.143		80	0.300	0.000		102	0.100	0.000	primer8	80	0.100	0.000
	62	0.100	0.000		70	0.100	0.071		82	0.100	0.000		103	0.100	0.071		82	0.200	0.000
	63	0.050	0.000		71	0.200	0.071		84	0.200	0.000		104	0.100	0.071		84	0.100	0.000
	64	0.100	0.036		78	0.050	0.000		89	0.100	0.000		109	0.000	0.071		86	0.150	0.000
	66	0.050	0.107		80	0.000	0.429	primer5	54	0.000	0.036		112	0.100	0.000		88	0.000	0.071
	67	0.100	0.071		82	0.000	0.143		66	0.000	0.036		114	0.000	0.071		90	0.000	0.143
	68	0.000	0.071		84	0.050	0.000		73	0.200	0.000		115	0.100	0.071		92	0.100	0.143
	70	0.000	0.036		88	0.050	0.000		79	0.300	0.179		116	0.100	0.000		94	0.100	0.071
	71	0.000	0.036	primer3	80	0.100	0.000		80	0.000	0.071		117	0.100	0.071		96	0.100	0.071
	73	0.000	0.036		85	0.100	0.000		81	0.000	0.071		120	0.000	0.071		98	0.000	0.214
	74	0.000	0.036		91	0.100	0.000		82	0.000	0.071		123	0.000	0.071		100	0.100	0.143
	76	0.000	0.036		93	0.300	0.000		83	0.100	0.000		124	0.000	0.214		102	0.000	0.071
	79	0.000	0.071		96	0.100	0.071		84	0.100	0.143		125	0.000	0.071		107	0.000	0.071
	89	0.050	0.000		98	0.200	0.071		86	0.000	0.036		130	0.000	0.071		121	0.050	0.000
	91	0.000	0.071		100	0.000	0.071		87	0.100	0.286		131	0.000	0.071	primer9	77	0.050	0.000
	93	0.050	0.000		102	0.000	0.143		88	0.000	0.071	primer7	129	0.000	0.143		80	0.100	0.000
	95	0.000	0.036		104	0.000	0.143		89	0.100	0.000		132	0.000	0.071		84	0.300	0.000
	97	0.100	0.000		106	0.100	0.071		93	0.100	0.000		136	0.000	0.143		86	0.100	0.000
	99	0.000	0.036		108	0.000	0.214						139	0.100	0.143		88	0.100	0.214
	103	0.050	0.036		114	0.000	0.071						143	0.000	0.143		90	0.200	0.143
	105	0.000	0.036		116	0.000	0.071						146	0.200	0.000		93	0.100	0.214
	109	0.000	0.071		118	0.000	0.071						150	0.200	0.143		95	0.000	0.143
	111	0.000	0.036										154	0.000	0.071		99	0.050	0.000
	114	0.050	0.036										158	0.100	0.000		109	0.000	0.143
	121	0.000	0.071										161	0.000	0.071		120	0.000	0.071
	134	0.000	0.036										166	0.200	0.000		151	0.000	0.071