

**ETIOLOGY AND POPULATION BIOLOGY OF SCLEROTINIA SPECIES CAUSING
STEM AND CROWN ROT OF CHICKPEA**

By

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ETIOLOGY AND POPULATION BIOLOGY OF SCLEROTINIA SPECIES CAUSING STEM AND CROWN ROT OF CHICKPEA

Abstract

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Sclerotinia spp. are destructive and cosmopolitan plant pathogens causing diseases on more than 400 plant species. In the 2005-2006 growing season, widespread occurrence of stem and crown rot of chickpea was observed in the Central and Sacramento Valleys of California. Previously *S. minor* and *S. sclerotiorum* were reported to infect chickpea in the US. However, the majority of Californian isolates differed from the reported species in growth rate and oxalic acid production. Isolates were characterized based on presence or absence of ascospore dimorphism, rDNA group I introns and ITS sequences. Most Californian isolates were identified as *Sclerotinia trifoliorum*. This is the first report of *S. trifoliorum* infecting chickpea in North America.

Since there are no existing molecular markers available to study the population biology of *S. trifoliorum*, efforts were made to develop microsatellite markers suitable for *S. trifoliorum* by constructing a microsatellite-enriched library. Thirty-three microsatellite loci were developed, the majority of which were transferable to *S. sclerotiorum* and to a less extent for *S. minor*, but not for *S. homoeocarpa*. This shows that *S. homoeocarpa* is genetically distant from other members of the genus *Sclerotinia*.

Microsatellite markers, mycelial compatibility groupings (MCG), rDNA introns and sequence related amplified polymorphism (SRAP) markers were used to study the diversity and genetic structure of a collection of 136 isolates of *S. trifoliorum*. The 136 isolates displayed high levels of genetic diversity. For example, 116 microsatellite haplotypes were found among the 136 isolates, with most haplotypes consisting of single isolates. No correlation between genotype and geography was found, and related genotypes were distributed across the sampling area, strongly suggesting human-assisted dispersal.

We tested the hypothesis of monocyclic spread in *S. trifoliorum* by strain typing 57 sclerotial isolates derived from crown infected plants in 17 disease foci. Although the existence of plant-to-plant spread was supported based on chi-square tests of the distribution of MCGs in infected plants, the majority of the isolate-pairs from adjacent plants were of different haplotypes and MCGs, suggesting that plant-to-plant spread is of secondary importance in the epidemic and *S. trifoliorum* is primarily a monocyclic pathogen of chickpea.

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DEDICATION

This dissertation is dedicated to my parents, Eston and Priscillah Njambere.

CHAPTER ONE

INTRODUCTION

The ascomycetous genus *Sclerotinia* includes some of the most important plant pathogens. There are three economically important species in *Sclerotinia* causing diseases on a wide range of crops: *Sclerotinia minor* Jagger, *S. sclerotiorum* (Lib.) de Bary and *S. trifoliorum* Erikss. They are necrotrophic plant pathogens characterized by production of variously shaped and sized sclerotia, lack of conidia, and production of brownish, cup-shaped apothecia arising from sclerotial stromata. The apothecia are lined with asci which are filled with eight hyaline ascospores. The ascospores are forcibly ejected in puffs and carried by wind currents (Kohn 1979; Holst-Jensen et al. 1998).

Although *Sclerotinia spp.* are considered not to be host specific pathogens, host ranges of the three species do vary considerably (Kohn 1979; Phillips et al. 2004; Boland and Hall 1994). Of the three species, *Sclerotinia sclerotiorum* is the most cosmopolitan and versatile. It infects more than 400 species of plants, including field crops, vegetables and weeds (Boland and Hall 1994). *S. trifoliorum* is also a worldwide fungus but with a narrower host range, mainly limited to the species of the family leguminosae and particularly to forage legumes such as alfalfa (*Medicago sativa* L.), red clover (*Trifolium pretense* L.) and white clover (*Trifolium repens* L.) (Kohn 1979). *S. minor* is primarily a flower and vegetable crops pathogen. Most hosts of *Sclerotinia minor* are in the class *Dicotyledonae*, but there are two documented occurrences of *S. minor* on hosts belonging to the class *Monocotyledonae* (Melzer et al. 1997). *Sclerotinia minor* is also worldwide in occurrence but limited to cool, moist regions (Melzer et al. 1997).

Separation of the three species has been based traditionally on morphological and physiological criteria such as gross cultural characteristics, sclerotial shape and size, ascus and

ascospore size, time of apothecial development in the field, and host association. However, these characteristics tend to be variable and unstable (Willettts and Wong 1980; Cother 1977). Even though the process of identifying members of the genus *Sclerotinia* through sclerotia and other morphological characteristics has been refined over time (Kohn 1979; Rehnstrom and Free 1993), there are limitations to the approach. For instance, the differentiation of *S. trifoliorum* from *S. sclerotiorum* based on sclerotial characteristics is difficult due to instability of some sclerotial characteristics with subsequent sub-culturing (Cother 1977). *S. minor* can be differentiated from *S. sclerotiorum* and *S. trifoliorum* relatively easily. It produces much smaller, but more numerous sclerotia in nature and in culture, and generally produces apothecia less frequently in nature (Kohn 1979). However, differentiation between *S. sclerotiorum* and *S. trifoliorum* is more complicated. *S. trifoliorum* is similar in biology and morphology to *S. sclerotiorum*. Research efforts have been made in searching for molecular techniques that are reliable and convenient to use. Power et al. (2001) reported that *S. trifoliorum* contains group I introns in the nuclear small subunit rDNA, whereas *S. sclerotiorum* and *S. minor* do not. Furthermore, Holst-Jensen (1998) used the ITS and rDNA region to develop an approximate phylogeny for members of genus *Sclerotinia* and related genera.

In 1983 Ohm and Fujii (1983) described heterothallism and mating type mutation in *S. trifoliorum*. Ascospores of *S. trifoliorum* show spore-size dimorphism (two different sized-ascospores in 4:4 ratio within a single ascus), whereas ascospores of *S. sclerotiorum* show no dimorphism (Kohn 1979; Uhm and Fujii 1983a and 1983b). This is the ultimate criterion for distinguishing *S. trifoliorum* from *S. sclerotiorum*. The small-spore strains (strains derived from single, small ascospores) are self-sterile but cross-fertile only with those from large-spore strains (strains derived from single, large ascospores). On the other hand, the large-spore strains are

always self-fertile but their asci again show spore-size dimorphism (4:4 segregation). This mating behavior of *S. trifoliorum* was described as bipor heterothallism (Uhm and Fujii 1983). Spore type difference is the pleotrophic expression of mating type (Uhm and Fujii 1983). The ascoprogenesis of this fungus was found to be that of a typical ordered tetrad which is represented by *Neurospora crassa* (Raju 1980; Uhm and Fujii 1983) and no sign of dikaryotic nucleus status nor diploidy (full or partial) was found in large ascospores. Thus fertility could not be explained based on the nucleus status (Uhm and Fujii 1983).

Among the three species, *S. sclerotiorum* is the most extensively studied in terms of genetic variability. Previous studies have shown that *S. sclerotiorum* populations are predominantly clonal (Kohn et al. 1991; Cubeta et al. 1997; Durman et al. 2003; Hambleton et al. 2002; Kohli and Kohn 1998) with some evidence of out-crossing (Atallah et al. 2004; Malvarez et al. 2007; Sexton and Howlett 2004; Sexton et al. 2006; Mert-turk et al. 2007). Kohn et al. (1991) and Cubeta et al. (1997) documented no evidence of recombination in *S. sclerotiorum* populations in canola and cabbage respectively. In canola the population structure consisted of a large population composed of a small number of clones and a single clone was repeatedly recovered over 2000 km within a 4-year period (Anderson and Kohn 1995). Clonality arises in two ways, through asexual reproduction or through sexual reproduction by self-fertilization. Individuals are grouped as clonal lineages when there is a strong association between two or more independent markers such as mycelial compatibility groups (MCGs), DNA fingerprints or microsatellite markers (Kohn et al. 1991; Cubeta et al. 1997). Mycelial compatibility is a phenomenon used by fungi for self or non-self recognition and is controlled by multiple loci in fungi (Leslie 1993). *S. minor* and *S. trifoliorum* have been much less intensively studied. Samples of *S. minor* collected from California over a three-year period were found to be even

less diverse than those of *S. sclerotiorum*. Very limited information is available on genetic variation and population structure of *S. trifoliorum*. Based on a population of 50 isolates of *S. trifoliorum* collected from USA, Powers et al. (2001) identified five subgroups based on number and position of group I introns in the rDNA region.

All of the three *Sclerotinia* species have been reported to infect chickpea (*Cicer arietinum* L.). Stem and crown rot caused by *Sclerotinia* species is an economically important disease of chickpea. Chickpea is a major food legume crop grown in tropical, subtropical and temperate regions. It is ranked second to beans among global pulse crop production (Vincent and Jimmerson 2005). Chickpea is valued for its high nutritive value comprised of high protein (25-29%) and carbohydrate (38-59%) contents. It is also rich in fiber, oil and minerals (Hulse 1991). In the U.S. chickpea is an agronomically attractive rotational or specialty crop in cereal or other production systems (Chen et al. 2006). This is because chickpea has the ability to fix atmospheric nitrogen in symbiosis with *Rhizobium spp.*, a benefit which is important in sustaining the productivity of farming systems (Rupela and Saxena 1987). When used in cereal production systems, it facilitates management of diseases, insect pests and weeds. Chickpea has a wide adaptability. It is mostly grown as a summer crop in United States, but in California chickpea is mostly grown as a winter crop for a specialty market for canning (Bosque-Perez and Buddenhagen 1990).

More than 70 pathogens have been reported to infect chickpea (Nene et al. 1984), but only a few cause serious economic losses. Among them are *Sclerotinia spp.* causing crown and stem rot, a disease of world-wide economic importance. *Sclerotinia* stem rot has been reported in Australia (Bretag and Mebalds 1987; Fuhlbohm et al. 2003), Canada (Hilton 2000), Chile, India, Bangladesh, Nepal, Iran, Morocco, Syria (Haware 1990), the United States (Buddenhagen et al.

1988; Chen et al. 2006; Matheron and Porchas 2000), and other countries (Boland and Hall 1994; Haware, 1990). In the United States, *S. sclerotiorum* has been reported on chickpea in Arizona (Matheron and Porchas 2000), California (Buddenhaggen et al. 1988), North Dakota, and Washington (Chen et al. 2006). In addition to *S. sclerotiorum*, *S. minor* has been reported in chickpea fields in Arizona (Matheron and Porchas 2000) and in Queensland, Australia (Fuhlbohms et al. 2003). So far among the three main species of *Sclerotinia*, *S. trifoliorum* is the only species which has not been reported as a causal agent of stem rot of chickpea in North America. Prior to this study, *S. trifoliorum* is only recorded as infecting chickpea in Australia, where it occurs side by side with *S. sclerotiorum* (Bretag and Mebalds 1987).

Management of *Sclerotinia* stem rot in various crops has produced mixed results. Although sources of resistance have been identified in some crops, field evaluations are difficult because of the disease's development pattern, specific environmental requirements (low temperatures and high level of moisture) for disease development and the high spatial variability associated with the disease (Delclos et al. 1997; Kim et al. 2000; Bolton et al. 2006). Moreover, the *Sclerotinia* spp. persist in soil for long periods of time through resistant sclerotia, and they are disseminated by a number of ways: airborne ascospores, mycelial bridges between plants, rain-splashed and washed sclerotia, infested soil debris, and infected and infested seeds (Bardin and Huang 2001; Bolton et al. 2006). A serious epidemic of stem rot of chickpea occurred in California in 2005 and 2006.

The present study was carried out to examine the species identity of this chickpea pathogen and to characterize its population biology. In chapter 2, I show that both *S. sclerotiorum* and *S. trifoliorum* infect chickpea in California but their relative importance may vary. I also refine various methods for distinguishing the three species based on carpogenic

germination, growth rate, rDNA introns and ITS sequence. Chapter 3 describes the development and characterization of microsatellite markers for the study of *S. trifoliorum*. It is shown that these markers can also be applied to *S. sclerotiorum*. In chapter 4, I describe the diversity and population structure of *S. trifoliorum*. In the final chapter (chapter 5), the microsatellite markers and MCGs were used to investigate the pattern of spread of various genotypes of *S. trifoliorum* in a chickpea field.

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CHAPTER TWO
STEM AND CROWN ROT OF CHICKPEA IN CALIFORNIA CAUSED BY
SCLEROTINIA TRIFOLIORUM

Plant Disease 92:917-922

ABSTRACT

The identities of *Sclerotinia* isolates obtained from chickpea plants showing stem and crown rot in California were determined using morphological characteristics, variation in group I introns and ITS sequences. Isolates could be separated into fast growing (~40 mm per day) and slow growing (~20 mm per day) groups based on growth rates at 22°C. All fast-growing isolates induced stronger color change of a pH indicating medium than did slow-growing isolates at 22°C. The slow-growing isolates contained at least one group I intron in the nuclear small subunit rDNA, whereas all fast-growing isolates lacked group I introns in the same DNA region. ITS sequences of the slow-growing isolates were identical to those of *Sclerotinia trifoliorum*. Those of the fast-growing isolates were identical to sequences of *S. sclerotiorum*. Finally, the slow-growing isolates showed ascospore dimorphism, a definitive character of *S. trifoliorum*, whereas the fast growing isolates showed no ascospore dimorphism. Isolates of both species were pathogenic on chickpea and caused symptoms similar to those observed in the field. This study not only associated the differences between *S. sclerotiorum* and *S. trifoliorum* in growth rates, group I introns, ITS sequences and ascospore morphology, but also represented the first report that *S. trifoliorum* causes stem and crown rot of chickpea in North America.

INTRODUCTION

Sclerotinia spp. are destructive and cosmopolitan plant pathogens that cause stem and crown rot on various agronomic and horticultural crops and wild species. There are three economically important species of *Sclerotinia*. Of the three species, *Sclerotinia sclerotiorum* (Lib.) de Bary is the most ubiquitous with a host range of over 400 plant species (Boland and Hall 1994). *Sclerotinia minor* Jagger causes diseases on a range of crops in at least 53 plant genera, and causes significant economic losses in peanut, sunflower and lettuce, while *Sclerotinia trifoliorum* Erikss. is reported to cause diseases in 21 genera with major losses occurring mainly in legumes, particularly forage legumes like *Medicago* spp. and *Trifolium* spp. (Kohn 1979). Although the three species are all reported to cause the stem rot of chickpea in different parts of the world, the most commonly reported species is *S. sclerotiorum*. The disease has been recorded in Australia (Bretag and Mebalds 1987; Fuhlbohm et al. 2003), Canada (Hilton 2000), Chile, India, Iran, Morocco, Syria (Haware 1990), US (Chen et al. 2006; Matheron and Porchas 2000), and other countries (Boland and Hall 1994; Haware 1990). In the United States, *S. sclerotiorum* has been reported on chickpea in Arizona (Matheron and Porchas 2000), California (Buddenhagen et al. 1988), North Dakota and Washington (Chen et al. 2006). In addition to *S. sclerotiorum*, *S. minor* has been reported in chickpea fields in Arizona, USA (Matheron and Porchas 2000) and in Queensland, Australia (Fuhlbohm et al. 2003). However, *S. trifoliorum* has not been reported infecting chickpea outside Australia, where it was reported to occur together with *S. sclerotiorum* (Bretag and Mebalds 1987).

Differentiation of the three *Sclerotinia* species is based on several criteria (Kohn 1979). *S. minor* can be relatively easily differentiated from the other two species based on its numerous, scattered small-sized sclerotia in culture and in the field. However, unequivocal differentiation

between *S. sclerotiorum* and *S. trifoliorum* requires observation of ascospore morphology. *S. trifoliorum* shows dimorphism in ascospore size (two different sized-ascospores within a single ascus), whereas ascospores of *S. sclerotiorum* show no dimorphism (Kohn 1979). Induction of carpogenic germination of sclerotia of *Sclerotinia* spp. is a time-consuming process, and may take up to several months. To further complicate the matter, some isolates of *S. trifoliorum* are heterothallic and require mating with a compatible strain for carpogenic germination and ascospore production (Uhm and Fujii 1983a; 1983b). To facilitate identification of *S. trifoliorum* and differentiation from *S. sclerotiorum*, efforts have been made in searching for reliable and easily applied techniques. Restriction fragment length polymorphism was used to separate three species of *Sclerotinia* (Kohn et al. 1988). Tariq et al. (1985) described differences in growth rates between *S. sclerotiorum* and *S. trifoliorum*. *S. sclerotiorum* grows faster than *S. trifoliorum* at 25°C. Power et al. (2001) reported that *S. trifoliorum* contains group I introns in the nuclear small subunit rDNA, and separated isolates of *S. trifoliorum* into five subgroups based on number and position of introns, whereas *S. sclerotiorum* and *S. minor* do not contain any introns in the same DNA region. Sequences of ITS region of the nuclear rDNA show two nucleotide differences between *S. sclerotiorum* and *S. trifoliorum* (Holst-Jensen et al. 1999). These studies documenting the different features between *S. sclerotiorum* and *S. trifoliorum* were performed on different sets of isolates. There is a need to relate these features on the same set of isolates.

In 2005 and 2006 widespread occurrence of stem and crown rot of chickpea was observed in the Central Valley and the Sacramento Valley of California. Disease symptoms in the field clearly exhibited two patterns of infection: infection of the stem near or below the soil line (crown infection) or infection of the stem that clearly started at least six inches above the soil line (stem infection). Most of the crown-infected plants initially were wilted, with occasional

yellowing and subsequent death. Some plants showed white mycelial growth from around the infected stem on soil surface (Fig. 2.1A). When the dying or dead plants were dug up, black irregularly shaped sclerotia usually were visible on the stem or in soil adjacent to the plant (Fig. 2.1B). On plants showing stem infection, symptoms usually included discoloration of stems, followed by girdling of the entire stem as the lesion expanded, wilting above the lesion, and finally stem breakage. Mycelial growth was not always visible on the stem. Sclerotia were usually not found in plants with stem infections. These two symptom types were characteristic of the previously reported pathogen *S. sclerotiorum*. It soon became apparent that the isolates from central California were very diverse, had different growth rates, and could be different species (Njambere et al. 2006). We therefore hypothesized that the isolates from stem and crown rot of chickpea from California were not *S. sclerotiorum*. The objective of this study was to determine the species identities of the causal agents, and to relate the three previously described features (growth rate, nuclear rDNA introns and ITS sequence) differentiating between *S. sclerotiorum* and *S. trifoliorum* using the same set of isolates.

MATERIALS AND METHODS

Collection and isolation of the causal agent. Sclerotia were collected from stems of infected chickpea plants from various locations in central California (Table 2.1). Where sclerotia were absent, segments (5 to 10 cm) of diseased stems were collected and brought to the laboratory for isolation. Ten isolates were obtained and used in this study. Four additional previously identified isolates of *S. sclerotiorum* from lentil (*Lens culinaris* Medik.) and pea (*Pisum sativum* L.) and *S. trifoliorum* from alfalfa were also included in the study for comparison (Table 2.1). For isolation, sclerotia or diseased stems were surface sterilized in 70% ethanol and

then 3% sodium hypochlorite from commercial bleach for one minute each. They were then rinsed in sterile distilled water and blotted dry in sterilized paper towels. Stems were aseptically cut into 1 cm pieces, and then plated onto potato dextrose agar (PDA, Difco laboratories, Detroit) containing 0.1% lactic acid (added after cooling). Sclerotia were cut in halves before plating. Plates were then incubated at room temperature (22°C) in the dark. Isolates were purified by hyphal tip isolation and placed onto new PDA plates. Only one isolate was obtained from each plant. Sclerotia were harvested from PDA plates, air-dried and stored in glass vials at -20°C for future use.

Morphological and physiological characterization. *Colony diameter and acid production.* To test for colony growth rates, a 7-mm diameter disc was cut from the edge of a 3-day old actively growing colony from each of the thirteen isolates. The disc was transferred to the center of a 9 cm diameter Petri dish containing PDA. Plates were incubated at room temperature (22°C) in the dark. Colony diameters were measured every 12 h up to 84 h or until colonies covered the whole plates. Colony diameter was calculated from two measurements taken at right angles to each other. Plates were arranged in a completely random design with three replicates (three plates per isolate) and the experiment was conducted two times. To test for possible production of oxalic acid, isolates were cultured on PDA plates containing the pH indicator bromophenol blue (50 mg per L) modified by excluding PCNB and antibiotics from the semi-selective medium of Steadman et al. (1994) in a similar manner as described above. The intensity of color change of the pH indicator medium (none, slight, or intense) was recorded after 36 and 72 h of incubation at 22 °C. Colony diameters were also measured every 12 h as mentioned above. Since there were no differences in growth rates between PDA and the pH

indicating medium, the data were combined for analyses. The growth rate of each isolate was determined from the average of twelve plates per experiment.

Carpogenic germination. In order to observe ascospore morphology, carpogenic germination of sclerotia was induced in the laboratory. Two methods were used: the method with preconditioning developed for *S. sclerotiorum* (Cobb and Dillard 2004), and the method without preconditioning developed for *S. trifoliorum* (Rehnstrom and Free 1993). Sclerotia were conditioned in aerated cold (4°C) water as described by Cobb and Dillard (2004). The conditioning was done for 4, 6, 8 and 10 wk to determine the optimum conditioning time. Conditioned sclerotia were then removed from water, air-dried overnight, and then subdivided for subsequent, repeated experiments. Sclerotia not used immediately were stored in a refrigerator (4°C) in glass Petri dishes. Subsequent production of apothecia and ascospores was done according to Cobb and Dillard (2004). Conditioned apothecia were embedded on the surface of autoclaved sand (~ 45 cm³) in 9-cm diameter glass Petri plates (6 to 20 sclerotia per plate), and incubated at 20°C with 12 h cool white fluorescent lights (100-132 microeinsteins m⁻² sec⁻¹). Observations were made daily and additional sterile distilled water was added when necessary to maintain moisture. The time in days required for the first appearance of apothecia was noted, before examining the apothecia and ascospores under a light microscope. In the method without preconditioning (Rehnstrom and Free 1993), sclerotia were directly placed in clean autoclaved sand as described above except that the plates were incubated at 15°C and light intensity was increased to about 200-300 microeinsteins m⁻² sec⁻¹. Each treatment had two replicate plates each containing 6 to 20 sclerotia. The preconditioning of sclerotia was done once and the experiment of carpogenic germination was performed twice.

Molecular characterization. *Group I introns.* To determine the presence of group I introns in the nuclear small subunit rDNA, total genomic DNA was extracted from sclerotia of the 14 isolates (Table 2.1) using the FastDNA® kit as described by Chen et al. (1999). Sclerotia were cut with razor blades into small pieces (2 mm slices) and placed into 2-ml centrifuge tubes containing a ceramic bead, following the manufacturer's instructions. DNA was finally eluted into 50 µl water, and DNA quality was initially checked by agarose gel electrophoresis. DNA was diluted 100 times before being used in PCR. PCR amplifications were conducted using the following primer pairs described by White et al. (1990): ITS1/ITS4 and ITS5/ITS4 for the ITS regions containing the 5.8S rDNA. Various combinations of the rDNA primers (NS3/NS6, NS5/NS6, NS3/NS8) were used to amplify different regions of the small subunit rDNA. The reaction conditions were hot started for 10 min at 92°C prior to 35 amplification cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min, and were followed with a final 10 min extension at 72°C. PCR products were separated and product sizes were estimated by 1% agarose gel electrophoresis along with standard DNA size markers, to confirm the presence of introns in the amplified DNA regions. Gels were stained with ethidium bromide and then visualized and photographed under UV light.

Polymorphism in ITS sequences. PCR products amplified with primer pair ITS1 and ITS4 were purified for direct DNA sequencing. Purification was done by treating 5 µl of the PCR product with 2 µl of ExoSAP-IT® (USB, Cleveland, Ohio) following the manufacturer's instructions to remove excess template, primer-dimers and oligonucleotides from the PCR product. Reaction mixture was incubated at 37 °C for 15 min followed by 15 min at 80 °C to inactivate the enzyme. Purified PCR products were subjected to direct sequencing for both strands using ABI PRISM 377 automatic sequencer (Applied Biosystems, USA) at the

Sequencing Core Facility of Washington State University. Sequence comparisons were carried out using BLASTn (<http://www.ncbi.nlm.nih.gov/BLAST>).

Pathogenicity tests. Five isolates, selected to represent the two different growth rates and acid production levels, were used in greenhouse pathogenicity assays (Table 2.1). This consisted of three isolates of *S. trifoliorum* and two of *S. sclerotiorum*. Inoculations were done on three-week old seedlings of chickpea cv. Spanish White and cv. Dwelley. A petiole inoculation method developed for soybean (Del Rio et al. 2001) was modified to inoculate a stem branch instead of a petiole. A branch of a stem just below the most newly expanded leaf node was cut at 3 cm from the main stem, and a 200- μ l pipette tip bearing an agar plug on the wider end from the edge of an actively growing colony was inserted over the cut through the agar plug. Control plants were inoculated with a plain agar block. Disease development was observed daily and disease severity recorded based on a 0-4 scoring system (0: no disease; 1: inoculated branch dried; 2: up or downward movement of the disease from inoculation site on the main stem; 3: both up and downward movement of the disease; 4: plant dead). The diseased plants were collected and used to isolate the pathogen at conclusion of the experiments. The experimental design was a randomized complete blocks with three replications (pots) consisting of three plants per pot. An observatory trial was also done whereby 4 to 6 weeks old seedlings were similarly inoculated and given the same treatment as the three old seedlings.

Statistical analysis. To test the hypothesis that isolates from California show different growth rates and reaction to chickpea plants from *S. sclerotiorum*, analysis of variance with SAS (version 9.1) statistical software was performed on data from the growth rate experiments and pathogenicity assays. Fisher's protected least significant difference (LSD, $P < 0.05$) was used to compare means of treatments.

RESULTS

Morphological and physiological characterization. All fourteen isolates were confirmed to have cultural features similar to those of other *Sclerotinia* species including white dense mycelial growth, no conidial formation, and abundant black sclerotia of various sizes and shapes formed in a ring pattern. Isolates could be categorized into two growth rates, fast (~40 mm diameter per day) and slow (~20 mm diameter per day) (Table 2.1). Fast-growing isolates covered the entire agar surface within 48 hours, whereas slow growing isolates took more than 60 hours to cover the plates. Of the 14 isolates tested, ten isolates were considered slow growing, while the remaining four were considered to be fast growing (Table 2.1). The four fast growing isolates induced a strong color change from blue to bright yellow of the pH indicating medium at 22°C, whereas slow growing isolates induced no color change or very faint color change of the pH-indicating medium under the same temperature condition (Table 2.1).

Apothecium and ascospore production. Sclerotia from the four fast-growing isolates produced apothecia at 20°C after the conditioning in water at 4°C. The time required for apothecium production decreased with increase of conditioning time period. The sclerotia took 7 days to start producing apothecia after 10 wk of conditioning, 9 days after 8 wk of conditioning, 14 days after 6 wk of conditioning, and 18 days after 4 wk of conditioning. No apothecia were formed within this time period by the slow-growing isolates. It was not until after almost three months that one slow-growing isolate (05WM6) conditioned for 10 wk started to produce apothecia. However, most of the sclerotia of the other slow-growing isolates had rotted by that time. For those sclerotia incubated at 15°C without conditioning, two isolates from the fast- and two isolates from the slow- growing started forming apothecia within 4 wk of incubation. It took isolates 05WM8 and 06CWM-A8 25 days to start forming apothecia, whereas the remaining

isolates except isolate 05WM21 started germinating carpogenically within a period of two months. Similar trend was observed in the repeat experiments. The ascospore arrangement within the asci was observed under a light microscope for both the slow- and fast- growing isolates. Ascospore dimorphism was consistently observed among the slow-growing isolates (typical of *S. trifoliorum*), but was not observed in fast-growing isolates. Slow-growing isolates exhibited 4 large and 4 small ascospores within each ascus (Fig. 2.2A and B), whereas the fast growing isolates had all the eight ascospores of the same size, as was expected for *S. sclerotiorum* (Fig. 2.2C). The arrangement of the large- and small-size ascospores varied, with majority exhibiting a 4:4 tetrad arrangement. Ascospore size was in the range of 9.5 to 13.2 by 4.0 to 7.0 μm for *S. sclerotiorum*, whereas, for *S. trifoliorum* it varied from 13.3 to 19.7 by 7.7 to 8.8 μm for large ascospores and 11.1 to 14.8 by 6.1 to 7.7 μm for the small ascospores.

Molecular characterization. *Group I introns.* PCR with primer pair ITS1/ITS4 amplified a DNA fragment of approximately 540 bp and was uniform size among all isolates tested. Seven of the isolates are shown in Fig. 2.3A. However, when the primer ITS1 was replaced with the primer ITS5 which is 24 base-pairs upstream of the primer ITS1, PCR with primers ITS4/ ITS5 amplified products of two different sizes, one of about 560 bp among the *S. sclerotiorum* isolates, and another of about 1000 bp from the *S. trifoliorum* isolates (Fig. 2.3B). The larger PCR amplicon from the *S. trifoliorum* isolates is due to presence of an intron located between primers ITS1 and ITS5 (Powers et al. 2001). Similarly, PCR amplifications with primer pair NS5/NS6 (nine representative isolates are shown in Fig. 2.3C) indicated presence of another intron in eight of the ten isolates of *S. trifoliorum*. PCR amplification with primer pairs NS3/NS6, and NS3/NS8 also showed presence of the same intron in the eight isolates of *S. trifoliorum* (data not shown).

Single nucleotide polymorphism (SNPs) in the ITS region. PCR with primers ITS1 and ITS4 amplified a 540-bp fragment from the ITS region. Sequencing both strands allowed unambiguous determination of sequences. Sequences of the four *S. trifoliorum* isolates were identical, and the sequences of the three *S. sclerotiorum* isolates were also identical among themselves. However, there were two SNPs between the sequences of the two groups. These two SNPs were located at position 120 (transversion T→G) and position 376 (transition T→C) of the amplicon (Table 2.1). BLASTn analysis of the ITS locus of slow-growing isolates (*S. trifoliorum*) showed 100% similarity to the sequence of *S. trifoliorum*. While the ITS locus of the fast-growing (*S. sclerotiorum*) isolates were identical to that of *S. sclerotiorum*. The ITS sequences of isolates 05WM6, 05WM21 and 05WM33 were deposited in GenBank and assigned accession numbers EU082464, EU082465 and EU082466, respectively.

Pathogenicity tests. All isolates tested were pathogenic to chickpea cultivars cv. Spanish White and cv. Dwelley causing symptoms typical of stem and crown rot. Inoculation initially produced symptoms typical of stem rot where lesion elongation, discoloration and drying of stem from point of inoculation were evident. The symptoms progressed indiscriminately in both directions on the main stem to other plant parts causing stems to become discolored, tan or bleached white. Expanding lesions often girdled the stem causing foliage to wilt and stems to break. White fluffy mycelium was occasionally formed on the soil surface next to an infected stem and at the point of inoculation. Inoculated plants died about a week after inoculation (Fig. 2.4). No sclerotia were formed on 3-wk-old seedlings, but sclerotia readily formed on plants inoculated when plants were 5 and 6 wk old (Fig. 2.1C).

DISCUSSION

This is the first report of *S. trifoliorum* causing stem and crown rot of chickpea in North America. *S. trifoliorum* was previously reported to be associated with chickpea in Australia based on cultural characteristics in the absence of apothecia or ascospores (Bretag and Mebalds 1987; Cother 1977). Our research provides a detailed characterization of the pathogen based on cultural characteristics and the definitive character of ascospore dimorphism. Furthermore, the morphological characters were associated with several distinctive molecular markers. One isolate (05CWM-F2) could be identified as *S. trifoliorum* based on colony morphology and molecular markers, but did not produce ascospores despite repeated attempts at inducing carpogenic germination. This isolate could be heterothallic and require mating with microconidia from a compatible strain for apothecium production (Uhm and Fujii 1983b) or it simply may be recalcitrant to carpogenic germination. Voucher specimens of isolates 05WM6, 06WM-A8, and 05WM33 used in this study were deposited in the Washington State University Herbarium and were assigned accession numbers WSP 71364, WSP 71365 and WSP 71366, respectively.

Although *S. trifoliorum* is mainly regarded a pathogen of forage legumes, our study demonstrated that *S. trifoliorum* can infect and cause considerable damage on chickpea. The semi – selective medium of Steadman et al. (1994), from which the pH-indicating medium was derived, was designed for detecting air-borne ascospores of *S. sclerotiorum* based on the fact that *S. sclerotiorum* produces oxalic acid in plants and in culture (Godoy et al. 1990; Maxwell and Lumsden 1970). Our study showed that this semi – selective medium may not be as effective for detecting ascospores of *S. trifoliorum* when used at 22°C.

The modified petiole inoculation method with agar plugs gave uniform contact between the inoculum and the host plants and there were no escapes of inoculated plants. It gave more

uniform inoculation than did the colonized oat kernel method (Blanchette and Auld 1978) in inoculating chickpea (unpublished data). When the inoculation was done near the crown of chickpea plants, the inoculation produced both stem rot and crown rot.

Powers et al. (2001) reported that isolates of *S. trifoliorum* contain group I introns in the nuclear small subunit rDNA, whereas isolates of *S. minor* and *S. sclerotiorum* do not, and that isolates of *S. trifoliorum* could be separated into five subgroups based on the location and number of introns in the small subunit rDNA. A high level of intraspecific variation is evident among isolates of *S. trifoliorum*. We detected two of the five subgroups even in our small sample size of ten isolates (Table 2.1). All *S. trifoliorum* isolates contained the intron located at the conserved DNA sequence region between primers ITS 1 and IST5. This is the same location of a group I intron that separated two formae speciales of *Phialophora gregata* (Chen et al. 1998). The two base-pair differences in the ITS region between *S. trifoliorum* and *S. sclerotiorum* reported by Holst-Jensen et al. (Holst-Jensen et al. 1999) are also confirmed in the isolates obtained from chickpea in California. Furthermore, the ascospore dimorphism, ITS sequence variation, and group I introns in the small subunit rDNA were associated with growth rates, and ability to induce pH changes in culture medium at 22°C. Willis (1971) might have recognized this phenomenon of the differential growth rates much earlier when he reported that the difference in growth rates between *S. sclerotiorum* and *S. trifoliorum* was temperature-dependent. *S. trifoliorum* grew more rapidly at low temperatures (5°C), and more slowly at higher temperatures (25°C) than did *S. sclerotiorum*.

We have shown that both *S. sclerotiorum* and *S. trifoliorum* can infect chickpea in central California. However, the relative importance of each species at various locations is still unknown. It would be impractical to use ascospore dimorphism as the criterion to identify

isolates into species for population studies because of the time required to induce carpogenic germination and the presence of heterothallism (hence require mating) in some isolates of *S. trifoliorum* (Uhm and Fujii 1983a; 1983b). Growth rate and group I intron and ITS sequence variations may be useful in separation of *S. sclerotiorum* and *S. trifoliorum* in the *Sclerotinia* populations on chickpea from central California. This information can be used to investigate the relative importance of the two species at various geographic locations. Additionally the two species can be investigated in relation to crown infection versus above ground stem infection, and other aspects of epidemiology.

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Table 2.1. Isolates of *Sclerotinia* spp. used in study along with their host origin, geographic location, and their cultural, molecular and morphological characterizations

Species /Isolate	Host	Location	Year of isolation	rDNA introns ^a	ITS haplotype SNP at position ^b		Apothecium production ^c	Ascospore dimorphism ^d	Growth rate (mm/day)	pH medium color change
					120	376				
<i>S. trifoliorum</i>										
05WM6 ^g	Chickpea	Robbins, CA	2005	Yes (2)	G	C	Yes	Yes	21.0	None
05WM8 ^g	Chickpea	Colusa, CA	2005	Yes (2)	G	C	Yes	Yes	18.7	Slight
05WM21 ^g	Chickpea	Five Points, CA	2005	Yes (2)	G	C	No	Nt	19.3	Slight
05WM42	Chickpea	Five Points, CA	2005	Yes (2)	Nt	Nt	Yes	Yes	21.3	Slight
06CWM-A8	Chickpea	Farmington, CA	2006	Yes (2)	Nt	Nt	Yes	Yes	22.0	Slight
06CWM-F2	Chickpea	Farmington, CA	2006	Yes (2)	Nt	Nt	Nt	Nt	19.3	None
06CWM-G3 ^f	Chickpea	Five Points, CA	2006	Yes (1)	Nt	Nt	Yes	Yes	21.0	Slight
06CWM-H6 ^f	Chickpea	Five Points, CA	2006	Yes (1)	Nt	Nt	Yes	Yes	20.5	Slight
05WMAL-1a ^g	Alfalfa	Visalia, CA	2005	Yes (2)	G	C	Nt	Nt	25.1	Slight
TP0506 ^g	Alfalfa	Prosser, WA	2006	Yes (2)	G	C	Nt	Nt	19.3	Slight
<i>S. sclerotiorum</i>										
05LWM-A5 ^g	Lentil	Colton, WA	2005	No (0)	T	T	Yes	No	46.2	Intense
05WM22 ^g	Chickpea	Five Points, CA	2005	No (0)	T	T	Yes	No	43.2	intense
05WM33 ^g	Chickpea	Five Points, CA	2005	No (0)	T	T	Yes	No	38.7	Intense
WM-A1 ^g	Pea	Central WA	2002	No (0)	T	T	Yes	No	39.8	Intense
LSD _{0.05}									1.6	

^a Detection of introns in the nuclear small subunit rDNA (nss rDNA) with PCR. Yes: introns were detected; No: no introns were detected. Number of introns detected in parentheses.

^b The single nucleotide polymorphism (SNP) position in the amplicon produced by PCR with the primer pair ITS1/ITS4. C: cytosine; G: guanine; T: thymine; and Nt: not tested.

^c Production of apothecia from sclerotia was induced either with conditioning in aerated water at 4°C or without conditioning. Nt: not tested

^d Presence of ascospore dimorphism. Nt: not tested.

^e Colony growth rate in mm/day on PDA at 22 °C measured after inoculation with a 7-mm diameter colonized agar disc

^f Isolate selected for pathogenicity test in the greenhouse.

^g Isolate from which the ITS regions was sequenced.

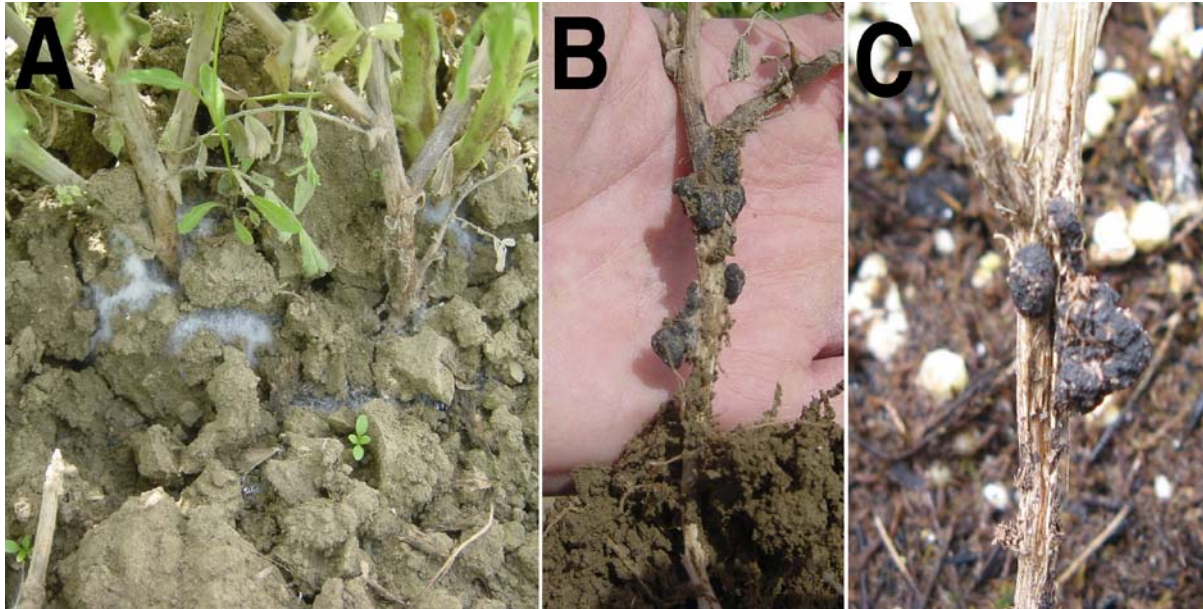


Fig. 2. 1 (A) Symptoms of crown rot caused by *Sclerotinia trifoliorum* with white mycelial growth including (B) black irregularly shaped sclerotia and (C) similar symptoms produced in greenhouse following artificial inoculation.

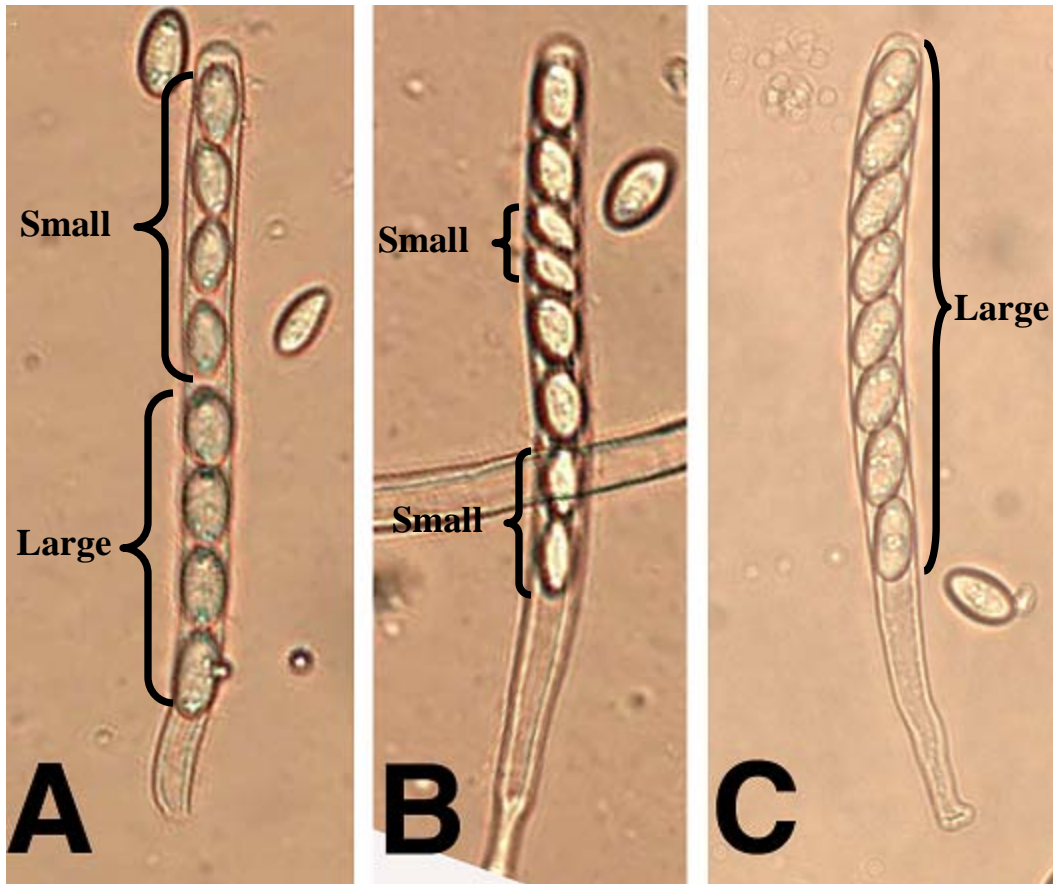


Fig. 2.2 Ascospore size dimorphism and arrangement within the asci. **A** and **B** show isolates of *S. trifoliorum* showing ascospore dimorphism with 4:4 and 2:2:2:2 tetrad arrangement respectively, and **C** is an isolate of *S. sclerotiorum* showing no ascospore size dimorphism.

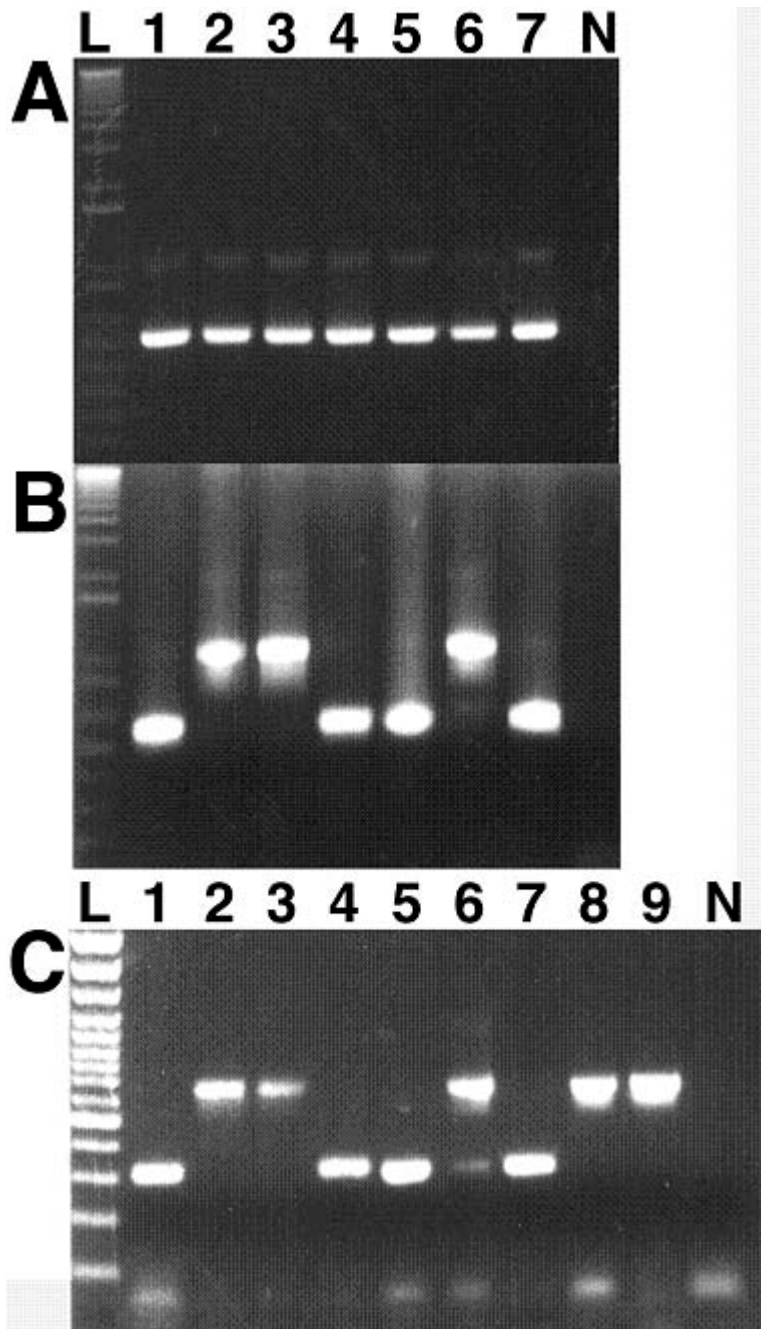


Fig. 2.3 Agarose gel electrophoresis of PCR products amplified with (A) primers ITS1 and ITS4 (White *et al.* 1990), (B) primers ITS4 and ITS5, and (C) primers NS5 and NS6. Larger product sizes in B and C indicate presence of introns. Lane L: DNA ladder; Lanes 1, 4, 5, and 7: *S. sclerotiorum* isolates 05LWM-A5, 05WM22, 05WM33, and WM-A1, respectively; Lanes 2, 3, 6, 8 and 9: *S. trifoliorum* isolates 05WM6, 05WM21, 05WMAL-1a, TP-0506, and 05WM8, respectively; Lane N: negative control.

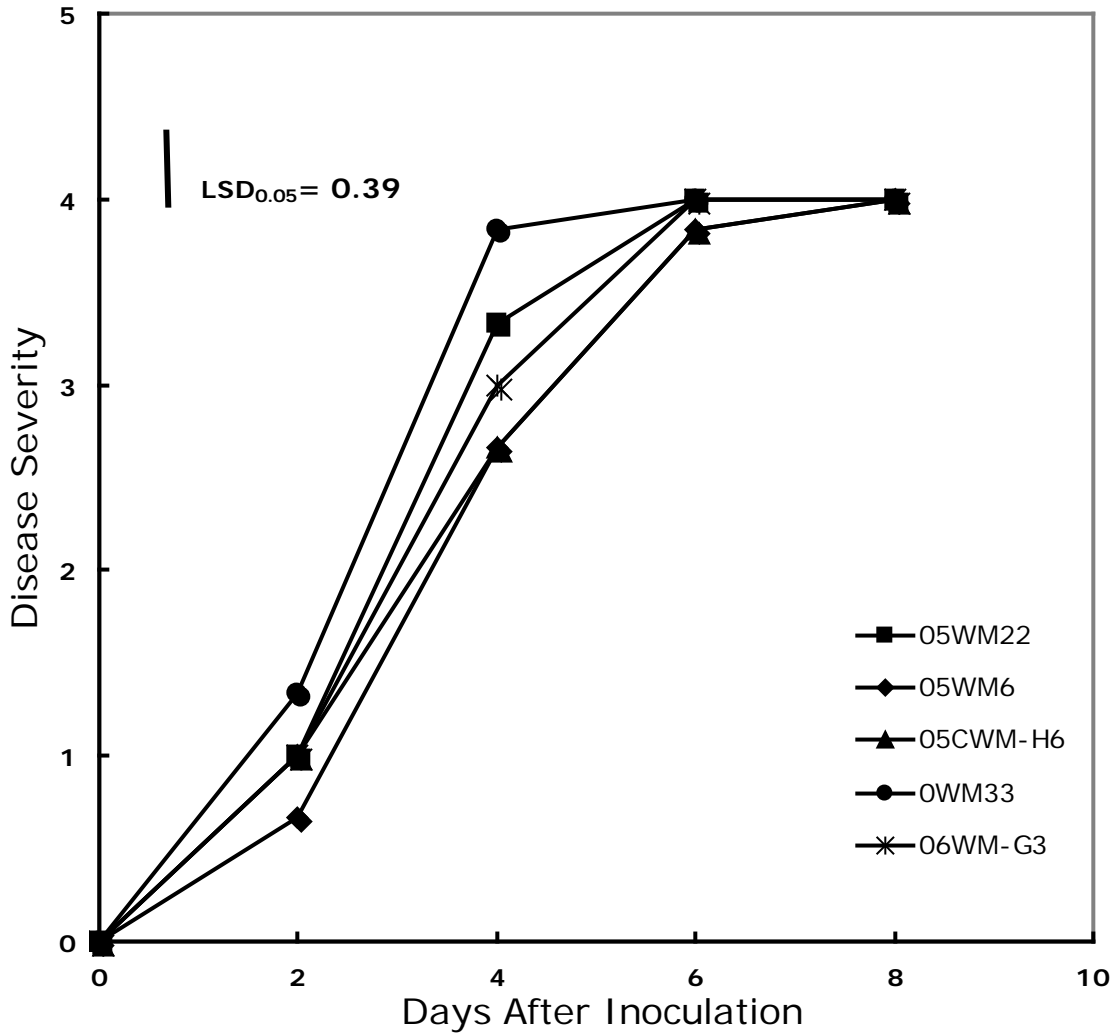


Fig. 2.4 Development of stem rot of chickpea following inoculation of 3 week-old plants of cv Dwelley with isolates 05WM6, 06CWM-G3 and 06CWM-H6 of *S. trifoliorum* and isolates 05WM22 and 06WM33 of *S. sclerotiorum*. The disease was rated on a scale of 0-4, 0: no disease; 1: inoculated branch dried; 2: up or downward movement of the disease from inoculation site on the main stem; 3: both up and downward movement of the disease; 4: plant dead. Control plants inoculated with plain PDA agar showed no disease (not shown).

CHAPTER THREE

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE MARKERS OF *SCLEROTINIA TRIFOLIORUM*

ABSTRACT

Sclerotinia trifoliorum is a major pathogen of leguminous forage and vegetable crops. Recently *S. trifoliorum* was found to infect chickpea (*Cicer arietinum*) in North America. Our attempts to study the population biology of this pathogen using previously developed microsatellite markers for the closely related species *S. sclerotiorum* and *S. subarctica* resulted in little or no amplification or low levels of polymorphism. This study reports the development, characterization and utility of microsatellite loci of *S. trifoliorum*. Thirty-three polymorphic microsatellite loci were developed from 56 clone sequences derived from a microsatellite-enriched library of *S. trifoliorum*. Scoring alleles at 33 microsatellite loci using 42 isolates of *S. trifoliorum* revealed an average of 6.5 alleles per locus (range 3-12) and an average expected heterozygosity of 0.63 (range 0.26-0.9). Based on the genome sequences of *S. sclerotiorum*, the microsatellite loci are dispersed throughout the genome. Fifty percent (265 of the 528) pairwise comparisons had significant linkage disequilibrium. This was not unexpected since *S. trifoliorum* is a partially outcrossing fungus. Thirty of the 33 loci were successfully applied to congeneric *S. sclerotiorum* and 28 loci were polymorphic, ten loci in *S. minor* and one loci in *S. homoeocarpa*. These markers are therefore useful for population structure assessment, QTL mapping and ecological analyses in *S. trifoliorum* and potentially in other *Sclerotinia* species.

INTRODUCTION

Sclerotinia trifoliorum is one of the three most economically important species of the genus *Sclerotinia*. The pathogen causes crown and stem rot of forage legume crops such as alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), and white clover (*Trifolium repens* L.), as well as several other legumes (Kohn 1979). Recently *S. trifoliorum* was shown to infect chickpea (*Cicer arietinum*), causing widespread epidemics (Njambere et al. 2008). Understanding the interaction between chickpea plants and *S. trifoliorum* requires knowledge of its genetic diversity and the mechanisms that lead to the development of new virulent genotypes. The development of molecular markers that allow monitoring of the dynamics of fungal populations is crucial in designing strategies for the disease control.

Very little is known about the genetic diversity of *S. trifoliorum*. Likewise, no genetic markers have been developed to study this pathogen. Microsatellites markers have been very informative in understanding the diversity and population biology of the sister species *S. sclerotiorum* (Atallah 2004; Sirjusingh and Kohn 2001). Above all, polymorphism at microsatellite loci can be efficiently assessed by PCR (Weber and May 1989; Brondani et al. 2000) by screening a pathogen population. Their detection is made easy by the fact that they are relatively abundant in eukaryotic genomes, co-dominant and multi-allelic. Allelic diversity at SSR loci are caused by variation in the number of repeats of the core sequence, probably caused by polymerase "slippage" and a lack of repair during DNA replication or through unequal cross-overs (Field and Wills 1996).

The benefits accruing from these markers prompted us to search for microsatellites. Sirjusingh and Kohn (2001) developed 24 polymorphic microsatellites markers for *S. sclerotiorum* of which 12 could cross amplify *S. trifoliorum*. Our attempts to use these markers

resulted in only one polymorphic locus. Further attempts to amplify *S. trifoliorum* using microsatellites developed for *S. subarctica* (Winton et al. 2007) resulted in no amplification, or unspecific priming or no polymorphism at all. This study was therefore initiated to develop microsatellite markers suitable for *S. trifoliorum*.

Our research hypothesis was that polymorphic microsatellite loci exist in *S. trifoliorum* and can be developed for use in addressing population and ecological questions concerning *S. trifoliorum*. Specifically the objectives of this study were i) to develop and characterize microsatellite markers suitable for *S. trifoliorum*, ii) determine their transferability (the ability of certain microsatellites loci to be applied to other species) to related species and iii) their resolving ability to genotype *S. trifoliorum* isolates from chickpea and alfalfa.

MATERIALS AND METHODS

Construction of microsatellite-enriched library. Genomic DNA was extracted from sclerotia of a chickpea isolate (06CWM-E10) collected from Woodland, Sacramento Valley, California using FastDNA kit (Chen et al. 1999). Microsatellite-enrichment was carried out at the Savannah River Ecology Laboratory of University of Georgia (<http://www.srel.edu/microsat>). Briefly, two micrograms of DNA were digested to completion with *RsaI* and size fractionated in 1% agarose gel electrophoresis. The recovered DNA fragments of approximate size 500bp were ligated to SuperSNX linker sequence (which is highly efficient for size selection of fragments) and amplified using the forward SuperSNX sequence as a primer (Hamilton 1999). Amplified fragments which contained the short tandem repeats were hybridized with 3'-biotinylated microsatellite oligonucleotide and captured on Dynabeads M-280 (Dyna Biotech). The following 3 mix of biotinylated oligos were used for enrichments, **Mix 2** - (AG)₁₂, (TG)₁₂, (AAC)₆,

(AAG)₈, (AAT)₁₂, (ACT)₁₂, (ATC)₈; **Mix 3** - (AAAC)₆, (AAAG)₆, (AATC)₆, (AATG)₆, (ACAG)₆, (ACCT)₆, (ACTC)₆, (ACTG)₆; **Mix 4** - (AAAT)₈, (AACT)₈, (AAGT)₈, (ACAT)₈, (AGAT)₈. The bound DNA was eluted from the magnetic beads. DNA amplification and cloning were performed at Pullman, Washington. Amplification from eluted DNA by PCR used the forward SuperSNX linker as a primer (Glenn and Schable, 2005). The resulting microsatellite enriched PCR products were purified using the sephadex columns, cloned with TOPO TA cloning kit (Promega, Madison, WI) and screened for recombinant.

Selection and sequencing of positive clones and primer design. Clones of the microsatellite-enriched library were identified and plasmid DNA of selected clones was extracted and prepared for sequencing. Sequencing reactions were performed with "Dye-Terminator" and "Big-Dye" kits (Applied Biosystems, Foster City, CA) using an Applied Biosystems 377 DNA sequencer. Specific primer pairs, unique and complementary to microsatellite flanking regions, were designed using the software Primer 3 (version 0.3.0; Rozen & Skatetsky, 2000), considering the following parameters: primer auto-annealing, G+C content of about 50% and annealing temperature in the 55–60°C range.

Screening for polymorphism and transferability of the microsatellite markers. The primer pairs were tested for applicability in PCR and for polymorphism using a panel of 42 unrelated isolates of *S. trifoliorum* from chickpea and alfalfa, and four isolates of *S. sclerotiorum* derived from chickpea, lentil, pea and potato. DNA for PCR amplification was extracted for all the isolates using FastDNA kit. PCR was performed in a 20- μ l reaction volume containing 4 μ l (10ng) genomic DNA, 1x PCR buffer, 1.5 mM MgCl₂, 200 μ M dNTPs, 0.5 μ M of each forward and reverse primers, 0.05 μ M IR labeled universal M13 primer, and 0.5 U *Taq* polymerase. The thermal cycling parameters were initially at 5 min denaturization at 95 °C followed by 35 cycles

of 95°C for 30 s, 45 s at the annealing temperature, as listed in Table 3.1 and 72 °C for 45 s with an addition extension of 30 min. Ten microliters were run initially on a 10% pre-cast polyacrymide gel (Bio-Rad), 1x TBE gel and visually scored under UV light. A run on a LI-COR 4300 sequencer required the IR labeled PCR products to be diluted at 1:12, denatured, and 1 ul of the dilution run on a 6.5% denaturing PAA gels on the LI-COR 4300 sequencer. To facilitate determination of allele sizes, the DNA size ladder (LI-COR size standard IRDye™ 700 and 800) was applied at each side of the gel and a reference isolate (06CWM-E10) used every 5 to 10 samples. The monomorphic bands of the reference isolate helped to calibrate the band sizes of the samples. On completion of the run (usually 100–150 min), the gel image was captured and stored for further processing. The Gene ImagIR 4.03 software (LI-COR Inc.) was used to determine allele sizes of individual isolates. Accurate fragment sizes were attained by a matching bands option of the software that generates allele bin classes with less than 0.5% standard deviation of sizing precision across the whole gel. A lower tolerance setting could even distinguish more subtle differences. Thus manipulating the match tolerance (standard deviation) helped detect two different bands and collapse bands with minor discrepancy to one allele.

Microsatellite loci that were polymorphic in *S. trifoliorum* and *S. sclerotiorum* were further tested and characterized using five isolates of *S. minor* and six isolates of *S. homoeocarpa*. Final allele size determinations for all the loci were performed on a LI-COR 4300 sequencer. PCR amplification of all the isolates was done using a M13 fluorescent labeling protocol (Schuelke 2000) where the universal M13 tail end is attached to the 5' end of the forward primer and an addition third primer, IR labeled universal M13 primer included in the reaction. The size of the repetitive region of each allele was confirmed in selected isolates through sequencing.

Data analysis. To ascertain the applicability of the developed microsatellite loci to address population genetics questions of *S. trifoliorum*, the software ARLEQUIN (Schneider et al. 2000) was used to calculate the number of alleles per locus and estimate the expected heterozygosities whereas MultiLocus 1.3 (Agapow and Burt 2001) was used for genotypic diversity and linkage disequilibrium analysis. The resolving power of microsatellite markers was investigated by plotting the number of genotypes identified vs. number of loci (i.e., the genotypic diversity vs. number of loci) using MultiLocus 1.3. Loci were randomly sampled 1000 times during the analysis. To further, characterize, the microsatellite loci, genomic locations were predicted and assigned by performing a BLASTN search of the *S. trifoliorum* SSR loci source sequence against the *S. sclerotiorum* genome www.broad.mit.edu/annotation/genome/sclerotinia_sclerotiorum. Clone sequences were used as query to locate the homologous contigs and super contigs in *S. sclerotiorum* and then linkage groups in the genome.

RESULTS

The cloning of the microsatellite-enriched library resulted in more than 300 positive clones based on blue-white selection. A total of 60 positive clones were randomly selected for sequencing. Four clones resulted in poor or unreadable sequences and three clones had the repeat region too close to the insertion site. Fifty three clones contained 56 microsatellite repeat motifs with flanking sequences suitable for primer design, and the sequences were deposited in GenBank (Table 3.1). Fifty-six primer pairs were designed based on the flanking sequences of the microsatellite loci. PCR amplification showed that 33 of the 56 loci (59%) were polymorphic (Table 3.1). The number of alleles per locus ranged from 3 to 12 (with an average of 6.5) among 42 isolates of *S. trifoliorum*. The expected heterozygosities for the loci ranged from 0.26 to 0.9

with an average of 0.63. The resolving ability of the 33 microsatellite loci was assessed by examining the relationship between the proportion of genotypes identified against the type and number of loci (with 1000 randomizations) as performed by Rossi et al. (1998). Permutation tests showed that three microsatellite loci were sufficient enough to detect 50% of the genotypes and scoring of seven loci could discriminate approximately 95% of the genotypes (Fig. 3.2).

Pairwise comparisons of linkage disequilibrium (LD) for all 33 loci revealed 265 of the 528 (50%) pairs of microsatellite were in significant LD. BLAST searching the genome of the closely related species *S. sclerotiorum*, using microsatellite sequences as query, 28 of the 33 loci had orthologs in the *S. sclerotiorum* genome (Table 3.1).

The 33 microsatellite markers developed from *S. trifoliorum* were applied to three other species of *Sclerotinia*: *S. sclerotiorum* (4 isolates), *S. minor* (5 isolates) and *S. homoeocarpa* (six isolates). Thirty of the 33 markers gave positive amplification and 28 of them were polymorphic in *S. sclerotiorum* (Table 3.2). Alleles sizes found in *S. sclerotiorum* were similar to those found in *S. trifoliorum*. Ten of the 33 microsatellite markers gave positive amplification in *S. minor*, and only one marker was polymorphic. Thirty-two of the 33 markers did not amplify any DNA in *S. homoeocarpa* and the remaining marker (ST4-1) produced a monomorphic allele (Table 3.2).

DISCUSSION

The high number of alleles observed per locus (average 6.5) and the high expected heterozygosity (mean 0.63) indicates that there is considerable variation among the 33 microsatellite loci, suggesting that a small set of selected markers should be sufficient to resolve genetic identity among isolates of *S. trifoliorum*. This is supported further by the resolution

power of up to 95% by using only 7 microsatellite loci in *S. trifoliorum*. In a purely out-crossing organism, an even lesser number of microsatellite loci have been noted to resolve the identity of genotypes whereas far more microsatellite loci are needed to determine genotypes in clonal species (Delmotte 2002; Fuller et al. 1999; Gomez and Carvalho 2000).

Assuming the microsatellite loci are homologous between the two closely related species *S. sclerotiorum* and *S. trifoliorum*, most of these *S. trifoliorum* loci are all located at different genomic regions (different scaffolds) of *S. sclerotiorum* (Table 3.1). It is reasonable to assume that most of the microsatellite loci are not physically linked despite the fact that half of the microsatellite pairs showed linkage disequilibrium. Fifty percent of pair-wise comparisons of microsatellite loci had displayed significant LD. This is not unexpected in *S. trifoliorum* as this fungus exhibits homothallism and heterothallism as well as clonal reproduction (Njambere et al. 2008; Uhm and Fujii 1983). Similar results have been reported in clonal species such as *S. subarctica* (Winton et al. 2007) and *Fusarium graminearum* (Vogelgsang et al. 2008).

The microsatellite loci for *S. trifoliorum* are readily applicable to *S. sclerotiorum* and to some extent to *S. minor*. However, such transferability is not reciprocal. Microsatellite markers developed for *S. sclerotiorum* appeared not to be transferable to *S. trifoliorum*. This may suggest that *S. trifoliorum* shares only part of the genome of *S. sclerotiorum* from their nearest common ancestor. In spite of our findings, transferability is a common phenomenon and has been observed in related species of *Botrytis cinerea* (Fournier et al. 2002) and *Fusarium graminearum* (Vogelgsang et al. 2008). Thus, this "transferability" of microsatellite markers may be a measure of phylogenetic relatedness. The inability to transfer *S. trifoliorum* microsatellite markers to *S. homoeocarpa* suggests that *S. homoeocarpa* is more distantly related to other *Sclerotinia* species

or may not be truly a species of genus *Sclerotinia*, supporting the on-going debate of re-classification of *S. homoeocarpa* (Powell and Vargas 1999).

In conclusion, these polymorphic microsatellite markers provide new tools to study population genetics, QTL mapping and ecological analyses of *S. trifoliorum*, and possibly in other *Sclerotinia* spp. and to infer phylogenetic relationships in *Sclerotinia*.

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Table 3.1 Characterization of 33 polymorphic microsatellite loci isolated from *Sclerotinia trifoliorum*

Locus (GenBank Accession)	Primer sequence (5'-3')	Repeat motif ^a	T _a (°C)	No. of alleles	Allele size range (bp)	H _E ^b	Location on ^c linkage group
ST2-2 (GQ901906)	F:CGTAATTGAGGGGAAGAGG R:GCCTAGCTAGCAGAATCAACC	(AAG) ₁₄	58	4	132-153	0.59	9(11)
ST2-4A (GQ901907)	F:TGTGCTTGTGCGTGTGAATA R:GGCGAGACATTACCTATCCTG	(TGAA) ₆	55	5	130-154	0.38	13(12)
ST2-5 (GQ901908)	F:CGTGTGTCCGCTGGATCTTTC R:TTCTCCTGAACTTCCATATCC	(CAA) ₉	55	3	143-158	0.40	R(18)
ST2-6 (GQ901920)	F:CAACACCACATACATCACATACG R:GGCGGAGAAGAGTGTAGGAT	(CATCAACAT) ₇ (CACCAA) ₄	55	12	134-194	0.74	7(6)
ST2-12 (GQ901922)	F:TGACTCACTCATGTCAATGGTT R:CACTCACACACTCCGACT	(CAAAT) ₁₆ (CACAAT) ₃ (CA) ₃	55	7	131-186	0.66	14(25)
ST2-12A (GQ901922)	F:TCGGAGTGTGTGTGAGTGTG R:CGATGTTGTGGTTTGGTGAT	(CATA) ₅ (CAGA) ₇ CACA(CCAT) ₃	55	3	127-135	0.38	14(25)
ST3-1 (GQ901923)	F:CATCCATCTACTACGCCTTTTC R:AGGCTATCCATCCTCACATCC	(GGTA) ₆	55	4	223-295	0.51	-
ST3-1A (GQ901909)	F:TTGATGAGGGTTGGAGGATG R:GAAGAGTAAGATAGGTGTTACG	(ATCT) ₅	55	8	158-190	0.75	-
ST3-3 (GQ901911)	F:TGGCAAAGTTACGGTTATGCT R:GGGACTATTTGTTGCATATCTGG	(ACCT) ₈	58	7	142-174	0.74	5(7)
ST3-4 (GQ901912)	F:TCAAAGGCCTCACTCTCACTC R:TGAAAGGAGAGACTGTAAGATTGC	(TGTTTTAA) ₃	55	3	150-158	0.56	3(1)
ST3-4A (GQ901912)	F:TATGACGCGACTTCTTCCTC R:TCCTGACTGACTGTAGTGTAGG	(GTCT) ₃ TCTGCCTG TT (GTCT) ₅	55	3	161-189	0.26	3(1)
ST3-6 (GQ901929)	F:GGTTTTGGTTTTGGCTTTGT R:CGAAGCGTACATTCATTTTCATT	(AGTG) ₁₁	58	6	78-102	0.69	11(20)
ST3-7 (GQ901930)	F:TTGTTGGTCTGTATGTCTGGATG R:AAAGGGACGGGAAGTTTTGA	(TGTC) ₇ TATC (TGTC) ₁ TATC (TGTC) ₅ (GTAG) ₁₀	55	8	160-204	0.81	4(3)
ST3-10 (GQ901932)	F:TCGGTAAGTAACAACACTATGACC R: GCAAAGAATTTAAACTACCTACCT	(GTAG) ₁₀	55	11	80-124	0.72	3(1)
ST3-13A (GQ901935)	F:CCTCAGGTTCCAAGTACTCTT R:ATCCGAGCCAACCAACTTAC	(CATA) ₁₀	55	4	96-112	0.60	Mixed ^d
ST3-14 (GQ901936)	F:GGAAGAGTAAGATAGGTGTTTCAGC R:CATTCTAAAAGGTTCTTGCGATA	(TAGA) ₅	55	11	88-132	0.90	-
ST3-15A (GQ901917)	F:CCTCAGGTTCCAAGTACTCTT R:ATCCGAGCCAACCAACTTAC	(CATA) ₁₀	55	6	100-140	0.68	Mixed ^d

ST3-18 (GQ901939)	F:TTTAACGATTGATTTGGAAATGG R:TCTCCTCTCCTCCCGTCCTA	(GAGT) ₁₀	55	6	96-124	0.67	8(4) ^e
ST4-1 (GQ901914)	F:GATATTCCATGCCAAACACG R:GGTGA CTCCATAACCATCTCG	(CATA) ₁₁	55	9	119-151	0.83	4(3)
ST4-2 (GQ901915)	F:CTCGTCGCATTACAATTTGC R:CGGATGCGTTCATCTCTTAG	(TATC) ₁₄	55	11	128-168	0.83	-
ST4-3 (GQ901916)	F:TGACCAACGATCGATTCC R:GCAATGCCTCTAATAATCTCG	(CATA) ₁₀	55	5	163-195	0.71	1(14)
ST4-4 (GQ901917)	F:CGGAGTAGGTAGTTCATTACGG R:GCTGCCTCTACTTCTGTACACAT	(GTAT) ₁₄	55	7	165-205	0.77	3(1) ^e
ST4-6 (GQ901919)	F:AGTGTGAGGCTGGTTGGTG R:ACCAACAATCCAATGACAGC	(TG) ₃₆	58	8	117-147	0.79	Mixed ^d
ST4-6A (GQ901941)	F:AGTGTGAGGCTGGTTGGTG R:ACCAACAATCCAATGACAGC	(ATGG) ₆	55	8	225-245	0.85	Mixed ^d
ST4-6C (GQ901941)	F:GGGTTTGGTTTGGTTTGTG R:AACCAGTACCTCGACGCCTA	(GTAT) ₂₀	55	8	133-193	0.79	-
ST4-9 (GQ901942)	F:CAAACCTTCAAGTGGGAGGA R:GTGCGGAGTACCTACGGAGT	(CATA) ₈	55	4	80-96	0.44	2(5)
ST4-11 (GQ901943)	F:GGATAGATATTTGATGCATGATGG R:CCTGCTTTTCGGGTAGAAGA	(CATA) ₇	55	4	79-91	0.55	5(7)
ST4-12 (GQ901944)	F:TGGGTTTGGATGGATGTATGG R:CATCCTTGGCAAAGTTACGG	(GTAT) ₉	55	6	97-129	0.42	5(7)
ST4-13 (GQ901945)	F:ACTGACCAACGATCGATTCC R:CGGCCATAAATTGAATGTGG	(CATA) ₁₀	55	3	101-113	0.66	1(14)
ST4-15 (GQ901947)	F:GCACACATACATACATACATCC R:CCAATCATTTTCACACGGTTC	(CATC) ₁₃	55	7	101-145	0.62	6(21)
ST4-16 (GQ901948)	F:TTACCGTACCTACAAATAAC R:ATGATGTACTTGAAGCAGGT	(CA) ₁₆	55	6	109-127	0.59	3(1)
ST4-19 (GQ901951)	F:CCTGCTTTTCGGGTAGAAGA R:GGATAGATATTTGATGCATGATGG	(GTAT) ₇	55	3	83-91	0.58	1(2)
ST4-20 (GQ901952)	F:GAACACGTCTCTTTGGGTCA R:CGTTCATAGATGGATGGATGG	(CATA) ₈	55	9	85-129	0.65	11(17)

^aRepeat motif is based on sequences of isolate 06CWM-E10 of *S. trifoliorum*.

^bExpected heterozygosity (H_E).

^cPredicted genome location based on available genome sequence of *S. sclerotiorum*. The digit or letter represents the linkage group while the digits in parenthesis represent the super contig number. Clone sequences were used to find the homologous super contigs and then linkage groups, even though in some instances we could not find the primer sequences in the homologous region despite good amplicons (not sequenced) in *S. sclerotiorum*. ^dImply more than one homologous region (super contigs) in *S. sclerotiorum*

^eThe homologous region in *S. sclerotiorum* was less than 50 bp.

- no corresponding region was found.

Table 3.2 Cross-species amplification of 33 microsatellite loci of *Sclerotinia trifoliorum* on *S. sclerotiorum*, *S. minor* and *S. S. homoeocarpa*

Locus	<i>S. sclerotiorum</i> (N=4)	<i>S. minor</i> (N=5)	<i>S. homoeocarpa</i> (N=6)
ST2-2	++*	-*	-
ST2-4A	++	+	-
ST2-5	++	++	-
ST2-6	++	-	-
ST2-12	++	-	-
ST2-12	++	-	-
ST3-1	++	-	-
ST3-1A	++	+	-
ST3-3	++	-	-
ST3-4	++	-	-
ST3-4A	++	+	-
ST3-6	++	-	-
ST3-7	++	-	-
ST3-10	++	-	-
ST3-13A	-	-	-
ST3-14	+	-	-
ST3-15A	-	-	-
ST3-18	++	-	-
ST4-1	++	+	+
ST4-2	+	-	-
ST4-3	++	+	-
ST4-4	++	-	-
ST4-6	++	+	-
ST4-6A	++	-	-
ST4-6C	++	-	-
ST4-9	++	-	-
ST4-11	-	+	-
ST4-12	++	-	-
ST4-13	++	+	-
ST4-15	++	-	-
ST4-16	++	-	-
ST4-19	++	+	-
ST4-20	++	-	-

* -, no amplification or inconsistent PCR product; +, amplification; ++, amplification and polymorphic.

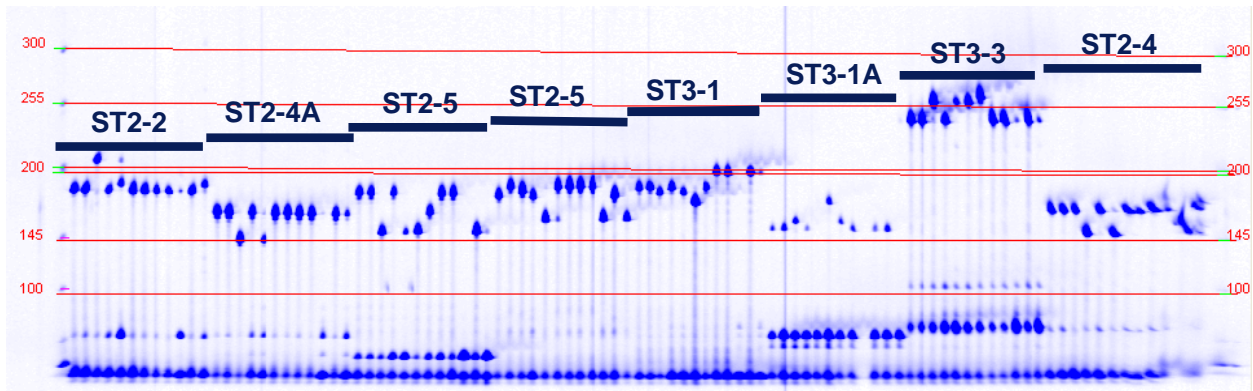


Fig 3.1 A LI-COR gel showing eight polymorphic microsatellite loci on 12 isolates of *Sclerotinia trifoliorum*. Each lane is a different isolates for each loci but the same isolates were repeated for all the eight loci. The isolates used were in different mycelial compatibility grouping and diverse rDNA intron groups.

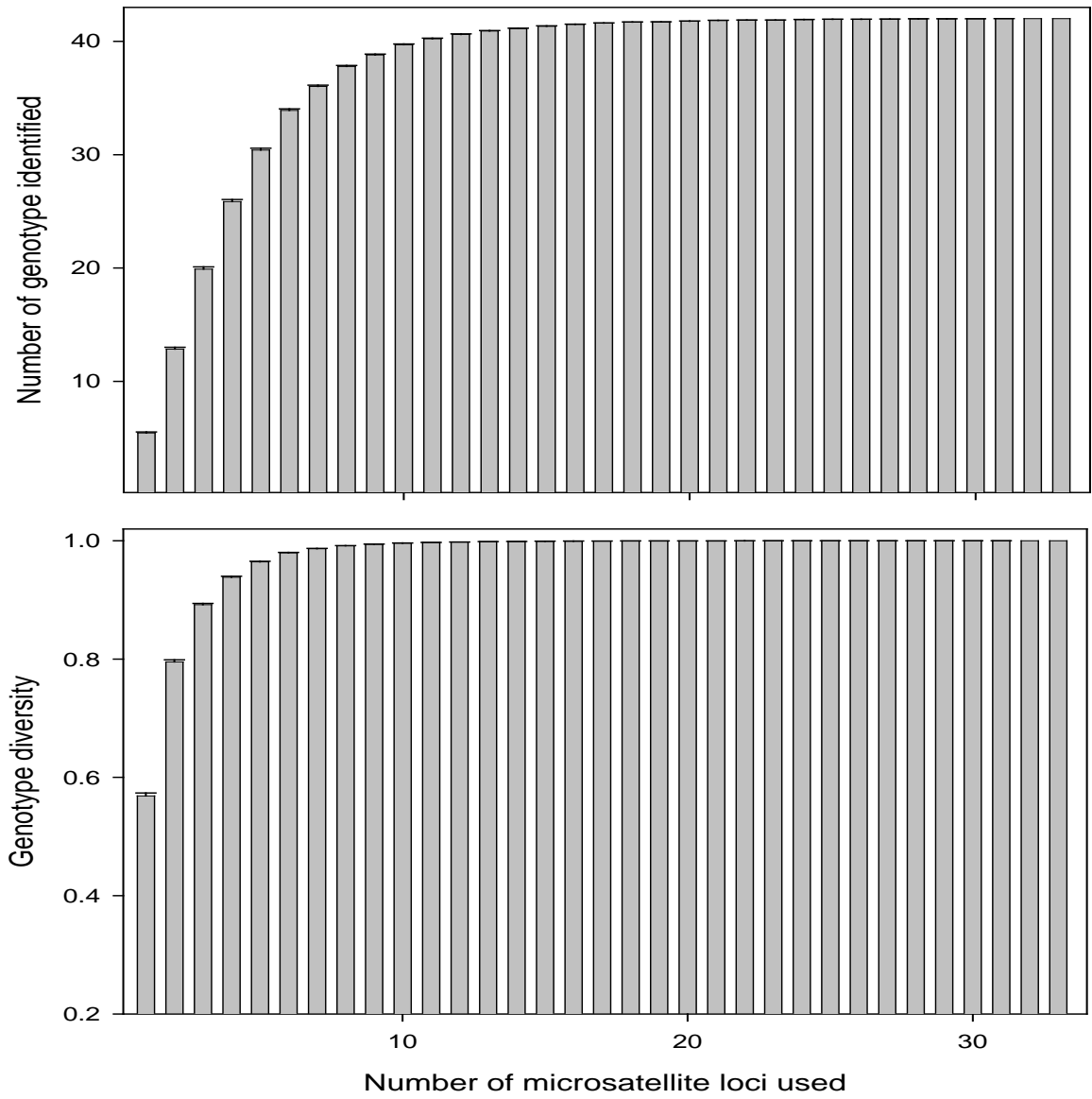


Fig. 3.2 Number and diversity of genotypes discriminated based on the number and type of loci used in the fungus *Sclerotinia trifoliorum*

CHAPTER FOUR

GENOTYPIC VARIATION AND POPULATION STRUCTURE OF *SCLEROTINIA*

TRIFOLIORUM INFECTING CHICKPEA

ABSTRACT

Sclerotinia trifoliorum is a destructive pathogen of many legume crops including chickpea, but very little is known about its genotypic variation and population structure, and the relationships among its populations have not been elucidated. We studied 136 isolates of *S. trifoliorum* infecting chickpea for diversity, patterns of variation and mating systems using 7 microsatellite markers, mycelial compatibility, sequence related amplified polymorphism (SRAP) markers and rDNA intron-derived markers. A high level of microsatellite haplotype diversity, SRAP haplotype diversity and mycelial compatibility group diversity was found among the isolates. However, only one out of three pooled populations had significant Index of Association (I_A) implying that recombination in *S. trifoliorum* is not the norm. No correlation between genetic distance and geographical distance was found, and related genotypes were distributed across the sampling area, suggesting human assisted dispersal plays an important role in the distribution of *S. trifoliorum*. Our data (based on genetic differentiation, R_{ST}) also provide evidence for shared close ancestry among geographically dispersed populations. The low to moderate R_{ST} values suggest that *S. trifoliorum* infecting chickpea could be a recent phenomenon. The high levels of haplotype diversity, population heterogeneity and the low to medium levels of population differentiation suggest that the population biology of *S. trifoliorum* in chickpea is complex. This study better defines some aspects of this complexity to aid in future epidemiological studies and, ultimately, to support suitable management strategies.

INTRODUCTION

Crown and stem rot is a destructive fungal disease of several economically important forage legumes as well as cool season grain legumes (Kohn 1979). Crown and stem rot disease is also a serious problem of chickpea (*Cicer arietinum* L.) in California, USA (Njambere et al. 2008). Crown and stem rot is caused by an ascomycete fungus *Sclerotinia trifoliorum* Erikss. Ever since Eriksson described and renamed (= *Peziza ciborioides* Rhem) the pathogen in 1878 based on diseased clover plants from Germany, Denmark and Sweden, this pathogen has been a subject of forage disease research (William and Western 1965; Kohn et al. 1979; Öhberg 2008). Work on this pathogen has focused mostly on biology, epidemiology and host range as way of distinguishing the pathogen from its sister species, *S. sclerotiorum* (Dixon and Doodson 1974; Kohn et al. 1979; Uhm and Fuji 1983a; 1983b; Rehnstrom and Free 1993; Njambere et al. 2008; William and Western 1965). Other studies resolved to elucidate the mechanism of host resistance, identify new sources of resistance, as well as investigate other disease management strategies to reduce yield losses (Halimi and Rowe 1998; Lithourgidis et al. 2005). Despite these studies, to our knowledge no work has been carried out to understand the pathogen diversity and genetic structure.

Information on the genetic diversity and population structure of *Sclerotinia* spp. causing stem rot (white mold) is widely available (Atallah et al. 2004; Carbone and Kohn 2001; Carpenter et al. 1999; Cubeta et al. 1997; Durman et al. 2003; Hambleton et al. 2002; Kohli and Kohn 1998; Sexton and Howlet 2004; Wu and Subbarao 2006; Malvarez et al. 2007) including the less common species *S. subartica* (Winton et al. 2006). *S. sclerotiorum* is the most intensively researched species, having been studied across a wide range of crops including canola, cabbage, soybeans, lettuce, chickpea, lentil, pea, potato, kiwifruit and common bean

(Atallah et al. 2004; Carbone and Kohn 2001; Carpenter et al. 1999; Cubeta et al. 1997; Durman et al. 2003; Hambleton et al. 2002; Kohli and Kohn 1998; Sexton and Howlet 2004; Malvarez et al. 2007). Of particular interest are Kohn et al. (1991) and Cubeta et al. (1997), who documented that populations of *S. sclerotiorum* in canola and cabbage respectively are clonal based on the evidence that a few clonal genotypes predominate in any locality and that the predominant clone is often recovered over many years and extended geographical distances. Further evidence of clonality is supported by strong association among unlinked molecular markers. This signifies extended generations of non recombining populations. Recently, Atallah et al. (2004), working with *S. sclerotiorum* from potatoes, demonstrated evidence of both clonal and outcrossing populations in Columbia basin of Washington state. This gave strong evidence of what was observed earlier in a limited scale by Kohli and Kohn (1998). More support for co-existence of clonal and outcrossing population of *S. sclerotiorum* has also been from Canada (Malvarez et al. 2007), Australia (Sexton and Howlett 2004; Sexton et al. 2006) and Turkey (Mert-turk et al. 2006). In the related species *S. minor*, Wu and Subbarao (2006) reported even much lower levels of genetic diversity than in *S. sclerotiorum*. The lower number of MCGs detected in *S. minor* was assumed to be due to lower recombination in *S. minor* than in *S. sclerotiorum*. In contrast, the levels of genetic diversity in *S. trifoliorum* are completely unknown except for the report of group I introns (Powers et al. 2001).

We hypothesized that populations of the soilborne pathogen *Sclerotinia trifoliorum* in California are clonal and geographically homogeneous. The objectives of this research were: 1) to assess genotypic variation in *S. trifoliorum* infecting chickpea; 2) to determine population structures of *S. trifoliorum* in California; and 3) to determine the prevailing reproductive mode of *S. trifoliorum*.

MATERIALS AND METHODS

Isolate collection. A total of 136 isolates of *S. trifoliorum* were collected for this study during 2005 and 2006. Of the 136 isolates, 133 were from infected chickpea plants from the Central and Sacramento valleys of California, representing 10 populations (Table 4.1; Fig. 4.1), and three isolates were from alfalfa in Central Valley, California (two isolates) and Prosser, Washington (one isolate). A population was defined as isolates collected from a single chickpea field (GPS coordinates of each field was noted). Sampling was carried out in a “zigzag” manner (successive samples at ends of transects that were at acute angles to each other) in each field, and whenever possible a distance of not less than 50m was maintained between sampled plants. Sampled areas spanned a range of about 350 km from south (Coalinga) to north (Colusa). Sclerotia from a single diseased plant were collected together, kept separate from those from other plants, and brought to the laboratory, where one isolate was obtained from each diseased plant. The sclerotia were first surface sterilized in 70% ethanol and then 3% sodium hypochlorite for 1 min each. They then were rinsed in sterile distilled water, blotted dry in sterilized paper towels and bisected before plating on PDA. Incubation was done at room temperature (22°C) in the dark. Isolates were purified by hyphal tip isolation, and sclerotia from each isolate were harvested, air dried, and either used for DNA isolation or stored in glass vials at -20°C for future use.

DNA Isolation. Total DNA was extracted from each isolate using the FastDNA® kit as described by Chen et al. (1999), and modified by Njambere et al. (2008). DNA concentration was quantified using a NanoDrop spectrophotometer ND-1000 (NanoDrop products, Wilmington, DE, USA) and diluted to 10µg/µl for use in PCR.

Mycelial compatibility grouping. Mycelial compatibility grouping was performed following the modified method by Kohn et al. (1990). Isolates were paired in PDA medium containing red food coloring and incubated at room temperature (22°C) in the dark (Kohn et al. 1990). Pairings were done by placing two 7-mm diameter agar plugs from the edge of actively growing colonies 2 cm apart in a 9 cm Petri plates. Compatibility was examined visually and recorded 7 and 14 days after inoculation. A compatible reaction was indicated by merging of two colonies with no distinct interaction zone. An incompatible reaction was indicated by a red reaction line and discontinuity between the two isolates (Fig. 4.2). Isolates that showed compatible reactions were placed in the same mycelial compatibility group (MCG). Isolates were paired in group of tens in all possible combinations including self pairing. Representatives of each MCG in the initial group of ten were paired with a representative of each of the other groups to determine MCGs of all isolates in the field. Then representatives of each MCG from a field were paired with representatives of other fields until all isolates were assigned to MCGs.

Group I introns in rDNA. To determine the variability in number, location and size of group I introns in rDNA among isolates, PCR amplifications were conducted using two universal primer pairs, ITS5/ITS4 and NS3/NS6, to detect introns of size 442bp (or 1565bp) and 311bp respectively (Njambere et al., 2008). A third primer pair NS-F (5'-GGAAGTTTGAGGCAATAACAGG-3') and ITS-R (5'-CCTTGTTACGACTTTTACTTCC-3') was designed to detect an intron of expected size 358bp. PCR product sizes were estimated by electrophoresis in 1% agarose gel along with standard DNA size ladder. Isolates with the same combination of number, location and size of introns were assigned to the same rDNA haplotype.

SRAP markers. Sequence related amplified polymorphism (SRAP) markers are known to target coding regions of the genome (Li and Quiros 2001). Based on initial screening of 60

pairs of SRAP primers, nine primer pairs were selected for this study based on their consistency in producing reliable and scoreable bands in repeated PCR reactions (Table 4.2). Reliability and scoreability were tested by repeating at least once the reproducibility of the selected markers using two different GeneAmp PCR System 9700 thermocyclers. All SRAP reactions were performed in 25 μ l volume containing 50 ng DNA; 200 μ M each dNTP; 1.5 mM MgCl₂; 2.5 units Amplitaq Gold DNA Polymerase (Applied Biosystems); 2.5 μ l 10x Amplitaq Gold Buffer, and 37.5 ng of both forward and reverse primers. The thermal cycling profile for all reactions was: 95 °C for 10 min, followed by 5 cycles of 94°C for 1 min, 35 °C for 1 min, 72 °C for 1 min, 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR products (15 μ l of each reaction) were electrophoresed on 10% PAGE-TBE gels (100V for 3 h), stained with ethidium bromide for 30 min and visualized with UV light. Amplicons were scored independently as being present or absent for each isolate. Each isolate was repeated once to test for reproducibility of the selected markers using two different GeneAmp PCR System 9700 thermocyclers. Isolates that had the same combination of alleles (across loci) were placed in the same SRAP haplotype.

Microsatellite markers. Microsatellites are usually found in non-coding regions of the genome and provide an independent marker system for the study of *S. trifoliorum*. Seven microsatellite loci that previously shown variation among isolates (Chapter 3) were selected (Table 3.1). PCR conditions used to amplify the DNA were the same as those described in Chapter 3. PCR amplification of all the isolates was done using a M13 fluorescent labeling protocol (Schuelke 2000) where the universal M13 tail end is attached to the 5' end of the forward primer and an addition third primer, IR labeled universal M13 primer included in the reaction. Analysis of fragment sizes was done using a LI-COR 4300 sequencer (LI-COR

BIOSCIENCES, Li-COR Inc.) and alleles determined using the gene ImagIR 4.05 software (Li-COR Inc.) as presented in Chapter 3. To ascertain the reproducibility of amplifying the microsatellite alleles, each isolate was run and analyzed at least twice. The size of the repetitive region of each allele was confirmed in selected isolates through sequencing.

Data analyses. To test if the populations were structured and the level of diversity within and among populations the program ARLEQUIN version 3.1 (Schneider et al. 2000) was applied to microsatellite data to estimate genetic diversity as the unbiased mean expected heterozygosity (H ; Nei 1987), number of total alleles, number of rare alleles (frequency <0.05), number of private alleles (those found in only one population), and values of genetic differentiation (R_{ST}). The R_{ST} statistic uses variation in allele sizes and incorporates the stepwise mutation model (SMM) to evaluate the proportion of the total genetic variation that is found in a subpopulation (Slatkin 1995). The significant difference of R_{ST} values was tested from zero ($p < 0.05$) was determined with 1000 permutations of individuals among populations. Pair-wise genetic distances of R_{ST} values among the populations were used to perform a hierarchical analysis of molecular variance (AMOVA) (Excoffier et al. 1992) with the ARLEQUIN program. The possible presence of geographic structure of genetic variation in *S. trifoliorum* was evaluated by testing for a pattern of isolation by distance (Rousset 1997). A Mantel test with 1,000 random permutations was performed with the matrix of pairwise genetic differentiation between populations, using $R_{ST}/(1-R_{ST})$, and a matrix of the $\ln M$ (M = geographic distance) with the software GENALEX version 6 (Peakall and Smouse 2006).

Genetically distinct populations were identified using a Bayesian clustering method implemented in STRUCTURE v. 2.0 (Pritchard et al. 2000; Falush et al. 2003). For each K number of populations modeled, individual genotypes are assigned membership in a population

as a function of allele frequencies and the proportion of an individual's genotype drawn from each of the K populations. Individuals are assigned to clusters to maximize linkage equilibrium and genetic homogeneity within each cluster. For each parameter value of K (from 1 to 8) we used a population ancestry model with admixture because most populations did not appear to be discrete based on the low R_{ST} values observed. We therefore also assumed that there was correlation of allele frequencies among populations. The analysis was done in five independent runs of 500 000 iterations with Markov Chain Monte Carlo simulation burn-in period of 100 000 iterations.

The index G:N (the ratio of the number of multilocus genotypes found over the sample size) was used to estimate the extent of clonal reproduction. A G:N ratio of 0 occurs under strict clonality when all individuals share the same genotype, whereas a value of 1 occurs when all individuals have distinct genotypes, under sexual reproduction (Ivey and Richards 2001). We further tested for potential outcrossing in *S. triflorum* by calculating the proportion of significant linkage disequilibria between pairs of loci, and then using the multilocus index of association (I_A) which estimates linkage disequilibrium using a Monte Carlo simulation when more than two loci are involved (Agapow and Burt 2001). To account for small population size we pooled the ten populations initially into one and then into three groups for those populations which were in close geographic proximity and had insignificant R_{ST} values. The null hypothesis was that the populations were randomly mating. Linkage disequilibria were computed in Genepop version 3.3 (Raymond and Rousset 1995). Indices of association were computed in MULTILOCUS version 1.3 with significance evaluated by permutation (Agapow & Burt 2001). This was done using the software to determine if I_A differed significantly from zero, by performing 1,000 randomizations of the data set.

RESULTS

rDNA introns. All the 136 isolates used in this study contained one or more group I introns in the small subunit rDNA. This confirmed that all the isolates were *S. trifoliorum* and not *S. sclerotiorum* because previous research showed that presence of introns in this DNA region differentiates *S. trifoliorum* from *S. sclerotiorum* and *S. minor* (Njambere et al. 2008). Introns were located in three locations and there were two different sizes of intron at one of the three locations. Combination of intron locations and intron sizes formed four haplotypes (Fig. 4.3), with the predominant haplotype containing 96 isolates.

Mycelial compatibility grouping (MCG). In MCG pairings, all the isolates were compatible with themselves showing a continuous growth of the mycelium (Fig. 4.2). However pairings between isolates among the 136 isolates produced only a few compatible reactions. In total, 80 MCGs were found among 136 isolates (Fig. 4.3) giving a G/N ratio of 0.6. Fifteen of the MCGs consisted of two or more isolates and the remaining 65 MCGs were made up of only one isolate each. Isolates belonging to the same MCG were not limited to any geographical location. The most frequent MCG had 19 isolates (14 % of the total) derived from seven populations.

SRAP markers. The nine selected primer pairs generated 56 scorable DNA bands, of which 51 (90%) of them were polymorphic. The number of polymorphic loci varied from 32.1% for the Davis population (D) to 62.5% for the Farmington population (A) with an overall average of 47.0%. A total of 78 haplotypes was found in 104 isolates (Fig. 4.3) with a G/N ratio of 0.75. Similar to the case of MCGs, members in the same haplotype were not restricted to any geographic area. The gene diversity (expected heterozygosity) varied from 0.02 to 0.20 for the ten populations.

Microsatellite markers. All seven microsatellite loci were polymorphic in each of the ten populations. The number of alleles per locus ranged between 6 in the ST3-4 locus to 13 in the ST4-20 locus (Table 4.3). The highest number of alleles found in a single population was nine for the ST4-20 locus in Farmington population (A). The mean number of alleles for the 10 populations was 9.4, while the estimated expected heterozygosity (H) values ranged between 0.54 and 0.72 per population with an average of 0.70. Most of the alleles were not unique to a population, but the Farmington population (A) had the highest number of total, rare and private alleles (Table 4.3).

A total of 116 multilocus haplotypes was identified among the 136 isolates. The frequency distribution of the haplotypes was highly skewed with 108 haplotypes (Fig. 4.3) consisting of single isolate. Most of the haplotypes were limited to single populations, but some haplotypes were found at multiple populations.

Population differentiation. To estimate the level of genetic differentiation among populations, we calculated R_{ST} values across all 7 microsatellite loci using ARLEQUIN and assuming SMM (stepwise mutation model) (Schneider et al. 2000). R_{ST} is an analog of F_{ST} and both estimate the amount of between – population differences. F_{ST} is calculated purely from differences in allele frequencies among populations. R_{ST} , however, takes into consideration differences in allele length and assumes a SMM to assess the extent of divergence between populations (Slatkin 1995). Most of the R_{ST} values obtained between pairwise comparisons were low to moderately differentiated (Freeland 2006), ranging from 0 to 0.15 (Table 4.4). Significant levels of genetic differentiation ($P = 0.05$) were obtained in 17/45 (38%) of the pairwise comparisons. Among 55 pair-wise comparisons, the Farmington population (population A) (R_{ST} values from 0.02 to 0.14) was the most differentiated population with all the pairwise

comparisons being significantly greater than zero except with Farmington (B) population. Interestingly, both F_{ST} (derived from SRAP markers) and R_{ST} results (derived from microsatellites) showed low to moderate levels of differentiation (Table 4.5).

Population clustering. When genetic variation was partitioned at three levels (within population, among populations and among groups of populations) using AMOVA, most genetic variability was found at the within-population level, which accounted for approximately 91% of the total genetic variation ($p < 0.001$). Variation among populations accounted for 5% of the total genetic variation ($p = 0.009$) and variation among groups of population accounted for 4% of the total genetic variation ($p = 0.046$) (Table 4.6). For comparative purpose, the SRAP markers AMOVA (Table 4.7) was also calculated and it proportioned 89% of the genetic variability to within-population differences, 9% to among populations and 1% among regional groups. The AMOVA analysis was only done for samples collected in 2006, as the 2005 samples were only from 2 populations. The test of isolation by distance showed that there was no significant spatial (geographic) differentiation among-populations (Mantel test; $p = 0.170$). This was reflected in the line of regression which explained only 0.2% of the total variance ($R^2 = 0.002$) (Fig. 4.4). Similar results were obtained with SRAP markers (not shown).

We next used STRUCTURE (which ignores the existing spatial boundaries) to detect any cryptic clustering in the Californian population. We found support for 3 distinct clusters among the Californian populations (Fig. 4.5). However these clusters were not concordant with geography and all the three clusters appeared to be fairly distributed within the chickpea growing areas (Fig. 4.6).

Linkage disequilibrium analyses. The G/N indices for MCG, SRAP and microsatellite markers showed that the 10 populations have a tendency towards outcrossing. However the

presence of some dominant genotypes within-populations implies also that isolates of *S. trifoliorum* also undergo clonal reproduction propagated either via mycelia or sclerotia. The importance of clonal versus sexual reproduction was assessed by calculating the two-locus analysis and multilocus index of association (I_A) (Multilocus, version 1.3) for each population separately, first before allowing for clone correction and with clone-corrected data, where only one representative isolate for each haplotype was included, to reduce bias due to clonality. These association measures test for multilocus linkage disequilibrium and the values are zero if alleles are recombining in the population (no linkage disequilibrium). When all 136 isolates were included, the two-locus analysis had significant linkage disequilibrium between many pairs of loci (15 out of the 21 two-locus combinations). There was also a significant departure from linkage equilibrium among seven out of 9 populations assessed in the multilocus analysis. Genetic disequilibrium may result from many causes, clonality being one of them. To assess for the potential effect of clonality/outcrossing these tests were performed with clone-corrected data. The two-locus LD analyses with clone correction showed only seven out of 21 combinations departed significantly from linkage equilibrium, while the I_A with clone correction reduced the populations with significant LD from seven to four. Various causes underlie apparent disequilibrium among populations; one reason being population size. To account for small population size we pooled the nine populations initially into one. Still, the null hypothesis of random mating was rejected. However, when we pooled seven of the nine populations which were in close geographic proximity and had insignificant R_{ST} values into three groups, we could not reject the null hypothesis of random mating in one of the three populations (Fig 4.7; Table 4.8).

DISCUSSION

In this study, we used four independent genetic markers to study population structure of *S. trifoliorum*: MCG, rDNA introns, SRAP markers and microsatellite markers. These markers target different regions of the genome. SRAP markers are known to target the coding region of the genome (Li and Quiros 2001), whereas microsatellite markers are usually found in the non-coding regions. Congruency was found in the results of SRAP markers and microsatellite markers. We provide evidence that the population structure of *S. trifoliorum*, a recently reported pathogen of chickpea in California (Njambere et al. 2008), is heterogeneous and show cryptic population structuring. These findings suggest that *S. trifoliorum* may have been introduced three times in chickpea and human assisted dispersal may have facilitated the distribution of the three groups in the chickpea growing areas in the recent past. Geographically defined populations imply evolutionary association (Grant and Bowen 1998); the fact that our inferred populations clusters do not attest to this definitive character may imply that the association between chickpea and *S. trifoliorum* in California could be recent. The epidemics of chickpea crown rot caused by *S. trifoliorum* in 2005 and 2006 typical of new virulent strains or novel pathogens (Macdonald 2004) further gives support to this opinion. The alternative is that the association between chickpea and *S. trifoliorum* could be old, but did not reached epidemic proportion until 2005. The pathogen diversity could have been facilitated by previous cropping histories of the area.

We identified 116 microsatellite haplotypes from 136 isolates of *S. trifoliorum*. Similarly, the diversity based on MCG (80 MCGS out of 136 isolates) and SRAP haplotypes (78 haplotypes out of 104 isolates) was equally high. This is the highest proportion of microsatellite haplotypes (85%) ever reported in the *Sclerotinia* genus. The closest previously reported was that by Sexton and Howlet (2004) who identified 51% different microsatellite haplotype based on

160 isolates of *S. sclerotiorum* in Australian canola fields. Phillips et al. (2002) reported high level of diversity (71%) in the U.S. in canola based on DNA fingerprints. It should be noted that the results are not directly comparable due to the different molecular markers used and sampling differences.

Central to this study was the question whether the genotypic diversity of *S. sclerotiorum* is structured in any manner. Results of AMOVA showed that 91% of genetic variation was within populations and only 5% was due to among population difference ($p=0.009$). This is contrary to the expectation of a well-structured population where we anticipate more differences among populations rather than (or in addition to) within populations. Closely related haplotypes were found scattered across geographic regions. There was also a tendency for a few related haplotypes to be in close proximity.

The low to medium level of population differentiation (R_{ST} varied from 0-0.15), with 42% of pairwise comparisons being between 0-0.05 and only 38% being significantly greater than zero reflect related populations but with a mixed ancestry (Avisé 2000). While these results may characterize a population with limited barrier to gene flow, it also reflects a tendency of exclusion by some populations. Similar predisposition has been observed in *Ophiostoma ips*; a beetle transmitted pathogen of conifers (Zhou 2008) and is indicative of admixture, phenomena attributed to anthropogenic disturbance (Wright et al. 2004).

A closer look at population differentiation based on R_{ST} showed a genetic structure characterized by low to moderate levels of population differentiation, with subtle significance differences. There was no progressive increase in differentiation with geographical distance as expected under an isolation-by-distance model. Despite the observed differences in some populations, we could not identify any corresponding barrier to movement in the field, further

reinforcing our anthropogenic admixture view of this population. Farmington (population A) was the most significantly differentiated population. Coincidentally this population had the highest number of private and rare alleles and has historically been used for field experimentation and seed multiplication trials. High genetic differentiation would have suggested that the individual populations have been reproductively isolated throughout their recent history, but these findings suggest otherwise. Our findings suggest that there is substantial gene flow among the populations. This pattern is typical of a population where there are limited barriers to geneflow and there has not been sufficient time to allow for lineage sorting (Freeland 2006).

The absence of isolation by distance (lack of correlation of genetic distance with geographic distance) could have several explanations. There is frequent and random gene flow among the populations, particularly facilitated by human assisted dispersal. The populations infecting chickpea are newly established, i.e., they are probably founder populations and are still devoid of geographic structure (Slatkin 1993); the high heterogeneity observed in *S. trifoliorum* isolates may thus be interpreted as a system not yet having reached drift-migration equilibrium after the establishment of the populations in the region or after a major mixing event.

The high level of haplotype diversity in *S. trifoliorum* as presented by G/N indices for MCG (0.62), SRAP (0.72) and SSR (0.85) imply that outcrossing may be occurring. However, calculation of the multilocus index of association (I_A) by pooling the populations into three groups showed significant departure from linkage equilibrium for two out of the three groups. This suggests a lack of random mating within the two groups. Grouping the populations together as one also portrayed the populations to be clonal (lack of random mating). Linkage disequilibrium may result from many causes including reduced gene flow, homothallism, clonality and geographic separation. These results suggest that outcrossing is not the norm in *S.*

trifoliorum infecting chickpea. There could be other factors shaping the genetic structure of *S. trifoliorum*. Based on work from other systems, researchers have observed that complex population histories play an important role in determining the population genetic structure. Tibayrenc and Ayala (2002) observed that the demography and genetics of a pathogen is affected by the rate of clonal versus sexual reproduction in natural populations. Whereas sexual populations pay a demographic cost by producing males (and will only produce males when it is necessary to do so), they take advantage of recombination that enhances the combination and spread of favorable mutations, relative to clonality (Macdonald 2004). In *S. trifoliorum* the male organs are represented by the microconidia (spermatia) which function only in sexual reproduction. Asexual propagation in *S. trifoliorum* occurs through mycelia and sclerotia. Sclerotia are the survival structures which carry the pathogen through the next generation (Kohn, 1979). Sexual structures are only produced under adverse weather conditions (winter temperatures) (Uhm and Fuji 1983a; 1983b; Rehnstrom and Free 1993; Njambere et al. 2008) which are rare in California. This creates a temporal barrier to gene flow. Non-random mating is also envisaged by the unidirectional mating system found in *S. trifoliorum*. Sexual reproduction in *S. trifoliorum* results in formation of dimorphic ascospores; 4 small and 4 large ascospores within an ascus. Isolates derived from large ascospores are homothallic and do not require another individual to complete their sexual cycle. Whereas those derived from small ascospore are heterothallic and must mate with spermatia coming from large ascospore individuals (Uhm and Fuji 1983; Rehnstrom and Free 1993). The fact that one group has undergone recombination (I_A) implies the possibility that *S. trifoliorum* is taking advantage of the two reproductive strategies to ensure survival (Taylor *et al.* 1999). Nonetheless, out-crossing can also occur occasionally in homothallic fungi although this might not be the overall trend (Marra & Milgroom 2001). Our

results show that the pattern of genetic differentiation in *S. trifoliorum* is complex. It is possible that a nested clade analysis (Templeton et al. 1992) could reveal the multiple founding events of the past *S. trifoliorum*-chickpea interaction.

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Table 4.1 Location where the 10 populations of *S. trifoliorum* were collected and the year of sampling

Population	Total No. of Isolates	Number of isolates		GPS Coordinates
		Year 2006	Year 2005	
Farmington (A)	32	32		037° 55' 096" N, 121° 03' 936" W
Farmington (B)	7	7		038° 01' 012" N, 121° 05' 029"W
Stockton/Machado (C)	6	6		037° 57' 111" N, 121° 07' 826" W
Davis (D)	7	7		038° 31' 758" N, 121° 50' 674" W
Woodland (E)	10	10		038° 39' 739" N, 121° 52' 928" W
Knights Landing (F)	10	10		038° 48' 367" N, 121° 40' 220" W
Five Points (G) (M)	31	12	19	037° 57' 111" N, 121° 07' 826" W
Lemoore (J)	11	11		036° 17' 101" N, 119° 54' 867" W
Stone Landing (K)	11	11		036° 07' 058" N, 120° 00' 260" W
Coalinga (W)	11		11	036° 08' 023" N, 120° 21' 037" W

Table 4.2 Primer sequences used for SRAP analyses in this study presented as forward primers/
reverse primers

Locus	Primer sequences	# of scored amplicons	# of polymorphic amplicons
L1	F9, 5'-GTAGCACAAGCCGGACC-3' R9, 5'-GACACCGTACGAATTTGA-3'	8	8
L2	F13, 5'-CGAATCTTAGCCGGATA-3' em1, 5'-GACTGCGTACGAATTAAT-3'	4	4
L3	F9, 5'-GTAGCACAAGCCGGACC-3' R10, 5'-GACTGCGTACGAATTCAT-3'	8	5
L4	F13, 5'-CGAATCTTAGCCGGATA-3' em5, 5'-GACTGCGTACGAATTAAC'-3'	6	6
L5	F6, 5'-GGATGAGTCCAAACCCA-3' em6, 5'-GACTGCGTACGAATTGCA-3'.	4	3
L6	F12, 5'-CGAATCTTAGCCGGAGC-3' em6, 5'-GACTGCGTACGAATTGCA-3'.	6	5
L7	F13, 5'-CGAATCTTAGCCGGATA-3' em6, 5'-GACTGCGTACGAATTGCA-3'.	6	6
L8	me1, 5'-TGAGTCCAAACCGGATA-3' R8, 5'-GACTGCGTACGAATTCAC-3	5	5
L9	me2, 5'-TGAGTCCAAACCGGAGC-3' R10, 5'-GACTGCGTACGAATTCAT-3'	9	9

Table 4.3 List of the seven microsatellite loci showing the number, mean and total number of alleles, expected heterozygosity, rare and private alleles for 12 populations of *Sclerotinia trifoliorum*

Locus	Primer sequence (5'-3')	A	B	C	D	E	F	J	K	M*	W	L*	G*	Mean	Total #	
															of alleles	H _E
ST3-1	F:CATCCATCTACTACGCCTTTTC R:AGGCTATCCATCCTCACATCC	4	4	3	2	3	4	2	2	2	2	2	3	2.75	9	0.53
ST3-3	F:TGGCAAAGTTACGGTTATGCT R:GGGACTATTTGTTGCATATCTGG	5	4	4	2	3	6	2	2	3	5	2	4	3.5	9	0.75
ST4-1	F:GATATTCCATGCCAAACACG R:GGTGACTCCATAACCATCTCG	3	2	2	2	2	2	2	2	2	5	2	2	2.33	6	0.52
ST4-2	F:CTCGTCGCATTACAATTTGC R:CGGATGCGTTCATCTCTTAG	6	3	2	3	4	3	5	3	4	5	2	5	3.75	8	0.78
ST4-4	F:CGGAGTAGGTTAGTTACATCGG R:GCTGCCTCTACTTCTGTACAT	4	2	4	3	5	4	4	2	3	6	3	4	3.67	11	0.7
ST4-6A	F:AGTGTGAGGCTGGTTGGTG R:ACCAACAATCCAATGACAGC	8	4	2	5	4	3	3	4	3	5	2	6	4.08	10	0.82
ST4-20	F:GAACACGTCTCTTTGGGTCA R:CGTTCATAGATGGATGGATGG	9	4	3	3	3	4	5	4	2	5	2	5	4.08	13	0.76
Mean		5.57	3.29	2.86	2.86	3.43	3.71	3.29	2.71	2.71	4.71	2.14	4.14	3.45	9.43	
H _E		0.68	0.7	0.71	0.61	0.65	0.71	0.58	0.58	0.53	0.6	0.66	0.71	0.65		0.69
Priv. A		2	-	-	-	-	-	-	-	-	-	-	2		4	
Rare A		5	-	-	-	-	-	1	2	2	-	-	-		5	

*The Five Points population has been split into two, 2005 (M) and 2006 (G). Additionally the three isolates of *S. trifoliorum* from alfalfa have been included as population L.

Table 4.4 Pairwise R_{ST} values and spatial distance among *Sclerotinia trifoliorum* populations based on microsatellite markers

Population	A	B	C	D	E	F	J	K	W	G&M
Farmington (A)	0.00	5	37	91	107	112	214	80	206	208
Farmington (B)	0.02	0.00	37	96	109	117	213	85	202	205
Stockton/Machado (C)	0.09*	0.04	0.00	110	125	133	197	101	184	179
Davis (D)	0.09*	0.05	-0.01	0.00	19	32	304	18	285	288
Woodland (E)	0.07*	0.05	0.05	-0.02	0.00	13	317	14	296	304
Knights Landing (F)	0.10*	0.04	0.02	0.04	-0.01	0.00	325	354	307	312
Lemoore (J)	0.14*	0.11*	0.07	0.06	0.04	-0.02	0.00	293	48	32
Stone Landing (K)	0.14*	0.12*	0.15*	0.06	0.02	0.06	0.07	0.00	280	259
Coalinga (W)	0.09*	0.08	0.10*	0.07	0.01	0.04	0.08*	0.07	0.00	38
Five Points (G&M)	0.10*	0.09*	0.10*	0.06	0.03	0.04	0.09*	0.09*	0.05	0.00

R_{ST} are reported in lower diagonal and the geographic distance (km) in the upper diagonal.

Asterisks indicate significant values ($p=0.05$).

Table 4.5 Pairwise F_{ST} values among *Sclerotinia trifoliorum* populations based on SRAP

markers

Population	A	B	C	D	E	F	K	J	G&M
Farmington (A)	0.000								
Farmington (B)	0.040	0.000							
Stockton/Machado (C)	0.022	0.040	0.000						
Davis (D)	0.013	0.049	0.024	0.000					
Woodland (E)	0.028	0.064	0.041	0.033	0.000				
Knights Landing (F)	0.035	0.059	0.055	0.044	0.035	0.000			
Stone Landing (K)	0.040	0.066	0.067	0.055	0.033	0.027	0.000		
Lemoore (J)	0.041	0.077	0.076	0.055	0.048	0.017	0.025	0.000	
Five Points (G&M)	0.045	0.119	0.071	0.050	0.072	0.112	0.106	0.097	0.000

Table 4.6 AMOVA of *S. trifoliorum* isolates (N= 104) using seven microsatellite loci

Source of variation	d.f.	Sum of squares	Variance components	Percentage of total variation	P value
Among regions	2	14.968	0.10657	4.29	0.05
Among populations (within regions)	6	20.157	0.11183	4.50	0.009
Within populations	95	215.192	2.26518	91.21	<0.001
Total	103	250.317	2.48358		

Table 4.7 AMOVA of *S. trifoliorum* isolates (N= 104) using SRAP markers

Source of variation	d.f.	Sum of squares	Variance components	Percentage of total variation	P value
Among regions	2	31.609	0.081	1	0.100
Among populations (within regions)	6	62.955	0.539	9	<0.010
Within populations	95	495.926	5.220	89	<0.010
Total	103	590.490	5.840		

Table 4.8 The observed indices of association (I_A) for the pooled populations of *S. trifoliorum* collected from California, with the p value indicating the deviation of the observed data from that of the artificially generated dataset

Population	No. of isolates	Observed I_A	P value
Farmington (A) and (B)	39	0.246	0.004*
Davis (D), Woodland (E), Knights Landing (F), and Stone Landing (K)	38	0.355	0.001*
Five Points (G&M)	31	0.175	0.212

*indicate significant values ($p=0.05$)

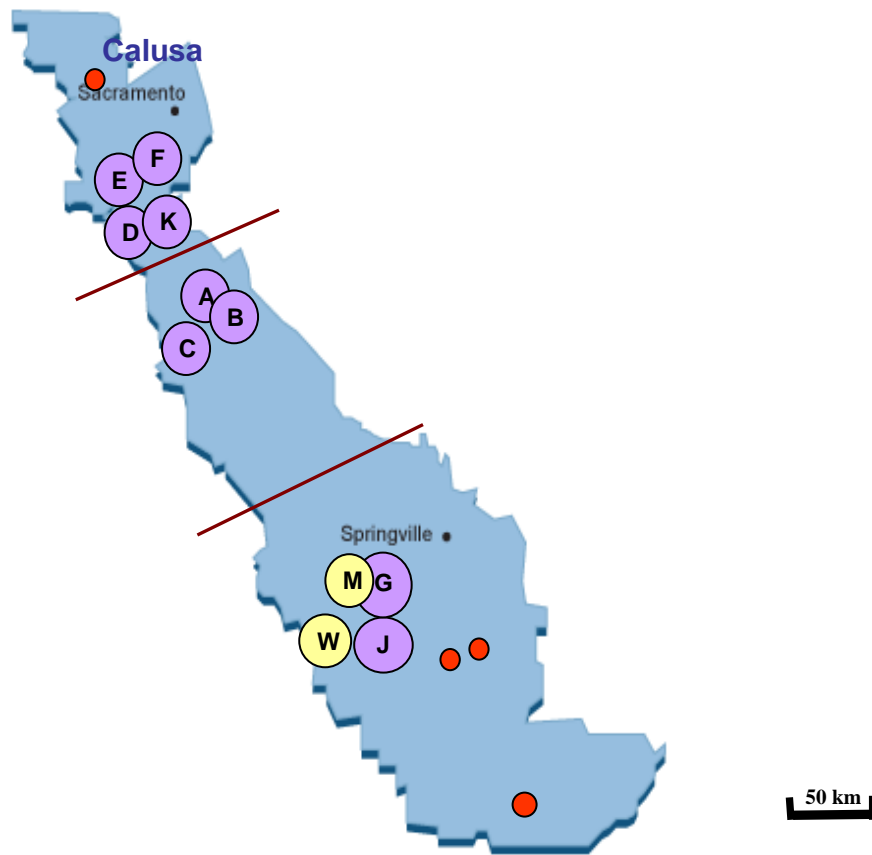


Fig. 4.1 Map of the study area in Central and Sacramento Valleys of California. The circle in purple marked with A to J represent areas sampled in 2006. Circle in yellow marked with M and W are areas sampled in 2005, while circles in red are areas where a single isolate was obtained in 2005. The sampled area was also divided into three regions based on geographic proximity (two parallel red lines).

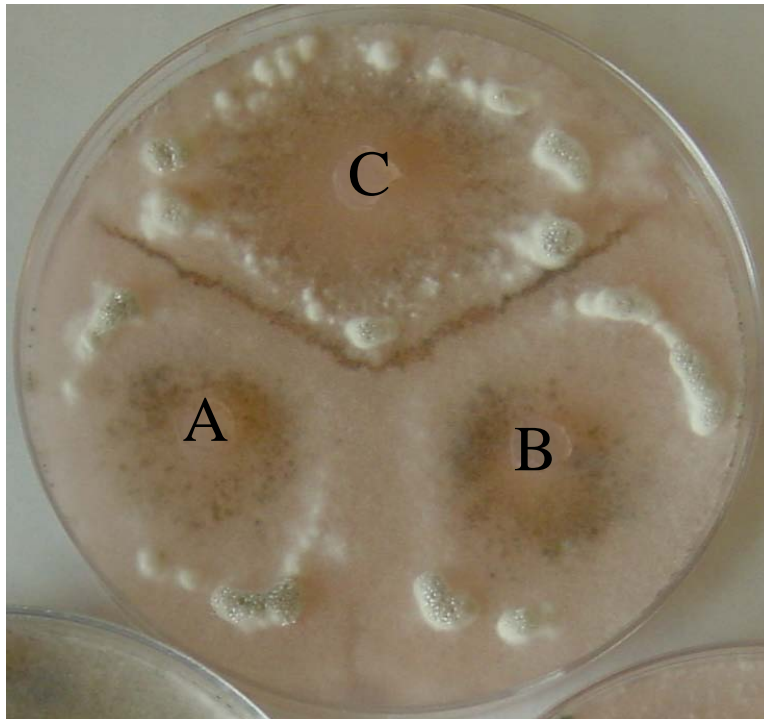


Fig. 4.2 Testing of mycelial compatibility groups. A compatible reaction shows the intermingling of mycelia with no interaction zone (between isolates A and B), while an incompatible reaction is characterized by accumulation of red food color which occurs between incompatible pairings (between isolates A and C; and B and C).

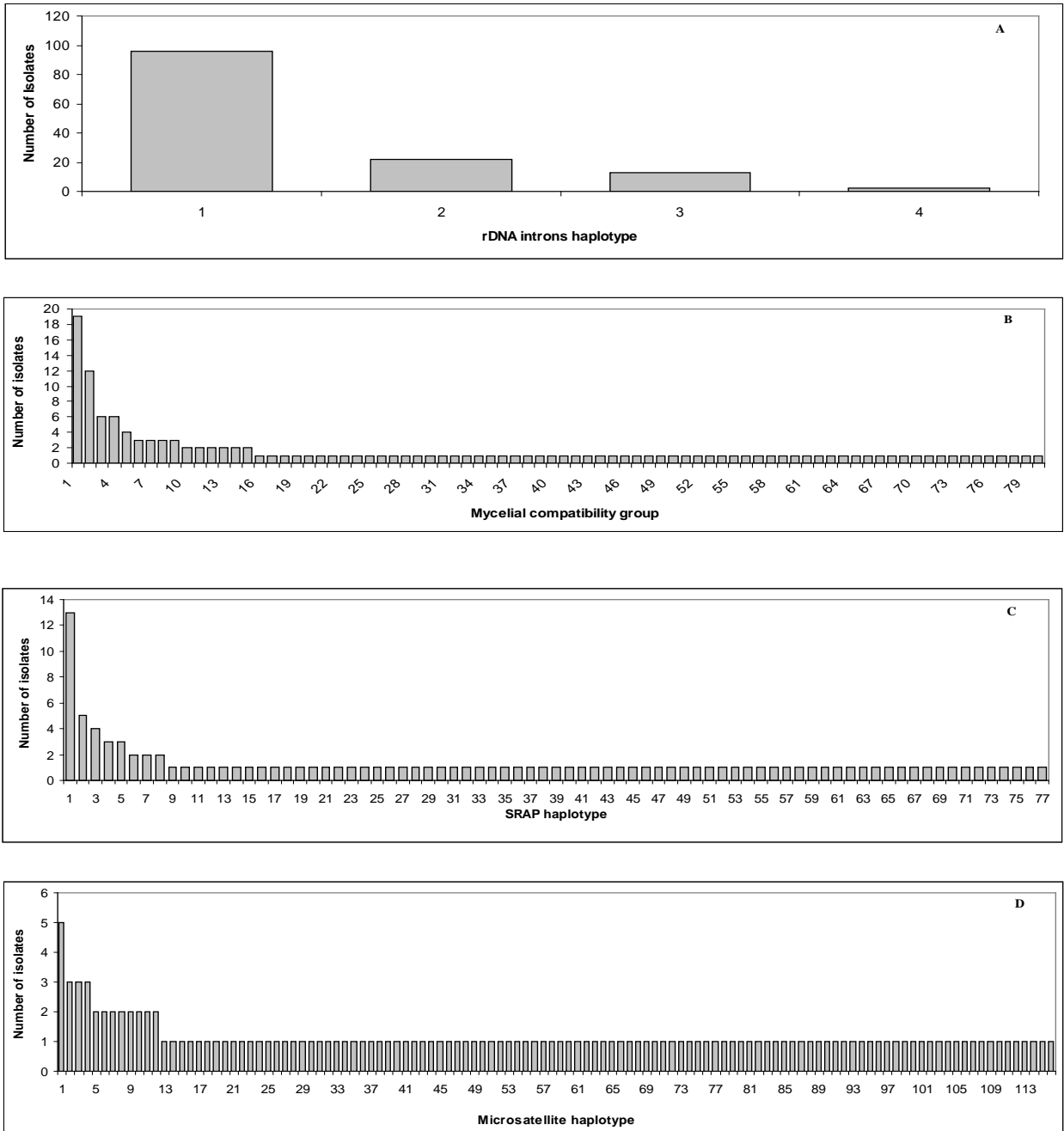


Fig. 4.3 Histograms showing genotypic diversity of *Sclerotinia trifoliorum*. **A:** the frequencies of the 4 rDNA haplotypes (N=136), **(B)** the frequencies of the 80 mycelial compatibility groups (N=136), **(C)** the frequencies of the 78 SRAP haplotypes (N=104), and **(D)** the frequencies of the 116 microsatellite haplotypes (N=136).

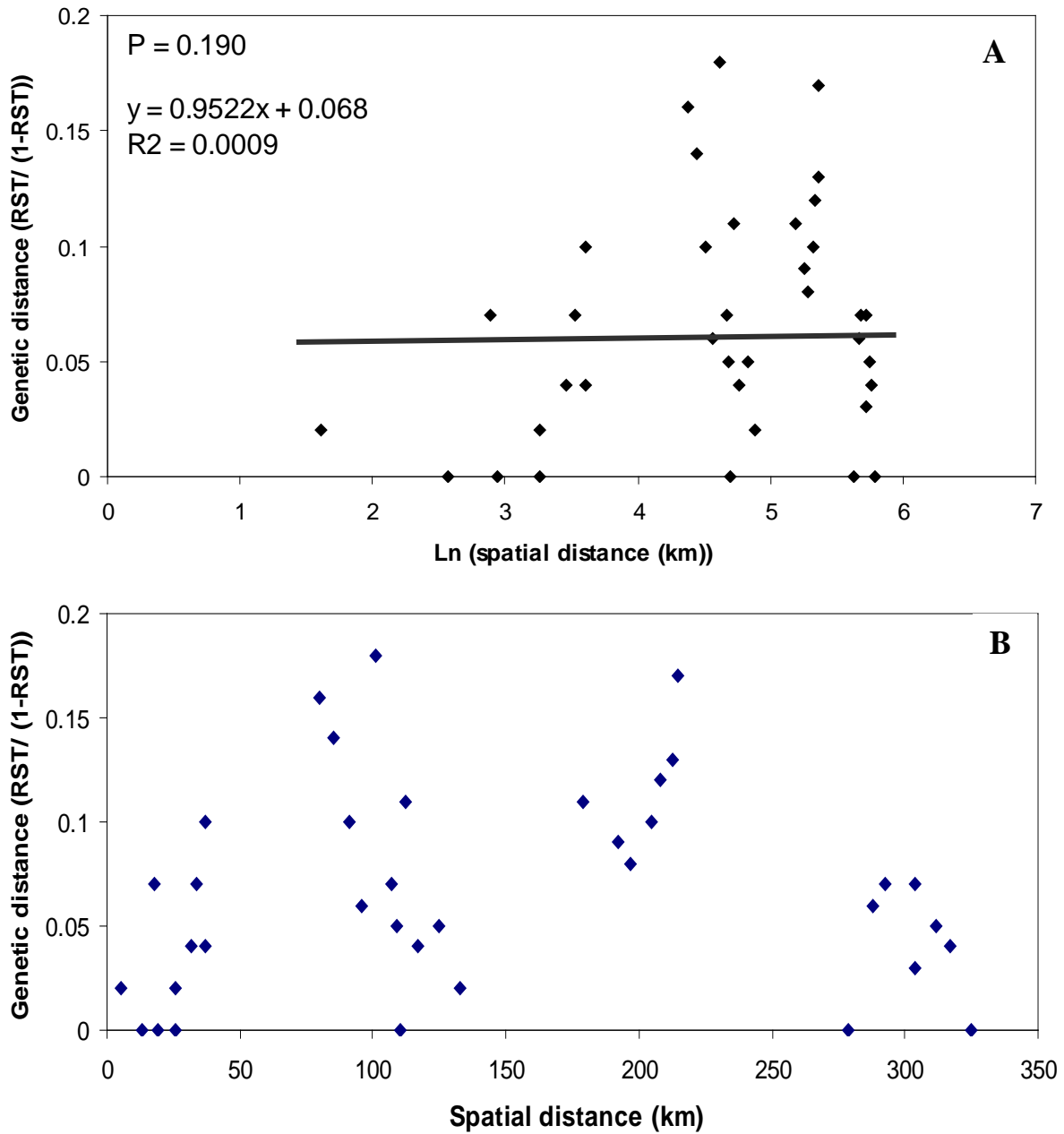


Fig. 4.4 Lack of isolation by distance between eight populations of *Sclerotinia trifoliorum* collected in 2006. Scatter diagram A is based on ln spatial distance while B is based on actual spatial distance between two population pairs. Mantel test with 999 permutations was not significant ($p=0.19$) indicating that there is no correlation between genetic distance and geographic distance.

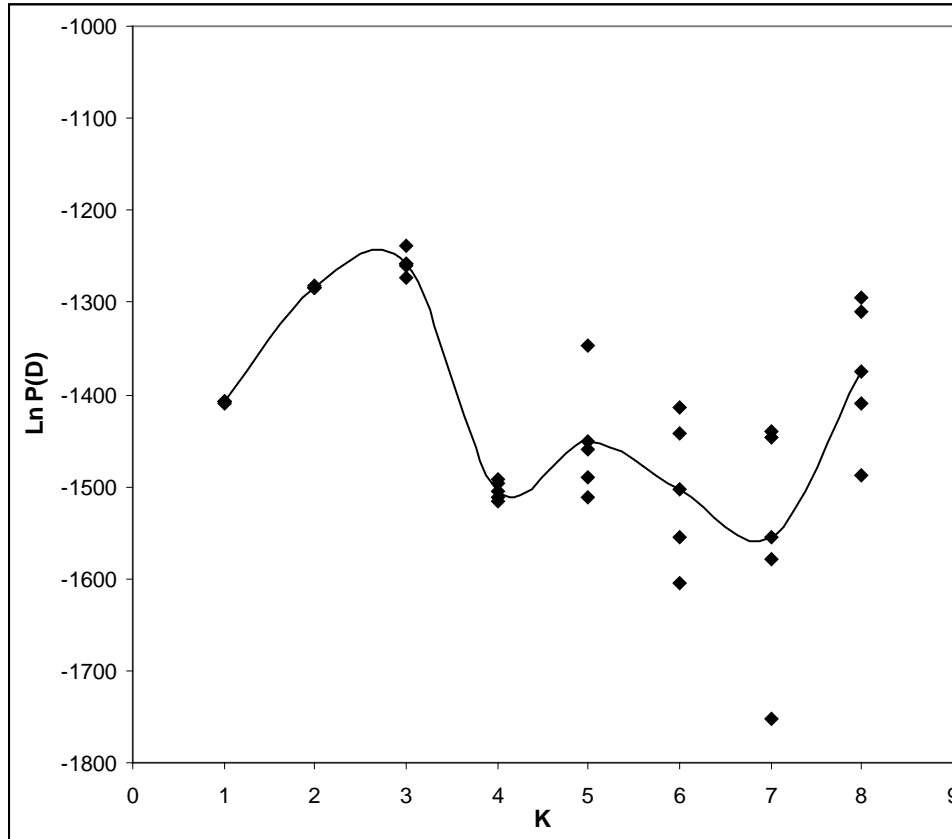


Fig. 4.5 Estimation of number of clusters (K) that best suited the data set, using the software STRUCTURE. The average likelihoods, ($\ln P(D)$) in five replications is shown for each value of K . The diagram is modeled based on admixture model of population ancestry and correlated allele frequency model. In this study we adopt $K=3$ at which $\ln P(D)$ is maximized. For values $K>3$, The $\ln P(D)$ dramatically decreases and the variance between independent runs increases.

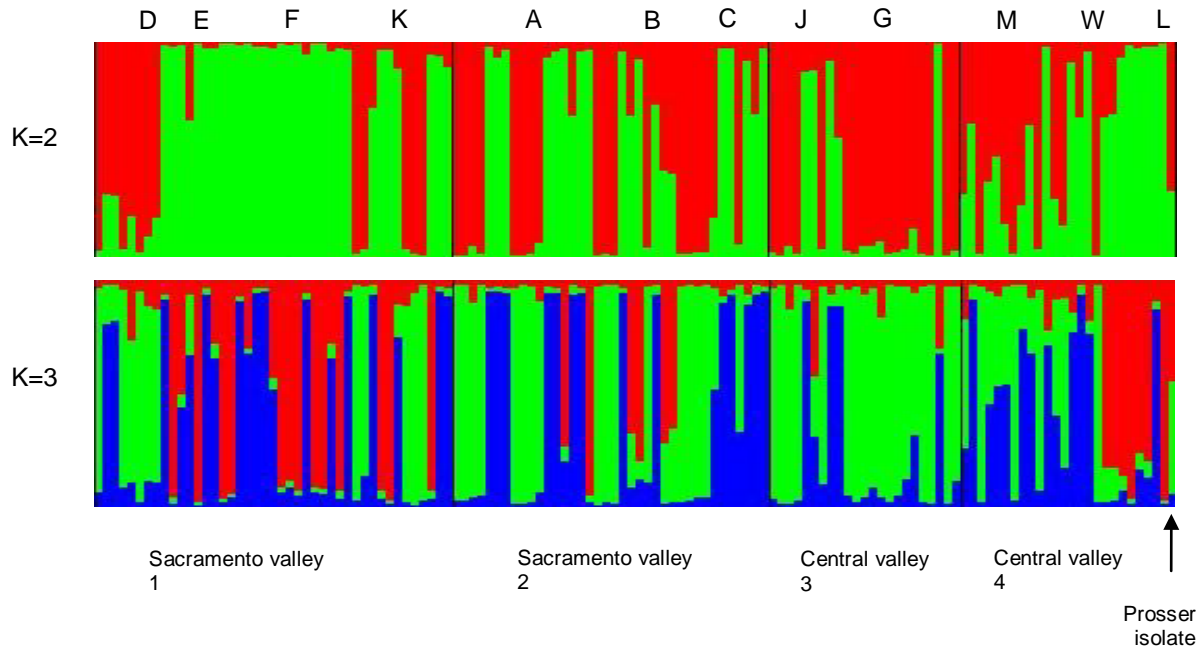


Fig. 4.6 STRUCTURE analyses of the *Sclerotinia trifoliorum* population with K= 2, and 3 using genotypes derived from seven microsatellite markers. The clustering is based on admixture model of population ancestry model and correlation between allele frequencies model done in five times runs of 500 000 iterations with a burn-in period of 100 000 iterations. Each vertical line represents an individual. Individuals were grouped into 3 regional groups and a fourth group in which the 2005 isolates were grouped together including the three alfalfa isolates (L), two from California and one from Prosser, WA. Colors represent the inferred ancestry from K ancestral populations. The clustering identified a value of K=3 as the optimal cluster size. The $\ln P(D)$ of the data is as follows, for K=1, $\ln P(D)=-1407.4$, K=2, $\ln P(D)=-1282.3$, for K=3, $\ln P(D)=-1256$ and, for K=4, $\ln P(D)=-1503.9$.

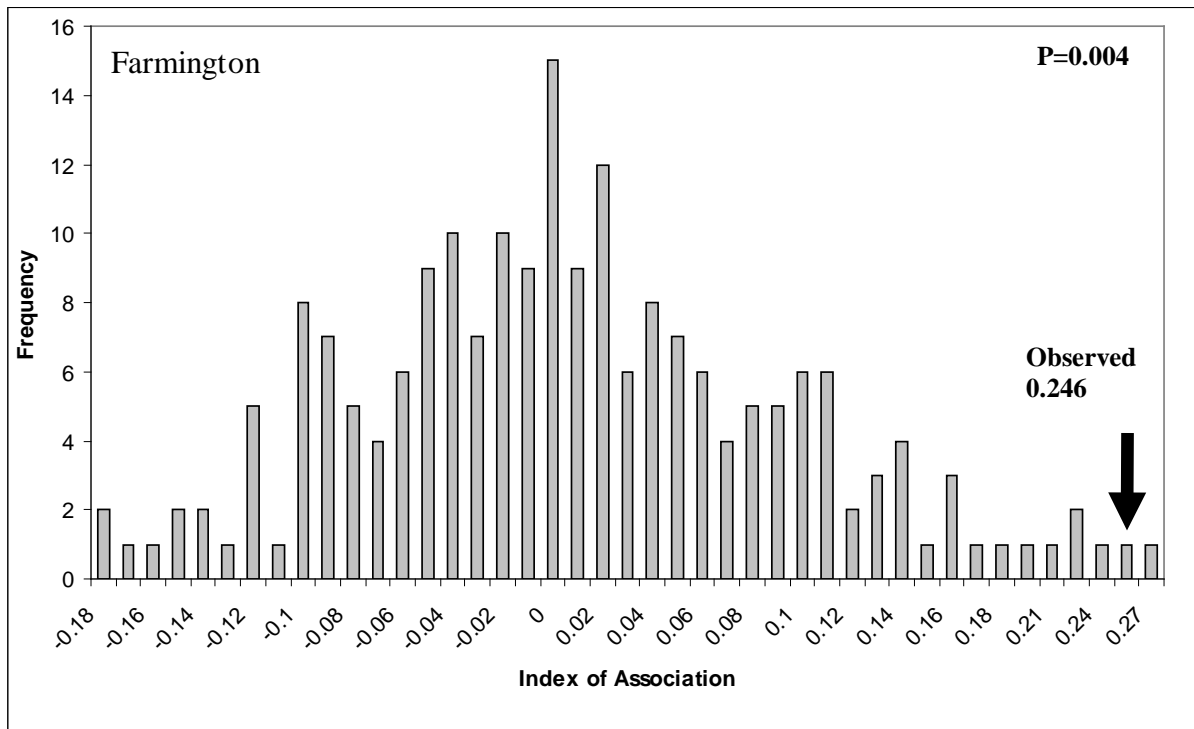
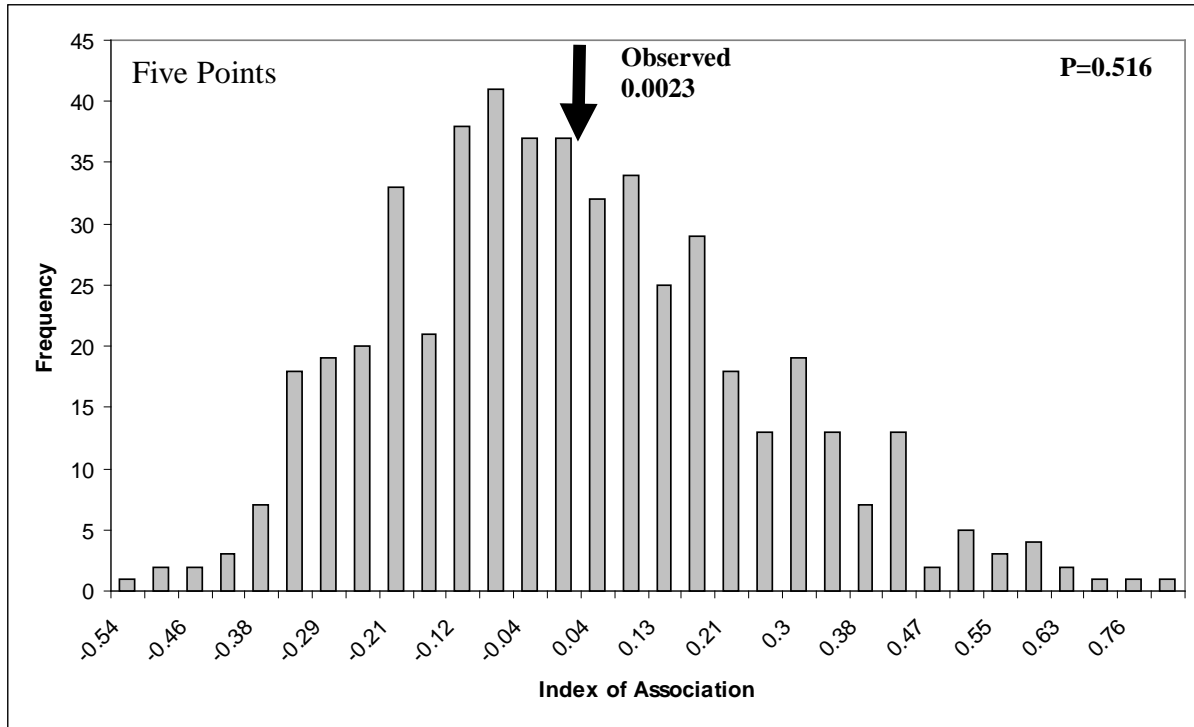


Fig. 4.7 Histograms representing the distribution range of simulated randomly recombining populations of *Sclerotinia trifoliorum* using the Index of Association (IA). In one of the three pooled populations, the IA of the observed data falls within that of the artificially randomized dataset (Five Point) indicating that we fail to reject random mating in this population while the other two populations were outside (example of Farmington) see also Table 4.8.

CHAPTER FIVE

APPLICATION OF STRAIN TYPING REVEALS MULTIPLE INDEPENDENT INFECTIONS WITHIN DISEASE FOCI OF CHICKPEA CROWN ROT CAUSED BY *SCLEROTINIA TRIFOLIORUM*

ABSTRACT

Sclerotinia trifoliorum causes crown rot of chickpea and other leguminous forage crops. The pathogen produces mycelial outgrowths on the soil surface from infected plants, and the disease is characterized by clearly defined disease foci. These two attributes in combination suggest possible secondary spread of the pathogen. In order to determine whether the pathogen is spreading from plant to plant during growing season (monocyclic versus polycyclic), isolates of *S. trifoliorum* were intensively sampled from a chickpea field near Five Points, California, in 2006. A total of 57 sclerotial isolates were obtained from 17 disease foci. Each isolate was obtained from a different diseased plant, and all sampled plants showed symptoms typical of crown infection. The isolates were genotyped based on mycelial compatibility groups (MCGs) and microsatellite haplotypes. Twenty-one MCGs were identified among the 57 isolates. The most numerous MCG contained 25 isolates and 14 MCGs consisted of single isolates. Thirty-five microsatellite haplotypes were identified. The most common haplotype contained seven isolates and there were 24 haplotypes of single isolates. Chi-square tests showed that the frequency of isolate pairs from adjacent plants with the same haplotype was not significantly higher than expected of random occurrence, although the frequency of the same MCG in isolate pairs from adjacent plants was significantly higher than expected because some MCGs contained multiple microsatellite haplotypes. The results suggest that plant-to-plant spread is rare and is therefore

not a major component of the epidemic of crown rot of chickpea. *Sclerotinia trifoliorum* therefore does not move efficiently in soil and is primarily a monocyclic pathogen.

INTRODUCTION

Plant pathogens are conceptually divided into monocyclic or polycyclic pathogens (Agrios 2005), with the latter having ability to produce secondary inoculum following primary infection within a growth season. Understanding such distinctions is essential for devising and implementing management strategies. Many foliar pathogens such as *Ascochyta rabiei*, causing Ascochyta blight of chickpea, or *Phytophthora infestans*, causing potato late blight, are polycyclic where secondary spread is important in disease progress (Tivoli and Banniza 2007; Mizubuti and Fry 2006). Control efforts are devoted to managing not only primary infection but also, often more importantly, secondary spread through repeated applications of fungicides during the growth season. With many of the soilborne vascular pathogens such as formae speciales of *Fusarium oxysporum* and *Verticillium* spp. (both of which are monocyclic), control efforts are focused on reducing primary infection through reduction of primary inoculum. For some other soilborne pathogens where clear disease foci (clusters of diseased plants) exist, the nature and extent of secondary spread is unknown. A case in point is the soilborne pathogen *Sclerotinia trifoliorum*, causing crown rot of chickpea.

Crown rot caused by *Sclerotinia trifoliorum* Erikss. is a serious disease of chickpea (*Cicer arietinum*) in California (Njambere 2008). The pathogen also infects other cool season forage legume crops such as alfalfa (*Medicago sativa* L.) red clover (*Trifolium pretense* L.), and white clover (*Trifolium repens* L.), as well as several other leguminous crops (Kohn 1979; Graham et al. 1979; Roupakias, 1983; Lithourgidis et al. 2005). Even though there are earlier

reports of *S. trifoliorum* isolated from chickpea seed lots in Australia, devastating outbreaks have only been reported in California, where the pathogen caused the 2005 and 2006 epidemics (Njambere 2008). During the epidemics, significant differences in susceptibility were observed among chickpea cultivars, suggesting interactions between chickpea genotypes and pathogen strains (Table 5.1).

The disease is favored by prolonged high humidity, frequent rains, and mild temperatures, which often occur during winter months in central California. *S. trifoliorum* survives during winter and during high summer temperatures as sclerotia in soil, embedded in diseased stems or adhering to plant surfaces. The sclerotia germinate during the cool wet weather to form mycelial strands, or they form apothecia in which asci and ascospores are produced. Ascospores are disseminated by wind to new hosts where they infect stems, flowers and leaves. In alfalfa, Nyvall (1999) reported that wind blown ascospores cause the primary infections and most of the subsequent crown and stem infection. However, even though mycelia are seen spreading between plants, mycelium only grows short distances in the soil and normally does not cause any infection (Nyvall 1999).

This raises the question of the role played by mycelium in the spatial spread of the disease in a chickpea fields as well. In epidemiological studies, plant to plant spread is often associated with mycelial spread, giving an aggregated (clustered) pattern of disease spread. Conversely, a random pattern of infected plants suggests that the disease is not spreading from plant to plant (Campbell and Madden 1990; Garret 2007; Madden et al. 1982). A nearest neighbor (doublet) sampling of infected plants followed by identification of the pathogen genotype would help to decipher the possible plant to plant spread.

Strain typing, the identification of individuals using their genotypes, provides a powerful tool for studying organisms that are difficult to observe or identify using conventional methods. The genotypes identified are permanent, enabling individuals to be followed throughout their lives, and provide a theoretically unambiguous means of identification (Palsboll 1999). Furthermore, the use of hypervariable microsatellite markers that are well distributed in eukaryote genomes and amplifiable from small amounts of DNA has made strain typing increasingly feasible. In fungal biology, determination of an individual's identity or 'DNA fingerprint' has a wide applicability in research, such as population diversity, levels of hybridization, or estimating the geographic range of a population. Despite its wide adoption in fungal biology, its full potential is yet to be realized. For instance, in epidemiological studies strain typing could in principle be combined with spatial data to study the spatial aspects of genotypes spread. This would help to elucidate how pathogen genotypes spread from plant to plant during the development of epidemics.

The pattern of genotype spread is an important factor that could explain variability in cultivar reaction in field disease screenings, and differential success achieved by various disease management strategies in stem and crown rot control. Most of the previous work related to spatial pattern of disease spread has focused on measuring the pattern and levels of disease spread by comparing infected plants to non-infected (Campbell and Madden 1990; Garret 2007; Madden et al. 1982). None of them have focused on strain identity to underscore the spatial distribution of various soilborne pathogen genotypes within a field.

Our research hypothesis was chickpea plants were infected independently by germinating sclerotia of *S. trifoliorum* and that there is no plant-to-plant spread. The objective of this study

was therefore to determine the pattern of in-field spread of *S. trifoliorum* on chickpea using genotypic identity based on MCG and highly discriminatory microsatellite haplotypes.

MATERIALS AND METHODS

Isolate collection and isolation. Isolates of *S. trifoliorum* were intensively sampled from three varieties (AWF1, AWF3 and Dylan) in agronomic trial plots near Five Points, California, in 2006. These three varieties were selected for sampling because they exhibited different levels of susceptibility or resistance to crown rot caused by *S. trifoliorum* (Table 5.1). No artificial inoculations were performed either in this experiment or in the cropping history of the field. All diseased plants were the result of crown infection. Within each disease focus, each diseased plant was sampled by extracting sclerotia from the crown region of the cultivars AWF1, AWF3 and Dylan. The cultivars were grown in a randomized complete block design plot with four replicates (Fig 5.1). A total of 57 sclerotial isolates were obtained from 17 disease foci (Table 5.2), and each isolate was obtained from a different diseased plant. Sampling was done at late pod filling stage of the chickpea plants. The isolates were brought to the laboratory for isolation. The sclerotia were first surface sterilized in 70% ethanol and then 3% sodium hypochlorite from commercial bleach for 1 min each. They then were rinsed in sterile distilled water, blotted dry on sterilized paper towels and then bisected before plating. Plates then were incubated at room temperature (22°C) in the dark. Isolates were purified by hyphal tip isolation and placed onto new PDA plates. Sclerotia were harvested from PDA plates, air dried, and either used for DNA isolation or stored in glass vials at –20°C for future use.

Mycelial compatibility grouping (MCG). Mycelial compatibility testing was performed following a modification of the method by Kohn et al. (1990). Isolates were paired in PDA

medium containing red food coloring and incubated at room temperature (22°C) in the dark. Pairings was via 7 mm diameter agar plugs with two isolates placed 2 cm apart in a 9 cm Petri plates. Pairings were examined with the naked eye 7 and 14 days after inoculation and scored as compatible when the two isolates merged to form one colony with no distinct interaction zone, or as incompatible when a red reaction line and discontinuity were observed between the two isolates. Isolates were paired in groups of tens, in all possible combinations, including self-self. Then representatives of each MCG were paired to determine all MCGs in the field. This process was repeated until all isolates were assigned to MCGs.

DNA Isolation. Total DNA was extracted from each isolate using the FastDNA® kit described by Chen et al. (1999), as modified by Njambere et al. (2008). DNA concentration was quantified using a NanoDrop spectrophotometer ND-1000 (NanoDrop products, Wilmington, DE, USA) and finally diluted to 10µg/µl before being used in PCR.

Microsatellite genotyping. Seven microsatellite loci which were at linkage equilibria in other populations (Chapter 3) were selected to represent the range of repeat motifs. The seven microsatellite loci were ST3-1, ST3-3, ST4-1, ST4-2, ST4-4, ST4-6A, ST4-20 (Table 3.1). PCR conditions used to amplify the DNA were the same as those described earlier in the dissertation (Chapter 3). PCR amplification of all the isolates was done using a M13 fluorescent labeling protocol (Schuelke 2000) where the universal M13 tail end is attached to the 5' end of the forward primer and an addition third primer, IR labeled universal M13 primer, included in the reaction. Analysis of fragment size was done using a LI-COR 4300 sequencer (LI-COR BIOSCIENCES, Li-COR Inc.) and alleles determined using the gene ImagIR 4.05 software (LI-COR, Inc.). Allele sizes of a locus in each isolate were confirmed in at least two independent amplifications. The size of the repetitive region of each allele was confirmed in selected isolates

through sequencing. Band size analysis and normalization of gel lanes were done as described in Chapter 3.

Data analyses. To test for the pattern of genotypes spread, microsatellite haplotypes were assembled using ARLEQUIN (Schneider et al. 2000) based on shared alleles across loci, whereas isolates were assigned to MCGs manually. Multilocus haplotypes and MCGs were mapped onto field locations where the isolates were obtained. The frequency of each genotype or MCG in the population was obtained and used to calculate the expected frequency of two random isolates being the same haplotype or MCG, using the formula for a haploid organism $\rho = \sum p^2$ where p is the frequency of a haplotype (Appendix 1). The value of p varied based on each multilocus haplotype frequency, whereas the ρ value remained the same in the whole field. ρ is the probability that two isolates drawn randomly from the population will have the same genotype. This is the expected frequency of sampling adjacent matching genotypes in a randomly disseminating population. To test whether diseased-plants were infected independently and randomly, Pearson's X^2 test was performed to determine whether the observed frequency of identical genotypes between adjacent plants was significantly different from the expected frequency of random occurrence. A significantly higher observed frequency would indicate plant-to-plant spread and possibly single sclerotia infecting more than one plant. Failure to reject the hypothesis would support the monocyclic nature of the pathogen and that plant to plant spread is rare.

RESULTS

Mycelial compatibility groups. All isolates were self-compatible. Out of the 57 isolates, 23 MCGs were identified. The largest MCG contained 25 isolates, and six additional MCGs had two or more isolates (ranged from 2 to 5 isolates). The remaining 14 MCGs had one isolate each (Fig. 5.2). Based on the frequencies of the MCGs in the population, the probability of having two random isolates belonging to the same MCG is 0.22. There were 39 pairs of isolates from adjacent plants, and twenty-three of the 39 pairs were of different MCGs (Appendix 2).

Microsatellite loci. Combinations of alleles at the seven microsatellites loci revealed 35 haplotypes among 55 isolates. The most common haplotype contained seven isolates, two other haplotypes had five isolates each, and eight haplotypes had two isolates each. The remaining 24 haplotypes were of single isolates (Fig. 5.2). The frequency of having two random isolates belonging to the same haplotype is 0.05 (Appendix 1). Among the 39 pairs of isolates from adjacent plants, thirty-six of them were of different microsatellite haplotypes (Appendix 2).

Plant-to-Plant spread. The basic question was whether the observed frequency of two isolates from adjacent plants being identical was significantly higher than expected by chance alone. When MCG was used as criterion, two isolates were considered to be identical if they were the same MCG. Likewise two isolates were considered identical if they were of the same microsatellite haplotype in the multilocus haplotype test. The Pearson's χ^2 test indicated that the observed frequency of two isolates from adjacent plants with the same MCG was significantly ($\chi^2 = 6.82$, $df = 1$, $P = 0.009$) greater than expected by random occurrence (Table 5.3). However, the observed frequency of two isolates from adjacent plants with the same microsatellite

haplotype was not significantly ($\chi^2 = 0.70$, $df = 1$, $P = 0.7913$) different from random expectation (Table 5.3).

DISCUSSION

Crown rot of chickpea caused by *S. trifoliorum* shows clear disease foci (clusters of diseased plants) in the field. In some cases outgrowth of mycelium on the soil surface from infected plants is clearly visible (Fig. 2.1) indicating the potential for spread from plant to plant. However, the nature and the extent of this spread are unknown. Because of the proximity of plants within rows and the frequent occurrence of clearly defined disease foci, it might be assumed that disease foci resulted from single primary infections. This study employed extensive and systematic sampling of diseased plants using highly discriminatory genotyping technique (microsatellite markers) in strain typing to test whether there is plant-to-plant spread. Chi-square tests showed that the frequency of isolate pairs from adjacent plants with the same haplotype was not significantly higher than expected by random occurrence, although the frequency of the same MCG in isolate pairs from adjacent plants was significantly higher than expected, suggesting that plant-to-plant spread is rare. Many of the disease foci with multiple diseased plants contained more than one MCG and all of the disease foci contained more than one microsatellite haplotype. In fact, majority of isolate pairs from adjacent plants were of different microsatellite haplotypes and MCGs. These results indicate that plant-to-plant spread is a limited mode of within season spread for *S. trifoliorum* and most of the diseased plants resulted from independent infections.

In traditional epidemiological studies involving spatial spread of a disease from one or more disease foci to another, emphasis is placed on measuring disease gradients and disease patterns without regard to strain types causing the infection. However, it is known that multiple

genotypes may be found in a single focus or even a single lesion (Macdonald and Martinez 1991). New infections may also be caused by exogenous inocula (Zhan et al. 2001). Our study shows that in many cases of *S. trifoliorum* crown infection, there are different independent infections in a disease focus. If secondary spread is an important component of the epidemic in a soilborne disease, most of the isolate pairs from adjacent plants should belong to the same haplotype or same MCG. The fact that majority of adjacent plant pairs were infected by different haplotypes (36/39) or different MCGs (23/39) demonstrate that secondary spread of the pathogen is limited and that *S. trifoliorum* is mainly a monocyclic pathogen. The proportion of infection through plant-to-plant spread is low, indicating that soil-borne mycelia grow for very short distances. This may imply that even though mycelial spread does occur, it is not important for rapid spread of disease outbreaks.

This study also shows that populations of *S. trifoliorum* in a single field are genetically heterogeneous with numerous MCGs/haplotypes occurring. There was also a strong correlation between MCG and microsatellite haplotypes. A strong significant correlation between two or more independent markers such as mycelial compatibility groups (MCGs) and microsatellites demonstrates the extent of clonal reproduction in a population (Auclair et al. 2004; Carbone et al. 1999; Hambleton et al. 2002; Kohn et al. 1991). Further, we showed that plant-to-plant spread is rare in *S. trifoliorum* and that it is mainly a monocyclic pathogen. These findings have implications for the control of the pathogen. Disease management strategies should aim at reducing primary inoculum as in managing other monocyclic pathogens. In addition, the information about the degree of heterogeneity (or its absence) of the inoculum and the primarily monocyclic nature of the pathogen will form the bases for developing plausible and testable hypotheses to account for the behavior of *S. trifoliorum* causing crown rot of chickpea.

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Table 5.1 Field disease incidence of 12 chickpea lines based on natural crown rot epidemics

Chickpea lines	Mean disease incidence (%, n=4)	Standard deviation
AWF1	90	6.1
AWF2	82.5	4.0
AWF3	17.5	13.0
BCC Sutter	12.5	5.6
BCC San Joaquin	7.5	2.5
1861W	7.5	2.5
0147W	60	30.0
AF46A	15	8.7
1869W	47.5	24.9
Dylan	22.5	8.3
HB-14	32.5	4.3
Sierra	16.25	13.8

Table 5.2 Field information for isolates of *S. trifoliorum* sampled from a chickpea field near Five Points, California showing the number of isolates sampled per foci and the variety from which they were collected

Focus	Sample size (number of diseased plants)	Variety
1	4	AWF1
2	5	AWF1
3	1	AWF1
4	4	AWF3
5	3	Dylan
6	3	Dylan
7	3	AWF3
8	4	AWF1
9	2	AWF1
10	1	AWF1
11	3	Dylan
12	3	AWF3
13	9	AWF1
14	1	AWF1
15	3	Dylan
16	5	AWF1
17	3	AWF3

Table 5.3 Chi-square analysis of observed frequencies of isolate-pairs from adjacent plants of the same microsatellite haplotypes or mycelial compatibility groups (MCG) against expected frequencies of the same haplotype or MCG drawn at random based on a single field

	Observed value		Expected		X ² value	P value
	Identical pairs	Non-identical pairs	Identical pairs	Non-identical pairs		
Microsatellite haplotypes	3	36	1.86	37.14	0.70	0.7913
MCG	16	23	8.42	30.58	6.823	0.0090*

*indicate significant values (p=0.05)

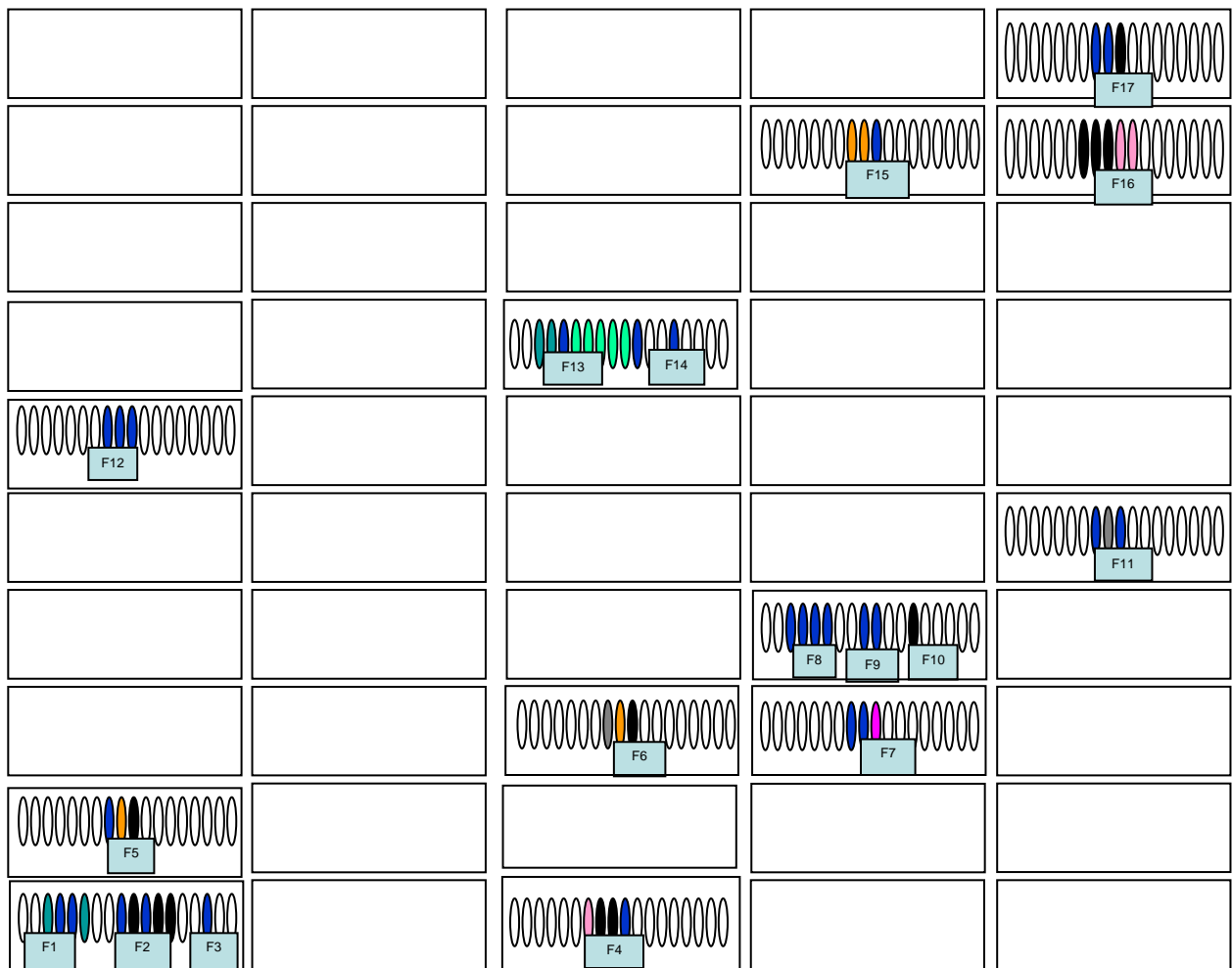


Fig. 5.1 Schematic intra-field distribution (not to scale) of *S. trifoliorum* based on mycelial compatibility groups (MCGs) in a chickpea field near Five Points, California. F1 to F17 refer to disease focus 1 to focus 17. An open oval represents a healthy plant; ovals with the same sample color are the same MCGs except black colored ovals for which each oval is a different MCG.

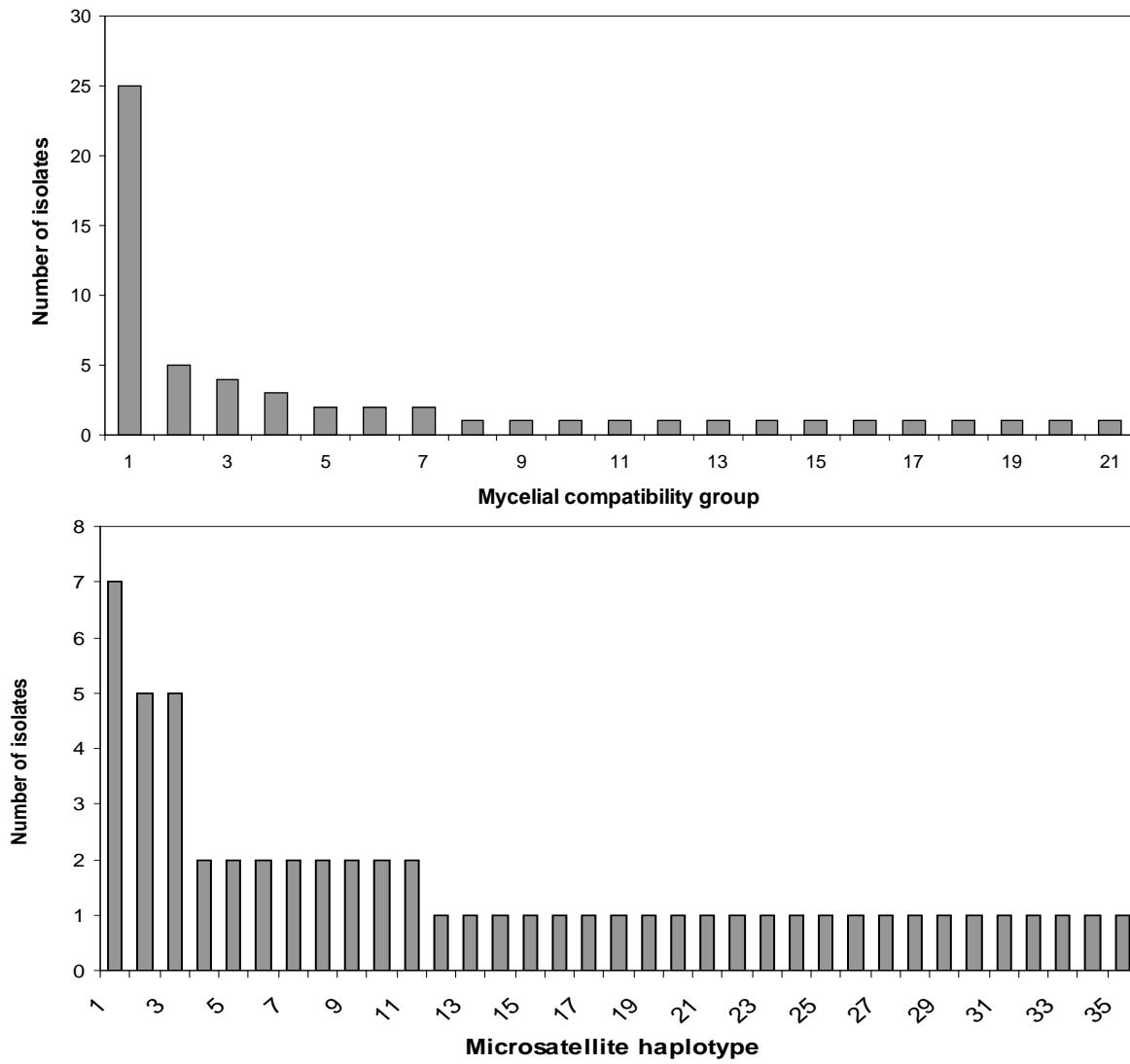


Fig. 5.2 Frequencies of mycelial compatibility groups and microsatellite haplotypes among 57 isolates of *Sclerotinia trifoliorum* collected from a single field in Five Points, California.

APPENDIX 1. Calculation of the probability that two genotypes picked at random will match based on microsatellite haplotypes and mycelial compatibility groups (MCG)

Haplotype	No. of isolates	Haplotype frequency	Probability adjacent haplotypes match	MCG	No. of isolates	MCG frequency	Probability adjacent MCG match
1	7	0.1228	0.0151	1	25	0.4386	0.1924
2	5	0.0877	0.0077	2	5	0.0877	0.0077
3	5	0.0877	0.0077	3	4	0.0702	0.0049
4	2	0.0351	0.0012	4	3	0.0526	0.0028
5	2	0.0351	0.0012	5	2	0.0351	0.0012
6	2	0.0351	0.0012	6	2	0.0351	0.0012
7	2	0.0351	0.0012	7	2	0.0351	0.0012
8	2	0.0351	0.0012	8	1	0.0175	0.0003
9	2	0.0351	0.0012	9	1	0.0175	0.0003
10	2	0.0351	0.0012	10	1	0.0175	0.0003
11	2	0.0351	0.0012	11	1	0.0175	0.0003
12	1	0.0175	0.0003	12	1	0.0175	0.0003
13	1	0.0175	0.0003	13	1	0.0175	0.0003
14	1	0.0175	0.0003	14	1	0.0175	0.0003
15	1	0.0175	0.0003	15	1	0.0175	0.0003
16	1	0.0175	0.0003	16	1	0.0175	0.0003
17	1	0.0175	0.0003	17	1	0.0175	0.0003
18	1	0.0175	0.0003	18	1	0.0175	0.0003
19	1	0.0175	0.0003	19	1	0.0175	0.0003
20	1	0.0175	0.0003	20	1	0.0175	0.0003
21	1	0.0175	0.0003	21	1	0.0175	0.0003
22	1	0.0175	0.0003	Total	57	1	0.2158 (exp)
23	1	0.0175	0.0003	Observed		16/39	0.41
24	1	0.0175	0.0003				
25	1	0.0175	0.0003				
26	1	0.0175	0.0003				
27	1	0.0175	0.0003				
28	1	0.0175	0.0003				
29	1	0.0175	0.0003				
30	1	0.0175	0.0003				
31	1	0.0175	0.0003				
32	1	0.0175	0.0003				
33	1	0.0175	0.0003				
34	1	0.0175	0.0003				
35	1	0.0175	0.0003				
Total	57	1	0.0477 (Expected)				
Observed		3/39	0.0769				

The expected value (shaded) is probability that two genotypes picked randomly will match.

