GENETIC DIVERSITY, POPULATION DIFFERENTIATION AND
INTRACHROMOSOMAL RECOMBINATION INFERRING
OUTCROSSING IN NATURAL POPULATIONS
OF SCLEROTINIA SCLEROTIORUM

By

RENUKA NILMINI ATTANAYAKE KITHUL-PELAGE

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Department of Plant Pathology

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of RENUKA NILMINI ATTANAYAKE KITHUL-PELAGE find it satisfactory and recommend that it be accepted.

________________________________
Weidong Chen, Ph.D., Chair

________________________________
Frank M. Dugan, Ph.D.

________________________________
Dennis A. Johnson, Ph.D.

________________________________
Tobin L. Peever, Ph.D.
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GENETIC DIVERSITY, POPULATION DIFFERENTIATION AND INTRACHROMOSOMAL RECOMBINATION INFERRING OUTCROSSING IN NATURAL POPULATIONS OF SCLEROTINIA SCLEROTIORUM

Abstract

by Renuka Nilmini Attanayake Kithul-Pelage, Ph.D.

Washington State University
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Chair: Weidong Chen

The main focus of this research was on the genetic diversity, population differentiation and evolutionary potential of the plant pathogenic fungus, Sclerotinia sclerotiorum. Populations isolated by distance evolve separately and might display great genetic and phenotypic differentiation. Two S. sclerotiorum populations obtained from canola fields in China and USA were genotyped using eight microsatellite markers and phenotyped for the production of oxalic acid, sclerotia and mycelium, sensitivity to fungicides benomyl, fluazinam and iprodione, and virulence. Significant population differentiation was observed for the genotypic and phenotypic traits except for virulence and fluazinam sensitivity. Half of the Chinese population was resistant to benomyl. Cross resistance between benomyl and iprodione was detected. Virulence was highly influenced by the environment, as evidenced by low heritability. Results suggest that even for a cosmopolitan pathogen like S. sclerotiorum, strict regulations on global pathogen movements should be applied.
The objective of the second project was to quantify genetic and phenotypic diversity of *S. sclerotiorum* from soil on a fine geographic scale. A high level of genetic and phenotypic diversity was observed among 40 isolates from 1 m² soil in an alfalfa field. Five genetic clusters were found and recombination was detected within a subpopulation.

In view of the high genetic diversity in soil, it was hypothesized that relatively lower genetic diversity is present in the infected alfalfa plants due to selection by host. The genetic diversity of soil populations sampled at the beginning of the season was compared with the stem populations sampled near the end of the growing season using microsatellite markers. Both stem and soil populations were equally diverse and not differentiated suggesting host selection is minimal or less important in the alfalfa- *S. sclerotiorum* patho-system.

Recombination among presumably unlinked markers has been previously detected in *S. sclerotiorum* populations. Tests of pair-wise Linkage Disequilibrium (LD) between physically linked markers in this study revealed that, LD decayed with increasing distance between markers on two supercontigs for most of the populations. Results provide new evidence of frequent outcrossing in natural populations of *S. sclerotiorum*. 
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Dedication

This dissertation is dedicated to my parents, uncle and aunt.
Preface

The title of this dissertation is “Genetic diversity, population differentiation and intrachromosomal recombination inferring outcrossing in natural populations of Sclerotinia sclerotiorum.” I used the term “natural population” to differentiate them from laboratory controlled populations. This dissertation has five chapters with the first chapter giving a general introduction and a brief overview of each research project followed by references. The title of the second chapter is “Genetic and phenotypic divergence of the Sclerotinia sclerotiorum populations infecting canola from China and USA.” The third chapter titled “Genetic and phenotypic diversity and random association of DNA markers of isolates of the fungal plant pathogen Sclerotinia sclerotiorum from soil on a fine geographic scale” was accepted by the “Soil Biology and Biochemistry” and formatted according to the journal requirements. The fourth chapter is titled “Genetic comparison of inoculum populations with infection populations of Sclerotinia sclerotiorum within alfalfa fields.” The fifth chapter is on “Intra-chromosomal recombination suggests outcrossing in natural populations of Sclerotinia sclerotiorum.” Citations and references in the general introduction, chapters two, four and five are in the same format.
CHAPTER ONE
GENERAL INTRODUCTION

1.1. *Sclerotinia sclerotiorum* and white mold

*Sclerotinia sclerotiorum* (Lib.) de Bary is a devastating fungal plant pathogen of more than 400 plant species worldwide including economically important crops such as potato, canola, soybean, alfalfa, pea, chickpea and lentil (54). The disease is known as Sclerotinia rot, stem rot, cottony rot, drop, white mold, white rot or crown rot depending on the crop and the infection type. Early events in pathogenicity of *S. sclerotiorum* involve oxalic acid production and secretion of cell wall degrading enzymes (13, 30). General symptoms include necrotic water soaked lesions covered with whitish mycelia. Necrotic tissues acquire a bleached and papery appearance as the tissues dry out. The pathogen produces recalcitrant survival structures, known as sclerotia, on or inside the infected plant tissues. Up to 95% of sclerotia remain viable in soil for two years (66) and some can remain viable in soil for about eight years (25). Sclerotia can germinate myceliogenically or can produce apothecia and ascospores via carpogenic germination. Carpogenic germination of sclerotia requires physiological maturation or preconditioning (60). Adequate moisture within crop canopies and cool temperatures (4-20°C) trigger dormant sclerotia to initiate a sexual cycle and release ascospores (60). Air-borne ascospores land on a new host to start a new disease cycle. However, long distance ascospore movement is limited by plant canopy and a majority of ascospores get deposited around a 100 m radius (6, 60) or even 3-4 km from the source (17).

1.2 Economic importance and disease management

*Sclerotinia sclerotiorum* causes extensive crop damage and resistant cultivars are currently not available for many crops. The annual average economic loss due to white mold in
the USA is estimated to be about $200 million (8). In 1994, Sclerotinia stem rot was ranked as the second most important soybean disease in the USA (67). Yield loss due to white mold on soybean could be up to 100% (32). One of the main constraints of canola production in North Dakota is white mold and the disease incidence was up to 14% causing $94 million loss during the period 1991-2002 (18). Due to economic impact of *S. sclerotiorum*, the National Sclerotinia Initiative was established by the US Department of Agriculture with the goal of coordinating research strategies to minimize the devastating effects of this pathogen on a variety of crops, including canola, lentils and peas. Fungicide application and cultural practices are important in controlling the disease. In the US Pacific Northwest (PNW), fungicides iprodione, dichloran, quintozene and fluazinam are registered for stem rot on potato and labels recommend that fungicide applications be made prior to row closure (33). For Sclerotinia stem rot of canola, azoxystrobin, boscalid, thiophanate-methyl and vinclozolin are currently registered in the USA (9). Iprodione is recommended on canola in Canada (9) and in China (42), and its potential use within the USA has been discussed (9). In the US dry pea and lentil cultivation systems, no chemical control is currently applied to control Sclerotinia white mold (W. Chen, personal communication). Depending on the weather, white mold could be a serious disease on alfalfa in the US PNW. However, no fungicides are currently applied on alfalfa to control the disease in the US PNW (L. Porter, personal communication).

1.3 Current understanding of population genetics of *Sclerotinia sclerotiorum*

*S. sclerotiorum* is one of the most extensively studied plant pathogens. Genetic variation, population genetics, ecology and molecular biology of *S. sclerotiorum* are subjects of many studies and numerous reviews (7, 8, 19, 30). Its genome sequence is available (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.html).
Current understanding of population genetic structure of *S. sclerotiorum* is that both clonal and recombining populations do exist around the world (1, 3, 5, 17, 24, 26, 31, 35, 46, 57). Early population genetic studies of *S. sclerotiorum* reported clonal population structure (1, 17, 26, 35). However, frequent outcrossing in *S. sclerotiorum* has been reported in recent population genetic studies from several crops worldwide despite its homothallic nature (3, 5, 24, 31, 46, 57). Inference of outcrossing in this homothallic fungus is largely based on random association of independent markers, and observation of random association could not be explained by migration and mutation alone. Atallah et al. (3) and Sexton et al. (57) discussed possible mechanisms of recombination. Atallah et al. (3) suggested that microconidia may act as spermatia, similar to other closely related genera. Sexton et al. (57) suggested that sclerotia with two different genotypes may produce recombinant ascospores. Ford et al. (22) reported the formation of recombinant ascospores from carpogenic germination of stable heterokaryotic sclerotia under laboratory conditions suggesting possible parsexual recombination in *S. sclerotiorum*. It has been suggested that *S. sclerotiorum* populations found in areas with mild winters could be frequently recombining in contrast to the populations found in areas of extreme winters (30). Even though not yet proven, it can be hypothesized that there is a gradual change from clonal to recombining population structures along a geographic cline ranging from strong winters to subtropical climate. Observations of clonal populations in Alaska and Canada and recombining populations in the Columbia Basin in the US PNW and in other countries might support this hypothesis.

### 1.4 Population structure and differentiation

Knowledge on population genetics of plant pathogens including *S. sclerotiorum* has largely improved due to the recent development of molecular biology and bioinformatics tools.
Molecular markers such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), microsatellite markers (Simple Sequence Repeats) and Single Nucleotide Polymorphism (SNPs) and several bioinformatics tools (a list of bioinformatics tools used in population genetics can be found at http://linkage.rockefeller.edu/soft/) have been developed during the past two decades and extensively used in population genetic studies of many plant pathogenic fungi (44, 48) including *S. sclerotiorum*. SNPs and microsatellite markers have been extensively used in many recent studies with the availability of high throughput analysis. Microsatellites are stretches of DNA that consist of tandem repeats of 1-6 bp and are frequently found in the non-coding regions of eukaryotic genomes (27).

Most natural populations are genetically substructured (4). Genetic structure of a population is defined as the amount and distribution of the genetic diversity within a population (45). Reliable measures of population differentiation and genetic structure are important in applying disease management, conservation biology and in population genetics studies (4). Genetic differentiation between populations has traditionally been quantified using Wright’s F-statistics using allele frequencies (68). F-statistics use inbreeding coefficients to describe the partitioning of genetic variation within and among populations. The most widely used F-statistic is *F*$_{ST}$ or fixation index. *F*$_{ST}$ is a measure of the degree of inbreeding within a subpopulation relative to the total population (all the subpopulations combined). It is calculated as,

\[
F_{ST} = \frac{H_t - H_s}{H_t}
\]

where, $H_s$ is the expected heterozygosity of the subpopulation and $H_t$ is the expected heterozygosity of the total population. For a bi-allelic locus considering two subpopulations, the *F*$_{ST}$ value would be zero if the two subpopulations have identical allele frequencies, whereas it
will be one, if two populations were fixed for different alleles. Nei (50) redefined the fixation index for multiple alleles and termed it as $G_{ST}$. $G_{ST}$ uses the average expected heterozygosity in the total population over loci and the average expected heterozygosity within subpopulations over loci (29). A similar measurement, $\theta$, was introduced by Weir and Cockerham (64) using the analysis of variance frame work taking the sample size differences into account and is suitable for many “real life” situations. A step wise mutation model (SMM) was introduced by Slatkin (58) for microsatellite markers and the pair-wise population differentiation index was termed as $R_{ST}$.

$R_{ST}$ is defined as (58),

$$R_{ST} = \frac{S - S_W}{S}$$

where, $S$ is the average squared difference in allele size between all pairs of alleles and $S_W$ is the average sums of squares of the differences in allele size within each population.

An improvement of the method of Weir and Cockerham was proposed by Excoffier et al. (20) to estimate population differentiation using a hierarchical analysis of molecular variance (AMOVA) following the analysis of variance (ANOVA) framework. To avoid confusion with other statistics, this statistic has been termed $\Phi$. In this modified estimate, hierarchical analysis of molecular variance is estimated from a matrix of squared distances between all pairs of haplotypes (20). The hierarchical analysis model incorporates a nested design and calculates variances within populations, among populations within groups and among groups. Different levels of the $\Phi$ statistics are available depending on the nested design (20). However, Jost (34) recently pointed out that the relative differentiation estimates, such as all the $F_{ST}$ type analyses do not estimate the real differentiation and introduced an absolute differentiation estimate, $D$.

Traditionally the $F_{ST}$ type measures were developed for systems/markers with low diversity, and
for such systems these estimates are adequate and can range from zero (no differentiation) to one (complete differentiation). Using a simulation study, Jost (34) pointed out that even though subpopulations are completely differentiated, $G_{ST}$ approaches zero (instead of one) if the gene diversity is very high in each population. Instead, Jost’s $D$ increases gradually with the increasing subpopulation differentiation and approaches near one. However, Jost’s $D$ may not be suitable for microsatellite loci since it is not based on the SMM. Each of these estimates has its own pros and cons and the researcher has to decide which one would best answer the research question at hand.

Understanding the driving forces that shape genetic structure of a population is important in both theoretical and applied research. In crop plants, population genetic structures and diversity is determined mostly by humans since farmers make decisions about cultivar selection and crop rotation (45). In the case of pathogen populations, a combination of factors, such as host resistance, cultural practices and control measures, environmental fluctuations, pathogen mode of reproduction, recombination rate and dispersal of propagules, genetic drift, mutations, population bottleneck and selection, influence the genetic structure. According to Fisher’s fundamental theorem, in a challenging environment with frequent fluctuations, populations with less genetic variation for fitness traits are vulnerable to extinction (45, 51). For pathogens, populations with high diversity are the most difficult to control since they have a great potential to adapt to new and challenging environments often created by farming practices (45).

1.5 Recombination and genetic diversity

As mentioned earlier, *S. sclerotiorum* could have clonal and/or recombining population structures. In a homothallic, haploid fungus like *S. sclerotiorum*, the homothallism (self-mating) is functionally equivalent to clonal reproduction. High genetic diversity has also been reported
by many researchers. Genetic variation is created by mutation and genotypic variation by recombination (11). Mutations are the ultimate source of introducing new alleles. Most mutations occur during DNA replication. Nucleotide substitutions, indels, DNA slippage and proof reading errors are the most common forms of mutations that occur at the nucleotide scale (40). Gene conversion, transposable elements mediated mutation, chromosomal brakeage and chromosomal non-disjunction (failure of chromosome pairs to separate properly during meiosis) are some forms of mutations that occur at the chromosomal level (29).

Recombination increases genotypic diversity via random shuffling of genes creating novel combinations of alleles in each generation. Recombination can be in two forms: sexual and parasexual. Parasexual cycles in fungi start with stable heterokaryon formation, which is the formation of colonies with hyphae containing different nuclei. Some haploid nuclei in the heterokaryon can fuse to form diploid nuclei at a low frequency. Mitotic crossover takes place even at a lower frequency during the haploidization (56). Theoretically, recombination can take place at any time when haploids form via diploid nuclei (56). Even though evidence of parasexual recombination has only been reported in a few fungal species under natural and laboratory conditions, the significance and relative importance of this kind of recombination in nature is not clear (15, 47, 53).

Genetic recombination via sexual reproduction remains the most important and common form of recombination in eukaryotes. Therefore, genetic recombination is the hallmark of sexual reproduction, manifested through random assortment of independent alleles and crossover between homologous chromosomes during meiosis (47). Meiotic recombination is extensively studied using classical genetic approaches in ascomycetous fungi including Sordaria spp., Saccharomyces spp. and Neurospora crassa (51). Sexual development and production of
ascospores in filamentous ascomycetes is regulated by a single genetic locus known as \textit{MAT}.

\textit{MAT} locus consists of two idiomorphs. The term “idiomorph” was introduced as an alternative to the term “alleles” due to the lack of sequence similarities between them (64). The two idiomorphs of the \textit{MAT} locus are designated as \textit{MAT1-1} with an open reading frame (ORF) that encodes a protein with an alpha box motif and, \textit{MAT1-2} with an ORF that encodes a protein with a high mobility group motif (HMG) (64). Heterothallic fungi possess single \textit{MAT} locus within an isolate whereas homothallic fungi have both of the \textit{MAT} loci within a single isolate. In the \textit{S. sclerotiorum} genome, both \textit{MAT1-1} and \textit{MAT1-2} idiomorphs coexist at the same \textit{MAT} locus. Malvárez et al. (43) studied nearly 300 isolates of \textit{S. sclerotiorum} and reported the presence of both \textit{MAT} idiomorphs in every isolate. Recently, however, Chitrampalam et al. (14) reported \textit{MAT1-1} primers failed amplifying \textit{MAT1-1} idiomorph in up to 50% of the isolates and mating type loci segregated in sibling ascospores of \textit{S. sclerotiorum}, suggesting some isolates of this fungus may not be truly homothallic.

1.6 Detection of recombination in natural populations

Detection of the reproduction mode and the recombination rate in natural pathogen populations is important in epidemic management. Fungi possess diverse mechanisms of reproduction from solely asexual, as seen in many plant pathogenic fungi, to obligatory out-crossing, as seen in the cedar apple rust resulting in clonal or highly diverse recombining population structures (47). However, the existence of extreme asexual organisms contradicts the evolutionary theory that sex is essential for generating genetic diversity. Supposedly, clonality reduces genetic diversity, hampering an organism’s ability to respond to frequently fluctuating environments and results in accumulation of deleterious mutations (23). Many plant pathogens maintain a mixed mode of reproduction with both sexual and asexual cycles (45). Tibayrenc et
al. (64) suggested that clonal genetic structure in natural populations does not imply that sexuality in the organism is completely absent and that population genetic approaches, rather than direct observation of sexuality are desirable in detecting sexual recombination. Therefore, an important research question would be how to infer random mating in a natural population (47).

Several indirect methods are available to infer random mating at the population level. One of the simplest methods is to search for a lack of association between neutral genetic markers and phenotypic markers as observed in some populations of Cryphonectria parasitica, Mycosphaerella graminicola (47) and S. sclerotiorum (57). Pair-wise linkage disequilibrium (LD) test (a detailed description of LD can be found in the next section) is another measure of detecting random association. Kohli and Kohn (36) detected random association among alleles in a population of S. sclerotiorum using pair-wise LD tests. The multilocus Index of Association ($I_A$) has been used to test deviation from random association of alleles among loci in many plant pathogens (47, 64). Ideally, purely asexual organisms with a clonal reproduction mode would be under perfect LD whereas a random mating population is expected to be in linkage equilibrium. Gandolfi et al. (23) searched for evidences of recombination in three taxonomic groups of ancient asexual organisms, arbuscular mycorrhizal fungi (Glomales), bdelloidea (rotifers) and darwinuloidea (crustacean ostracods) and, recombination was inferred by the reticulations in networks and low levels of LD (23).

Recently, approaches based on statistical models have been developed for DNA sequence data to detect the rate of recombination. Also known as model-based inference, these methods are based on coalescent theory and could be computationally intensive (62) and are reviewed by Stumpf and McVean (62).
1.7 Linkage Disequilibrium (LD)

Clonal reproduction results in LD in populations. LD is defined as “non random association of alleles among loci”. The term is quite misleading since the loci could be in LD even though they are physically unlinked and therefore the term “gametic disequilibrium” has been introduced to avoid confusion with physiological linkage (28). Conversely, loci in linkage may exhibit equilibrium. Mathematically, LD for a bi-allelic system is defined as the deviation of observed frequency of haplotypes from the expected frequency as (28),

\[ D_{ij} = x_{ij} - p_i q_j \]

where, \( x_{ij} \) is the observed haplotype frequency with \( i^{th} \) and \( j^{th} \) alleles and \( p_i \) is \( i^{th} \) allele frequency and \( q_j \) is \( j^{th} \) allele frequency and \( p_i q_j \) is the expected haplotype frequency. Fisher’s exact test is used to test the statistical significance of LD. The main disadvantage of the above LD estimate is its allele frequency dependence. Lewontin (39) normalized the estimate as,

\[ D'_{ij} = D_{ij}/D_{max} \]

where, \( D_{max} \) is the maximum value for \( D_{ij} \) with the given allelic frequency. However for multiallelic loci, simple LD estimation is not possible. Different coefficients have been proposed for measuring the extent of overall disequilibrium among all possible pairs of alleles between multiallelic loci (28). Most common measures are \( D' \) and \( r^2 \) (21). Hedrick’s \( D' \) for the total disequilibrium across multiallelic loci is defined as,

\[ D' = \sum_{i=1}^{k} \sum_{j=1}^{l} p_i q_j |D'_{ij}| \]

where, \( D'_{ij} \) is Lewontin’s \( D \) and \( p_i \) is \( i^{th} \) allele frequency and \( q_j \) is \( j^{th} \) allele frequency. Hedrick (28) compared several coefficients of LD and found that \( D' \) performs better over several other estimates.
Index of association ($I_A$), another traditional measure of multilocus LD, was first introduced by Brown et al. (10) for *Hordeum spontaneum* and later applied to microbial populations to test LD (59).

The amount of LD in a population decreases with time and the recombinational distance between markers (2). This relationship can be shown as,

$$D_t = (1-r)^t D_0$$

where, $D_t$ is the amount of disequilibrium, $D_0$ is the disequilibrium at the beginning, $t$ is time and $r$ is recombination distance (2). Recombination distance is an estimate of the amount of crossover events and is related to the physical distance. Thus it is the basis of quantitative trait locus (QTL) mapping done in controlled breeding experiments. Therefore, in general, the amount of LD is expected to be inversely proportionate to the physical distance and proportionate to the amount of crossover frequency. Conway et al. (16) observed decaying LD with the increasing distances between the pairs of nucleotides within a 5 kb region of merozoite surface protein 1 gene of *Plasmodium falciparum* populations. Thus, the presence of homologous recombination in natural populations of the malaria parasite was inferred. However, this relationship is not always expected due to one or more reasons, such as, an epidemic population structure, natural selection, variable recombination rates (recombination hot spots and cold spots at genomic as well as population scale), variable mutation rates, population subdivision (Wahlund effect) and genetic drift (2). The Wahlund effect greatly influences LD leading to erroneous conclusions of the clonality of populations. Bayesian analysis is useful in such instances to detect hidden population structures (55) and several researchers were able to detect recombination within subpopulations whereas it would otherwise be concluded as clonal
populations (12, 37, 49, 61). Study and detection of recombination and LD in populations has theoretical as well as practical importance.

1.7 Research objectives and findings

Many population genetic studies of *S. sclerotiorum* have been conducted using isolates obtained from within fields, between fields of the same region/country and in a few studies, isolates between countries have been compared (38, 41). To our knowledge, all of the population genetic studies of this cosmopolitan pathogen have been conducted using isolates obtained from infected plants, and genetic structure of this pathogen in soil where primary inoculum resides has largely been neglected. The pathogen population genetic structure of *S. sclerotiorum* infecting alfalfa in the US PNW is not well understood even though white mold is a serious disease of alfalfa. Genotypic and phenotypic variation of this pathogen is well reported in many studies. However, differences between populations for variance in quantitative traits and their heritabilities have not been thoroughly studied. Therefore, the overall objective of this study was to improve our understanding of *S. sclerotiorum* population diversity and genetic structure from between continents to at the fine geographic scale. In chapter two of this thesis on population differentiation between China and the USA, high genetic and phenotypic diversity and recombination are reported and discussed. To our knowledge, there is no study investigating genetic and phenotypic diversity of *S. sclerotiorum* inhabiting the soil habitat. Therefore, the next research question asked was “If a high genetic and phenotypic diversity is observed in geographically distant populations obtained from infected plants, what is the level of diversity in soil at a smaller geographic scale?” Chapter three answers this question. Genetic and phenotypic diversity of *S. sclerotiorum* isolates obtained from one square meter of soil was quantified, and the results suggested high genetic and phenotypic diversity among isolates and random association among the markers. This study is unique in that it reports the genetic diversity of *S.*
sclerotiorum in soil in an area as small as 1 m$^2$ revealing a hidden population structure within the soil. Many such small scale diversity studies have been conducted on soil bacteria, but this is the first study to quantify the genetic diversity of *S. sclerotiorum* in soil on a fine geographic scale. Since soil serves as a reservoir for sclerotia accumulated over years, genetic diversity in the soil-borne sclerotia (inoculum population) should be higher than that of populations collected from plants (infection population), due to host selection. To test this hypothesis, isolates from soil collected at the beginning of the growing season and isolates collected near the end of the growing season of the same alfalfa fields were compared for genetic diversity in Chapter four. However, no significant difference in the amount of genetic diversity and no significant population differentiation were found between the soil and stem populations indicating a lack of or minimal host selection in alfalfa. While testing pair-wise LD between loci in the studies of the previous chapters, we found physically linked microsatellite markers were in linkage equilibrium, which suggests homologous recombination. Therefore, the last chapter (Chapter five) was designed to test the extent of homologous (intrachromosomal) recombination in eight natural populations using multiple loci on the same chromosomes. Results showed that LD decayed with increasing distance between loci, suggesting the decay in LD is due to crossover (not due to mutation alone), providing new evidence of outcrossing in the homothallic fungus, *S. sclerotiorum*.

This research has several theoretical and practical implications. First, significant genetic and phenotypic differentiation was detected between the *S. sclerotiorum* populations from China and the USA. Benomyl resistant genotypes were detected in the Chinese population even after a decade from the discontinuation of the fungicide. Development of potential cross resistance between benomyl and iprodione was also detected. Restrictions on global movement of *S.*
sclerotiorum should be taken seriously since it could introduce new genes to the existing gene pool and recombination could generate new genotypes. Currently, quarantine restrictions are applied on the movement of pathogens by species names regardless of the genotypes. New and novel genotypes could be imported potentially causing severe economic loss if an epidemic occurs. High genetic and phenotypic diversity exists not only at the scale of continents, but also on a fine geographic scale in alfalfa field soils in the US PNW. Host selection seems to be less important on S. sclerotiorum populations of alfalfa in the US PNW. We were able to detect intrachromosomal recombination. LD decayed with the increasing distance between physically linked markers of S. sclerotiorum. Therefore, the observed random association of alleles is due to crossover and cannot be simply attributed to random mutation. This provides an additional evidence of outcrossing in the homothallic fungus, supporting the high genotypic diversities and recombination reported in many previous studies.
References


CHAPTER TWO
GENETIC AND PHENOTYPIC DIVERGENCE OF \textit{SCLEROTINA SCLEROTIORUM}
POPULATIONS INFECTING CANOLA FROM CHINA AND USA

Abstract

Genetic and phenotypic diversity, and population differentiation of \textit{Sclerotinia}
\textit{sclerotiorum} isolates collected from two commercial canola fields in Anhui province, China (30 isolates) and North Dakota, USA (29 isolates) were investigated. Genetic diversity between the populations was compared with eight microsatellite markers and mycelial compatibility tests (MCGs). Phenotypic diversity was assessed by estimating the sensitivity to three different fungicides, production of oxalate, sclerotia and mycelium and virulence on two canola cultivars. There were no shared multilocus haplotypes or MCGs between the two populations and genetic differentiation was significant ($p < 0.001$). The populations differed significantly for all of the phenotypic traits except for sensitivity to fluazinam and virulence ($p < 0.05$). A higher recombination rate was detected in the Chinese population compared to the USA population. In addition, insensitivity to benomyl and iprodione, presence of darkly pigmented mycelia, production of less sclerotia and mycelia and the production of high levels of oxalate were some of the unique features among the Chinese isolates. Regardless of all the differences, both populations were equally virulent on two canola cultivars as measured by the mycelial colonization on detached canola leaves. Of all the traits studied, virulence had the least heritability indicating a strong environmental influence. Virulence was not significantly correlated with any other phenotypic trait ($r < 0.5$). Some isolates possessed only a single \textit{MAT} idiomorph as detected by a PCR based assay. This indicated that \textit{S. sclerotiorum} populations
may harbor heterothallic individuals and out-crossing could be possible in nature. Results suggest that environmental management is critical to control the disease. Cross resistance between benomyl and iprodione was detected, indicating the difficulties in devising disease management strategies. Results also suggest that restrictions on global pathogen movements should be taken seriously.

Introduction

*Sclerotinia sclerotiorum* is a cosmopolitan fungal plant pathogen, which causes white mold on more than 400 plant species including canola around the world (5). In the USA, North Dakota produces about 90% of the total USA canola production and white mold is one of the major constraints (9). The disease incidence on canola in ND was up to 14% causing $94 million loss during the period 1991-2002 (9, 31).

*S. sclerotiorum* employs a diverse array of mechanisms as a devastating plant pathogen on a wide variety of hosts. Early pathogenicity events include oxalic acid production and secretion of cell wall degrading enzymes (12, 24). Once the disease is established, pathogen survival structures, sclerotia, found inside the pith or on plant tissues can fall to the soil during threshing or other disturbances. Sclerotia can survive in soil for approximately eight years. When conditions are favorable for the pathogen, myceliogenic or carpogenic germination of sclerotia initiates another disease cycle. Therefore, production of oxalic acid, sclerotia and mycelia, virulence and fungicide resistance are the key elements required for disease establishment, survival and completion of the pathogen’s life cycle. Both uniformity and significant differences among isolates for oxalic acid production, growth rate, sclerotia production, pathogenicity and/or fungicide sensitivity, have been reported in several studies (3, 11, 28, 40, 46). Clonality (2, 8, 22, 29) and recombination have also been detected in many studies (3, 8, 20, 25, 39, 51).
According to Fisher’s fundamental theorem of natural selection, in a challenging environment with frequent fluctuations (e.g. an agricultural ecosystem with frequent application of pesticides, crop rotation and short cropping cycles), populations with less genetic variation for fitness traits may be vulnerable for extinction (36). Populations with high genetic variation can easily adapt to adverse environments (37). Conversely, a highly adapted population may have low genetic variance for certain traits because of the erosion of genetic variance that occurs under selection (19). However, none of the known population genetic studies of *S. sclerotiorum* have attempted to estimate the genetic variance of agronomically important phenotypic traits to infer the adaptive potentials of geographically distant populations of different environmental conditions. Geographically distant populations with restricted/lack of gene flow evolve separately and result in significant population differentiation. Specific ecologically beneficial characteristics (morphological, physiological or genetic) may be selected in different geographical locations resulting in populations undergoing adaptive differentiations. Therefore, geographically isolated populations may display genetic and phenotypic differentiation due to differential selection resulting from environmental variation and differences in crop rotation and control practices.

Populations from the USA and China were compared for genetic and phenotypic diversity using microsatellite markers and five agronomically important quantitative traits. This choice of populations allowed us to test the hypothesis that, geographically distant populations with distinct environmental conditions would display significant genetic and phenotypic differentiation and have different abilities to respond to selection. The first objective of this research was to study the population genetic structure, genetic diversity and differentiation between the populations of China and the USA. The second objective was to assess whether each
population differs in the ability to respond to selection by estimating the genetic variance and heritability of each trait. The ultimate goal is to employ better control strategies.

Materials and methods

Sample collection

Sclerotia of *S. sclerotiorum* were obtained from infected plants of commercial canola fields in the USA and China: 29 from North Dakota, USA in 2009, and 30 from Anhui province, China in 2010. Sclerotia were collected at least 6 m apart to ensure that isolates were from different infection foci. Sclerotia were surface sterilized (one min. each in 70% ethanol and 3% sodium hypochlorite), rinsed three times with sterile distilled water, aseptically cut into halves and plated on potato dextrose agar (PDA, Difco Laboratories, Detroit). One isolate was obtained from each sclerotium and kept at 4°C for short-term storage.

Mycelial compatibility grouping (MCGs)

Mycelial compatibility grouping (MCGs) was performed by pairing isolates in all possible combinations on PDA plates amended with red food coloring (49). Actively growing mycelial discs (6 mm diameter) were placed 2 cm apart in a 9-cm diameter Petri plate and incubated at room temperature in the dark for one week. Self-pairing was included as a control in every plate. The presence of an antagonistic barrage zone at the contact points was recognized as an incompatible reaction and a compatible reaction was characterized as merging colonies and formation of a confluent uniform colony. Compatible isolates were grouped into one MCG and incompatible isolates were placed in different MCGs. Each pair was tested at least twice.

PCR detection of MAT idiomorphs

A Polymerase Chain Reaction (PCR) screening was performed to determine if isolates carried one or both of the *MAT* idiomorphs. The full genomic sequence is available for the *S.*
sclerotiorum strain 1980 and the MAT1-1 alpha and MAT1-2 HMG idiomorphs are at a single MAT locus (35). Primers flank a 673-bp region in the MAT1-1 alpha and a 650-bp MAT1-2 HMG box region from a previous study (35), were used to detect the presence of MAT1-1 and MAT1-2. PCR conditions were as described in Malvárez et al. (35).

**Microsatellite genotyping**

Total genomic DNA was extracted from sclerotia of each isolate using FastDNA®Spin Kit (MP Biomedicals, Solon, OH) following the manufacturer’s instructions with minor modifications. Eight microsatellite loci developed by Sirjusingh and Kohn (52) were used to investigate the genetic diversity. PCR was performed with one of the four fluorophores (Vic, Pet, Ned and Fam), multiplexed and genotyped using an ABI3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) at USDA-ARS Western Regional Small Grain Genotyping Laboratory, Pullman. GeneMarker software (SoftGenetics, State College, PA) was used for the fragment analysis. PCR conditions were similar to those described by Sirjusingh and Kohn (52). Each isolate was genotyped at least twice for each locus.

**Statistical analysis of genotypic data**

Programs GenAlEx ver. 6.3 (43) and ARLEQUIN ver. 3.5 (16) were used to estimate gene diversity: number of polymorphic loci, mean number of alleles per locus (allelic richness), expected heterozygosity (41) and number of private alleles (those found only in one population) of each population. Allele frequencies between the populations were compared. Multilocus genotypic diversity was estimated using several indices: number of unique haplotypes observed in each population (g), Stoddart and Taylor’s G (53) and number of genotypes per isolate (g/N) using GENODIVE ver.1.0 (38).
STRUCTURE ver. 2.2 (47) was used to determine the most probable number of genetic clusters using the Bayesian clustering approach and the admixture model (individuals are allowed to have ancestry from multiple populations). Five independent runs were conducted to assess the consistency between the runs for each k value with 100,000 Markov Chain Monte-Carlo (MCMC) iterations followed by burning period of 500,000 iterations. The most probable number of genetically homogenous clusters (k) was estimated as described in Evanno et al. (15) using STRUCTURE HARVESTER ver. 0.6 (13). A principal coordinate analysis (PCoA) was performed using GenAlEx to test the population clustering using microsatellite data. PCoA has the advantage over the STRUCTURE due to a lack of strong assumptions of underlying genetic models such as linkage equilibrium.

The level of population differentiation was evaluated using analysis of molecular variance (AMOVA). AMOVA uses an analysis of variance framework to partition the total variance into covariance components due to between and within population differences (18). Variances were computed with a matrix of genetic distances between all pairs of haplotypes using the step wise mutation model (18). The significance of the null hypothesis of AMOVA, *S. sclerotiorum* isolates from China and the USA were not genetically differentiated, was tested with 1,023 permutations by a non-parametric approach implemented in ARLEQUIN.

Evolutionary relationships among haplotypes were graphically represented using a minimum spanning network (MSN) obtained from a pair wise distance matrix assuming the step wise mutation model (17) as implemented in ARLEQUIN. To visualize and edit the MSN, HapStar ver. 0.5 (54) and Inkscape ver. 0.48 (http://www.inkscape.org), a free graphics editing software, were employed.
Linkage disequilibrium indices, Index of association ($I_A$) and a modified statistic for the differences of the number of polymorphic loci, $r_d$, were estimated with MULTILOCUS ver. 1.3 (1). Briefly, the observed variance of distances (estimated by the number of different loci) between all pairs of individuals is compared to the expected variance of a simulated data set having unlimited recombination. The variance of distances of a recombining population is normally distributed whereas the variance of distances of a clonal population is skewed. The statistical significance of the null hypothesis of random mating was tested with 1000 randomizations of the data set.

**Fungicide sensitivity**

All of the experiments for phenotypic diversity estimations were designed as a completely randomized design (CRD). Sensitivities of all the isolates to three different fungicides were evaluated. Technical grade fungicides iprodione (BASF, Research Triangle Park, NC) and fluazinam (Syngenta Crop Protection Inc., Greensboro, NC) were used. Formulated product of benomyl, benlate 50WP (50% a.i., DuPont Agricultural products, Wilmington, DE), was used because technical grade benomyl was not available. Single discriminatory concentrations, which were determined in a preliminary study, were used for benomyl (0.2 µg a.i./ml), fluazinam (0.005 µg a.i./ml) and iprodione (0.25 µg a.i./ml) in sensitivity tests. In the preliminary study, S. sclerotiorum isolates displayed the highest variance of growth rates at these concentrations. Fungicide-amended PDA plates were inoculated with 5-mm agar disks from the edge of actively growing colonies and incubated in the dark at room temperature (23-25°C). Radial growth on control PDA plates without fungicides was measured for each isolate. Percent inhibition of each isolate was calculated based on the difference between radial growth on control PDA plates and the radial growth on fungicide-amended plates.
divided by the radial growth on control PDA plates after 36 h of inoculation. Each isolate was tested on three replicate plates and the experiment was repeated once.

**β-tubulin gene sequencing**

Partial sequence of the β-tubulin gene was amplified by modifying the primers B1 and B3 described in Li et al. (32). Degenerate nucleotides of the primers, B1 and B3, were replaced with the nucleotides of β-tubulin gene sequence in the *Sclerotinia sclerotiorum* genome database (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.html). Newly designed primers were TubF (5’-AAATCACCCACTCTCTCG-3’) and TubR (5’-CTCCATCTCGTCCATACC-3’). PCR products of all 59 isolates were sequenced (Elim Biopharm Inc., Hayward, CA) in both directions and sequences were manually edited, aligned and compared with the sequence of β-tubulin gene of *S. sclerotiorum* obtained from the genome database (SS1G_04652) using BioEdit ver. 7.1.3 (Tom Hall Ibis Biosciences, Carlsbad, CA). Single Nucleotide Polymorphisms (SNPs) and amino acid substitutions were searched and compared with the phenotypes of benomyl sensitivity.

**Oxalic acid production**

Production of oxalic acid in potato dextrose broth (PDB) was quantified by reverse phase High-Performance Liquid Chromatography (HPLC). PDB (25 ml, Difco laboratories, Detroit) in 125-ml flasks were inoculated with three 5-mm actively growing mycelial disks from PDA. Three replicate flasks were prepared for each isolate. Control flasks were inoculated with plain PDA plugs. Cultures were statically incubated for 4 days at room temperature (23-25°C) with a 12 h photoperiod. Mycelium was removed by vacuum filtration through Whatman no. 1 filter papers and culture filtrates were filtered through 0.45-µm membrane filters and used for HPLC analysis. Oxalate present in 10 µl of the sample was separated by an Eclipse XDB-C18 (4.6x 250
mm, 5 micron) column (Agilent technologies, Waldbronn, Germany) with the mobile phase of 2.8 mM H$_2$SO$_4$ and ethanol (9:1 ratio) at 55°C, at 0.5 ml/min flow rate and detected with UV light at 210 nm, a method modified from Rassam and Laing (48). Mycelial dry weight was determined after drying at 60°C for three days. The amount of oxalic acid produced by each isolate was expressed as total oxalate per flask and as per unit dry weight of mycelia (OA mg/mg). Mycelial dry weight produced in PDB was used as a measure of growth rate to compare two populations. The experiment was repeated once.

**Virulence assay**

A detached leaf assay was conducted in moist chambers prepared by placing a filter paper moistened with 4 ml of sterilized distilled water in a Petri dish (15 cm diam.). Fully expanded youngest leaves of 6 weeks old canola cvs. Dekalb 30-42 (susceptible) and Hyola 357 Magnum (moderately resistant) were used in the virulence assay. Fungal cultures were grown on V-8 juice agar (20% V8 juice, 0.3% CaCO$_3$, 80% deionized water, 2% Bacto agar (Becton, Dickinson and Company, Sparks, MD). A 5-mm diameter mycelial plug from the actively growing edge of a 2-day old culture was placed mycelial side down on one half of the leaf. A reference isolate obtained from pea was inoculated on the other half of the leaf and incubated at room temperature (23-25°C) with a 12 h photoperiod as described in Sexton et al. (50). Lesion areas 24 h after inoculation were measured with the digital image analysis software Assess 2.0 (APS Press, St Paul, MN). Lesion area and its difference in comparison with the reference isolate were referred to as absolute area and relative lesion area, respectively. Four replicate leaves per isolate were used and the experiment was performed twice.
Determination of colony color and sclerotial dry weight

Mycelial plugs (5 mm dia.) from the edge of actively growing colonies on PDA were transferred into the center of PDA (Difco laboratories, Detroit, MI) plates (20 ml per plate, four plates per isolate). Plates were incubated with a 12 h photoperiod at room temperature (23-25°C) for two weeks and then placed in the dark for another week before recording the colony color. Colony colors were visually evaluated and recorded as white, beige or black. The plates were then incubated in the dark at room temperature for two months before harvesting sclerotia. Sclerotia were dried under vacuum for three days and dry weight per plate was measured. The experiment was performed twice with three replicates per isolate.

Statistical analysis of phenotypic data

Statistical analyses were performed using SAS ver. 9.1 (SAS Institute, Cary, NC) and Minitab 16 Statistical Software (State College, PA). Significant difference between the first and second trials of each phenotypic trait was tested using PROC MIXED procedure in SAS using trials as the random effect. If there was no significant difference between the repeated experiments, data were pooled for further analyses. Significant differences between trials were detected for the virulence data and therefore it was analyzed separately. Significance of the differences between the populations at $p = 0.05$ was tested using nested ANOVA. Isolates and populations were treated as random and fixed, respectively. Non-parametric Mann-Whitney U test was used when violation of model assumptions was detected in the residual plots since data transformation did not improve the homogeneity of variance. Twenty-eight correlation analyses were performed between pairs of all the phenotypic traits to assess the possible relationships among them. Principal components analysis, a multivariate method to reduce a large number of interrelated variables to retain the highest amount of variation present in the data set via a new
set of variables known as principal components (PC), was performed with eight phenotypic variables (sensitivity to three fungicides, total oxalic acid production, sclerotial dry weight, mycelial dry weight and virulence on two canola cultivars). One way ANOVA was conducted with the PC scores to determine if the population is a significant predictor for the PC scores using PROC GLM procedure in SAS.

The phenotypic variance of an organism can be partitioned into several components: genetic variance, dominance effect, epistasis (among loci interaction) and environmental variance (19). Dominance effect does not occur in haploid organisms (55) like *S. sclerotiorum* and if epistasis is assumed to be negligible, the phenotypic variance is only caused by environmental and genetic variances (44, 55). Therefore, the total variance (*V*<sub>P</sub>) for *S. sclerotiorum* was partitioned using the modified equation of Falconer and Mackay (19), as *V*<sub>P</sub> = *V*<sub>A</sub> + *V*<sub>E</sub> where, *V*<sub>A</sub> is the genetic variance and *V*<sub>E</sub> is the environmental variance. Environmental variance can be estimated using the variance among replicates (55). Thus, genetic variance in each population can be estimated as the phenotypic variance minus the environmental variance. The Chinese population displayed two distinct phenotypic groups in sensitivity to benomyl, and therefore, *V*<sub>A</sub> was estimated for the two groups separately. The variances in quantitative traits were partitioned into among replicates and within population components using the PROC VARCOMP procedure in SAS software. The environmental variance was obtained from the among replicates variance component. Heritability of each trait in each population was estimated as *V*<sub>A</sub>/*V*<sub>P</sub> (19) accordingly.

**Results**

**Genetic analysis**
No shared MCGs were detected between the two populations. All of the isolates were self compatible. There were 27 MCGs within the Chinese population (Table 1) with the largest represented by three isolates. The next largest MCGs had two isolates and there were 25 single-isolate MCGs. There were 19 MCGs within the USA population (Table 1): 14 were of single isolates, two contained four isolates each, one had three isolates, and two had two isolates each (Appendix 1).

All the genotypic data for eight microsatellite markers were shown in the Appendix 1. Microsatellite marker data showed that the total number of alleles and the number of private alleles was higher in the USA population than in the Chinese population. However, the USA and Chinese populations did not differ in the mean number of alleles per locus or in the mean expected heterozygosities (Table 1). The frequencies of individual alleles varied between the two populations (Table 2). Locus 12-2 was monomorphic in the Chinese population. However, the Chinese population was genotypically richer than the USA population as indicated by the number multilocus haplotypes \((g)\), number of haplotypes per isolate \((g/N)\) and Stoddart and Taylor’s \(G\) (Table 1). MCGs were in concordance with multilocus haplotypes in the USA population but not in the Chinese population. Cluster analysis performed using STRUCTURE indicated that the populations were best explained by two genetic clusters in which all of the Chinese isolates were clearly assigned to one group and all of the USA isolates into the other group (Fig. 1). PCoA of microsatellite data also supported the presence of two clusters of \(S.\ sclerotiorum\) isolates, each cluster from one of the two continents (Fig. 2). In the PCoA analysis, components one and two explained 43.4% and 17.5% of the total genetic variation, respectively. The third component accounted for 12.2% of the total variation (not shown). The separation of the two populations was also confirmed by AMOVA, indicating a significant population
differentiation ($p < 0.001$). Over 37% of the total genetic variance could be explained by the genetic differentiation between the populations. Population clustering was also evident in the minimum spanning network. Two distinct groups were observed, with more reticulations within the Chinese population (Fig. 3). The null hypothesis of random association of alleles could not be rejected in Multilocus Linkage Disequilibrium estimates, $I_A$ and $r_d$, for the Chinese population indicating linkage equilibrium (Table 1). However, random association of alleles was detected for both the populations when clone corrected data were used.

**PCR detection of the variation in MAT idiomorphs**

Homothallic fungal species such as *S. sclerotiorum* harbor both *MAT1-1* and *MAT1-2* idiomorphs within a genome. Twelve of the 30 Chinese isolates (40%) had both the *MAT* idiomorphs and 11 of the 29 (38%) USA isolates had both *MAT* idiomorphs. The remaining isolates amplified only the *MAT1-2* idiomorph (Fig. 4). Repeated attempts to amplify *MAT1-1* from those isolates with different amplification conditions were unsuccessful. Variation among isolates for the MAT idiomorphs was shown in the Appendix 1.

**Phenotypic characterization**

**Fungicide sensitivity**

Sensitivity of the isolates to the three fungicides measured as percent inhibition of mycelial growth is shown in Fig. 5 and Appendix 1. Significant differences among isolates within populations were observed ($p < 0.05$) for all three fungicides. However, significant population differentiation ($p < 0.001$) was observed only for the percent inhibition by benomyl and iprodione. Two distinct groups of phenotypes were found in the Chinese population for benomyl sensitivity. The isolates that displayed 20% or less growth inhibition in presence of 0.2 a.i. µg/ml benomyl were considered as putatively resistant isolates (Fig. 5A). On average the
Chinese population displayed lower percent inhibition compared to the USA population for benomyl and iprodione (Table 3). However, there was no significant difference in percent inhibition for fluazinam between the populations \( (p = 0.49) \) (Table 3).

**β-tubulin gene sequence analysis**

PCR with the TubF and TubR primers produced an 874 bp sized fragment from all of the isolates. Sequences were 99-100% similar to each other and corresponded to the β-tubulin gene of the *S. sclerotiorum* genome database. There were three single nucleotide polymorphisms (SNPs), making four types of sequences among the 59 isolates (Fig. 6). The type I sequence was identical to the same sequence region in the *S. sclerotiorum* genome database, and was found only in the USA population. The type II sequence consisted of 15 isolates with a transversion mutation \((A \rightarrow C)\) at position 193 (Fig. 6). This mutation is non-synonymous changing the amino acid from Glu to Ala. Isolates with the type II sequence corresponded to all the 15 isolates showing insensitivity to benomyl (Fig. 5A, Appendix 1). The type III sequence, found in 13 of the Chinese isolates, had a transition event \((T \rightarrow C)\) located in the 560\(^{th}\) position of an intron region. Only five of the USA isolates had this transition event. Two of the 30 Chinese isolates had a mutation at the 368\(^{th}\) position, which was identified as the type IV sequence (Fig. 6). No detectable phenotypes were associated with isolates harboring the types III and IV sequences. The four types of sequences were deposited in the GenBank under the accession numbers JX181759, JX181760, JX181761 and JX181762.

**Oxalic acid and mycelial dry weight production**

Oxalic acid production was expressed as either total oxalate produced or oxalate per unit mycelial dry weight. An interval plot showing the mg of oxalate produced per mg mycelial dry weight is shown in Fig. 7a. Significant differences among isolates were detected \((p < 0.05)\).
Significant population differentiation was observed for the total oxalate production, oxalate per mg mycelial dry weight and for the mycelial dry weight \((p < 0.05)\) (Table 3). On average, the Chinese isolates produced higher levels of total oxalate per mg mycelial dry weight compared to the USA isolates (Table 3). Mean mycelial dry weight of the Chinese population was smaller than that of the USA population (Table 3).

**Mycelial color**

Among the 29 US isolates, two isolates were white in color and 27 isolates were beige in color. However, 13 of the 30 Chinese isolates had unique black/darkly pigmented mycelia. Seventeen Chinese isolates were beige color. No white colored mycelia were observed among the Chinese isolates.

**Sclerotial dry weight**

Mean sclerotial weight for the USA population was higher than that of the Chinese population (Fig. 7B). Significant differences among isolates, as well as between populations, were observed at \(p < 0.05\) (Table 3).

**Virulence assay**

All of the isolates were pathogenic on the two canola cultivars as measured by mycelial colonization of detached leaves. Mean absolute lesion areas of the isolates were 1.49±1.04 and 2.76±1.07 cm² on moderately resistant (Hyola 357 Magnum) and susceptible (Dekalb 30-42) cultivars respectively and significant cultivar differences were detected \((p < 0.05)\). Relative lesion area for each isolate was determined by the difference in lesion area between the isolate and the paired reference isolate. Pearson’s correlation analysis between the repeated experiments found that stronger correlations exist for relative lesion area compared to the absolute area; therefore, the rest of the virulence analyses were conducted with the relative lesion area (Table
3). First and second trials were analyzed separately because there was a significant difference between the trials. In both trials, significant difference among isolates for the virulence was found \((p < 0.05)\); however, no significant difference was detected \((p > 0.05)\) between the populations (Table 3, Fig. 7C).

**Correlation and Principal Components Analyses among phenotypic traits**

Out of 28 Pearson’s correlation analyses between all possible comparisons among eight phenotypic traits, 10 were significant (Table 4). Significant positive correlation was observed for the benomyl and iprodione insensitivities, even though they belong to different fungicide families. None of the traits were significantly correlated with virulence on either canola cultivar. Results from principal components analysis of phenotypic traits revealed that 65% of the total variance among isolates was attributable to the first three components and nearly 77% was attributable to the first four components. When principle components used as the response variable, population was a significant predictor for the first principal component. Sensitivity to benomyl and iprodione, mycelial weight, sclerotial weight and oxalate production were largely contributed to the total variance (Table 5).

**Comparison of populations for genetic variance and estimates of heritability**

In general, genetic variances of phenotypic traits were higher in the Chinese population than in the US population (Table 6). The genetic variances for percent inhibition of benomyl and iprodione, oxalate production, mycelial dry weight and sclerotial dry weight in the Chinese population were several-fold higher than those of the USA population. There was a high genetic variance for fluazinam sensitivity in the USA population. Despite the drastic difference in genetic variances for the traits studied, heritability of each trait was approximately similar between the populations, suggesting that the Chinese population had high levels of
environmental variance as well as high levels of genetic variance. Heritability of virulence was lower than that of all other phenotypic traits (Table 6), suggesting a larger impact of environmental variance on virulence than on the other traits.

**Discussion**

Sampling of *S. sclerotiorum* isolates from China and the USA enabled us to study genetic and phenotypic differentiation of geographically isolated populations from two continents; each population associated with discrete environments and cultural practices. There were no shared multilocus haplotypes or shared MCGs between the two populations indicating a restricted/lack of gene flow between them. In addition, AMOVA, STRUCTURE, MSN and PCoA analysis all supported the conclusion of population differentiation and restricted gene flow. This could be due to the tight quarantine regulations and/or long geographic distance between the populations. Although higher gene diversity was observed in the USA population, the Chinese population had a higher genotypic diversity. This suggests that recombination rates might be higher in the Chinese population than in the USA population. Signatures of high recombination rates in the Chinese population was found in the $I_A$ and $r_d$ tests, frequent reticulations in MSN, and the disagreement between MCGs and multilocus haplotypes. The hot and humid climate in Anhui province in China might have been conducive for frequent recombination in *S. sclerotiorum*. Huang (26) reported that sclerotia exposed to sub-freezing temperatures often germinated myceliogenically. Similarly, extreme winters in ND might have been favorable for myceliogenic germination of sclerotia resulting relatively a low level of recombination. The fact that some isolates possessed only a single MAT idiomorph as detected by the PCR based assay might suggest the existence of heterothallic isolates in natural populations and the outcrossing. However, a more reliable method to detect the presence and absence of MAT idiomorphs should
be employed. Recombination can generate new genotypes better adapted to the environment and might challenge disease management strategies.

Significant population differentiation was detected not only in the molecular markers, but also in phenotypic traits. Significant population differentiation was detected for all of the studied phenotypic traits except virulence and fluazinam sensitivity. Chinese isolates were unique in many aspects: presence of isolates with darkly pigmented mycelia, reduced growth rate and sclerotia production, high levels of oxalic acid production, benomyl and iprodione insensitivity. Small scale farming practices with high crop diversity, environmental variations and poor weed control in the Chinese agricultural system may explain the observed differences.

Detection of cross resistance between benomyl and iprodione was another interesting finding of this research. Benomyl belongs to the benzimidazole (MBC) group which inhibits β-tubulin assembly during cell division, whereas iprodione belongs to the dicarboximide group which affects on osmotic signal-transduction ([http://www.frac.info/frac/index.htm](http://www.frac.info/frac/index.htm)). During the 1990s, benomyl application was a common practice to control white mold in China and resistance was detected in 1996 (32). Half of the Chinese population we studied was resistant to benomyl, even after a decade without a selection pressure. Resistance to benomyl was not detected in the USA population or among the other 300 isolates collected from various crops in the US PNW (unpublished data). However, benomyl resistance in *S. sclerotiorum* has been detected in Canada (21) and resistance to both benomyl and iprodione has been detected in *Sclerotinia minor* in the USA (7, 27). Even though benomyl is currently prohibited for canola, thiophanate and thiophanate methyl, which belong to the same fungicide group, are recommended in the USA (6). Iprodione is recommended for *S. sclerotiorum* white mold on lettuce in the USA and on canola in China (34). Therefore, considering the possibility of
development of cross resistance is important in choosing fungicides for managing Sclerotinia white mold. Significant differences were not detected between the populations for sensitivity to fluazinam, although one isolate in the US population was significantly more insensitive to this fungicide. The multisite mode of action of fluazinam and a lack of selection pressure in both countries could explain the lack of population differentiation.

In addition to the correlation with benomyl sensitivity, iprodione sensitivity was also found to be positively correlated with sclerotial production and growth rate (Table 4). An earlier report showed that laboratory-induced iprodione resistant mutants produced pigmented mycelia with reduced sclerotial production, and were associated with two point mutations on a growth rate related os-1 gene (34). Here we report such a relationship of iprodione sensitivity with low sclerotial production and growth rate and the presence of pigmented mycelia in natural populations, although the genetic changes in the isolates remain to be determined.

Despite the significant genetic and phenotypic differentiations, the populations were not differentiated in virulence as measured by mycelial colonization of canola leaves. Although variation was observed among the isolates, virulence was not correlated with other phenotypic traits. Virulence of S. sclerotiorum has been a subject of many studies, and uniformity (3, 4, 10, 30, 40, 45, 50) as well as significant difference among isolates and among geographic locations (30, 33, 42) have been reported. Similar to our study, Sexton et al. (50) also reported a lack of significant difference in virulence among isolates from different canola fields, as measured on detached canola leaves. The lack of population differentiation in the current study could be due to the nature of detached leaf assay and a detached leaf assay might not replicate an in-planta assay. However, Irani et al. (28) found a significant correlation between detached leaf assays and in-planta assays of sunflower plants. Detached leaf assays are necessary to accommodate the
large number of isolates and to minimize environmental variation. Our results suggest that the virulence might be under different selection than the other phenotypic traits and virulence may not be related to the geographic origin. Virulence was not correlated with any other phenotypic trait studied. However, conflicting reports are available reporting significant correlations as well as lack of correlations among phenotypic traits and virulence (14, 28, 33, 40).

In the current study we observed that the Chinese population had greater variance for most traits (several folds higher than the USA population), but also had higher environmental variance, as demonstrated by the fact that heritabilities did not differ between populations. Because the response to selection depends on both the genetic and environmental variance (i.e., the magnitude of the heritability), it seems that each population has roughly equivalent abilities to respond to selection. Interestingly, heritability was high in both populations for most traits except virulence. This suggests two things. First, it is likely that virulence has been under strong selection in both populations, because an erosion of heritability is expected under selection. Two, the low heritabilities mean that currently virulence is largely influenced by the environment in both of these populations. This might explain the variable results of virulence in the repeated experiments as well as reported by other studies as mentioned earlier.

This research has several practical and theoretical implications. First, we found that natural populations of *S. sclerotiorum* can display a high level of genetic diversity and a range of phenotypic variation indicating that disease management could be challenging. Second, pathogen virulence has a major environmental component and therefore, when screening breeding materials for resistance, controlled environmental conditions should be adapted for better results. To manage the disease, cultural practices including environmental management could be important due to the heavy influence of environment on virulence. Third, cross resistance may
develop in *S. sclerotiorum* for fungicides (especially to benzimidazole and dicarboximide fungicides) and care in fungicide selection should be exercised. Fourth, a single discriminatory concentration for fast benomyl resistance screening was found possible and can be used at locations where molecular facilities are not available. Finally, the results support the importance of restrictions on movement of pathogens. Currently, quarantine restrictions are applied on movement of pathogen by species names without regard to genotypes. New and novel genotypes could be imported potentially generating the best fit genotypes via recombination. Since we were able to detect cross resistance and recombination, restrictions on global movement of *S. sclerotiorum* should be taken seriously. A precise genotype based method would be favorable for quarantine regulations.

**Acknowledgements**

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References


Table 1. Comparison of *Sclerotinia sclerotiorum* populations from China and USA for gene diversity, genotypic diversity and random association of alleles

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<th></th>
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<td>Mean number of alleles per locus</td>
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<td>Expected heterozygosity&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>$I_A$ (p)</td>
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<sup>a</sup>Nei’s expected heterozygosity (41) over eight microsatellite markers calculated using ARLEQUIN (16)

<sup>b</sup>Estimates of multilocus linkage disequilibrium and significance of the null hypothesis tested using MULTILOCUS
Table 2. Comparison of allele frequencies \((P)\) and gene diversities \((H_e)\) between the populations of *Sclerotinia sclerotiorum* from China and USA

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</table>
|      |      |� as defined by Sirjusingh and Kohn (52)
Table 3. Comparison of population mean, standard deviation and the significance of non-parametric Mann-Whitney U test of the phenotypic traits between the populations of *Sclerotinia sclerotiorum* form China and USA

<table>
<thead>
<tr>
<th>Trait</th>
<th>China</th>
<th>USA</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fungicide inhibition (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benomyl</td>
<td>42.4±29.5</td>
<td>79.1±4.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Iprodione</td>
<td>45.0±13.9</td>
<td>64.8±9.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fluazinam</td>
<td>71.2±6.5</td>
<td>69.8±8.2</td>
<td>0.49</td>
</tr>
<tr>
<td><strong>Oxalate production</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (mg)</td>
<td>15.9±4.3</td>
<td>13.1±3.5</td>
<td>&lt;0.05a</td>
</tr>
<tr>
<td>mg/mg mycelial dry weight</td>
<td>0.26±0.2</td>
<td>0.1±0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Sclerotia dry weight (mg)</td>
<td>0.15±0.02</td>
<td>0.2±0.04</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Mycelial dry weight (mg)</td>
<td>74±39</td>
<td>125.5±32</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Virulence (relative lesion area)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cv. Dekalb 30-42</td>
<td>0.58±0.8</td>
<td>0.52±1.00</td>
<td>0.62</td>
</tr>
<tr>
<td>cv. Hyola 357 Magnum</td>
<td>0.46±0.82</td>
<td>0.55±0.85</td>
<td>0.57</td>
</tr>
<tr>
<td>Virulence (relative lesion area)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cv. Dekalb 30-42</td>
<td>1.3±0.58</td>
<td>1.15±0.62</td>
<td>0.61</td>
</tr>
<tr>
<td>cv. Hyola 357 Magnum</td>
<td>0.98±0.66</td>
<td>0.89±0.56</td>
<td>0.79</td>
</tr>
</tbody>
</table>

*a Significant in both ANOVA and Mann-Whitney U test*
Table 4. Pearson’s correlations (r) between phenotypic traits of *Sclerotinia sclerotiorum* isolates from the populations of China and USA

<table>
<thead>
<tr>
<th></th>
<th>Fluazinam</th>
<th>Benomyl</th>
<th>Iprodione</th>
<th>Sclerotial weight</th>
<th>Mycelial weight</th>
<th>Oxalate production</th>
<th>Virulence cv. Dekalb 30-42</th>
<th>Virulence cv. Hyola 357 Magnum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluazinam</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benomyl</td>
<td>0.02</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iprodione</td>
<td>-0.04</td>
<td>0.62**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sclerotial weight</td>
<td>-0.01</td>
<td>0.33**</td>
<td>0.58**</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycelial weight</td>
<td>-0.18</td>
<td>0.57**</td>
<td>0.49**</td>
<td>0.34*</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total oxalate</td>
<td>-0.01</td>
<td>-0.32*</td>
<td>-0.26</td>
<td>-0.47**</td>
<td>-0.36*</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virulence cv. Dekalb 30-42</td>
<td>0.02</td>
<td>0.049</td>
<td>-0.17</td>
<td>-0.02</td>
<td>-0.13</td>
<td>-0.083</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Virulence cv. Hyola 357 Magnum</td>
<td>0.13</td>
<td>-0.002</td>
<td>-0.12</td>
<td>-0.17</td>
<td>-0.22</td>
<td>0.23</td>
<td>0.13</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*p < 0.05

**p < 0.001
**Table 5.** Eigenvectors of the first three principal components of eight phenotypic traits

<table>
<thead>
<tr>
<th>Variable</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iprodione</td>
<td>0.466</td>
<td>-0.164</td>
<td>0.295</td>
</tr>
<tr>
<td>Mycelial weight</td>
<td>0.451</td>
<td>0.158</td>
<td>0.062</td>
</tr>
<tr>
<td>Benomyl</td>
<td>0.448</td>
<td>-0.289</td>
<td>0.095</td>
</tr>
<tr>
<td>Sclerotial weight</td>
<td>0.433</td>
<td>-0.167</td>
<td>-0.075</td>
</tr>
<tr>
<td>Total oxalate production</td>
<td>-0.354</td>
<td>0.065</td>
<td>0.571</td>
</tr>
<tr>
<td>Fluazinam</td>
<td>-0.106</td>
<td>-0.537</td>
<td>0.173</td>
</tr>
<tr>
<td>Virulence cv. Dekalb 30-42</td>
<td>-0.147</td>
<td>-0.426</td>
<td>-0.672</td>
</tr>
<tr>
<td>Virulence cv. Hyola 357 Magnum</td>
<td>-0.183</td>
<td>-0.183</td>
<td>0.294</td>
</tr>
</tbody>
</table>
Table 6. Comparison of *Sclerotinia sclerotiorum* populations from China and USA for genetic variance and heritability of phenotypic traits

<table>
<thead>
<tr>
<th>Trait</th>
<th>Genetic variance</th>
<th>Heritability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chinese population</td>
<td>USA population</td>
</tr>
<tr>
<td>Benomyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sensitive*</td>
<td>61.52</td>
<td>14.58</td>
</tr>
<tr>
<td>insensitive</td>
<td>24.61</td>
<td>-</td>
</tr>
<tr>
<td>Iprodione</td>
<td>155.8</td>
<td>66.5</td>
</tr>
<tr>
<td>Fluazinam</td>
<td>38.33</td>
<td>59.325</td>
</tr>
<tr>
<td>Total oxalate (mg)</td>
<td>23.68</td>
<td>12.17</td>
</tr>
<tr>
<td>Oxalate mg/mg mycelia</td>
<td>0.028</td>
<td>0.002</td>
</tr>
<tr>
<td>Sclerotial dry weight (mg)</td>
<td>0.004</td>
<td>0.0014</td>
</tr>
<tr>
<td>Mycelial weight (mg)</td>
<td>1630.9</td>
<td>326.6</td>
</tr>
<tr>
<td>Virulence trial 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cv. Dekalb 30-42</td>
<td>0.19</td>
<td>0.2</td>
</tr>
<tr>
<td>cv. Hyola 357 Magnum</td>
<td>0.21</td>
<td>0.4</td>
</tr>
<tr>
<td>Virulence trial 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cv. Dekalb 30-42</td>
<td>0.02</td>
<td>0.08</td>
</tr>
<tr>
<td>cv. Hyola 357 Magnum</td>
<td>0.31</td>
<td>0.27</td>
</tr>
</tbody>
</table>

*Due to bimodal distributions in sensitivity to benomyl, the Chinese population was divided into two groups, sensitive and insensitive, for the calculation of additive genetic variance and heritability.*
Fig. 1. STRUCTURE analysis showing the isolates divide into two genetic clusters (from left to right, USA and China). Y axis shows the Bayesian posterior probability of population assignment.
Fig. 2. Principal coordinates analysis (PCoA) of *Sclerotinia sclerotiorum* isolates using eight microsatellite markers divides the isolates into two groups, USA and China. Each point represents each isolate based on the pair-wise distance matrix. Two axes with the highest percent variances are shown.
Fig. 3. Minimum spanning network (MSN) of multilocus haplotypes based on eight microsatellite markers. Open circles represent haplotypes from USA whereas closed circles represent haplotypes from China, and the size of a circle is proportionate to the frequency of the haplotype. Black dots represent missing haplotypes. The distance between nodes are proportionate to the genetic distance based on the step wise mutation model.
Fig. 4. Variation in PCR amplification of the mating type idiomorphs, *MAT1-1* (a) and *MAT1-2* (b) of 11 of the Chinese isolates (numbers 1-11). Only *MAT1-2* was amplified in isolates 3, 4, 7, 9, 10 and 11 whereas both *MAT1-1* and *MAT1-2* were amplified in isolates 1, 2, 5, 6 and 8. L denotes Hyper ladder II.
Fig. 5. Variation of percent inhibition of *Sclerotinia sclerotiorum* isolates from China (C01-30) and USA (U01-29) with three fungicides: Benomyl (A), Iprodione (B), and Fluazinam (C). Each point is the mean of six measurements and vertical bars represent one standard error of the mean.
**Fig. 6.** Sequence types, Single Nucleotide Polymorphisms (SNPs) and their relative locations on the partial sequence of β-tubulin gene and the number of isolates of each genotype in each population.
Fig. 7. Variation of *Sclerotinia sclerotiorum* isolates from China (C01-30) and USA (U01-29) for oxalic acid production (A), sclerotial production (B), and relative virulence on cv. Dekalb 30-42 (C). Each point in A and B is the mean of six measurements and mean of four measurements is shown in the C. Vertical bars represent one standard error of the mean.
Abstract

Sclerotia of the soil-borne plant pathogen *Sclerotinia sclerotiorum* were collected from 1 m² area of the top 1.27 cm layer of soil in an alfalfa field in southeastern Washington state of the US. Out of 272 sclerotia collected, 40 were randomly selected and analyzed for genetic diversity in terms of microsatellite loci, mycelial compatibility groups (MCGs) and phenotypic diversity using five phenotypic traits (fungicide sensitivity, oxalic acid production, growth rate, colony color and virulence). Sixteen microsatellite haplotypes and 15 MCGs were found among the 40 isolates. The isolates showed three colony colors (beige, dark and white) on Difco PDA and exhibited significant differences in growth rate, oxalic acid production, and sensitivity to three fungicides, benomyl, fluazinam and iprodione. However, these isolates did not show differences in their ability to colonize detached pea leaves. No apparent relationship among the neutral genetic markers and the phenotypic traits was detected. Two of the haplotypes accounted 40% of the isolates, suggesting isolates of these haplotypes might be better adapted to the environmental conditions in this alfalfa field. Several lines of evidence indicated high levels of genetic diversity and potential outcrossing within the population of *S. sclerotiorum*: 1) high likelihood of five genetic populations based on Bayesian probability and the presence of admixed isolates; 2) random association of alleles in every pair-wise linkage disequilibrium test.
among eight independent microsatellite loci; 3) discordances between microsatellite haplotypes and MCGs and 4) lack of correspondence among the genetic markers and phenotypic traits. Multilocus Index of Association test suggested that outcrossing occurs only within interbreeding subpopulations of *S. sclerotiorum*.

1. Introduction

Soil is the primary reservoir of inoculum for many soil-borne plant pathogens including the necrotrrophic fungal pathogen *Sclerotinia sclerotiorum*. *S. sclerotiorum* causes diseases called white mold, stem rot, crown rot or head rot of more than 450 plant species (Boland and Hall, 1994), and causes significant economical losses worldwide. The fungus produces melanized resting mycelial aggregates called sclerotia. The recalcitrant sclerotia can remain up to 95% viable in soil for two years (Williams and Western, 1965) and some can survive in soil for eight years (Grogan, 1979). Infected seeds may produce sclerotia when planted and sclerotia themselves may be mixed with and transmitted along with seeds (Dueckz and Sedun, 1983; Mueller et al., 1999). Thus, soil serves not only as a reservoir for sclerotia, but also as a medium for sclerotial development and regeneration, providing sustained inoculum of *S. sclerotiorum*.

Because of its enormous economical importance, *S. sclerotiorum* is one of the most extensively studied fungal plant pathogens. Its biology, ecology, epidemiology, genetics, physiology and its pathogenic mechanisms and interactions with host plants are well documented (Boland and Hall, 1994; Hegedus and Rimmer, 2005; Bolton, 2006; Erental et al., 2008). Its genome sequence is available (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.html). Genetic variation and population genetics of *S. sclerotiorum* is also a subject of many studies and numerous reviews. It was initially found that most of its populations are clonal (Anderson and
Kohn, 1995; Kohli, et al., 1995; Hambleton et al., 2002). However, frequent outcrossing has been reported in many *S. sclerotiorum* populations from several crops worldwide despite its homothallic reproduction (Atallah et al., 2004; Sexton et al., 2006; Mert-Türk et al., 2007; Hemmati et al., 2009; Barari et al., 2010; Gomes et al., 2011). Inference of outcrossing in this homothallic fungus is largely based on random association of independent markers which could not be explained by migration and mutation alone. All the population genetic and diversity studies of *S. sclerotiorum* have focused on isolates obtained from diseased plants collected on geographic scales ranging from within fields to transcontinental locations (Kohn et al., 1991; Cubeta et al., 1997; Carpenter et al., 1999; Atallah et al., 2004; Sun et al., 2005; Sexton et al., 2006; Mert-Türk et al., 2007; Hemmati et al., 2009; Li et al., 2009). Although its sclerotial densities in soil have been well documented (Williams and Western, 1965; Merriman et al., 1979; Holley and Nelson, 1986; Gilbert, 1987; Ben-Yephet et al., 1993), the genetic diversity and population structure of *S. sclerotiorum* in soil, where overwintering inoculum resides, have not been studied. Because the soil population of *S. sclerotiorum* represents the primary inoculum of the pathogen, understanding the genetic diversity in soil is important toward a complete understanding of the pathogen diversity, to evaluate the fitness of soil-borne sclerotia and to estimate hidden genetic structure in soil. This research was undertaken to investigate phenotypic and genetic diversity and population structure of *S. sclerotiorum* obtained from soil on a fine geographic scale.

Microbial components are important factors of biological processes in soil. Therefore, the importance of studying the diversity of soil microorganisms cannot be ignored. Even though microbial diversity in soil has been a subject of many studies, almost all the studies focused on species diversity of bacteria and some fungi (see reviews: Torsvik et al., 1996; Anderson and
Cairney, 2004). To our knowledge, there is no study investigating genetic and phenotypic diversity of *S. sclerotiorum* inhabiting the soil habitat. Within-species diversity shall present a new dimension of the soil habitat contributing to overall biodiversity.

The objective of this study was to estimate and understand the genetic diversity and genetic structure of *S. sclerotiorum* in soil. It was hypothesized that soil-borne sclerotia harbor high genetic and phenotypic diversity. Genetic diversity was studied in terms of neutral microsatellite markers, and several phenotypic characters such as mycelial compatibility groupings (MCGs), sensitivity to three fungicides, oxalic acid production, growth rate, colony color and virulence.

2. Materials and Methods

2.1 Isolate collection and identification

Sclerotia were collected from 1 m² area of soil in an alfalfa seed production field with a known history of Sclerotinia stem rot near Touchet, WA. White mold caused by *S. sclerotiorum* on alfalfa has been reported in this area since 1982 (Gilbert, 1987). The top 1.27 cm layer of soil was collected on March 17, 2010 and air-dried for two weeks in a greenhouse at 18-24°C. The soil was then placed in a cold room (15°C) until processing. To collect sclerotia, the soil was repeatedly rolled lightly with a plastic rolling wheel and sieved through an 850-µm sieve until there was no soil left on the sieve. Sclerotia or sclerotium-like objects in the remaining debris were searched manually. From this collection of sclerotia, 40 were randomly selected, surface sterilized (1 min each in 70% ethanol and 3% sodium hypochlorite), rinsed three times with sterile distilled water, aseptically cut into halves and plated on potato dextrose agar (PDA, Difco Laboratories, Detroit). One isolate was obtained from each sclerotium, purified by hyphal tip isolation and maintained on PDA at 4°C.
Species identity of the isolates was determined using three methods. First, general colony morphology and sclerotia production were used to determine if the isolates were *Sclerotinia*-like. Second, lack of group I introns in the small subunit rDNA was used to differentiate *Sclerotinia sclerotiorum* from *Sclerotinia trifoliorum* (Powers et al., 2001, Njambere et al., 2008), two morphologically similar species reported to cause stem and crown rot of alfalfa (Gilbert, 1987; Boland and Hall, 1994). Third, nucleotide sequences of the ITS region of four selected isolates (LP-10-18, LP-10-19, LP-10-30, LP-10-40) with different genotypes and phenotypes were determined as described by Njambere et al. (2008). The resulting sequences were compared with ITS sequences of *S. sclerotiorum* available at GenBank.

### 2.2 Mycelial compatibility

Mycelial compatibility grouping (MCG) was performed by pairing each isolate with itself and with every other isolate on PDA plates amended with red food coloring (Kohn et al., 1990). Mycelial discs (6-mm diameter) taken from the edge of an actively growing colony were placed 2 cm apart in a 9-cm diameter Petri plate and incubated at room temperature (23-25°C) in the dark for 1 week. Self-pairing was included as a control on each and every plate. Isolates that formed compatible reactions characterizing by merging colonies with no barrage zone were placed into one MCG, whereas isolates that formed incompatible reactions (the presence of an antagonistic barrage zone) at the areas of contact were assigned to different MCGs. Isolates were tested in all possible combinations and each pair was tested at least twice.

### 2.3 Microsatellite allele determination

Nine microsatellite loci from those developed by Sirjusingh and Kohn (2001) were used to investigate the genetic variation (Table 1). The fluorescent labeling method of Schuelke (2000) was followed for multiplex PCR except that the M13 sequence used was
CACGACGTTGTAAAACGAC. Total genomic DNA was extracted from sclerotia of each isolate using FastDNA®Spin Kit (MP Biomedicals, Solon, OH) following the manufacturer’s instructions with minor modifications as described by Njambere et al. (2008). PCR reaction mixtures included 750 pg of template genomic DNA, 1.5 units of Taq DNA polymerase (Promega, Madison, WI), 0.2 mM dNTPs and 1x PCR buffer containing 1.5 mM MgCl₂, 50 nM forward primer, 200 nM of each of the four dyes, Vic, 6-Fam, Pet or Ned, and 250 nM reverse primer in a total reaction volume of 12 µl. PCR parameters were similar to those described by Sirjusingh and Kohn (2001). PCR products labeled with each fluorophore were multiplexed and diluted with molecular grade water (G-Biosciences/Genotech: St. Louis, MO) by 0.12 times for Vic and Fam, 0.16 times for Ned and 0.24 times for 6-Pet. Three micro liters of this mixture was mixed with 9.9 µl Hi-Di formamide and 0.1 µl of LIZ-445 size standard in a 96 well plate and denatured for 5 min at 95°C before assembling in ABI plate sandwich and run on an ABI3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) for fragment analysis using GeneMarker (SoftGenetics, State College, PA), carried out at the USDA-ARS Western Regional Small Grain Genotyping Laboratory, Pullman. Each isolate was tested at least twice for each locus.

2.4 Genetic diversity

ARLEQUIN ver. 3.1 (Excoffier et al., 2010) was used to determine the number of alleles, gene diversity, expected heterozygosity (Nei, 1987), allele frequency and haplotype frequency. Minimum spanning tree (Rohlf, 1973) was obtained from the matrix of pair-wise distances assuming a step wise mutation model, as implemented in ARLEQUIN, and visualized with TreeView ver. 1.6.6 (Page, 1996). STRUCTURE ver. 2.2 (Pitchard et al., 2000) was used to determine the most probable number of genetic populations using the Bayesian clustering approach. The clustering method determines that there are k populations, each characterized by
allele frequency data at each locus. Each individual isolate is assigned to one of the $k$ populations without consideration of the geography or location. A population admixture model was used and each simulation consisted of 100,000 Markov Chain Monte-Carlo (MCMC) iterations followed by a burning period of 50,000 iterations. Five independent runs of one to eight subpopulations ($k=1-8$) were performed to estimate the most probable number of genetically homogenous clusters ($k$).

Linkage disequilibrium tests were performed using the clone corrected data set, where only one representative of each haplotype was included to reduce the bias due to over representation of clones. First, the null hypothesis of independent association of alleles between loci was tested with pair-wise linkage disequilibrium analysis using MULTILOCUS ver. 1.3 (Agapow and Burt, 2001) and Fisher’s exact test using in GENEPOP ver. 4.0.10 (Raymond and Rousset, 1995). Second, to test multilocus linkage disequilibrium, a modified measure of multilocus Index of Association, $r_d$, was estimated as implemented in MULTILOCUS for the whole population as well as for the subpopulations defined by STRUCTURE (without admixed isolates LP-10-25, LP-10-25A and LP-10-31). In addition to the $r_d$ test, Parsimony Tree Length Permutation Test (PTLPT), a phylogeny-based recombination detection method, was performed for the largest subpopulation defined in STRUCTURE (without the admixed isolate LP-10-31). Both pair-wise comparison and multilocus Index of Association analyses involved comparing the observed variance of distances (estimated by number of different loci) between all pairs of individuals to the expected variance of a simulated random mating population. Simulated data sets were generated by randomly shuffling the alleles for each locus among the members of the subpopulation.
2.5 Oxalic acid production and growth rate

A pH-indicating medium (PDA amended with bromophenol blue at 60 mg/L; called BPDA) was used as a semi-quantitative measure of oxalic acid production (Steadman et al., 1994). The intensity of yellow color was assumed to be related to the amount of oxalic acid production. Mycelial plugs of 5 mm dia. from actively growing colonies (on PDA) were placed on BPDA plates (20 ml per plate, four replicate plates per isolate). Plates were incubated with a 12 h photoperiod at room temperature (23-25°C). Sixty hours after inoculation, the color intensity of the medium was scored using a + (low) to +++ (high) rating system (Fig. 1). It was initially determined that the bromophenol blue at the experimental concentration did not affect the growth rate of *S. sclerotiorum* (data not shown). Colony diameters on the pH-indicating medium were measured at a 90° angle, and the average was recorded as growth rate measurements. Each experiment was performed at least twice.

Two isolates (LP10-18 and LP10-40) representing the extremes of oxalic acid production based on the pH-indicating medium were selected for quantitative measurements of pH change and oxalic acid production in a liquid medium. Isolates were grown statically at room temperature (23-25°C) with a 12 h photoperiod in 125-ml flasks containing 25 ml of potato dextrose broth (PDB, Difco Laboratories, Detroit) with three replicates. At 2, 3, 4, 5 and 6 days after inoculation, mycelium was removed by vacuum filtration through Whatman no. 1 filter paper, and pH values and oxalate content in the medium was measured with a pH meter (Beckmen Coulter, Inc., Brea, CA) and reversed phase High Performance Liquid Chromatography (HPLC), respectively. Culture filtrates were passed through a 0.45 µm Millipore membrane filter (Carrigtwohill, Co., Cork, Ireland) and a sample of 10 µl of the filtrate was chromatographed at 55°C using an Eclipse XDB-C18 (4.6x 250 mm, 5 micron) column.
(Agilent Technologies, Waldbronn, Germany) with the mobile phase consisting of 2.8 mM H$_2$SO$_4$ and methanol pumped at a 9:1 ratio at a flow rate of 0.5 ml/min. Oxalate was detected at 210 nm using a diode array detector, a method modified from Rassam and Laing (2005). Differences in pH values and oxalic acid produced by the two isolates were tested using the Student’s unpaired “t” test in Minitab 16 statistical software (State College, PA).

2.6 Colony color

Mycelial plugs (5 mm dia.) from the edge of actively growing colonies on PDA were transferred into the center of PDA (Difco laboratories, Detroit) plates (20 ml per plate, four plates per isolate). Plates were incubated with a 12 h photoperiod at room temperature (23-25°C) for two weeks and transferred into the dark for another week before recording the colony color. Colony colors were recorded as dark, beige or white (Fig. 1) and the experiment was repeated once.

2.7 Fungicide sensitivity tests

All the isolates were tested for sensitivity to three different fungicides. Technical grade iprodione (BASF, Research Triangle Park, NC) and fluazinam (Syngenta Crop Protection Inc, Greensboro, NC) were used. Since technical grade benomyl was not available, formulated product, Benlate 50WP (50% a.i.), (DuPont Agricultural products, Wilmington, DE) was used. Stock solutions of the fungicides were made in acetone at 300 µg a.i. /ml and subsequently diluted and added to PDA (previously autoclaved and cooled to 55°C) to achieve desired concentrations. Control plates were amended with an equal amount of acetone without fungicides. To determine an appropriate discriminatory dose of each fungicide, ten isolates were tested for growth rates on PDA plates amended with the respective fungicide at a concentration gradient. The fungicide concentration that gave the largest variance in growth rate among the ten
isolates was selected and used to test all the isolates. Mycelial plugs (5 mm dia.) from the edge of actively growing colonies on PDA were transferred to the center of PDA plates amended with the fungicides, iprodione, benomyl, or fluazinam (one plug per plate and three replicate plates per treatment). Petri plates were arranged in a completely randomized design (CRD) and incubated in the dark at room temperature. Colony diameters were measured at a 90° angle every 12 h for three days and mean diameters were recorded. Growth rate was calculated using the measurements before colonies reached the edge of the Petri dish. Experiments were repeated two times, and freshly prepared fungicide-amended PDA plates were used at each time. Data were analyzed by one-way analysis of variance (ANOVA) in Minitab. Hsu’s multiple comparison (highest growth rate was chosen as the best) was performed (Hsu, 1984) and isolates that did not significantly differ from the highest were considered as putatively insensitive isolates for each fungicide.

2.8 Virulence assay

Virulence of the isolates was measured as mycelial colonization of pea leaves using a detached leaf assay in moist chambers as described in Sexton et al. for canola (2004). Fully expanded pea (*Pisum sativum* cv. Guido) leaves of 5-week-old plants obtained from the third node from the bud were placed in moist chambers formed by placing a moist filter paper in 9-cm Petri dishes. Five-mm diameter plugs from actively growing edges of colonies of 2-day-old cultures on V8 medium (80% deionized water, 20% V8 juice, 0.3% CaCO₃, and 2% Bacto agar), were placed on one side of the midvein of the leaf. A reference isolate obtained from pea was inoculated on the other side of the midvein of the same leaf for comparison for every isolate (four replicated leaves in different moist chambers per isolate). The moist chambers were incubated at room temperature with a 12 h photoperiod. Lesion size was captured with a digital
camera 24 h after inoculation and lesion area was measured with the digital image analysis software ASSESS 2.0 (APS Press, St Paul, MN). Difference in lesion area in comparison with the reference isolate was used in one-way analysis of variance (ANOVA) in Minitab. The experiment was performed twice.

3. Results

3.1 Species identity of the isolates

A total of 272 sclerotia were collected from the one square meter of soil. Cultural characters of the forty randomly selected sclerotia were similar to *Sclerotinia sclerotiorum* or *S. trifoliorum* (Kohn, 1979). All the isolates produced the 560-bp PCR product with the ITS4/ITS5 primer pair indicating that none of the isolates had an intron near the 3’ end of the small subunit rDNA. *S. sclerotiorum* can be distinguished from *S. trifoliorum* by the absence of an intron at this location (Powers et al. 2001; Njambere et al., 2008) and all the 40 isolates tested in this study were *S. sclerotiorum*. Additionally, the DNA sequences of the ITS region of the four selected isolates (LP-10-18, LP-10-19, LP-10-30, LP-10-40) were identical to those of *S. sclerotiorum*.

3.2 Mycelial compatibility

All the isolates were self compatible. Many isolates produced an incompatible reaction when paired with another isolate. There were 15 MCGs among the 40 isolates. The largest two MCGs each contained 8 isolates, and the next two largest MCGs each had 4 isolates. One MCG had three isolates. There were three MCGs each with 2 isolates and there were seven single-isolate MCGs (Fig. 2).
3.3 Genetic diversity

3.3.1 Haplotype diversity and STRUCTURE analyses

All of the nine microsatellite loci tested were polymorphic among the 40 isolates with allele numbers ranging from 3 to 6, and a total of 33 alleles were detected (Table 1). All the isolates showed a single allele in every locus, indicating they were homokaryotic. Nei’s gene diversity ($H_e$) for loci ranged from 0.27 (locus 110-4) to 0.79 (locus 55-4) with the average gene diversity of 0.59±0.16. There were 16 unique multilocus haplotypes (Fig. 2).

Analysis of the population structure using STRUCTURE software gave the highest likelihood $k$ value of 5 (Ln P(D) = -153.45 with variance of 3.5) meaning that there were 5 possible genetic clusters within the sample of 40 isolates. Three isolates (LP-10-25, LP-10-25A and LP-10-31) had multiple population assignments and were admixed (population assignment was <50%). The biggest cluster (pink colored in Fig. 2) contained 12 isolates with six haplotypes (assignment probability >50%). The next two clusters contained nine and eight isolates with one and two haplotypes, respectively. Another cluster contained six isolates with three haplotypes and the smallest cluster contained four isolates with two haplotypes (Fig. 2).

3.3.2 Pair-wise linkage disequilibrium and multilocus Index of Association

In 36 pair-wise linkage disequilibrium tests, the null hypothesis of random association of alleles between loci could not be rejected except for the locus pair 7-3 and 12-2 (Table 1). Similar results were obtained by Fisher’s exact test in GENEPOP (data not shown). A close examination of the loci sequences in the GenBank revealed that loci 12-2 (AF377906, 217 bps) and 7-3 (AF377909, 206 bps) are of the same microsatellite repeats and actually one locus. Alleles of the two loci had complete correspondence, and therefore, only locus 7-3 was included in subsequent analyses.
Because the population is subdivided, Index of Association analyses of the whole population will skew toward clonality as pointed out in the program manual and demonstrated in other fungi (Koufopanou et al., 1997; Campbell et al., 2005; Montarry et al., 2010; Stewart, 2011). Multilocus Index of Association test was applied to the five subpopulations defined by STRUCTURE and \( r_d \) value was 0.30 \((p = 0.076)\), indicating random association of alleles within subpopulations. In addition, length of the most parsimonious tree generated in PTLPT was not significantly different between the observed data and the simulated data, also supporting random association of alleles within the subpopulation.

### 3.4 Oxalic acid production and growth rate

All isolates changed the color of the pH-indicating medium from blue to yellow 48 h after inoculation except isolate LP-10-18. For isolate LP-10-18, color change was not only consistently delayed, but also the final color was not as intense as the other isolates (Fig. 1). In liquid media both isolates LP-10-18 and LP-10-40 reduced pH of the medium over time (Fig. 3). However, the pH values of isolate LP-10-40 were always lower \((p < 0.001)\) than those of isolate LP-10-18 (Fig. 3), consistent with results of the pH-indicating plate assay. Amount of oxalic acid produced by isolate LP-10-18 in cultures were consistently lower \((p < 0.001)\) than those of isolate LP-10-40 (Fig. 3). Oxalic acid concentration in the liquid medium increased with decreasing pH values.

In terms of growth rate, there were significant differences among the isolates \((p < 0.001)\). Most of the isolates grew very fast on PDA and covered the 9-cm Petri plates after 48 h except for isolate LP-10-18 which took 60 h to cover the Petri plate. Hsu’s multiple comparison with the highest mean growth rate \((3.5 \text{ cm per day})\) resulted in 16 isolates \((\text{LP-10-02, LP-10-08, LP-10-11, LP-10-12, LP-10-13, LP-10-15, LP-10-17, LP-10-19, LP-10-21, LP-10-23, LP-10-24, LP-10-25, ..., LP-10-29})\).
26, LP-10-29, LP-10-31, LP-10-36, and LP-10-40) designated as fast growth whereas the remaining isolates had slow growth.

3.5 Variation in mycelial color

Based on mycelial color on Difco PDA plates, isolates were classified into three groups as beige, dark and white. Nineteen isolates were beige, whereas 13 and eight isolates were dark and white, respectively (Fig. 2).

3.6 Fungicide sensitivity

The highest variance of growth rate among isolates was obtained at 0.2 µg a.i./ml of benomyl, 0.005 µg a.i./ml of fluazinam and 0.25 µg a.i./ml of iprodione. These concentrations of respective fungicides were selected and used as the discriminatory doses to test all the isolates. All the isolates displayed reduced growth rates in the presence of fungicides compared to that in the controls (absence of fungicides). On control plates, all the isolates except isolate LP-10-18 completely colonized the plates in 48 hrs after inoculation. Percent growth inhibitions compared to control at 36 hrs after inoculations were 32-80%, 42-80% and 66-86% for iprodione, fluazinam and benomyl, respectively. Significant differences (p < 0.001) of growth rates among isolates on fungicide-amended plates were observed. For benomyl, Hsu’s multiple comparisons yielded that 11 isolates (LP-10-02, LP-10-03, LP-10-08, LP-10-11, LP-10-12, LP-10-28, LP-10-30, LP-10-31, LP-10-33, LP-10-34 and LP-10-40) were not significantly different from the highest mean (p < 0.05). For iprodione, two isolates (LP-10-27 and LP-10-36) did not differ from the highest mean growth rate. Ten isolates (LP-10-17, LP-10-18, LP-10-20, LP-10-22, LP-10-23, LP-10-24, LP-10-26, LP-10-29, LP-10-36 and LP-10-40) did not differ from the highest mean growth rate in presence of fluazinam (p < 0.05). Consistent results were observed in repeated experiments. Isolate LP-10-40 showed relatively high growth rate (putative
insensitivity) to both benomyl and fluazinam, and isolate LP-10-36 was putatively insensitive to both fluazinam and iprodione. Many of the isolates displayed resistance development by growing sectors on PDA plates amended with fungicides. However, isolate LP-10-18 was unique in that it had an equal growth rate along the periphery of the colony in the presence of fluazinam (Fig. 4).

3.7 Variation in virulence

There were variations in lesion area as measured by mycelial colonization among replicates. However the lesion area of the test isolate was positively correlated with the lesion area of the corresponding paired reference isolate (Pearson correlation = 0.452, $p < 0.05$). Therefore, the difference of lesion area between the test isolate and the paired reference isolate was used in the analysis. No significant differences in lesion size on detached pea leaves among the isolates were detected. Interestingly, even though isolate LP-10-18 grew slower and produced less oxalic acid and, isolate LP-10-40 produced high oxalic acid, there was no significant difference in lesion size between the two isolates on detached leaf ($p = 0.128$). However, isolate LP-10-18 always produced lesions with less browning compared with that of isolate LP-10-40 or any other isolate (Fig. 5).

3.8 Relationship among genetic markers and phenotypic traits

There were some congruence between MCGs and microsatellite haplotypes. For example, MCG 3 contained four isolates of the same haplotype. Five single-isolate MCGs, 9, 10, 11, 13, and 15, each contained a distinct haplotype. But there were several instances where MCGs and microsatellite haplotypes did not similarly correspond. There were isolates of the same haplotype that belonged to different MCGs. For instance, isolates LP-10-18 and LP-10-30 belonged to the same microsatellite haplotype, but were in different MCGs. Similarly, isolates LP-10-03, LP-10-17, LP-10-32 and LP-10-35 were in the same haplotype, but belonged to two different MCGs.
(Fig. 1). Also there were isolates of the same MCGs that belonged to different haplotypes. For instance, isolate LP-10-07, a single-isolate haplotype, belonged to an MCG with seven other isolates (Fig. 2).

The eight isolates producing white mycelium belonged to MGC 2, and seven of the eight isolates belonged to one haplotype (Fig. 2). However, the beige and dark-colored mycelium groups had no discernible relationship with either MCGs or haplotypes. Likewise, oxalic acid production and virulence showed no detectable relationship with MCGs, microsatellite haplotypes, or genetic clusters obtained from Bayesian posterior probability or mycelial color.

4. Discussion

Sclerotinia stem and crown rot of alfalfa can be caused by either *S. sclerotiorum* or *S. trifoliorum* or both (Boland and Hall, 1994). Thus, it was important to confirm the species identity of the sclerotia obtained from alfalfa-cultivated soil for population genetic studies. Evidence showed that all the isolates obtained in this study were devoid of the diagnostic intron in small subunit rDNA, thereby conforming to characteristics of *S. sclerotiorum*, not *S. trifoliorum*. Complete ITS sequences of selected isolates further confirmed the *S. sclerotiorum* identity. The fact that all the nine microsatellite markers generated expected alleles on all the isolates also supports the conclusion that the isolates were *S. sclerotiorum*, because a previous report showed that these markers do not work on isolates of *S. trifoliorum* (Njambere et al., 2010). The conclusion is in agreement with the previous report that alfalfa crown and stem rot in southeastern Washington is caused by *S. sclerotiorum* (Gilbert, 1987).

Studies on recovery of sclerotia of *Sclerotinia* spp. from soil to estimate inoculum density and to study survival of the pathogens have been reported (Merriman et al., 1979; Holley and Nelson, 1986; Gilbert, 1987; Ben-Yephet et al., 1993; Alexander and Stewart, 1994; Wu and
Sclerotial density in soil varies greatly depending on cropping systems and soil depth (Holley and Nelson, 1986; Gilbert, 1987; Ben-Yephet et al., 1993). Sclerotia were present at high densities in soil (more than 270 sclerotia per 1 m² area) in this study, similar to the result reported by Gilbert (1987). The actual sclerotial density could be higher considering that sclerotia smaller than 0.85 mm were not collected. Since sclerotinia disease incidence is positively correlated with sclerotial density in soil (Holley and Nelson, 1986; Ben-Yephet et al., 1993; Chitrampalam et al., 2010), the high density of sclerotia in the soil could present tremendous disease pressure on crops.

Several lines of evidence show high levels of genetic diversity from such a small geographic area: 1) high expected heterozygosity, 2) presence of a high number of haplotypes and MCGs and the presence of admixed isolates, 3) high likelihood of five genetic populations based on Bayesian probability and 4) high levels of variation in fungicide sensitivity, growth rate and oxalic acid production.

STRUCTURE analysis showed a subdivided population in such a small geographic scale. Several factors may contribute to shape the genetic structure of the soil population. *Sclerotinia sclerotiorum* can clonally propagate through production of sclerotia or through strictly homothallic ascospore production (equivalent to clonal reproduction). Clonal reproduction is exhibited in this population as evidenced by a restricted number of haplotypes, each with multiple isolates. Mutations also contribute to genetic diversity since microsatellite loci are of short sequence repeats in which unequal cross over may occur generating new alleles and DNA slippage can occur during DNA replication (Li et al., 2002). The microsatellite loci with high number of alleles may have higher mutation rates than loci with low number of alleles. It is conceivable that closely related haplotypes could be easily generated through mutations. New
genotypes may also be introduced into the soil through infected seeds, infested seed lots with sclerotia, carry over by machinery or through receiving air-borne ascospores from adjacent fields. Thus migration may introduce new distantly related as well as closely related haplotypes, plausibly explaining the five distinct genetic clusters.

However, potential outcrossing cannot be ruled out even in this homothallic fungus because mutation and migration alone cannot explain the random association of alleles and discordance between haplotypes and MCGs. Although no empirical evidence of outcrossing has been shown for *S. sclerotiorum*, there are numerous reports of inferred outcrossing in *S. sclerotiorum* through random association of independent markers (Atallah et al., 2004; Sexton et al., 2006; Mert-Türk et al., 2007; Hemmati et al., 2009). Hemmati et al. (2009) studied 276 isolates mostly from canola from Iran and found linkage equilibrium among microsatellite alleles and suggested occurrence of outcrossing. Also based on random association of alleles and disagreement between microsatellite haplotypes and MCGs, Sexton et al. (2006) suggested the occurrence of clonal reproduction and outcrossing in *S. sclerotiorum* from canola in Australia. Recently, Chitrampalam et al. (2011) reported segregation of mating type loci in sibling ascospores of *S. sclerotiorum*, suggesting some progenies of this fungus may not be homothallic and may require outcrossing for sexual reproduction. In studying four potato populations of *S. sclerotiorum* collected not far from our study site (within 100 km), Atallah et al. (2004) found evidence of outcrossing (linkage equilibrium) in two of the four populations and observed multiple MCGs of ascospore isolates from individual apothecia substantiating occurrence of outcrossing. Results from this study also suggest that isolates of *S. sclerotiorum* may not freely outcross with every member of the species, only with members of interbreeding subpopulations. Similarly, several other studies have reported the recombination in subdivided populations, while
the whole population is in linkage disequilibrium (Koufopanou et al., 1997; Campbell et al., 2005; Montarry et al., 2010; Stewart, 2011). Campbell et al. (2005) detected the recombination of Cryptococcus gattii isolates within genetically differentiated subgroups of sample size as small as five isolates and found recombination was independent of sample size. Although the mechanisms governing outcrossing in S. sclerotiorum are not known, possible mechanisms are discussed by Atallah et al. (2004) and Sexton et al. (2006).

Oxalic acid is an important pathogenic factor of S. sclerotiorum and other fungal pathogens (Godoy et al., 1990; Dutton and Evans, 1996). However, the variation in oxalic acid production did not correlate with the virulence as measured by mycelial colonization of pea leaves. Oxalic acid production in culture may not represent the ability to produce it in-situ, and the oxalic acid produced by the isolates may be above the threshold needed for colonization of pea leaves. Even for isolate LP-10-18 that always showed the lowest level of oxalic acid production, there was no significant difference in lesion size between this and any other isolate.

Conflicting reports exist about variation in virulence among isolates of S. sclerotiorum. Pratt and Rowe (1995) and Atallah et al. (2004) reported phenotypic uniformity for aggressiveness on plant inoculations. On the other hand, significant difference among isolates for virulence has been reported (Kull et al., 2004; Otto-Hanson et al., 2011). Forty isolates in this study did not show significant variation in lesion area in detached pea leaves despite showing wide variation in growth rate and in oxalate production. This may reflect the nature of the pea leaf assay itself. The pea leaves could be super susceptible and offered no resistance to any isolates possessing minimum threshold of disease causing abilities, even for the isolate LP-10-18 which produced the least amount of oxalic acid. This is consistent with the report that
oxalate-deficient mutants caused similar levels of disease as the wild type strain on detached pea leave (Xu et al., 2011).

High levels of genetic diversity and clonal reproduction as well as evidence of outcrossing have been widely reported for *S. sclerotiorum* as mentioned earlier. What is unique in this study is that it documents genetic diversity and potential outcrossing in soil at a small geographic scale. The high level of genetic diversity is exemplified by existence of not only 16 haplotypes but also five genetic clusters in 1 m² of soil. In contrast to this soil population, Hemmati et al. (2009) found only one genetic cluster (panmictic population) of 276 isolates from infected plants in 37 field populations of canola from four provinces in northern Iran. The patterns of genetic diversity of this soil population give us a window to infer processes happened in the past. The two largest haplotypes consisted of 9 and 7 isolates, respectively, roughly (not exactly) corresponding to the largest MCGs, and are likely progenies of clonal reproduction. The dominance of the two haplotypes implies that these two haplotypes are plausibly more adapted to the environment of the alfalfa field/soil than the other haplotypes. Although the isolates did not show variation in mycelial colonization of pea leaves, the isolates of these two largest haplotypes may be more effective in the other aspects of field infection such as carpogenic germination. Future study shall determine if these isolates are more efficient in apothecial germination and ascospore release and ascospore infection, which are required for successful infection in addition to mycelial colonization.

Soil presents a habitat for diverse micro- and macro-organisms and an environment for dynamic biological processes. Most biodiversity studies have focused on soil bacterial communities or mycorrhizal fungi, and on the diversity at the species level (Anderson and Cairney, 2004). The current study is unique and presents a new dimension characterizing fungal
biodiversity in soil at genetic and phenotypic levels within a species on a fine geographic scale. For soil-borne plant pathogens including *S. sclerotiorum*, the genetic diversity presents not only the history of the pathogen propagated in the soil, but also the potential of the pathogen to evolve and adapt to changing agricultural practices like crop rotation and fungicide application. Although the precise mechanisms creating and maintaining such high levels of genetic and phenotypic diversity of *S. sclerotiorum* in soil remain unknown, documenting the levels of phenotypic and genetic diversity in soil is the first step toward that goal.

**Acknowledgments**

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Table 1. Number of alleles in each locus and pair-wise linkage disequilibrium among the nine microsatellite loci estimated using MULTILOCUS. Significance levels are in parenthesis (Note loci 7-3 and 12-2 amplified the same microsatellite repeats.).

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*Locus definition from Sirjusingh and Kohn (2001)*
**Fig. 1.** Top. Color change of the pH-indicating medium rated from left to right as +, ++ and +++ 3 days after inoculation. Bottom. Variation in colony color on Difco PDA from left to right dark, beige and white three weeks after inoculation.
Fig. 2. (A). Minimum spanning tree based on eight independent microsatellite loci showing the relationship among 40 isolates of *Sclerotinia sclerotiorum* from 1 m² of soil. Scale bar equals one mutational step under step wise mutation model. (B). Posterior probability of population assignment from STRUCTURE analysis. (C). Mycelial compatibility groups. (D). Colony color (W-White, B-Beige, D-Dark), and (E). Oxalic acid production on a pH-indicating medium (+ least, +++ highest).
Fig. 3. Comparison of isolates LP-10-18 and LP-10-40 for pH change and oxalic acid production in a liquid medium over 6 days. Bars represent 1 standard errors.
Fig. 4. Isolate LP-10-18 (left) showing continuous growth around the periphery of the colony, compared with another isolate LP-10-40 (right) on fluazinam amended PDA.
**Fig. 5.** Pathogenicity on detached pea leaves. Isolate LP-10-18 displayed water-soaked but green lesion (right) compared with isolate LP-10-40 (left) showing brown discoloration of the lesion.
CHAPTER FOUR

GENETIC COMPARISON OF INOCULUM POPULATIONS WITH INFECTION POPULATIONS OF SCLEROTINIA SCLEROTIORUM WITHIN ALFALFA FIELDS

Abstract

White mold caused by Sclerotinia sclerotiorum can be a serious problem in alfalfa seed production system in the eastern Washington state. Isolates of S. sclerotiorum were collected from two commercial alfalfa seed production fields located at Lowden and Moses Lake, WA, before the beginning of the growing season from soil and from infected alfalfa plants at the end of the growing season. This sampling strategy allowed us to compare inoculum populations (isolates from soil) with infection populations (isolates from infected plants) and study genetic structure and population dynamics of the two most important stages of the life cycle. In A total of 171 isolates were genotyped using eight microsatellite markers. Genetic diversity between the soil and stem populations were compared to test the hypothesis of population differentiation and stem populations have low genetic diversity compared to the soil populations due to host selection. Both stem and soil populations were equally diverse and no significant population differentiation of each location was found. Recombination was detected in three out of the four populations. Lack or minimal host selection could explain a lack of population differentiation between the populations. However, a significant population differentiation was observed between the two field locations, Lowden and Moses Lake. The disease management will be challenging due to the presence of high genetic diversity in WA. Washington is one of the leading alfalfa seed producers in the USA and alfalfa seed stocks are distributed to the domestic
and foreign markets. Sclerotia of highly diverse genotypes could also be distributed with the infested seed stocks. Therefore, special attention should be paid to reduce sclerotial contamination of alfalfa seeds to prevent the distribution of highly diverse genotypes around the country/world.

**Introduction**

Alfalfa (*Medicago sativa* L.) is a perennial legume grown mainly for animal feed/hay. However, in eastern Washington state alfalfa is grown primarily for seed production and Washington state is one of the leading alfalfa seed producers in the USA. In the eastern Washington State, alfalfa seed harvest takes place between August and early September. Any remaining alfalfa stubble die in the winter and the plant goes into a dormant state from November to March. In spring, plants germinate and fully bloom between the middle of June and July. White mold is one of the major diseases of alfalfa and the disease is caused by *Sclerotinia sclerotiorum* (Lib.) de Bary in the US PNW (13), although the disease could be caused by *Sclerotinia trifoliorum* Erikss. elsewhere (5). *S. sclerotiorum* infection occurs from May to July in this region and can cause severe crop damage (13). The pathogen survives in soil as recalcitrant sclerotia, a vegetative structure consisting of compacted masses of mycelia. Sclerotia can survive in soil for up to 8 years (14) and may regenerate new sclerotia in soil in the absence of a host plant (40), prolonging the survival of sclerotia in soil. In fields of perennial crops such as alfalfa, sclerotia of *S. sclerotiorum* can accumulate over years resulting in high sclerotial densities in soil. Over 700 sclerotia per square meter of soil have been reported in alfalfa fields in WA (13). Gilbert (13) studied the pathogen disease cycle and chronology in this region and reported that the overwintering sclerotia in soil germinate and form apothecia between late March and mid May. Infection, mycelial growth on plants, and disease symptoms can be seen
from early May through mid-July. However, whether the infection is initiated by ascospores or by mycelium from sclerotia has not been determined (13). Sclerotia are produced on alfalfa plants in June and July, and majority of the sclerotia formed on alfalfa stems fall to the ground during harvest and complete the disease cycle (13). This chronology allows us to sample the inoculum population – the sclerotia in soil before the start of the new growing season, and the infection population – sclerotia produced on infected plants near the end of the growing season.

A few investigators have compared *S. sclerotiorum* populations from the same field, sampled at different disease stages or from different infection types (2, 9, 26, 32). Previous comparisons have been made between isolates from ascospores trapped from air with isolates from disease lesions on potato (2), isolates from lesions caused by ascospore infection with isolates obtained from crown/root infection on sunflower (9), isolates causing rosette infection with isolates causing stem infection on canola (26) and isolates from floral infections of canola caused by ascospore with isolates from secondary stem infections caused by mycelia (32). However, direct comparison of soil isolates within a field to those causing stem infections in the same field to determine the selection pressure exerted by the host and/or the environment during the infection process has not been made in any known studies. Earlier, we observed the presence of high genetic and phenotypic diversity of *S. sclerotiorum* isolates from soil on a fine geographic scale (3). With the observations of our previous research (3), it was hypothesized that less genetic diversity exists in isolates originating from stem infections compared to those originating from sclerotia in the soil located in the same fields. The hypothesis was based on the assumption that only a portion (or the most fit isolates) of the accumulated sclerotia in soil can successfully initiate a carpogenic germination, release ascospores, overcome host defense and initiate infection. Similarly, even if the disease initiates via mycelial germination of sclerotia,
host defense mechanisms should be overcome for the disease to establish. Therefore, whether the ascospore or mycelia initiates the infection, the pathogen has to face the selection pressure applied by the host and may not achieve 100% success in infection. The objective of this research was to compare the genetic and genotypic diversity of \textit{S. sclerotiorum} isolates collected from soil (hereafter referred to as inoculum population or soil population interchangeably) before the beginning of the season (in March) with those from stem infections (hereafter referred to as infection population or stem population interchangeably) at the end of the growing season (in August) from the same fields to study the population dynamics of the most important two stages of the disease cycle, survival vs. infection.

\textbf{Materials and methods}

\textbf{Isolates}

Sclerotia of \textit{S. sclerotiorum} were collected from soil at the beginning of the growing season in March 2011 and from infected alfalfa plants near the end of the growing season in August 2011 from two commercial alfalfa seed production fields. The two fields were fourth-year stands of cultivars D-222 and Pioneer 54V09, located in the Columbia Basin approximately 125 km apart near Lowden and Moses Lake, WA, respectively. Sclerotia from soil were collected from the top 1.27 cm layer of a one square meter area near the center of the field as described in the previous chapter. Sclerotia from stems were collected from infected plants at 1-meter intervals to avoid collection from the same infection foci. Crown infection of alfalfa is not common in eastern Washington State (13) and was not observed in the sampling locations (L. Porter, \textit{personal communication}). Sclerotia were surface-sterilized by soaking first in 70% ethanol for 1 min and then in 3% sodium hypochlorite for an additional min. Sclerotia were then rinsed three times in sterile distilled water, aseptically cut into halves and plated on acidified
PDA. Pure cultures were obtained by hyphal tip isolation and maintained on PDA at 4°C for short-term storage. One isolate was obtained from each sclerotium. Species identity of the isolates were determined based on colony morphology and the absence of group I introns in the ITS region of *S. sclerotiorum*, thereby distinguishing isolates from those of *S. trifoliorum* as previously described (24, 27). Isolates were first grouped by location (Lowden and Moses Lake). Isolates from each location were then separated by the source, stem or soil. Thus, the isolates constituted four populations: Lowden soil, Lowden stem, Moses Lake soil and Moses Lake stem.

**Microsatellite genotyping**

Total genomic DNA was isolated from each isolate using the FastDNA® Spin Kit (MP Biomedicals, Solon, OH) following the manufacturer’s instructions with minor modifications as described by Njambere et al. (24). Eight microsatellite markers from those developed by Sirjusingh and Kohn (34) were used to compare the gene and genotypic diversity among populations. The 5’ end of the forward primers were labeled with the M13 sequence, (CACGACGTTGTAAAACGAC) and a fluorescent labeled multiplexed PCR approach (30) was used for genotyping. PCR parameters were similar to those described by Sirjusingh and Kohn (34). PCR products labeled with each fluorophore were multiplexed and genotyped using the ABI3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) at the USDA-ARS Western Regional Small Grain Genotyping Laboratory, Pullman. Fragment analysis was performed using GeneMarker software (SoftGenetics, State College, PA) to determine allele (fragment) sizes. Microsatellite repeat numbers were confirmed by sequencing representative sizes of each allele.

**Gene diversity, genotypic diversity and population structure**

ARLEQUIN ver. 3.5 (11) was used to determine Nei’s gene diversity, $H_e$, (23). Allelic Diversity Analyzer (ADZE) ver. 1.0 (39) was used to estimate the number of alleles per locus and the
number of private alleles per locus using the rarefaction approach (17) to facilitate comparison of populations with different sample sizes.

Genotypic richness, the number of genotypes observed in each population \( (g) \), and the number shared genotypes between populations were estimated using ARLEQUIN. A rarefaction approach, which estimates the number of multilocus haplotypes expected (denoted as \( g_{exp} \) in the Table 1) in a population corresponding to the smallest sample size, was used due to the unequal population sizes (17) as suggested by Grünwald et al. (15),

\[
E(g_n) = \sum_{i=1}^{g} \left[ 1 - \left( \frac{N_i}{n} \right) / \left( \frac{N}{n} \right) \right]
\]

where, \( E(g_n) \) is the expected number of individuals (multilocus haplotypes in this case) corresponding to \( n \) (the smallest population size, \( n=20 \) in this case) selected at random from \( N \) isolates (sample size of the population of interest). \( N_i \) is the individuals of the \( i^{th} \) genotype and \( i \) can range between one and the number of unique genotypes in the smallest sample size. The \( g_{exp} \) value was verified by using 10,000 jackknife replicates in GenoDive ver.1.0 (20) and 95% confidence intervals were estimated. Genotypic diversity was also estimated using a similar measure as expected heterozygosity (Nei’s gene diversity at genotypic scale), the probability that two randomly chosen haplotypes are different in the sample, as implemented in ARLEQUIN with 1000 randomizations. A modified Stoddart and Taylor’s \( G \) (37) scaled to the differences of sample sizes as suggested by Grünwald et al. (15) was estimated by \( G/g \) where \( G \) is the Stoddart and Taylor’s \( G \) and \( g \) is the number of haplotypes. Shannon-Wiener’s index (33) \( H’ \) and genotypic evenness as measured by Hill’s modified evenness index (also known as modified Hill’s ratio) (16, 19), \( E_5 = (G-1)/(e^{H’}-1) \), where \( G \) is the Stoddart and Taylor’s \( G \) and \( H’ \) is the Shannon-Wiener’s index (33), and 95% confidence intervals were determined using SAS macro <jackboot.sas> and 2000 bootstrap replicates (15) in SAS (version 9.2; SAS Institute, Cary, NC).
Genotypic diversity indices were considered significantly different if there was no overlap of 95% confidence intervals. The highest value for $G$ is the number of haplotypes where as $G/g$ can have the maximum of one. $H'$ ranges from zero, if only one haplotype is present in the population, to its maximum when every isolate is a unique haplotype. However, $H'$ has a bias towards sample size (19). $E_5$ measures the evenness of the distribution of genotypes in a sample and ranges from zero (if a single genotype is dominant) to one (if genotypes distributed evenly). $E_5$ has the advantage in that it does not depend on the sample size (15, 19).

Pair-wise comparisons among populations were performed and Slatkin’s $R_{ST}$ (35) was calculated, which assumes the step wise mutation model for microsatellite data, to detect genetic differentiation between soil and stem generated populations. Significant difference of $R_{ST}$ value from zero ($p < 0.001$) was estimated with 1023 permutations as implemented in ARLEQUIN. An $R_{ST} = 0$ indicates a panmictic population (no sub-groups and all individuals are potential recombining partners) whereas $R_{ST} = 1$ indicates complete differentiation.

STRUCTURE ver. 2.2 (29) was used to determine the most probable number of genetic clusters using the Bayesian clustering approach. The clustering method determines that there are $k$ populations, each characterized by allele frequency data at each locus. Each individual isolate is assigned to one of the $k$ populations without consideration for geography or location. Population admixture model was used and each simulation consisted of 500,000 Markov Chain Monte-Carlo (MCMC) iterations followed by a burning period of 100,000 iterations. Five independent runs of one to eight subpopulations ($k = 1-8$) were performed to estimate the most probable number of genetically homogenous clusters ($k$). True $k$ value was identified as the maximum value of posterior probability of ln likelihood, lnP(D), as described in Evanno et al.
(10) using the software STRUCTURE HARVESTER ver. 0.6 (8). Briefly, LnP(D) plateaus after reaching the true $k$ value and the most likely number of genetic clusters was determined.

A matrix of pair-wise genetic distance and Eigenvectors were computed and principal coordinate analysis (PCoA) was performed as implemented in GenAlEx ver. 6.41 (25). PCoA has the advantage over STRUCTURE due to lack of underlying assumptions such as linkage equilibrium, and was used to confirm the genetic structure inferred by the STRUCTURE.

The relative distribution of genetic variance at three levels (within population, among populations, and among groups of populations) was estimated using a hierarchical analysis of molecular variance (AMOVA) (12) based on the analysis of variance frame work using ARLEQUIN. Genetic distance matrix was based on the step wise mutation model (35). Significance of the null hypothesis that soil and stem populations are not genetically differentiated, was tested using a non-parametric approach in ARLEQUIN with 1023 permutations of the data set among and within population groups. Soil and stem populations from each location (Lowden and Moses Lake) were grouped together and the significance of the distribution of variance components were recorded.

**Linkage disequilibrium**

Recombination was tested using the Index of Association ($I_A$) test implemented in MULTILOCUS ver. 1.3 (1). This method was first developed for barley by Brown et al. (6) and adapted to microbes by Maynard and Smith (36). To remove the dependency on the number of polymorphic loci, instead of $I_A$ a modified measure, $r_{st}$, was used. Briefly, the observed variance of distances (estimated by the number of different loci) between all pairs of individuals is compared to the expected variance of a simulated data set of random mating. Significance of the null hypothesis, no linkage disequilibrium, was tested with 1000 randomizations of the data set.
In an out-crossing population, \( r_d \) is expected to be zero. Clone corrected data set of each population, where only one representative of each haplotype was included, was used to reduce the bias due to over representation of clones. A Parsimony Tree Length Permutation Test (PTLPT), which is based on a phylogenetic method, was also adapted to detect recombination using MULTILOCUS. Phylogenetic trees were built from the multilocus genotypes using PAUP ver. 4.0b10 (38) by treating the loci as characters and the alleles as character states. This test is based on the observation that clonal populations have significantly shorter tree lengths than recombining populations (1). Significance of the test was determined by comparing the observed tree length to the distribution of tree lengths of 1000 data sets that have been artificially generated from a random mating population as mentioned above.

**Gene flow among populations**

Since no significant differences between soil- and stem-generated populations were found in each location in pair-wise population analyses, both inoculum population and stem-infection population from each location were combined in the gene flow analysis. In addition, a previously studied population of 40 *S. sclerotiorum* isolates from alfalfa field soil in Touchet, WA (3) was included. Therefore, three populations: Lowden, Moses Lake and Touchet, WA were analyzed for gene flow. The approximate distance from Touchet to Lowden is 7 km, from Touchet to Moses Lake is 150 km, and from Lowden to Moses Lake is 125 km. MIGRATE ver. 3.2.1.6 (4), which uses the coalescence theory to implement maximum likelihood estimation of population size and immigration rate, was used. Brownian motion model, which assumes the step wise mutation, was used in all runs. Ten initial short chains with 20,000 sampled genealogies (20 steps) and three final long chains with 200,000 sampled genealogies (20 steps) were used as Markov Chain settings. MIGRATE estimates the amount and direction of gene flow between
populations (4) with the mutation scaled population size, $\Theta = 2Ne\mu$ (for haploids) where $Ne$ is the effective population size and, the mutation scaled immigration rate $M = m/\mu$ where $m$ is the immigration rate and $\mu$ is the mutation rate per generation. The number of immigrants per generation was estimated with 95% confidence intervals as $N_e m = \Theta M/2$.

Results

Germination rates of sclerotia collected from soil ranged from 25-72%, whereas 91-93% germination rates were observed for sclerotia collected from stems. A total of 117 isolates were obtained from the two locations (46 from Lowden, and 71 from Moses Lake, WA). Twenty of the 46 Lowden isolates were from soil whereas 31 of the 71 Moses Lake isolates were from soil (Table 1). All the isolates produced colony morphology similar to Sclerotinia spp. and lacked the group I intron in the small subunit rDNA confirming that all the isolates were indeed S. sclerotiorum (24, 27).

Gene and genotype diversity

The four populations (Lowden stem, Lowden soil, Moses Lake stem and Moses Lake soil) were analyzed separately for gene and genotypic diversity. All of the loci tested were polymorphic in the four populations, except locus 114-4, which was fixed in the Lowden soil population. The number of alleles per locus ranged from 2.4 to 3.9 among populations. The number of alleles per locus and the number of private alleles per locus in the stem populations were greater than that of the soil populations. However, due to the differences in sample sizes, these numbers are not directly comparable and a rarefaction correction was required. After rarefaction correction, allelic richness estimates were not significantly ($p = 0.05$) different between stems and soil populations from each location (Table 1). Stem and soil populations were
not different for the expected heterozygosity, $H_e$, (23) and both stem and soil populations from each location had approximately equal levels of gene diversity (Table 1).

Genotypic diversity was estimated using several indices. Most haplotypes were represented by single isolates and a total of 81 haplotypes were detected among the 117 isolates. Five haplotypes were shared between soil and stem populations at each location, but shared haplotypes were not found between the two geographic locations (Fig. 1, Table 2). At the Lowden location, 16 unique haplotypes were detected in the stem population and the genotypic diversity was greater (57% of total) than that of the soil population. Similarly, at the Moses Lake location, 38 unique haplotypes were found in the stem population and genotypic diversity was higher (60% of total) than that of the soil population. However, due to sample size differences these numbers are not directly comparable, as mentioned earlier. Soil and stem originated populations from each location did not differ for the population size-scaled Stoddart and Taylor’s $G$, the number of haplotypes after rarefaction correction ($g_{exp}$), haplotypic diversity estimate and a genotypic evenness index, $E_5$, based on the non overlapping 95% confidence intervals (Table 1). Soil and stem populations did not differ for the estimate $H'$ in the Moses Lake location. However, in Lowden location, $H'$ in stem population was significantly higher than the soil population (Table 1). $H'$ estimate has a bias towards sample size differences (15, 19). Both soil and stem populations from each location were equally rich in genotypic diversity.

Pair-wise population differentiation estimates, Slatkin’s $R_{ST}$ (35) values, ranged from 0.018-0.19 (Table 2). No significant population differentiation ($p < 0.001$) was observed between the stem and soil populations in either the Moses Lake or Lowden locations even after Bonferroni corrections ($p = \alpha/6$). This was further evident by the shared haplotypes between the stem and soil populations (Table 2). All of the 117 isolates were clustered into two groups in
STRUCTURE analysis (LnP(D) = -840.3 ± 0.34) (Fig. 2). Population clusters generated by STRUCTURE corresponded with the geographic locations. Two isolates in the Lowden population had Moses Lake affiliation, whereas three isolates from Moses Lake had high affiliation to the Lowden population (approximately q > 70%) (Fig. 2). One isolate from the Moses Lake population showed admixture.

Soil and stem populations from each location were grouped together in AMOVA analysis due to the lack of significant differentiation in each location. The highest percent of the total genetic variance (> 80%) resided within populations and was statistically significant (p < 0.001) (Table 3). A significant proportion (p < 0.001) of the total genetic variance (> 18%) was due to between two geographic locations (Table 3) and was significant (p < 0.001). The differentiation between populations within groups (soil and stem from each location) was not significant (p = 0.83).

Population differentiation between the two geographic locations was confirmed by the PCoA. PCoA analysis, a method to visualize the patterns of genetic relationships, showed that the isolates from the two locations were separated by the first and second principal coordinates (Fig. 3). As observed in the Bayesian clustering method implemented in STRUCTURE (Fig. 2), PCoA also confirmed that some isolates from Moses Lake clustered with the Lowden population (Fig. 3). Haplotypes were separated primarily by three coordinates and explained 35.4, 20.9 and 16.0% of the total variation (72.3% cumulative).

Both PTLPT and $r_d$ analysis produced approximately similar results (Table 1). The observed $r_d$ value for the soil population from Lowden was well within the distribution of $r_d$ values for the 1000 randomized data sets and could not reject the null hypothesis of random association of alleles (at $p < 0.05$). Similarly the null hypothesis of recombination could not be
rejected for the stem populations from Lowden and Moses Lake (at $p < 0.001$) (Table 1). The length of the tree generated from the observed data set was not significantly different from the distribution of tree lengths estimated for 1000 randomizations of the data set for the soil population from Lowden (at $p < 0.05$) and for the stem populations from Lowden and Moses Lake (at $p < 0.001$). In both the tests, Moses Lake soil population was found to be clonal (Table 1).

**Gene flow among populations**

Though no evidence for shared haplotypes between Lowden and Moses Lake was detected (Tables 2, 3, Fig. 1), it cannot be assumed that there was no migration between populations and among neighboring fields. Therefore, a previously studied population obtained from another alfalfa field (3) located in Touchet, WA (the distance between Lowden and Touchet is 7 km) was included in the gene flow analysis. Number of migrants and 95% confidence interval was estimated for each direction. The highest migration rates (approximately symmetrical) were found between Moses Lake and Touchet populations based on maximum-likelihood analysis using MIGRATE [$N_e m_{\text{Moses Lake} \rightarrow \text{Touchet}} = 2.3$ (1.2 - 4.0) and $N_e m_{\text{Touchet} \rightarrow \text{Moses Lake}} = 2.6$ (1.3 - 3.4)]. Intermediate migration rates (but asymmetrical) were found between Lowden and Touchet [$N_e m_{\text{Lowden} \rightarrow \text{Touchet}} = 1.6$ (0.15 - 3.2) and $N_e m_{\text{Touchet} \rightarrow \text{Lowden}} = 0.61$ (0.3 - 2.5)]. The least amount of gene flow was detected between Moses Lake and Lowden [$N_e m_{\text{Lowden} \rightarrow \text{Moses Lake}} = 0.7$ (0.05 - 1.8) and $N_e m_{\text{Moses Lake} \rightarrow \text{Lowden}} = 0.2$ (0.15 - 0.28)]. The migration rates and directions were depicted in the Fig. 4. However, the effective population sizes (indicated by $\Theta$) were different and it might have an effect on the gene flow estimates (Fig. 4). Further observations found that eight haplotypes were shared between Moses Lake and Touchet.
whereas six haplotypes were shared between Touchet and Lowden populations (data not shown) supporting the results of MIGRATE.

**Discussion**

Genetic structure and population differentiation of *S. sclerotiorum* have been extensively studied (2, 9, 26, 31, 32). However, all the previous studies (except Atallah et al. (2)) have been conducted on populations obtained from infected plants even though the source of infection may be different (air-borne ascospore infection vs. infections from mycelial germination of sclerotia). Host selection has not been taken into account since the samples originated from infected plant tissues. Considering the longevity (two to eight years) of sclerotia in soil (14, 40) and the ability to regenerate new sclerotia in the absence of host plants (40), the sclerotia in soil represent populations of the pathogen infecting the crops that have grown in the fields for several years, whereas the stem isolates represent a subset of the population found in the soil that are putatively better adapted to infect the current crop, alfalfa in this case. If the alfalfa plants exerted a strong selection pressure, the stem populations would exhibit less genetic diversity than the soil populations. Stem infection of alfalfa plants can occur from air-borne ascospores or from mycelia from germinated sclerotia when stems touch the ground after the row closure (13). However, the exact proportion of inocula originating from ascospores and sclerotia is not well understood in alfalfa seed production systems of the US Pacific Northwest (13), and mycelial infection of stems in contact with soil was frequently observed (L. Porter, unpublished information). Crown infection was not observed in PNW alfalfa cultivation systems (L. Porter, unpublished information). For an ascospore to infect a plant it has to overcome several barriers like carpogenic germination of sclerotia (those escaped from mycoparasites), release ascospores and the ascospores must land on suitable substrates prior to the infection. Therefore, it is
conceivable that a higher selection pressure acts on populations from ascospore infection than on populations from mycelial infection by mycelial germination of sclerotia. Even if sclerotia in soil germinated via mycelial germination, the pathogen has to overcome its host defense mechanisms. Therefore it is intuitive to hypothesize that soil isolates harbor greater genetic diversity than the stem isolates. However, the proportions of ascospore infection and mycelial infection causing disease were not determined in this study and in either case host selection affects the genetic structure of the pathogen and can result in less diversity in infection populations.

Both soil and stem populations in each location were equally diverse. The high genetic diversity in the studied populations could have been due to recombination (recombination was detected in three out of four populations) and gene flow. Even though the gene flow between Moses Lake and Lowden populations was limited, a relatively high level of gene flow was detected between Moses Lake and Touchet populations (Fig. 4). Air-borne ascospores receiving from neighboring fields, movement of sclerotia by agricultural machinery and through contaminated seed lots would explain possible gene flow and sources of genetic diversity since, dissemination of *S. sclerotiorum* via seed-born inoculum has been reported (7, 22). Atallah et al. (2) reported that migration could contribute to the observed high levels of diversity in the US PNW as they were able to capture isolates belong to different MCGs from air-borne ascospore-samples at a study site not very far from the current fields (about 100 km). All these factors may explain the high diversity in soil and stem populations observed in the current study.

Our sampling strategy allowed us to investigate the population dynamics at the most important stages of the disease cycle (infection vs. survival) and compare the pathogen populations before and after the cropping season and to observe population sub structuring.
Significant population differentiation or a population structure was not observed between soil and stem populations as indicated in AMOVA, pair-wise $R_{ST}$, STRUCTURE and PCoA analyses. In addition to these estimates, an absolute pair-wise population differentiation measure of Jost’s $D$ (18) also indicated no population differentiation (data not shown). The results suggest that the alfalfa plants might exert low selection pressure on the pathogen or the soil populations were already selected for infecting alfalfa plants since alfalfa is a perennial crop and the sampled fields were fourth year stands. Sclerotia collected from soil had much lower germination rates compared with sclerotia collected from plant stems. It could be that the sclerotia accumulated from previous crops or years were severely parasitized by mycoparasites and failed to germinate, as reported by Merriman (21). Therefore, the germinated sclerotia were likely recently accumulated in the current crop, and might have been adapted to alfalfa and/or to survive in soil. However, considering the high levels of genetic diversity and the numerous single isolate haplotypes in all four populations, it can be reasonably concluded that the selection pressure exerts by alfalfa plants on *S. sclerotiorum* is low. Diverse genotypes of *S. sclerotiorum* seem to be equally able to infect alfalfa plants. A previous study of 40 isolates of *S. sclerotiorum* from alfalfa soil found no variation in virulence on detached leaves of pea, also a cool season legume just as alfalfa, despite high levels of genotypic variation among the isolates (3). Sexton et al. (32) also found no significant difference in isolate virulence between populations from ascospore infection and mycelial infection.

Lack of differentiation between inoculum population and infection population is not unique to the alfalfa - *S. sclerotiorum* pathosystem. Sexton et al. (32) compared isolates collected from ascospore-infected petals, which are the source of inoculum for secondary infection, with those collected from infected stems of canola resulted from secondary infection, and found no
genetic differentiation between the inoculum population and the stem-infection population. Ekins et al. (9) compared head rot (initiated by ascospores) and basal stem rot (initiated by sclerotia) and found no population differentiation. Lack of population differentiation is not surprising considering the wide host range of *S. sclerotiorum* and lack of variation in virulence among isolates on a range of crops demonstrated in previous studies (2, 28, 31). However, our sampling strategy that the areas from which the stem isolates were collected were larger than the areas from which the soil isolates were collected could have under-estimated the genetic diversity of the inoculum populations relative to infection populations. Another possibility is that alfalfa might be super susceptible to *S. sclerotiorum* and if sampling was done from a resistant crop or cultivar, the results could have been different.

Despite the lack of differentiation between the soil populations before the beginning of the season and the stem populations near the end of the season, a significant differentiation was observed between the two geographic locations (Tables 2, 3, Fig. 1). Similarly, Sexton et al. (31) also observed population differentiation between two canola fields approximately 400 km apart in Australia, even though no population differentiation between ascospore and infection populations of the same fields were detected. Currently, no resistant alfalfa cultivars are available for white mold. Cultivars D-222 and Pioneer 54V09 were grown in Lowden and Moses Lake, respectively, and were very susceptible and moderately susceptible based on the growers experience (L. Porter, *personal communication*). It is unlikely that the different alfalfa cultivars exerted the selection and differentiation of populations from two geographic locations because of their susceptibility to white mold and low selection pressure, as discussed above.

The present study was unique since it compared soil populations (inoculum population) and stem populations (infection population) of *S. sclerotiorum* to infer potential host selection
pressure impacting diversity between the two populations. A high level of genetic diversity of S. sclerotiorum isolates collected from alfalfa plants and from soils in the US PNW was observed. Although population differentiation was detected at the geographic level, we were unable to find differences in the level of genetic diversity between soil and stem-collected populations, indicating that host selection could be of minimal importance in alfalfa-white mold system in the US PNW. Results suggest that the studied pathogen populations in the US PNW alfalfa fields were stable from season to season. Since we observed high genetic diversity in WA alfalfa seed production system, the disease management will be challenging. Washington state is one of the leading alfalfa seed producers in the USA. Seed stocks are distributed to the domestic and foreign markets and sclerotia of highly diverse genotypes could also be distributed with the infested seed stocks. Therefore, special attention should be paid to control white mold on WA alfalfa seed production systems to prevent the distribution of diverse genotypes around the country/world, since S. sclerotiorum can infect a wide variety of crops and newly introduced genotypes may challenge the existing agricultural systems.

Acknowledgements

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*Sclerotinia sclerotiorum* in an Australian canola field at flowering and stem-infection

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Table 1. Gene diversity, genotypic diversity and tests of recombination in stem and soil populations of each location.

<table>
<thead>
<tr>
<th>Location</th>
<th>Source</th>
<th>N</th>
<th>Gene diversity</th>
<th>Genotypic diversity</th>
<th>Evenness</th>
<th>Recombination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td># of alleles per locus</td>
<td># of private alleles per locus</td>
<td>g_{obs}</td>
<td>g_{exp}</td>
</tr>
<tr>
<td>Lowden</td>
<td>Stem</td>
<td>26</td>
<td>3.1 (2.4-3.3)</td>
<td>0.37 (0.0-0.5)</td>
<td>16</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>Soil</td>
<td>20</td>
<td>2.4 (1.7-2.9)</td>
<td>0.12 (0-0.4)</td>
<td>12</td>
<td>0.81</td>
</tr>
<tr>
<td>Moses</td>
<td>Stem</td>
<td>40</td>
<td>3.9 (2.9-3.7)</td>
<td>0.37 (0.0-0.5)</td>
<td>38</td>
<td>0.91</td>
</tr>
<tr>
<td>Lake</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Soil</td>
<td>31</td>
<td>3.8 (2.8-3.7)</td>
<td>0.12 (0.06-0.3)</td>
<td>25</td>
<td>0.85</td>
</tr>
</tbody>
</table>

124
a Number of isolates in each population
b Values in parenthesis represent 95% confidence intervals of the means across loci estimated by ADZE ver. 1.0 (39) with 1000 bootstrap replications
c Observed numbers of alleles per locus
d Expected number of alleles per locus corrected for the smallest sample size (20 in this case)
e Nei’s expected heterozygosity estimated by ARLEQUIN (11)
f The number of observed multilocus haplotypes in each population
g Stoddart and Taylor’s G scaled to the sample size according to Grünwald et al.(15).
h Expected number of genotypes corrected for the smallest sample size (n=20 in this case) using GenoDive ver. 1.0. and 95% CIs with 10,000 jackknife replicates within parentheses (20).
i Haplotypic diversity, the probability that two randomly selected haplotypes are different in the sample, using ARLEQUIN (11). Values in parenthesis are sampling variance with 1000 randomizations.
j Shannon Wiener index of diversity and values in parenthesis are 95% CIs calculated using SAS macro <jackboot.sas> and 2000 bootstrap replicates (15)
k Evenness estimate (an estimate indicating how genotypes are distributed within a sample) and values in parenthesis represent 95% CIs calculated using SAS macro <jackboot.sas> and 2000 bootstrap replicates (15)
l Index of Association among loci and the significance (within parenthesis) as measured by MULTILOCUS (1).
m Statistical significance of the Parsimony Tree Length Permutation Test (PTLPT) as implemented in MULTILOCUS (1) and performed in PAUP ver. 4.0b10 (38)
Table 2. Population pair-wise comparison of *Sclerotinia sclerotiorum* as measured by Slatkin’s $R_{ST}$ (upper diagonal) and the number of shared haplotypes between populations (lower diagonal).

<table>
<thead>
<tr>
<th>Location</th>
<th>Lowden</th>
<th>Moses Lake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soil</td>
<td>Stem</td>
</tr>
<tr>
<td>Populations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil</td>
<td>0.000</td>
<td>0.018</td>
</tr>
<tr>
<td>Stem</td>
<td>5</td>
<td>0.000</td>
</tr>
<tr>
<td>Soil</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stem</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

An $R_{ST} = 0$ indicates no differentiation and $R_{ST} = 1$ indicates complete differentiation.

* significant at $p < 0.001
Table 3. Analysis of molecular variance (AMOVA) within and among groups of *Sclerotinia sclerotiorum* populations from Lowden and Moses Lake, WA

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f</th>
<th>Sum of squares</th>
<th>Variance components</th>
<th>% of the total variance</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups (Lowden &amp; Moses Lake)</td>
<td>1</td>
<td>34646.9</td>
<td>609.3</td>
<td>18.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Between populations within groups (stem and soil)</td>
<td>2</td>
<td>1193.4</td>
<td>-74.9</td>
<td>-2.28</td>
<td>0.83</td>
</tr>
<tr>
<td>Within populations</td>
<td>113</td>
<td>311165.6</td>
<td>2753.6</td>
<td>83.75</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>116</td>
<td>371,016.1</td>
<td>3288.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1. Frequency distribution of 81 multilocus haplotypes among 117 isolates of *Sclerotinia sclerotiorum* from four alfalfa field populations: Lowden stem (A), Lowden soil (B), Moses Lake stem (C), Moses Lake soil (D).
Fig. 2. Assignment of individual isolates of *Sclerotinia sclerotiorum* from four populations into genetic clusters using STRUCTURE. Isolates are separated into vertical bars that are organized by each population (from left to right, Lowden Stem, Lowden Soil, Moses Lake Soil and Moses Lake stem respectively). Y axis shows the Bayesian posterior probability of population assignment. $k = 2$ was determined to be the most likely number of genetic clusters and population affiliation is indicated by each color.
Fig. 3. Principal coordinate analysis (PCoA) performed by GenAlEx using multilocus haplotypes of *Sclerotinia sclerotiorum* from the four populations: Lowden stem, Lowden soil, Moses Lake soil and Moses Lake stem. Each point represents a multilocus haplotype based on the pair-wise genetic distance matrix of eight microsatellite loci. Two axes with the highest percent variances are shown here.
Fig. 4. A diagrammatic depiction of gene flow among three geographic populations of alfalfa fields in WA. Arrow lengths are proportionate to the number of immigrants per generation and direction of migration between sink and source populations are indicated by arrow heads. Θ value is a measure of effective population size ($2N_e\mu$, where $N_e$ is effective population size and $\mu$ is mutation rate inferred for each microsatellite loci). Approximate distances between populations in km are shown below the arrows.
CHAPTER FIVE

INTRACHROMOSOMAL RECOMBINATION SUGGESTS OUTCROSSING IN NATURAL POPULATIONS OF SCLEROTINIA SCLEROTIORUM

Abstract

Sclerotinia sclerotiorum is a homothallic fungal plant pathogen. Clonal as well as recombining population structures have been reported in many studies on S. sclerotiorum around the world. We previously observed random association between three physically linked loci in a S. sclerotiorum population suggesting intrachromosomal recombination or high mutation rates at these loci. In the same study, we observed a decay of pair-wise linkage disequilibrium (LD) with the increasing physical distance. The objective of this study was to test whether intrachromosomal recombination and LD decay with increasing physical distance between markers is frequent in natural populations of S. sclerotiorum. Twelve microsatellite markers distributed over four chromosomes were used and 269 isolates sampled from eight populations in the USA and China, from a variety of crops, were genotyped. Each isolate carried a single allele for each of the 12 loci suggesting that the isolates were haploid and homokaryotic. Pair-wise LD tests between linked loci showed relationships ranging from linked to random association. For most of the populations, LD decayed with increasing physical distance between loci in two out of three supercontigs. Therefore, the observed random association of alleles cannot be simply attributed to random mutation. Different recombination rates in various DNA regions (recombination hot spots and cold spots) and different evolutionary histories in different populations could explain the observed differences among the supercontigs and among populations. The majority of the isolates exhibited mycelial incompatibility, likely minimizing the possibility of heterokaryon formation and mitotic recombination. Thus, the observed high
intrachromosomal recombination could be due to meiotic recombination following outcrossing in these populations.

**Introduction**

Genetic diversity (gene diversity and genotypic diversity) of a population is generated and maintained by mutations and recombination. Recombination could be sexual or asexual (parasexual). Since parasexual recombination is relatively rare and limited in nature (14, 37), sexual reproduction plays a key role in generating recombinant genotypes, increasing genotypic diversity in populations (34). Therefore, genetic recombination is the hallmark of sexual reproduction manifested through random assortment of independent alleles and crossover between homologous chromosomes (34). Recombination can be detected by linkage disequilibrium (LD) analysis. Linkage Disequilibrium is defined as non-random association of alleles among loci and the term could be misleading since physically unlinked loci can also be in perfect LD in some populations. LD has been frequently used to distinguish clonal from sexual reproduction in populations (34, 46). While LD is frequently attributed to clonal reproduction, evolutionary forces like selection and preferential mating also cause LD. However, random association of alleles is unequivocally considered as a result of sexual reproduction (34). At a multilocus scale, Index of Association ($I_A$), a traditional measure of multilocus LD, was first introduced by Brown et al. (10) for *Hordeum spontaneum* and later applied to microbial populations (44). The term linkage disequilibrium will be used throughout this chapter since physically linked loci are the subject of this study and recombination via random assortment of alleles is not a consideration.

In the homothallic haploid fungus *Sclerotinia sclerotiorum*, the homothallism (self-mating) is functionally equivalent to clonal reproduction. Earlier population studies of S.
*sclerotiorum* found association of independent traits like haplotypes and mycelial compatibility groups (MCGs) and widespread distribution of few haplotypes over time and space, and concluded that *S. sclerotiorum* is clonal (2, 16, 20, 26). However, recent studies found random association of presumably independent molecular markers and discordance between haplotypes and MCGs, and inferred occurrence of outcrossing in *S. sclerotiorum* (6, 9, 19, 22, 33, 42).

During our previous study on genetic diversity of a *S. sclerotiorum* population from alfalfa in WA, random association of alleles between pairs of loci were observed (7). Further investigations found that three markers located on the supercontig-3 (physically linked loci) were in significant linkage equilibrium suggesting random association. Such random association of alleles could be either due to crossover at chromosomal locations between two loci or due to high mutation rates at these loci or both. Ascertainment of the cause has important implications. If the observed random association between linked loci is simply due to high mutation rates, that would suggest the Index of Association test (*I*<sub>A</sub>), frequently used to test reproductive mode, cannot be applied to *S. sclerotiorum* and, thus, the previous conclusions of outcrossing based on *I*<sub>A</sub> test could be erroneous. This is very important since many recent studies were conducted using microsatellite marker loci (simple sequence repeats) which are known to have high mutation rates (30). Mutations in microsatellite markers could occur by slippage and proofreading errors during DNA replication (30). This process primarily changes the number of repeats. On the other hand, if the random association of alleles between linked loci is due to crossover between homologous chromosomes, it would provide another line of evidence for the occurrence of outcrossing in *S. sclerotiorum*. Because *S. sclerotiorum* is haploid and homokaryotic (natural heterokaryons of *S. sclerotiorum* have not been found and proven), the source of variation must have come from outcrossing followed by crossover.
Crossover is best studied in controlled populations and commonly practiced in Quantitative Trait Locus (QTL) mapping in plant and animal breeding experiments, and only a few examples are available in fungi (13). QTL mapping requires controlled populations which simply reflect recombination and segregation of DNA markers and phenotypes. This process is time consuming and limited in resolution (25). Due to the homothallic nature, techniques for controlled crosses of *S. sclerotiorum* are not available. Association mapping (also known as linkage disequilibrium mapping) is an alternative to QTL mapping. In association mapping, marker-trait associations in natural populations are sought, and haplotype diversity is used to infer the recombination events that have happened not only in a single family, but accumulated over generations in the history of the population (25). Currently, association mapping has widely been used in human and plant genetics (17) and in a few fungal genetics studies (4, 31). Association mapping is based on the LD among loci and uses a dense set of evenly spaced markers across the genome/chromosomes to search for LD blocks. This approach has been studied in searching candidate disease loci in humans (17).

The amount of LD decays with time \( t \) and recombinational distance between markers \( r \). This relationship can be shown as \( D_t = (1-r)^t D_0 \) where \( D_t \) is the extent of disequilibrium at time \( t \), \( D_0 \) is the disequilibrium at the beginning, \( t \) is time and \( r \) is recombinational distance (3). Physical distances between loci are usually estimated from recombinational distances, which in turn are estimated from crossover events. Therefore, physical distance is proportionate to the amount of recombination (crossover) and inversely proportionate to the LD. In other words, LD would decay with the increasing physical distance between markers and with frequency of crossover events. Therefore, LD decaying with the physical distance is a direct result of meiotic recombination. However, this relationship depends on several factors such as mutation rates,
selection, long evolutionary history of the chromosomal region (or the entire genome) and genetic drift (5). In human genetics this phenomenon was first used to identify the recombination host spots near the beta globin gene by observing a relationship between non-random association and physical distance between the loci (11). Detection of such relationships in *S. sclerotiorum* would add additional new evidence to the inferred recombination which would explain the high genetic diversity found in various studies as mentioned earlier.

Another interesting aspect of studying intra-chromosomal scale LD (the genetic rearrangements between linked homologous sequences) is that it would estimate whether selection is acting on a particular loci or a region of a chromosome providing information on recombination “hotspots” and “cold spots”. For a devastating pathogen like *S. sclerotiorum*, this type of analysis could provide useful information related to pathogenicity and fungicide resistance development and evolution.

With the results of our previous study that the existence of significant linkage equilibrium among microsatellite markers (7), it was hypothesized that homologous recombination events take place in *S. sclerotiorum* populations and can be detected in some regions of the genome. The first objective of this study was to develop additional microsatellite markers in such a way that at least three markers were on the same supercontig. The second objective was to test the pair-wise LD between microsatellite marker loci on each chromosome and detect the relationship of LD and the physical distances among markers to infer homologous recombination and outcrossing. Third objective was to see whether recombination rates differ among different populations and among different chromosomes.
Materials and methods

Isolate collection and Mycelial Compatibility Groups (MCGs)

Two hundred and thirty nine isolates of *S. sclerotiorum* were collected from pea, lentil, potato and gourd fields from the US Pacific Northwest (PNW) and from a canola field in ND, USA. Another 30 isolates were collected from canola from Anhui province, China (Table 1). Infected plant materials or sclerotia were collected from infected plants at least 6 feet apart. Isolates were obtained from the sclerotia as previously described (8). Isolates were tested for mycelial compatibility groups (MCG) by pairing isolates in all possible combinations (within each population) on PDA plates amended with red food coloring (28). Each pair was tested at least twice.

Development of multiple microsatellite loci on the same chromosomes in *S. sclerotiorum*

Genomic locations of the microsatellite markers developed by Sirjusingh and Kohn (43) were determined by BLAST searching of the *S. sclerotiorum* genomic database (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.html). Three loci located on the supercontig-3 were chosen (Appendix 2). In order to develop multiple microsatellite loci located on the same supercontig, twelve new primer pairs flanking microsatellite repeats on three supercontigs were developed using the genomic database and the software WebSat (32). These 12 new primer pairs along with four additional loci from Sirjusingh and Kohn (43) formed multiple loci on three additional supercontigs (Appendix 2). A total of 19 markers were tested for polymorphisms among isolates of *S. sclerotiorum*. PCR amplification was performed at initial denaturation at 95°C for 2 min followed by 35 cycles of 95°C for 30s, 30s at the annealing temperatures (55-57°C) and 72°C for 30s with final extension for 10 min at 72°C. The reaction mixtures (20 μl) contained 40 ng of template genomic DNA from *S.
sclerotiorum, 1.5 units of Taq DNA polymerase (Promega, Madison, WI), 0.2 mM dNTPs and 1x buffer containing 1.5 mM MgCl$_2$ (Promega, Madison, WI), and 0.5 μm of each primer. Nucleotide sequences were determined from both strands using an ABI PRISM 377 automatic sequencer (Applied Biosystems, Foster City, CA) at the Sequencing Core Facility at Washington State University to confirm the presence of tandem repeats.

Total genomic DNA from each of the 269 isolates was extracted using FastDNA® Spin Kit (MP Biomedicals, Solon, OH) as previously described (36). The 5’ ends of forward primers were tagged with M13 tail sequence, CACGACGTTGTAAAACGAC for multiplexing as described in Schuelke (39). PCR was performed with one of the four fluorophores (Vic, Pet, Ned and Fam), multiplexed and genotyped using an ABI3730xl DNA analyzer (Applied Biosystems, Foster City, CA) at the USDA-ARS Western Regional Small Grain Genotyping Laboratory, Pullman. GeneMarker software was used for fragment analysis (SoftGenetics, State College, PA). Each isolate was genotyped at least twice for each locus. Variations in allele sizes were confirmed by sequencing.

**Detection of Linkage Disequilibrium (LD) between loci**

LD between pairs of loci on the same supercontig was tested using three methods. First, LD was tested by Fisher’s exact probability test implemented in GenePop ver. 4.13 (38). The null hypothesis was that the alleles of one locus are independent of the alleles of another locus. The software estimates the significance of the null hypothesis with Markov Chain (MC) algorithm for the contingency tables with all possible pairs of loci.

Second, the LD measure based on Lewontin’s $D’$ (29), a normalized multiallelic LD coefficient, was estimated according to Hedrick (21). LD for a bi-allelic system is defined as the deviation of observed frequency of haplotypes from the expected frequency as, $D_{ij} = x_{ij} - p_iq_j$
where \( x_{ij} \) is the observed haplotype frequency with \( i^{th} \) and \( j^{th} \) allele and \( p_i \) is \( i^{th} \) allele frequency and \( q_j \) is \( j^{th} \) allele frequency and \( p_i q_j \) is the expected haplotype frequency. The main disadvantage of the above LD estimate is its allele-frequency dependence. Lewontin (29) normalized the estimate as,

\[
D'_{ij} = \frac{D_{ij}}{D_{\text{max}}}
\]

where, \( D_{\text{max}} \) is the maximum value for \( D_{ij} \) with the given allelic frequency. However for multiallelic loci such as microsatellite loci, a simple LD estimation is not possible. Different coefficients have been proposed for measuring the extent of overall disequilibrium between all possible pairs of alleles between multiallelic loci (21). Most common measures are \( D' \) and \( r^2 \) (17). Hedrick (21) compared several coefficients of LD and found that \( D' \) performs better over several other estimates. Contingency tables for genotypes of each pair of loci on each supercontig were generated using GenePop (38) and Hedrick’s \( D' \) for the total disequilibrium was estimated as follows.

\[
D' = \sum_{i=1}^{k} \sum_{j=1}^{l} p_i q_j |D'_{ij}|
\]

where, \( D'_{ij} \) is Lewontin’s \( D \) and \( p_i \) is \( i^{th} \) allele frequency and \( q_j \) is \( j^{th} \) allele frequency. An Excel spreadsheet with built-in functions for the calculation of \( D' \) is available from the author.

Third, pair-wise LD between all pairs of loci in each supercontig was estimated as implemented in MULTILOCUS ver.1.3 (1). MULTILOCUS estimates Indices of Association (\( I_A \) and \( r_d \)), traditional measures of multilocus LD (10), using the variances of distances generated by the number of different alleles among all pairs of isolates and compared to a variance of distances of a hypothetical population with unlimited random association. The \( r_d \) is a modified statistic corrected for the differences of the number of polymorphic loci. Significance of the null
hypothesis, no LD, was tested with 1000 randomizations of the data set. Pearson’s correlation coefficient between $D'$, $r_d$ and $p$ values from Fisher’s exact test was performed to detect if these estimates were correlated.

**Results**

Many isolates exhibited mycelial incompatibility. All the populations displayed a high diversity for MCGs ranging from 16-22 and MCG per isolate ranged from 0.45-0.7 (Table 1).

**Microsatellite loci and pair-wise linkage disequilibrium**

All the isolates showed single allele amplification in each PCR reaction for every microsatellite locus. Twelve of the 19 microsatellite markers were found to be polymorphic and selected for further analyses [five of them were developed in this study and seven were from Sirjusingh and Kohn (43)]. These 12 loci were located on four supercontigs (two on supercontig-15, three each on supercontigs-3 and 19, four on supercontig-9). Polymorphic markers developed in this study were deposited in the GenBank (GenBank accession numbers (JX181754, JX181755, JX181756, JX181757 and JX181758). Except for the locus SC9-2, the number of alleles in each locus ranged from two to seven. Sequencing of different alleles of each locus confirmed that the differences were due to the number of repeat motifs. However, locus SC9-2 had an unusually large number of alleles ranging from 4-16 in the populations, and sequencing of alleles from eight isolates found that the allele sizes were due to inconsistent sequence variations outside the microsatellite repeats. Consequently locus SC9-2 was not included in further analysis, rendering three effective loci on supercontig-9. For the supercontig-15, eight newly designed primers were tested and only one (SC15-9) was polymorphic.

**Relationship between the significance of Fisher’s exact test and physical distance**
Pair-wise LD as measured by Fisher’s exact tests for all the pairs of loci on the same supercontigs were generated (Table 2). Overall, more populations showed significant LD on pairs of loci close to each other than on loci further apart on the same supercontig, particularly for supercontigs-3 and 9 (Table 2). On supercontig-3, the closest pair of loci (3A vs. 3B, 56 kb apart) showed significant LD in six of the nine populations ($p < 0.001$), whereas for the loci that were further apart (3A vs. 3C, 212 kb) only two of the eight populations had significant LD. This relationship showed that recombination rate increased as the distance between markers on the supercontig-3 increased (Table 2). In general, the $p$ values of Fisher’s exact tests increased as the distance between the locus pair increased (Table 2).

Likewise, on supercontig-9, the closest pair of loci (9B vs. 9C, 1000 kb apart) showed significant ($p < 0.001$) LD in four of the eight populations (Table 2), and pair-wise comparisons of other loci that were further apart (9A vs. 9B, 4000 kb apart, and 9A vs. 9C, 5000 kb, apart) showed no significant LD in any of the eight populations failing to reject the null hypothesis of random association (Table 2). Again, a trend of increasing $p$ values of Fisher’s exact test with increasing distance between loci on supercontig-9 was observed in six of the eight populations.

However, the three loci on supercontig-19 behaved differently. One locus was monomorphic in the Canola population from China and therefore one could not observe a relationship with the physical distance. The two closest loci (19B vs. 19C, 14.2 kb apart) showed significant LD in four of the eight populations, whereas the two loci that were furthest apart (19A vs. 19C, 261.2 kb apart) showed significant LD in two of the eight populations. A trend of increasing $p$ values with increasing distance between loci was detected only in three of the eight populations (Table 2).
All the primer pair sequences and their genomic locations used in the current study are shown in Appendix 2 and all the data points generated for the three supercontigs are shown in Appendix 3.

**Relationship between pair-wise linkage disequilibrium and physical distance**

The relationship of pair-wise LD measured with Hedrick’s $D'$ and the physical distance between loci on the three supercontigs for the eight populations were shown in the Figure 1. In general, linkage disequilibrium $D'$ decayed with increasing distances along supercontigs in half of the instances (Figure 1).

For supercontig-3, four of the eight populations showed a clear relationship of decaying $D'$ value with the increasing physical distance. The remaining four populations showed no apparent strong relationship between $D'$ values and physical distances. Similarly, for supercontig-9, six of the eight populations displayed decaying LD (decreasing $D'$ values) with increasing physical distance. An apparent relationship between $D'$ value and physical distance was not detected in the remaining two populations. However, for supercontig-19, majority of the populations (seven out of eight) showed no apparent relationship between linkage disequilibrium and physical distance. Only two out of nine populations showed such relationship of LD decay with increasing physical distance (Figure 1). For supercontig-15, significant LD ($p < 0.001$) was observed in three out of eight populations and the two loci were 73.9 kb apart.

An estimate of pair-wise LD, $r_d$, estimated with MULTILOCUS also produced similar pattern of results as Fisher’s exact test (data not shown). In comparing the relationships among the three measurements, Pearson’s correlation analyses found that for supercontigs 3 and 9, $D'$ and $r_d$ were significantly correlated ($p < 0.05$, $r > 0.5$). A negative correlation was found between $D'$ and Fisher’s exact test $p$ values as well as $r_d$ and Fisher’s exact test $p$ values ($p < 0.05$, $r >$
0.5). However, for supercontig 19, no significant correlations were detected except the negative correlation coefficient between $r_d$ and Fisher’s exact test $p$ value.

Discussion

Even though *S. sclerotiorum* is homothallic, genetic recombination has been reported in many population genetic studies around the world ([6, 22, 33, 41, 42]). Kohli and Kohn ([27]) reported random association of pair-wise marker loci in a supposedly clonal population of *S. sclerotiorum* as estimated by Index of Association. Atallah et al. ([6]) and Sexton et al. ([42]) detected recombination and discussed the possible mechanisms of outcrossing in *S. sclerotiorum* populations. Even though the exact mechanism for recombination is not known, outcrossing of this pathogen has been largely inferred using Index of Association of independent markers. However, high mutation rates would confound tests of index of association and markers located on different chromosomes or supercontigs do not estimate the chromosomal scale LD.

We found significant LD between the markers on the same supercontigs indicating intra-chromosomal recombination. In addition, we found decay of pair-wise LD with increasing physical distance between loci in several instances on two of the three supercontigs. Conway et al. ([15]) also observed significant decline in LD (estimated by Fisher’s exact test and Lewontin’s $D'$) with the increasing distance between nucleotide pairs of the merozoite surface protein 1 antigen (*msp1*) gene and concluded that meiotic recombination is frequent in certain populations of *Plasmodium falciparum*. In the current study we used microsatellite markers and detected the decline of LD with increasing physical distance, inferring outcrossing. However, microsatellite markers are known to have high mutation rates ([30]) and may interfere with the estimated LD. If the observed random association of alleles between linked loci is due to random mutation, the measured LD should be independent of physical distance between loci. Therefore, the decay of
LD along the supercontigs observed in the studied populations here cannot be attributed to random mutations alone. Another possibility is mitotic recombination events. However, majority of the isolates exhibited mycelial incompatibility, likely minimizing the possibility of heterokaryon formation, and moreover, mitotic recombination in nature is rare (14). Heterokaryon formation has been reported only in laboratory conditions for *S. sclerotiorum* (18). Thus mitotic recombination is unlikely or very rare in nature for *S. sclerotiorum*.

We detected a relationship between decay of LD and physical distance on supercontigs-3 and 9 for most of the populations, but only two out of eight populations had such a relationship in the case of supercontig-19. There are possible explanations. DNA region on supercontig-19 covered by the three microsatellite markers could be a recombination hotspot, or the three microsatellite markers might have high mutation rates. Recombination hotspots (3, 5, 11, 23, 25, 35, 40, 45) as well as mutation hotspots (24) have been reported in other genomes. In DNA regions of recombination hotspots, crossover occurs frequently and even closely linked loci do not show linkage disequilibrium. Also the frequent crossover events in the recombination hotspots may interfere with each other, also known as interference. Thus the relationship between LD and physical distance cannot be observed in DNA regions of recombination hotspots or in mutation hotspots. In such instances markers in close proximity may not be in LD or vise versa (25).

Even though LD is expected to be lower with increasing recombination and marker distances, such a relationship may not be achieved always in natural populations due to the unknown histories of different populations (5). It is obvious that the crossover rates even for a given DNA region are influenced by many other factors. The eight natural populations have different histories, and have faced different environmental conditions. Thus a combination of
ecological, molecular and evolutionary forces such as mutation events, natural selection, genetic drift, population bottleneck, gene flow, mutation and recombination rates in different populations could explain the lack of linear relationship between LD and the distance on a given chromosome/supercontig in all the populations studied (23, 25). There are reported recombination activators in other fungal genomes (23) and similar recombination activators may be in different states in various natural populations of *S. sclerotiorum*, presenting another possible explanation of the observations.

While different recombination rates were detected among DNA regions of the genome, different mutation rates were also detected in *S. sclerotiorum* in this study. Locus SC9-2 located on supercontig-9 had unusually high number of alleles, indicating a mutation hotspot. Occurrence of mutation hotspots is the norm for many organisms and has been detected in human genome analysis (24). On the other hand, only two polymorphic loci were found among eight different microsatellite markers tested on supercontig-15 (Appendix 3) indicating that this DNA region might be resistant to mutation/recombination and could be a mutation/recombination cold spot within the *S. sclerotiorum* genome. Lack of mutations indicates strong selection pressure, suggesting highly conserved, and likely critical functions of this DNA region for *S. sclerotiorum*.

It is known that intra-chromosomal recombination frequently occurs during meiosis through crossovers between homologous chromosomes, generating recombinant genotypes and potentially increasing fitness of the species and such recombination is unevenly distributed throughout genome (23). What is unique in this study is that recombination occurs in such high frequency in a homothallic species like *S. sclerotiorum*. In a truly haploid homothallic species intra-chromosomal recombination should not be detectable using the conventional techniques
baring mutations. The finding of intra-chromosomal recombination suggests frequent outcrossing in *S. sclerotiorum* in nature. Recently Chitrampalam et al. (12) reported segregation of mating type loci in sibling ascospores of *S. sclerotiorum*, suggesting some progenies may not be really homothallic and may require outcrossing for sexual reproduction. We also found that while all isolates of *S. sclerotiorum* contain MAT1-1 idiomorph, some (about 40%) isolates do not contain the MAT1-2 idiomorph (Chapter two) indicating that all the isolates in nature are not homothallic and outcrossing could be possible in some populations. Additionally, in this study we found that the three measures of multi-allelic pair-wise linkage disequilibrium (Hedrick’s $D'$, $r_d$ and Fisher’s exact test) were correlated in two out of three supercontigs. This indicates that $r_d$ measure and Fisher’s exact test are also important and reliable in quantifying the amount of LD between multi-allelic loci. This study also provides baseline information about recombination cold spots and hotspots in *S. sclerotiorum* genome. Future studies need to design equally spaced markers to investigate the nature and frequency of recombination to gain further insight about outcrossing of *S. sclerotiorum*.

**Acknowledgments**

I would like to thank Drs. Austin Burt (Imperial Collage, London), Michael G Milgroom (Cornell University, USA) and Tobin Peever (Washington State University, USA) for their suggestions and frequent communications. Special thanks to Dr. Vidhura Thennakoon, Department of Economics (Washington State University, USA) for helping in mathematical analysis of $D'$. 
References


149


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<th>Location</th>
<th>Number of isolates</th>
<th>MCGs</th>
<th>G:N&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup> Number of MCGs per isolate
Table 2. Physical distance and significance of Fisher’s exact test (GenePop ver. 4.0.10(38)) between alleles on three supercontigs of *Sclerotinia sclerotiorum* in eight populations

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n/d – due to the presence of monomorphic markers, no contingency tables were produced
Figure 1. Relationship between pair-wise linkage disequilibrium ($D'$) and the physical distance between markers on three supercontigs for eight populations. Trend lines were shown only if $R^2 > 0.5$. (A) supercontig-3. (B) supercontig-9. (C) supercontig-19.
APPENDIX 1

Genotypic data for 8 microsatellite loci for the canola isolates from USA (WM) and China (AH)

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? - Missing data

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\(^a\) % inhibition
\(^b\) β tubulin sequence type correspond to the figure 6 in page 64
\(^c\) Mating type idiomorphs. 1&2 indicates the amplification of both MAT1-1 and MAT1-2, whereas 2 indicates the amplification of MAT1-2.
Appendix 2. Primer sequences, chromosomal locations, microsatellite repeat motifs and annealing temperatures for the markers used in this study.

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R: CAGGGATGACTTTTGGAATGG | n/a  
92 | 111 | (TTA) | 55 | 351 |
| 114-4 3B | F: GCTCCTGTATA ACCATGTCTTG  
R: GGACTTTCCGACATGATGAT | 55958  
55610 | 55978  
55629 | (AGAT)(AAGC) | 55 | 368 |
| 7-2 3C | F: TTTGCGTATTATA GTGGTGGGC  
R: ATGCGGCAACTCTCAATAGG | 1612091  
1612243 | 1612109  
1612262 | (GA) | 55 | 171 |
| **Supercontig 9 (Chromosome 6)** | | | | | | | |
| 110-4 9A | F: ATCCCTAACATCCC TACAGCGC  
R: GGAGAATTGAAGAATTGAATGC | 1083935  
1084289 | 1083954  
1084310 | (TATG) | 55 | 375 |
| 13-2 9B | F: TCTACCCCAAGCTCC TACATTCC  
R: GAACCTGGTAATTGTCTC CGG | 1492929  
1492647 | 1492944  
1492666 | (GTGGT) | 55 | 263 |
| SC9-1* 9C | F: CGGTCTTTTA ACTCTTCCCATCTG  
R: TCACCATTCTCTTCTACTCCC | 1503300  
1503620 | 1503321  
1503641 | (ATCC) | 55 | 341 |
| SC9-2* 9D | F: TCGCAATGTAGTAAACC CGACAC  
R: ATTTGGGTC GTTGAGATGAGAT | 1590634  
1590981 | 1590655  
1591002 | (TAGA) | 55 | 368 |
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| SC19-2* | 19B | F: CAAAATATCATCATCCCATCCC | R: ATCAAGCAAGCGCCACTAAT | 763733 | 764010 | 763754 | 764029 | (TCTCA) | 57 | 296 |
| 12-2 | 19C | F: CGATAAATTTCCCCTCAGTTC | R: GGAAGTCCTGATATCGTTGAGG | 777981 | 778176 | 778001 | 778197 | (CA) | 55 | 216 |

* Polymorphic primer pairs used in this study

n/a Genomic designation is not available in the database
**Appendix 3.** Data points generated for all three supercontigs and for all the isolates used in the analysis (fragment size includes the M13 tail sequence 5’ CACGACGTTGTAACGAC 3’)

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