Identification of the Teleomorph *Mycosphaerella graminicola* in Manitoba by Direct Observation and Molecular Polymorphism

By

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FORWARD

This thesis is presented in the format of two manuscripts that include an abstract, introduction, methods and materials, results and discussion. Preceding the manuscripts are a general abstract, a general introduction and a review of the literature. The manuscripts are followed by a general discussion, a list of references cited and appendices.

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1. GENERAL ABSTRACT

Mycosphaerella graminicola (Fückel) J. Schröt. in Cohn causes speckled leaf blotch (septoria leaf blotch) in wheat, a major leaf-spotting disease found in many wheat-growing regions. The sexual structures of *M. graminicola* were identified in wheat stubble during the summer of 2001 in Carman, Manitoba. Ascospores were isolated from pseudothecia and formed conidial colonies similar to the pycnidiospores of *Septoria tritici* Roberge in Desmaz., the asexual state, on agar medium. Wheat plants inoculated with conidia derived from ascospore isolates developed symptoms typical of speckled leaf blotch. The pathogen was reisolated and budding conidial colonies identical to those produced by the germination of the ascospores were obtained. This represents the first time that the teleomorph *M. graminicola* was identified by direct observation in Canada. Amplified fragment length polymorphism (AFLP) was the molecular marker system used to indirectly identify the sexual state, and the objective was to detect polymorphism among *M. graminicola* isolates due to sexual recombination. Isolates from Manitoba field locations at Carman and Minnedosa were collected using a hierarchical procedure and were divided into two separate sets. Set I consisted of 44 isolates representing the two field locations and different stations within each of the fields. Set II was a control test, with 42 isolates representing single spores and their respective pycnidia picked from different leaves and lesions at one station in each of Carman and Minnedosa. Both Set I and II AFLP banding patterns showed polymorphism among isolates and no distinct clustering of populations was observed in the dendrograms. Analysis of molecular variance (AMOVA) of Set I revealed that 99.02% ($P<0.05$) of the total genetic diversity

was due to within population variation and 0.98% to between population variation. This uniform pattern of inoculum distribution within fields suggests that wind-blown ascospores are present in Manitoba and contribute significantly to speckled leaf blotch epidemics.

2. GENERAL INTRODUCTION

Mycosphaerella graminicola (Fückel) J. Schröt. in Cohn (anamorph, *Septoria tritici* Roberge in Desmaz.) is a fungal pathogen of worldwide importance (Van Ginkel and Scharen 1988). It incites speckled leaf blotch (also known as septoria leaf blotch), a foliar disease of wheat that causes significant yield losses in many wheat-growing regions (Eyal et al. 1985, Royle et al. 1986, Gilbert and Woods 2001). Severe damage occurs when the upper leaves of the crop are infected (King et al. 1983, Shaw and Royle 1989a).

Sanderson (1972) was the first to identify the ascogenous state of *M. graminicola* in New Zealand. Since then, the teleomorph was confirmed in many other wheat-producing regions. Only the anamorph, *S. tritici*, had been isolated from wheat fields in Manitoba (Gilbert et al. 1998), and no region of Canada has yet to report the sexual state using a direct method of observation.

Occurrence of the sexual state, *M. graminicola*, in Canada has been inferred based on indirect methods of observation. Different molecular tools were employed; Razavi and Hughes (2001) used microsatellites and McDonald et al. (1999) used restriction fragment length polymorphism (RFLP). A molecular marker system can detect differences in DNA sequences, or polymorphisms, among individuals. Identical DNA fingerprints result when isolates are clonal and unique genotypes occur when isolates are genetically diverse, due to sexual recombination (Kohli 1992).

In many wheat-growing regions, it is believed that air-borne ascospores are the primary inoculum initiating infection of wheat plants in the spring, whereas the rain-splashed pycnidiospores of the asexual state are believed to be the secondary inoculum

that continues disease development during the growing season (Shaw and Royle 1989b). A large amount of diversity was reported in populations of *M. graminicola*, suggesting that wind-dispersed ascospores spread evenly across a field (Razavi and Hughes 2001). Population studies showed that extensive genotypic diversity could be distributed on a small scale within a field and that the genetic structure of *M. graminicola* populations around the world is very similar (McDonald et al. 1995). The involvement of ascospores in the epidemiology of speckled leaf blotch has serious implications on the current methods of control, specifically crop rotations and development of genetic resistance to the pathogen (Hunter et al. 1999). Ascospores could blow in and infect a wheat crop even though good rotation practices were followed to reduce inoculum. Also, sexual recombination increases how rapidly a pathogen can evolve and resist control measures such as resistant cultivars (Schnieder et al. 2001).

To date, there are no reported studies on the sexual state *M. graminicola* in Manitoba. The objective of this study was to assess the presence of the sexual state *M. graminicola* in Manitoba, using direct observation and DNA polymorphism.

3. REVIEW OF THE LITERATURE

Mycosphaerella graminicola (Fückel) J. Schröt. in Cohn is the causal agent of speckled leaf blotch, a leaf-spotting disease capable of causing devastation on a global scale (Eyal et al. 1987). Severe infection of the flag and second leaf can cause serious yield losses (Shaw and Royle 1989a, Thomas et al. 1989) and wheat kernels can become shriveled and unsuitable for milling (Eyal et al. 1987).

Speckled leaf blotch is a relatively new leaf spotting disease of wheat in Manitoba and the other prairie provinces. In a 1990 wheat disease survey of Manitoba, speckled leaf blotch was by far the least prevalent foliar pathogen, occurring in only 1.3% of fields surveyed (Gilbert et al. 1991). Prevalence was determined by the number of fields in which the pathogen was detected. *Stagonospora nodorum* (Berk.) Castellani and E.G. Germano and *Septoria avenae* (Frank), the other pathogens of the speckled leaf blotch complex, occurred in 46.5% and 9.7% of fields, respectively. Tan spot (*Pyrenophora tritici-repentis* (Died.) Drechs.) and spot blotch (*Cochliobolus sativus* Ito & Kuribayashi Drechs. ex Dastur) were the most prevalent foliar leaf diseases in 1990, accounting for 83.9%, 72.3%, respectively. However, 1993 showed a dramatic increase of *M. graminicola* in Manitoba and by 1994, this pathogen became the most prevalent of all the foliar pathogens of hard spring wheat (Gilbert et al. 1998). *Pyrenophora tritici-repentis* remained the most prevalent in durum. *Mycosphaerella graminicola* was the most frequently isolated fungal pathogen in hard red spring wheat (68%) in 1994, while the closest competitor was 8-10% (Gilbert and Woods 2001). Frequency of isolation was obtained by summing the number of leaf pieces from which a pathogen grew. In the

following three years (1995-1997), the prevalence and frequency of isolation of *M. graminicola* remained the highest of all foliar pathogens of hard red spring wheat in the west (and for the most part in the east) but decreased overall. In 2001, the prevalence (67%) and frequency of isolations (27%) of speckled leaf blotch were lower (Gilbert et al. 2002) than in 2000 (93% and 48.7%, respectively) (Gilbert et al. 2001). Spot blotch now accounts for the highest prevalence and frequency of isolation in wheat fields (Gilbert et al. 2002).

3.0 The Pathogen

3.0.1 The Teleomorph

Mycosphaerella graminicola is part of the Class Ascomycetes and the Order Dothideales (Kempken and Küch 1998). This haploid fungus (Schnieder et al. 2001) was first linked with the imperfect state *Septoria tritici* by Sanderson in New Zealand (Sanderson 1972). Its fruiting body is a perithecioid pseudothecium (ascocarp) (Eyal et al. 1987), which typically measures 68-150 µm in diameter (Sivanesan 1990), dark brown, globose and immersed in dead leaf tissue (Sanderson 1972, 1976). *Mycosphaerella graminicola* ascospores are hyaline, 1-septate with one cell slightly longer and broader than the other, and approximately 9-16 x 2.5-4.5 µm in size (Sivanesan 1990). The ascus is thick-walled, bitunicate, 30-40 x 11-14 µm and typically contains eight ascospores (Sanderson 1972, 1976).

Mycosphaerella graminicola is a bipolar, heterothallic fungus and therefore requires two mating types to cross for meiotic recombination to occur and for the sexual structures (ascocarps, asci, ascospores) to develop (Wang and Szmidt 1998). To date, no

successful *in vitro* crosses have been made and Kema et al. (1996) was the only group able to generate *M. graminicola* ascospores *in planta*.

Shaw and Royle (1989b) demonstrated that ascospores can move at least several hundred meters and are probably capable of reaching distances of tens of kilometers (Boeger et al. 1993, Chen and McDonald 1996), which is in agreement with Scott et al. (1988) and Sanderson (1972) who suggested ascospores could be carried for many miles. Species dispersed by air-borne spores exhibit higher rates and distances of migration than species with only rain-splash dispersal of spores (Zhan et al.1998). Ascospores are liberated by changes in relative humidity, which could be rain, dew or fog while pycnidiospores are dependent on rain dispersal (Sanderson et al. 1985). The viability of ascospores was drastically reduced when exposed to maximum sunlight for one day (Brown et al. 1978), but remained viable for 1-2 weeks when stored in the shade.

Mycosphaerella graminicola ascospores are a source of primary inoculum wherever they occur (Eyal et al. 1987) and they provide a different genetic composition in each generation (Kema et al. 1996). The pycnidia of the asexual state and the pseudothecia of the sexual state look very similar. Consequently, pycnidiospores are often mistakenly thought of as the sole source of inoculum (Eyal et al. 1987). In many countries, the air-borne ascospores are considered to be the most important source of primary inoculum and the pycnidiospores maintain disease progression for the rest of the growing season (Boeger et al. 1993, Sanderson et al. 1985, Scott et al. 1988, Shaw and Royle 1989b, 1993, Schuh 1990a). It is necessary to know for certain which is responsible for primary inoculum in order to create effective approaches to disease

control (Shaw and Royle 1989b). The even distribution of the disease within crops (Shaw and Royle 1987), the random disease patterns as opposed to clumped (Schuh 1990a, 1990b) and the frequent occurrence of speckled leaf blotch in fields previously cropped to non-wheat species (King 1977) all point to an air-borne source of primary inoculum. Research outside Canada concluded that ascospores play a more important role in the epidemiology of speckled leaf blotch than previously believed (Shaw and Royle 1993, McDonald et al. 1995).

Recent studies by Kema et al. (1996) suggest an expanded role of *M. graminicola* ascospores in disease development. They confirmed that ascospores do not originate exclusively from overwintering stubble or volunteers but also from infected leaves of the current crop, suggesting that they can also serve as secondary inoculum. Two important implications can be drawn: i) *M. graminicola* is able to complete several sexual cycles per season (5 weeks to complete one sexual cycle under conducive conditions) and ii) disease progress does not depend only on rain-splashed *S. tritici* pycnidiospores alone (Kema et al. 1996). Hunter et al. (1999), in southwest England, confirmed that ascospores could be produced from ascocarps of the current crop but they reported that the development of the sexual cycle took longer (94-140 days). Research by Shaw (1999) suggested that ascocarps on old wheat leaves and stems are exhausted of ascospores by mid season, and to account for ascospore release over an extended period, an additional source of inoculum is probably provided by the growing crop.

The genetic structure of fungal populations is affected by the relative contributions of sexual and asexual reproduction to each generation (Chen and McDonald

1996). Populations that are largely asexual will display a high degree of clonality while random mating populations are expected to exhibit a high degree of genotypic diversity (Chen and McDonald 1996). McDonald et al. (1995, 1999) suggested that sexual reproduction has a more significant impact on the genetic structure and biology of *M. graminicola* populations than asexual reproduction. In addition, sexual recombination and ascocarp development occurring during the growing season would greatly affect the genetic variation in the population structure (Hunter et al. 1999) and it was suggested that ascospore release and their contribution to the genetic population may be much higher than realized (Kema et al. 1996). Sexual reproduction is the primary source of genetic variation in an organism and the amount of genetic variation maintained in a population indicates how rapidly a pathogen can evolve and resist control measures (Schnieder et al. 2001). The large population size of *M. graminicola* enables the appearance of new races with virulence to deployed resistance genes, (McDonald et al. 1999), as well as the generation of individuals with greater fitness (Wang and Szmidt 1998). The presence of the perfect state introduces new dimensions to wheat breeding for resistance, pathogen variability and the study of speckled leaf blotch epidemics (Nelson and Marshall 1990). Sexual recombination provides the potential for the appearance of unique isolates and new, virulent races, thus reducing the effectiveness of resistant wheat cultivars and fungicides (Hunter et al. 1999).

3.0.2 The Anamorph

Septoria tritici, the asexual state of the pathogen, is part of the Class Deuteromycetes (Fungi Imperfecti) and the Order Sphaeropsidales (Eyal et al. 1987).

Pycnidia are globose to elliptical, brown to black, immersed in the leaf tissue, and are arranged longitudinally between veins (Sutton and Waterston 1966). Pycnidia range in size from 80-180 μm in diameter and are the fruiting structures that contain the conidia (Sutton and Waterston 1966). Pycnidiospores (conidia) are colourless, typically curved, flexuous, obtuse at the base and gradually tapering to an acute apex. They are 3-5-septate, thin structures that measure 30-80 x 1.5-2 μm in size (Sivanesan 1990).

Hilu and Bever (1957) suggested that pycnidiospores can remain viable in the pycnidia on infested stubble for several months, while Wiese (1977) stated that pycnidiospore production can occur in undisturbed stubble for up to three years. Gough and Lee (1985) reported that pycnidiospores held in cirrhi are tolerant to dessication and are viable at low relative humidity. Pycnidia can act as overwintering structures and provide a source of primary inoculum for the next wheat crop. In Manitoba, pycnidiospores of the anamorph were thought to be the only source of primary inoculum to initiate disease development in spring as well as the secondary inoculum that contributes to further disease cycles during the growing season (Grieger 2001).

Asexual reproduction plays an important role within an area of a few centimeters (Chen and McDonald 1996) or square meters (Boeger et al. 1993). In a field setting, there is a fine-scaled mosaic of overlapping clusters of clones, resulting from splash dispersal of pycnidiospores (Boeger et al. 1993). Conidia released by rain-splash could become air-borne, as demonstrated by *Stagonospora nodorum* (Faulkner and Colhoun 1976) or debris could be blown by wind (Holmes and Colhoun 1975). Pycnidiospores that do become air-borne are deposited within short distances (Shaw and Royle 1989b)

and few spores could leave a field entirely (Brennan et al. 1985). Overall, *M. graminicola* ascospores are much better adapted to air-borne dispersal (Scott et al. 1988), as they are ejected forcefully out of their ascus.

3.1 The Host

Mycosphaerella graminicola infects both hexaploid bread wheat (*Triticum aestivum*) and tetraploid durum wheat (*T. durum*). Brokenshire (1975a) also reported a wide host range of other graminaceous and weed species that can act as alternative hosts and promote sporulation. These include cultivated crops such as triticale and rye (Jones and Clifford 1983), several grasses such as annual bluegrass (Ao and Griffiths 1976), foxtail barley, wall barley, tall oatgrass, squirreltail fescue (Brokenshire 1975a) and chickweed (King et al. 1983, Eyal et al. 1987).

There is some disagreement from one region to another as to which wheats are more resistant to speckled leaf blotch. Brokenshire (1976), Yechilevich-Auster et al. (1983) and Eyal (1981) reported that diploid and tetraploid wheat cultivars are more resistant to speckled leaf blotch than hexaploid wheat cultivars in England, Turkey and Israel, respectively. In Tunisia, bread wheats provide much more resistance to the local *S. tritici* population than *T. durum* (Eyal et al. 1987). In Manitoba, Gilbert and Woods (2001) reported higher levels of *M. graminicola* in hard red spring wheat than in durum, even though Grieger (2001) found that isolates originating from hexaploid wheat in Manitoba could infect both bread and durum wheats. Contrasting findings were reported in Australia, Uruguay and the U.S.A., where isolates secured from *T. aestivum* were generally avirulent on *T. durum*. This supports the findings of Kema et al. (1996), who

reported that pathogen isolates originating from bread wheat and durum wheat appeared to be adapted to their hosts, respectively. This indicated that specificity is an important characteristic of the pathosystem in some regions, possibly due to differences in the cultivars studied.

To breed new cultivars, a source of germplasm resistant to speckled leaf blotch can be found in wild *Triticum* species such as *T. monoccocum, T. boeoticum*, *T. turgidum dicoccoides* (wild emmer), *T. longissimum*, *T. speltoides* and *T. tauschii* and also *Agropyron elongatum* (Gough and Tuleen 1979). Eyal et al. (1985) suggested that wheat and triticale cultivars that display a high level of resistance to one region's virulent isolates should be considered as a source of germplasm in breeding for resistance in another region.

The fact that cultivars currently recommended for the Canadian prairies have little or no resistance to speckled leaf blotch adds to the epidemic problem (Chungu et al. 2001). There has been a lag in developing genetically resistant cultivars to speckled leaf blotch and this must be addressed in the near future to help minimize losses due to this disease (Eyal 1999). Resistant cultivars have already been developed for the other major leaf diseases such as rusts and powdery mildew, so speckled leaf blotch can take their place on the wheat leaves (Eyal 1999). Breeders must be aware that the pathogen population is not only highly diverse, but also discontinuous within one season and between seasons, probably with respect to virulence (Kema et al. 1996). Kema et al. (1996) suggests that the vast genotypic diversity of *M. graminicola* is due to a mixed population of genotypes with qualitative differences.

Under severe epidemics, plant structure is irrelevant to the pathogen, however, vertical distance between leaf layers becomes important in moderate to light epidemics (Eyal et al. 1987). In young seedlings, the distances between the first 3-4 leaves is similar in short and tall varieties. However, when plants mature, differences in height can produce significant differences in disease development. In taller wheat varieties, the distance between each leaf is greater toward the flag leaf compared to dwarf cultivars and the greater the distance, the more remote the chance of spread (Eyal 1971). However, inoculum transport from the lower leaves to the upper leaves may be a limiting factor in disease development because of the long latent period of this pathogen (approximately 3 weeks) (Lovell et al. 1997). Infection of the leaves must occur early for infection to become severe when a pathogen has a long latent period (Lovell et al. 1997).

3.2 Symptoms

Speckled leaf blotch symptoms are characterized by chlorotic lesions eventually turning into grayish, rectangular necrotic lesions (Eyal et al. 1987). The necrotic lesions usually contain numerous pycnidia. Necrotic symptoms develop on wheat leaves approximately 14-21 days after infection with *S. tritici* conidia (Eyal et al. 1987). Garcia and Marshall (1992) observed symptoms one week after inoculation with conidia, and pycnidia with pycnidiospores appeared on wheat leaves after two and a half weeks. By contrast, in the same experiment with ascospore infections, the earliest symptoms were seen after 2 weeks and pycnidia with pycnidiospores were observed three and a half weeks after inoculation. Time was needed for ascospores to produce a number of infective units comparable to those produced by conidia, because ascospore inoculum did

not initiate infection directly, but through conidial proliferation (Garcia and Marshall 1992).

One of the problems in studying the sexual state of speckled leaf blotch is that the symptoms that appear on the wheat leaves are identical to the symptoms that the asexual state would produce (Scott et al. 1988). In one experiment, the pathogenicity of several isolates, each from a single *M. graminicola* ascospore, was tested by spraying pots of different wheat varieties with a conidial suspension. Many leaves developed necrotic lesions with pycnidia, indistinguishable from the lesions produced by the *S. tritici* anamorph on naturally infected crops (Scott et al. 1988). Scott et al. (1988) made estimates of the percentage of fruiting bodies that contained *S. tritici* conidia and *M. graminicola* ascospores in winter wheat in England. Ascospore incidence rose in autumn and was at its peak during the winter months of December to January while conidia incidence declined to zero in December (Scott et al. 1998).

3.3 Epidemiology

Infected debris from previous wheat crops and volunteers are the source of primary inoculum (Brokenshire 1975a, Holmes and Colhoun 1975, Eyal et al. 1987, Shaw 1999) for this polycyclic disease. Alternative hosts probably support little sporulation (Brokenshire, 1975b) and the pathogen is rarely seed-borne (Brokenshire 1975c, King et al. 1983). Furthermore, Brokenshire (1975c) never demonstrated that infected seeds could give rise to infected seedlings. Seed transmission by *Stagonospora nodorum* is more of a concern (Cunfer and Johnson 1981, Griffiths and Ao 1976), however, the potential role of infected seed deserves additional research (Boeger et al.

1993).

Increased levels of straw on the field may induce higher levels of speckled leaf blotch (Eyal et al. 1987, Schuh 1990b). Farmers in New Zealand leave wheat plants as standing stubble and this cultural practice is considered to be the main factor for the development of the sexual fruiting bodies during favourable environmental conditions (Eyal et al. 1987). Since standing stubble is predominately dry and also dries out quickly after wet conditions, it is not subject to rapid breakdown by saprophytic organisms and can provide a much better physical structure for pseudothecia development and ascospore release (Eyal et al. 1987). This helps explain why New Zealand was one of the first countries to identify the teleomorph. Cultural practices that reduce residue, such as plowing, stubble-burning and crop rotation, should remove the major source of primary inoculum (Sanderson 1978). A contradictory finding by Gilbert and Woods (2001) reported that there were higher levels of *M. graminicola* in conventional till fields with less stubble than conservation till fields. This was probably due to an increased presence of inoculum in those areas surveyed, because equal levels of *M. graminicola* were found in southeastern Manitoba in both types of tillage.

Different variables affect symptom development, with temperature being the most important in determining the latent period (time from infection to sporulation) (Chungu et al. 2001). There seems to be a compensation effect between moisture and temperature in susceptible wheat varieties. When the moisture period is short, an increase in temperature may result in severe levels of disease and conversely, high levels of disease occur when low temperatures are complemented by long moist periods (Hess and Shaner

1985). *Mycosphaerella graminicola* populations cannot compensate for reduced or delayed infection when the uppermost leaves are young, probably because conditions become less suitable for the pathogen (hotter and drier) later in the summer (Shaw 1999). Eyal et al. (1987) suggested that asexual spores are the most likely source of primary inoculum when there is an absence of summer rains and high temperatures, conditions favourable to the development of the sexual state. Ascospores are more readily found during mild autumns and winters in Australia, the United States and Europe (Eyal et al. 1987). However, Shaw and Royle (1993) indicated that weather conditions required for infection are not very stringent and are regularly fulfilled during the early growth of winter crops.

Moisture is required for all stages of infection (Hooker 1957, Shaner and Finney 1976), however, there is some disagreement about the length of time moisture is needed to produce comparable levels of disease. Hess and Shaner (1985) reported that twenty-four hours of moisture is insufficient to cause any disease symptoms. Garcia and Marshall (1992) observed the same effect when ascospores were the source of inoculum. However, 24 hours of humidity was sufficient for conidia to infect and produce abundant pycnidia, quantitatively comparable to those obtained at 48 hours or more (Garcia and Marshall 1992). Hess and Shaner (1985) reported that pycnidiospores exposed to 72 hours of moisture caused the same level of infection as when they were exposed to more than 72 hours. However, 48 hours or less resulted in significantly less disease (Hess and Shaner 1985).

Speckled leaf blotch increased dramatically in Manitoba in the 1990s, and the

main reason was thought to be environmental change that favoured its development. There was an increase of precipitation from June to December in the 1990s compared to the previous 30 year average, and more specifically, July had more precipitation than June (Chungu et al. 2001, Gilbert and Woods 2001). Also, Manitoba was experiencing warmer summer daytime temperatures in these years, averaging between 22^{0}C and 25^{0}C (Chungu et al. 2001). Weber (1922) and Eyal et al. (1987) reported that temperatures between 22 - 24^{0}C and 20 - 25^{0}C were the most favourable for spore germination and ranged from a minimum of 2-3^{0}C to a maximum of 33-37^{0}C. A study by Gilbert and Woods (2001) supported these findings, as the isolation frequency of *M. graminicola* increased in sampling times with higher mean temperatures. The warmer temperatures favoured by this pathogen on spring wheat in Manitoba is in contrast to the lower temperatures favoured in the UK (Jenkins and Jones 1981) on winter wheat. Greenhouse experiments performed by Magboul et al. (1992) also demonstrated that the most successful infection occurred at cooler temperatures around 16 to 19^{0}C when leaves were wet for 48 h, and that temperatures above 25^{0}C suppressed pathogen growth. However, Magboul et al. (1992) also found that the temperature range at which infection occurs increases when the leaf wetness period is prolonged.

3.4 Disease Control

Developing resistant wheat varieties is the ideal method of control (Eyal 1999). However, the success of breeding projects depends on the genetic variability in the pathogen population and the genetic system through which they regulate their genetic structure and interact with the host (Wang and Szmidt 1998). Genetic flexibility allows

the fungus to adapt readily to changing environmental conditions, including new host genotypes. It is suggested that the *M. graminicola* - wheat pathosystem follows the gene-for-gene theory (Brading et al. 2002), and in a pathogen with a high level of sexual reproduction, such as *M. graminicola*, every sexual generation produces new combinations of virulence genes that can be selected by the corresponding host resistance genes (Chen and McDonald 1996). In addition, the population size is large enough to ensure ample mutations and rapid response to selection (mutation from avirulence to virulence) (McDonald et al. 1999). Since pyramiding resistance genes into common varieties will likely fail, Chen and McDonald (1996) recommend that plant breeders incorporate nonspecific resistance that is often inherited as a quantitative trait. Breeding programs have to consider the genetic structure of the pathogen population and the relative contributions of sexual and asexual reproduction.

Fungicides are still the most important method of controlling speckled leaf blotch, even in the United Kingdom where resistant cultivars are available (Hollomon et al. 1999). The correct fungicide must be applied at the optimum time (Eyal 1981) and timing is guided by growth stage rather than by any identification of disease risk (Lovell et al. 1997). Research conducted by Cook and Thomas (1990) demonstrated that an application of a systemic fungicide (triazole) at flag leaf emergence almost always gave a profitable yield response and was more effective than earlier or later sprays. Cook (1999) recommended spraying the wheat crop when the third leaf was emerged, regardless of sowing date, and that inoculum should be controlled during and after stem extension, but not before. However, the continuous development of the sexual phase, especially during

the growing season, increases opportunities for the pathogen to respond quickly to selection pressures from fungicides (Hunter et al. 1999).

Cultural control methods, such as crop rotation and elimination of stubble, are other methods used to reduce the incidence of speckled leaf blotch. Since *M. graminicola* is a stubble-borne pathogen, tillage is likely to reduce the source of primary inoculum from infected fields (Brokenshire 1975a).

3.5 DNA Fingerprinting

Studies of any organism with mixed modes of reproduction rely on the ability to unambiguously identify sexually produced individuals and asexually produced clones (van der Werf 2002). Molecular markers can detect a large number of polymorphic loci that represent sites where differences in DNA sequences occur between individuals (van der Werf 2002), and this set of markers is known as a fingerprint. Molecular markers, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites, have become essential tools used to characterize fungal pathogens (McDonald et al. 1995, Kema et al. 1996, Czembor and Arseniuk 1999) and for plant taxonomy (Czembor and Arseniuk 1996, Menkir et al. 1997). They can identify particular races and pathotypes and are not subject to selection pressure (Schnieder et al. 2001). Considerations such as the extent of genetic polymorphism needed and the time and cost required for each technique help to determine which molecular marker tool is the most appropriate (Parker et al. 1998).

For *M. graminicola*, DNA markers that assay genotypic variation directly have

made it possible to measure the relative impact of sexual and asexual reproduction. Random mating (sexual) populations display a high degree of genotypic diversity while populations that are largely asexual will have a high degree of clonality, with a few genotypes present at high frequencies (Chen and McDonald 1996). Strains are considered to belong to the same clone if they have identical DNA (Kohli et al. 1992, Chen and McDonald 1996) and clonal variability within and among field populations isolated from wheat leaves can be determined by DNA fingerprinting with a high degree of certainty (McDonald and Martinez 1991, Goodwin et al. 1992). If the physical structures containing ascospores (pseudothecia) cannot be found, analysis of DNA polymorphism of isolates collected from commercial fields can yield valuable information about pathogen populations and taxonomy (Mills et al. 1992).

Razavi and Hughes (2001) inferred that the sexual state *M. graminicola* exists in Canada by using microsatellites to detect polymorphism among isolates. McDonald et al. (1999) used RFLP to analyze populations of *M. graminicola* from several countries, including Canada. They demonstrated that the genetic structure of *M. graminicola* is similar around the world and that the populations had a high degree of genetic stability over time. Genetic structure refers to the amount and distribution of genetic variation within and between populations (Schnieder et al. 2001), and will be affected by the relative contribution of sexual and asexual reproduction in each generation. A field experiment was conducted by Zhan et al. (1998) to determine the relative contributions of immigration and sexual reproduction to the genetic structure of *M. graminicola* populations during the course of an epidemic. Control plots naturally infected by

ascospores were compared to artificially inoculated plots. RFLP loci of all the isolates in the control plots had unique genotypes and were at gametic equilibrium, which is consistent with random mating. The proportion of isolates in the inoculated plots that differed from the inoculated isolates was 3% in the early season and 34% in the late season, demonstrating that the proportion of infections caused by ascospores increased over the growing season. Immigrants (10%) and sexual recombinants (24%) accounted for the 34% of unique genotypes, with 66% of the remaining isolates being asexual progeny of the inoculated isolates (Zhan et al. 1998).

The genetic variation of *M. graminicola* populations is vast (Schnieder et al. 2001). For example, in a collection of 711 isolates from one field in Oregon, there were 654 different genotypes (Chen et al. 1994) and in another collection of 673 isolates, there were 617 different DNA fingerprints (Chen and McDonald 1996). Chen and McDonald (1996) reported that the isolates originated from random mating and that genetic recombination must occur frequently and play a major role in the genetic structure of *M. graminicola* populations. Furthermore, McDonald et al. (1995) reported that when isolates of the same genotype were identified, they were almost always clustered in the same location. Nearly every lesion on the same leaf had a different genotype, and moreover, different genotypes were present in the same lesion in 25% of the lesions assayed (McDonald et al. 1995), indicating co-infection by two or more isolates (Schnieder et al. 2001, McDonald et al. 1995).

When comparing the Oregon and California populations separated by 750 kilometers, McDonald et al. (1995) noted that the most common alleles at each RFLP

locus were present at similar frequencies and overall levels of gene diversity were nearly identical. Also, no genotypes were shared between populations, implying that the similarity was not due to movement of particular clones. The simplest interpretation of uniting two geographically separate *M. graminicola* populations into a single genetic population of nearly identical genetic structures was gene flow between wheat fields (Boeger et al. 1993, McDonald et al. 1995). Pathogens that can be dispersed over long distances display greater genetic uniformity across local populations than pathogens which are not dispersed over long distances (Schnieder et al. 2001, Razavi and Hughes 2001). Boeger et al. (1993) reported that the movement of one individual per generation is adequate to prevent populations from diverging significantly by genetic drift. However, to account for the high degree of genetic similarity between the Oregon and California *M. graminicola* populations, at least 12 individuals per generation would have had to move this distance, which is more than sufficient to make these geographically separated populations a co-evolving unit. Since *M. graminicola* can infect many common grass species, alternative hosts likely play a major role in ascospore movement from field to field of a few hundred kilometers (Boeger et al. 1993, McDonald et al. 1995). However, another possible theory for long distance travel of *M. graminicola* is seed transmission (Boeger et al. 1993). Even though seed transmission appears to be low, movement of one or two individuals would make an impact (Boeger et al. 1993).

To a much larger extent than just 750 km separations, *M. graminicola* populations around the world have a high degree of genetic similarity and very similar genetic structure. The most likely mechanism for gene flow is through infected seed or some

other human transport (McDonald et al. 1995, 1999). The high gene and genotype diversity found in most populations around the world suggest that population sizes are large and genetic drift effects are negligible (McDonald et al. 1999). Large population sizes ensure population stability over time and facilitate the occurrence of many mutations, allowing for rapid selection response to resistant cultivars and fungicides. McDonald et al. (1999) suggested that new resistant wheat varieties and fungicides be screened against hundreds of strains of isolates due to the huge diversity for virulence in natural populations. Due to the limited dispersal distance of conidia, no one particular isolate will reach a high frequency. The genes in the fittest individuals will persist and be recombined to create novel genotypes each season and selection will change the frequency of genes that affect adaptation to the wheat host (McDonald et al. 1999).

When analyzing different conidia from the same pycnidium, McDonald et al. (1995) and Schnieder et al. (2001) reported identical genotypes. In contrast, Czembor and Arseniuk (1999) discovered genetic variation when analyzing conidia originating from the same pycnidium. They suggested that sexual recombination is not the only cause of different genotypes and that the asexual reproduction of *S. tritici* may contribute more as a possible source of genetic variability among populations of the pathogen than realized. Czembor and Arseniuk (1999) hypothesize that heterokaryosis resulted from a high mutation rate of microsatellites and transposon activity. Heterokaryosis results from mutation in a homokaryon or hyphal fusions (anastomoses) between genetically different individuals that enables the exchange of genetic information (Czembor and Arseniuk 1999). Newton et al. (1998) noticed there was genetic variation among *S. nodorum*

monopycnidiospores discharged from a single pycnidium, however, the pycnidia were not uninucleate in origin. Griffiths and Ao (1980) reported large differences in cultural characteristics and pathogenicity among *S. nodorum* monopycnidiospore isolates derived from the same pycnidium. Overall, exchange of genetic information via anastomosis is limited due to vegetative incompatibility and the parasexual cycle appears to be non-significant in the generation of recombinant nuclear genotypes (Newton et al. 1998).

3.5.1 Random Amplified Polymorphic DNA (RAPD)

RAPD analysis is a useful genetic tool to study fungal isolates. They are used to detect random nucleotide sequence polymorphisms in a DNA amplification-based assay using only a single, short primer. The primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. If the sites are close enough, this region is amplified and a discrete DNA product is produced through thermocyclic amplification (Williams et al. 1990). Polymorphisms between individuals are detected as differences between the pattern of DNA fragments amplified using a given primer (Micheli et al. 1994). RAPDs are relatively low cost (Raffle and Hsiang 1998) and simple to perform (Tingey and del Tufo 1993, Raffle and Hsiang 1998). Analyses are rapid, using small amounts of DNA (O'Neill et al. 1997, Micheli et al. 1994), and therefore, desirable to use when working with a large number of isolates (Tingey and del Tufo, 1993). RAPDs have proven beneficial in examining the genetic variation of many fungi, including gramineous crop pathogens, *S. tritici* (Czembor and Arseniuk 1996), *Pyrenophora tritici-repentis* (Aung 2001), *Fusarium graminearum* (Walker et al. 2001), *F. oxysporum* (Grajal-Martin et al. 1993, Kelly et al. 1994) and *Puccinia recondita* (Kolmer et al. 1995).

However, there are many criticisms of RAPD analysis as a genetic tool, such as inconsistent reproducibility (Staub et al. 1996), lack of positional homology (Van de Zande and Bijlsma 1995) and preferential amplification of DNA fragments that can mask relatedness between populations (Wilkerson et al. 1993). RAPDs are sensitive to differences in experimental conditions, and DNA preparation from one time to the next can influence primer annealing and consequently reproducible amplification (Smith et al. 1994).

3.5.2 Restriction Fragment Length Polymorphism (RFLP)

RFLP markers detect polymorphism through restriction enzyme digestions and probe hybridizations (McDonald et al. 1995). Restriction enzymes cut DNA wherever they find the appropriate nucleotide sequence (recognition sequence) and if there is a mutation at this sequence, no cleavage occurs and the resulting DNA fragment is longer. Conversely, a mutation can give rise to a new recognition sequence between pre-existing sites, and on digestion, result in a pair of shorter fragments. Genetic differences are therefore differences in the fragment length, as the name of this molecular marker implies, and are detected by Southern hybridization of a radio-labeled probe (van der Werf 2002).

RFLPs have been a popular method of genetic analysis because they give highly reproducible results (Schnieder et al. 2001) and are codominant markers (Wang and Szmidt 1998). However, there are some major disadvantages. RFLPs are labour and time intensive (Tingey and del Tufo 1993) and in particular, the blotting and hybridization steps are tedious (Schnieder et al. 2001). If there are no known probes, they

must be generated by creating clone libraries and screening for suitable inserts. Other important limitations are the difficulty in standardization, the variation in sensitivity, the high amount of high quality DNA required, the relatively small numbers of polymorphisms generated (O'Neill et al. 1997) and the use of radioisotopes (Czembor and Arseniuk 1996).

3.5.3 Amplified Fragment Length Polymorphism (AFLP)

Another PCR-based technique for analyzing genomic DNA is based on restriction fragment length polymorphism. AFLPs are essentially a combination of RAPDs and RFLPs. Genomic DNA is digested with two restriction endonucleases and end specific adapters are added to the fragments. Sequence specific primers, containing selective nucleotides homologous to the adapters, are added and the fragments are then amplified by PCR and separated on a polyacrylamide gel (O'Neill et al. 1997).

There are many advantages to using the AFLP approach. Abundant markers are generated from small quantities of DNA (O'Neill et al. 1997), 10-100 times greater than with microsatellites or RAPD (van der Werf 2002). AFLP fingerprinting is not sensitive to variation of reaction mixtures such as template concentration (Schnieder et al. 2001, Vos et al. 1995). Also, the primers used in AFLP are long and are homologous to the adapter and restriction site sequence, avoiding non-specific annealing. Therefore, they are more reliable than the non-specific primers used in RAPD analyses. It is a very complex technique and every step must be successful, however, this improves reproducibility and reliability (O'Neill et al. 1997, Schnieder et al. 2001). Reagents and equipment are costly, but not as expensive as for RFLPs. Like RAPDs, AFLP analyses

require no prior knowledge of DNA sequence of the species under study and are less expensive and easier to develop than microsatellites.

3.5.4 Microsatellites or Simple Sequence Repeats (SSR)

Microsatellites, or simple sequence repeats (SSR), are ideal DNA markers for genetic mapping and population studies because of their abundance and high polymorphism between individuals within populations (Cordeiro et al. 1999). Microsatellites are DNA regions with variable numbers of short tandem repeats flanked by a unique sequence. They make good genetic markers because each DNA region can have many different alleles (many different lengths of the repeat region). Through the PCR reaction, which uses the unique sequences on either side of the repeat sequences as primer binding sites, microsatellite DNA can be specifically amplified. The alleles that an individual carries at a particular locus can be determined by noting the size of the amplified fragment through agarose gel electrophoresis. As mentioned previously, microsatellites were used by Razavi and Hughes (2001) to study *M. graminicola* isolates and to provide evidence that the sexual state of this pathogen exists in Saskatchewan.

4. RESULTS OF RESEARCH

4.0 First report of *Mycosphaerella graminicola*, the sexual state of *Septoria tritici*, in Manitoba, Canada

4.0.0 Abstract

Mycosphaerella graminicola, the sexual state of the pathogen that causes speckled leaf blotch in wheat, was found in wheat stubble during the summer of 2001 in south central Manitoba. This is the first time that the teleomorph of the pathogen has been identified by direct observation in Canada. Ascospores isolated from pseudothecia formed similar conidial colonies on agar medium as the pycnidiospores of *Septoria tritici*, the asexual state. Wheat plants inoculated with conidia derived from ascospore isolates developed symptoms typical of speckled leaf blotch. Single cirrhi were picked from the necrotic lesions in order to reisolate the pathogen. Budding conidial colonies identical to those produced by the germination of the ascospores were obtained, proving that the pseudothecia found in the wheat stubble were those of *M. graminicola*. Therefore, it can be assumed that *M. graminicola* ascospores contribute to the primary inoculum of speckled leaf blotch in Manitoba. The presence of the sexual state in Canada has been anticipated and is consistent with reports from other regions of the world.

4.0.1 Introduction

Speckled leaf blotch, caused by the fungal pathogen *Mycosphaerella graminicola* (Fückel) J. Schröt. in Cohn (anamorph, *Septoria tritici* Roberge in Desmaz.), is an important foliar disease in many wheat-growing regions and is capable of reducing grain yields by as much as 40% (Brown and Rosielle 1980). This disease is also known as septoria leaf blotch.

Speckled leaf blotch is gaining in importance in western Canada, especially in Manitoba, where summer daytime temperatures average between 22°C and 25°C and above-average rainfall has been occurring in recent summers (Chungu et al. 2001). According to a recent wheat disease survey, incidence of speckled leaf blotch increased from less than 2% of fields in 1989 to 93% of fields in 2000, when it accounted for more than 48% of isolations of pathogenic fungi from foliar lesions (Gilbert et al. 1991, 2001). In 1993, the increasing prevalence of this disease was mainly attributed to higher rainfall in combination with conservation tillage practices (Gilbert et al. 1994). However, more recently, there was evidence that the disease levels are higher in fields with conventional till rather than conservation till (Gilbert and Woods 2001).

Only the anamorph has been isolated from wheat fields in Manitoba (Gilbert et al. 1998), therefore clonal populations resulting from asexual reproduction (pycnidiospores) were thought to be the most prevalent means of pathogen increase (Grieger 2001). In many countries, ascospores are considered to be the most important source of primary inoculum and pycnidiospores maintain disease progression for the rest of the growing season (Scott et al. 1988; Shaw and Royle 1989b). Sanderson (1978) established the

relationship between the teleomorph and anamorph of the pathogen and suggested that the initial speckled leaf blotch infections in crops in New Zealand were from ascospores produced on stubble. The perfect state plays an important role in the epidemiology of speckled leaf blotch because wind-borne ascospores are capable of initiating infection in distant crops (Sanderson 1978).

The ascogenous state of *S. tritici* has been reported from most wheat producing regions. Sanderson (1972) was the first to identify *M. graminicola* in New Zealand in 1972 and link it to *S. tritici.* The teleomorph has since been reported in several regions, including Australia (Brown 1975), the United Kingdom (Scott et al. 1988), the United States (Garcia and Marshall 1992) and the Netherlands (Kema et al. 1996). In Canada, the teleomorph *M. graminicola* has been inferred from DNA polymorphism studies (McDonald et al. 1999, Razavi and Hughes 2001). In this report, we provide direct evidence of the occurrence of the teleomorph *M. graminicola* in Manitoba.

4.0.2 Materials and Methods

Stubble sampling

The source of stubble used in this study originated from a field in Carman, Manitoba, with seven years of wheat monoculture. The disease developed every year from naturally occurring inoculum. One-and two-year-old stubble was collected weekly from May to August, 2001. The samples were surface sterilized using a 0.13% sodium hypochlorite solution for one minute and cut into 3-cm pieces. Six pieces were placed on wet filter paper in each of eight glass Petri dishes, with two or three stubble pieces yielding *M. graminicola* pseudothecia a week.

After one week of rehydration, ascocarps resembling those of *M. graminicola* were picked off the straw with a needle, mounted in distilled water on a microscope slide and pressed open. The slide was then examined under a Zeiss compound microscope (Zeiss, Oberkochen, Germany) to identify *M. graminicola* asci and ascospores. Measurements of ten asci and ascospores were taken for each pseudothecium identified.

Isolation of *M. graminicola* ascospores

Once a pseudothecium was confirmed to be that of *M. graminicola*, ascospores were scraped off the slide with a sterile needle and streaked across a Petri dish containing Yeast Malt agar and 0.25% chloramphenicol (Appendix 1a) (YMA^+) (Sigma, St. Louis, MO). Single ascospores were cut out and transferred to a new YMA^+ plate, sealed with parafilm tape and grown in the dark at 22^0C for four days. The growing colony was then spread into a larger area on the plate and left for three more days. At this time, spores were transferred and spread onto new YMA^+ plates using a wire loop, the plates were

then sealed with parafilm and placed under continuous, fluorescent light for one week to increase inoculum for pathogenicity tests. A modified method described by Kema et al. (1996) was also used for ascospore isolation. A piece of wheat straw bearing ascocarps resembling those of *M. graminicola* was placed in the lid of a Petri dish lined with wet filter paper. The Petri dish was turned upside down so the lid containing the straw piece was at the bottom. The dish containing YMA^+ was placed over the lid and rotated every 15 minutes for 1 hour, then covered with a new sterile lid and sealed with parafilm. This method assumes ascospores will be forcibly ejected from the asci and stick to the agar lid. The ascospores were allowed to germinate overnight in an incubator (20^0C). They were then individually transferred to YMA^+ plates and grown as described above. The plates were observed for the development of colonies resembling those of *S. tritici*.

Wheat lines used in pathogenicity tests

A differential set consisting of two bread wheat (Erik, Salamouni) and two durum wheat genotypes (Coulter, 4B1149), with known reactions to *S. tritici* (Grieger 2001) was used in this experiment. Erik/4B1149 and Salamouni/Coulter are known to be, respectively, highly susceptible and near-immune to all tested isolates from Manitoba and Saskatchewan. Six seeds of each wheat line were planted in 10-cm-diameter clay pots, with two wheat lines sown as two separate clumps in each pot. A 2:1:1 mixture of soil, sand and peat (v:v) was used. The seedlings were grown in a greenhouse at 22°C and a 16-hour photoperiod (181 $\mu E.m^{-2}s^{-1}$) for three weeks and watered as needed.

Inoculum production and inoculation

Four conidial cultures derived from single ascospores were used as a source of

inoculum for this study. Conidia from these isolates and from isolate 96-36, originally obtained from a sporulating pycnidium (Grieger 2001), were harvested from YMA^+ plates containing 7-day-old cultures by pouring approximately 10 ml of sterile distilled water into each plate and suspending the spores using a wire loop. The suspension was then filtered through two layers of cheesecloth and the spore concentration measured with a hemacytometer and adjusted to10^7 pycnidiospores/ml by addition of sterile, distilled water. Two drops of the surfactant Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma) were added per 100 ml of suspension. This suspension was sprayed onto 3-week-old seedlings until runoff, using a deVilbis-type sprayer.

After inoculation, the plants were placed in a misting chamber and kept under continuous leaf wetness for 72 hours. In the misting chamber, barriers were set up between the wheat differential lines inoculated with different isolates to prevent cross-contamination. The plants were then transferred to a growth room maintained at the same temperature and light conditions as the misting chamber. The plants were watered daily and additional water was poured under the bench to maintain a high level of humidity. Plants were scored for disease 21 days after inoculation. Symptomless leaves or those with minute necrotic or chlorotic flecks were rated as resistant while the presence of necrosis and pycnidia on the leaves indicated susceptibility.

Reisolation of the pathogen

Leaves with necrotic lesions and abundant pycnidia were selected to reisolate the pathogen from plants inoculated in the tests described above. Leaf segments (2- to 3- cm) were placed on wet filter paper inside a sealed glass Petri dish and incubated under

continuous fluorescent light overnight. Single cirrhi were transferred to microscope slides and observed under a compound microscope or transferred to YMA^{+} plates and incubated.

4.0.3 Results

Pseudothecia, asci and ascospores of *M. graminicola*

Pseudothecia resembling those described by Sanderson (1972) for *M. graminicola* were found on leaf sheaths (Fig. 1A) of wheat straw collected on a weekly basis from June 7 to August 22, 2001. There was no difference related to age of stubble in detection of *M. graminicola* ascocarps. Also, *M. graminicola* pseudothecia were found on approximately two to three pieces of stubble each week from June to August, regardless of speckled leaf blotch development in the field.

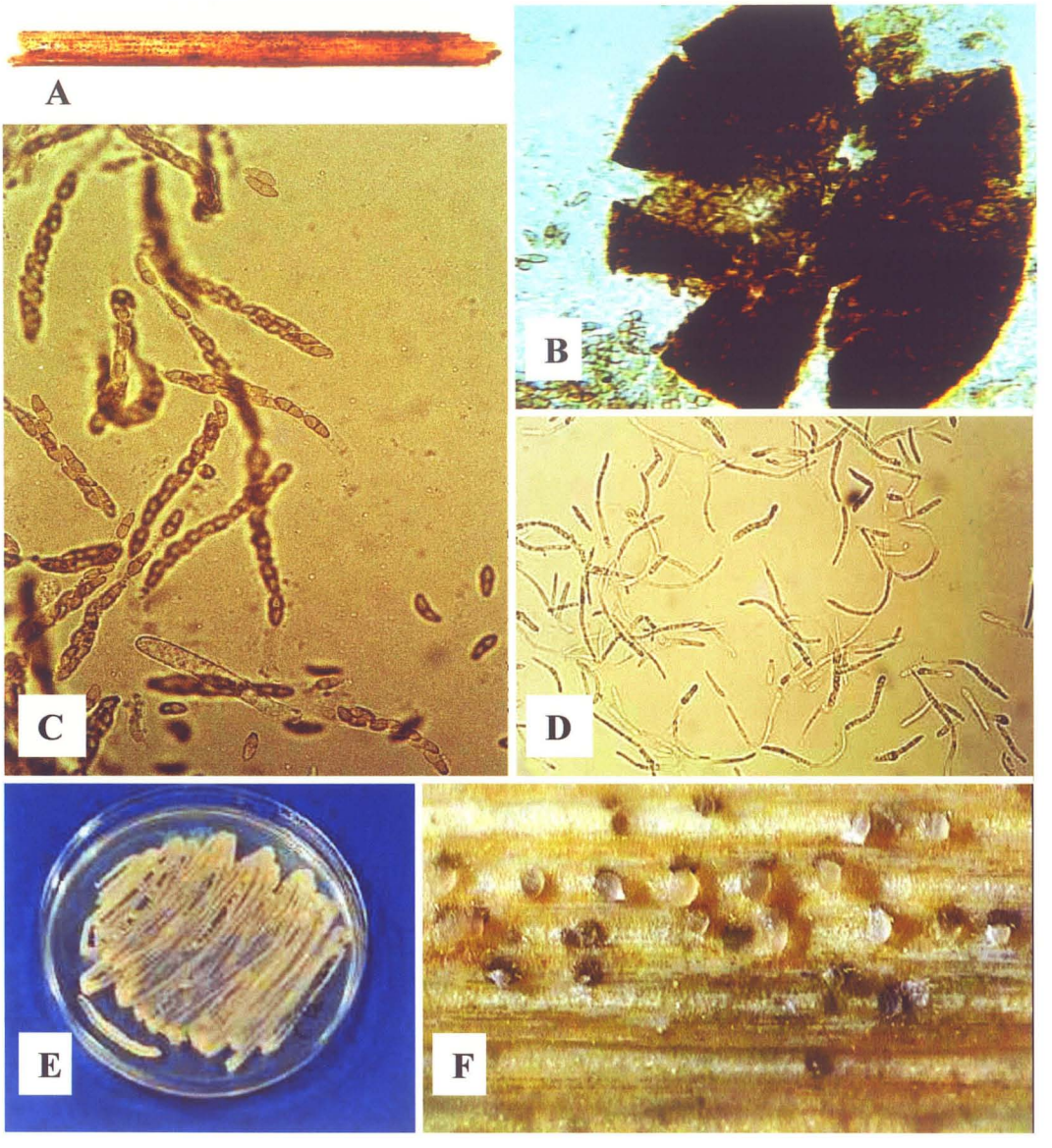
Pseudothecia (Fig. 1B) were dark brown, mostly circular, slightly immersed in the leaf sheath and ranging in size from 110 to 130 μm. When found, measurements of 10 asci and ascospores were taken every week until August 22, 2001. Asci were obpyriform (pear-shaped), approximately 42 μm long and each contained eight ascospores. The two-celled ascospores were irregularly shaped with variable sizes ranging from 8 to10 μm long and from 2 to 2.5 μm wide (Fig. 1C).

The morphology and measurements of asci and ascospores reported in this study are consistent with the observations reported by Sanderson (1972, 1976). He reported ascocarps are superficially immersed in dead leaf blade tissue of wheat stubble, globose, dark brown and 68-114 μm in diameter when mature. Asci are obpyriform and range in size from 30 to 41 μm long x 11-14 μm wide. Ascospores are two-celled, with one cell

FIGURES 1A-F

Figure Captions

Figure 1. *Mycosphaerella graminicola*. Structures of the teleomorph and the anamorph. **A.** Pseudothecia of *M. graminicola* on stubble. **B.** Crushed pseudothecium of *M. graminicola* with ascospores (400x). **C.** Asci and ascospores of *M. graminicola* (630x). Note the irregularly shaped, two-celled ascospores. **D.** *S. tritici* pycnidiospores (400x). Note the multi-septate, curved spores. **E.** Budding, pinkish colony of *S. tritici* growing on YMA^{+}. **F.** Cirrhi emerging from pycnidia on a leaf of susceptible wheat cv. Erik.


A
B
C
D
E
F

tending to be slightly larger, hyaline, and 9-15 μm x 2.5-3 μm (Sanderson 1972).

Ascospore-derived cultures

Four single ascospores were isolated and grown on plates containing YMA^+ until typical pinkish, budding colonies of *S. tritici* were visible. Microscopic observations revealed that the colonies consistently produced 2- to 3- septate, mostly flexuous and filiform spores, similar to those of *S. tritici* (Sanderson 1976) (Fig. 1D).

Inoculations and reisolation

Wheat plants were scored three weeks after inoculation with the ascospore-derived conidial cultures (Fig. 1E). Erik and 4B1149 were susceptible to all four *M. graminicola* isolates and the control isolate 96-36, and developed rectangular-shaped necrotic lesions and abundant pycnidia (Fig. 1F), typical of speckled leaf blotch. Salamouni and Coulter were resistant and either displayed some chlorotic flecking or remained symptomless (Table 1).

Leaf segments from the susceptible lines Erik and 4B1149 were placed on wet filter paper in glass Petri dishes overnight to allow cirrhi to develop (Fig. 1F). Single cirrhi were individually picked, mounted on a microscope slide, and observed under the compound microscope. Pycnidiospores were identified as those of *S. tritici* (Fig. 1D). Individual cirrhi were transferred to YMA^+-containing plates and produced budding colonies similar to the ones used in inoculations (Fig. 1E), thereby associating the ascospores with the anamorph *S. tritici* and the development of speckled leaf blotch.

Table 1. Disease reaction to four single ascospore isolates of *Mycosphaerella graminicola* on a differential set of bread (hexaploid) and durum (tetraploid) wheat lines.

	Isolate				
Wheat lines	C-1	C-2	C-3	C-4	96-36 (control)
Hexaploid					
Salamouni	R	R	R	R	R
Erik	S	S	S	S	S
Tetraploid					
4B1149	S	S	S	S	S
Coulter	R (chl)	R (chl)	R (chl)	R (chl)	R

Note: S, susceptible, abundant pycnidia; R, resistant, minute necrotic flecks or symptomless; chl, chlorotic flecks.

4.0.4 Discussion

The teleomorph *M. graminicola*, originally described by Sanderson (1972), was reported in several countries by direct observation (Scott et al. 1988, Garcia and Marshall 1992, Halama 1996) and inferred from studies of DNA polymorphism (McDonald and Martinez 1990a, 1990b). Even though the occurrence of the sexual state of the pathogen in the Canadian prairies was inferred from DNA polymorphism studies (McDonald et al. 1999, Razavi and Hughes 2001), no direct observations were reported as of this writing.

The pseudothecia, asci, and ascospores of *M. graminicola* characterized in this study matched those reported in the literature (Sanderson 1972) and provide a direct proof of the occurrence of the sexual state of the pathogen in Manitoba. Ascospore-derived pycnidiospore cultures were similar to *S. tritici* in morphology (Fig. 1E) and disease reaction on differential wheat lines (Table 1). The symptoms produced by ascospore-derived cultures in susceptible Erik and 4B1149 were similar to those produced by isolate 96-36, which was previously obtained from the anamorph *S. tritici* (Grieger 2001) (Fig. 1F). The reactions of the wheat lines/cultivars used in this study were similar to those reported previously (Grieger 2001). Single cirrhi were reisolated from lesions produced in susceptible Erik and 4B1149 and pycnidiospore morphology and growth on YMA^{+} medium were those of *S. tritici*.

Sanderson (1972); Sanderson et al. (1986) tested the pathogenicity of isolates from single ascospores of *M. graminicola* with conidia that formed abundantly in culture. Cultures grown from *M. graminicola* ascospores were typical of, and produced conidia of, *S. tritici* (Sanderson 1978), as was found in the present study. The demonstration of Koch's postulates for *M. graminicola* presents difficulties as ascospores of the pathogen

are not readily produced under controlled conditions. With the exception of Kema et al. (1996), who uses a combination of laboratory and natural environment, there are no reports of the production of ascospores by any researcher. Furthermore, the fungus is heterothallic and ascospore production would require co-inoculation with two isolates of opposite mating types. The association of naturally occurring sexual structures and ascospores with the anamorph *S. tritici*, the reproduction of typical speckled leaf blotch symptoms under controlled conditions, and the reisolation of the pathogen from inoculated plants reasonably satisfy Koch's postulates.

Mature pseudothecia were found with similar frequencies in 1-and 2-year-old stubble. However, it is not clear from our study if the sexual state is formed during a single growing season, as was the case in the Netherlands and United Kingdom (Kema et al. 1996, Hunter et al. 1999). Under our conditions, it is likely that ascospores are released in the summer to act as initial inoculum and that they could be discharged throughout the growing season because of staggered maturation dates of the pseudothecia. Hunter et al. (1999) reported that the length of time from the first observed symptom of *S. tritici* in the upper leaves of the crop to first detection of ascocarps in the sampled plants ranged from 94-140 days. However, the growing season in Manitoba is only 90-100 days, suggesting that staggered maturation dates of ascospores is the most likely situation. The involvement of ascospores is consistent with the high degree of polymorphism reported in Canada (Razavi & Hughes 2001, McDonald et al. 1999) and deserves further investigation.

The presence of the teleomorph *M. graminicola* in Manitoba raises important concerns. Sexual recombination will likely play a major role in the virulence and fitness

of the pathogen and may provide an effective mechanism for the pathogen to adapt to fungicides or newly developed resistant cultivars (Hunter et al. 1999).

Even though the occurrence of the teleomorph in western Canada was anticipated, the results of this study provide the first conclusive evidence of the presence of *M. graminicola*, the sexual structure of *S. tritici*, in western Canada. This evidence is supported by direct observation and proof of association between field-produced ascospores, laboratory-produced pycnidiospores, and pathogenicity tests.

4.1 Molecular analysis of the teleomorph *M. graminicola* in Manitoba.

4.1.0 ABSTRACT

Mycosphaerella graminicola is the causal agent of speckled leaf blotch, a foliar disease of wheat responsible for serious economic losses in many wheat-growing regions. The objective of this study was to provide evidence of the sexual state *M. graminicola* in Manitoba using DNA polymorphism. Isolates of *M. graminicola* were collected from wheat fields in Carman and Minnedosa, Manitoba, during the summer of 2001 and were analyzed with amplified fragment length polymorphism (AFLP) genetic markers. A hierarchical sampling procedure was used, and different locations, leaves and lesions were represented. *M. graminicola* isolates were analyzed as two sets: Set I was comprised of 44 isolates representing different stations within a field and different field locations; Set II was comprised of 42 isolates representing different leaves and lesions from a single station in each of two fields. A high degree of polymorphism was detected using AFLP markers and no distinct clustering patterns were evident in either set. Each single spore isolate from Set I had a unique genotype and only two isolates in Set II shared an identical genotype. As visualized in the AFLP gels and dendrograms, single spores and cultures from individual pycnidia always shared identical genotypes. Analysis of Molecular Variance (AMOVA) showed high levels of genetic diversity in each field population of Carman and Minnedosa: within population (isolate-to-isolate) variation accounted for 99.02% and 88.96% of the total genetic variation for Set I and Set II, respectively. The findings of high and uniform genetic diversity within field populations suggests that the primary source of inoculum consists of air-borne ascospores, which are evenly distributed across a field. Our results are consistent with the involvement of

ascospores and confirms our previous findings that the teleomorph *M. graminicola* exists in Manitoba.

4.1.1 INTRODUCTION

Speckled leaf blotch (also known as septoria leaf blotch), caused by the pathogen *Mycosphaerella graminicola* (Fückel) Schröeter (anamorph: *Septoria tritici* Rob. ex Desm.), is a wheat disease known to cause severe epidemics and substantial yield losses (Stewart et al. 1972, King et al. 1983). Since the early 1990s, its prevalence in Manitoba has been increasing due to the occurrence of favourable environmental conditions and the fact there are no resistant wheat varieties available to farmers (Gilbert et al. 1998, Chungu et al. 2000). Plant breeders require a better understanding of the pathogen population in order to develop these resistant cultivars, and molecular markers can provide some important information about the genetic diversity of the pathogen population (McDonald et al. 1995)

The teleomorph (sexual state) *M. graminicola* has recently been reported in Manitoba using a direct method of observation (Hoorne et al. 2002). Ascospores were isolated and pathogenicity tests were performed on a wheat differential set using ascospore-derived conidial cultures. Since we know that the sexual structures of *M. graminicola* are present in Manitoba and that sexual recombination is most likely occurring, it would be beneficial to have a general idea of how much is occurring in a field setting. This can be accomplished by using molecular tools to detect polymorphism, an indicator of unique individuals and, therefore, sexual recombination (Mills et al. 1992). Clonal populations are identified when pycnidiospores of the asexual state are present (McDonald et al. 1995). Haploid tissue, such as that of *M. graminicola*, is easy to work with and interpretation of the resulting marker patterns, in relation to genotypes, is straightforward (Wang and Szmidt 1998). Interpreting the genotype patterns of diploid

and dikaryotic tissues can be difficult, since in both cases only the dominant allele at any specific locus will be detected (Wang and Szmidt 1998).

There are several molecular techniques available to analyze genetic diversity, such as RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA and AFLP (amplified fragment length polymorphism). Each technique has its advantages and disadvantages, depending on its objectives. RFLP markers are reproducible, co-dominant and locus-specific. The disadvantages of RFLP (the use of radioactive elements, laboriousness of the technique and the amount of DNA required) are overcome by RAPD markers (Saliba-Colombani et al. 2000). However, RAPD markers present the disadvantage of being dominant, non-locus-specific and having low reproducibility (Reiter et al. 1992). AFLP is a restriction enzyme, polymerase chain reaction (PCR)-based molecular marker assay that combines the features of RFLP and RAPD techniques (Vos et al. 1995). It is locus-specific at the species level (Saliba-Colombani et al. 2000) and produces dominant markers (Powell et al. 1996). Saliba-Colombani et al. (2000) and Powell et al. (1996) compared the efficiency of RAPD, RFLP and AFLP techniques. They reported that AFLP is the most advantageous regarding polymorphism because it allows the simultaneous analysis of a large number of DNA bands per gel. AFLP can detect a higher number of loci and, therefore, a higher rate of polymorphism in a single assay than RFLP or RAPD (Powell et al. 1996).

In this study, AFLP markers were used to detect the degree of polymorphism in a collection of *M. graminicola* isolates taken from various stations in two distantly separated fields. The objective of this study was to investigate the genetic structure of two populations of *M. graminicola* and to indirectly determine the involvement of

ascospores in the epidemiology of the disease in Manitoba.

4.1.2 MATERIALS AND METHODS

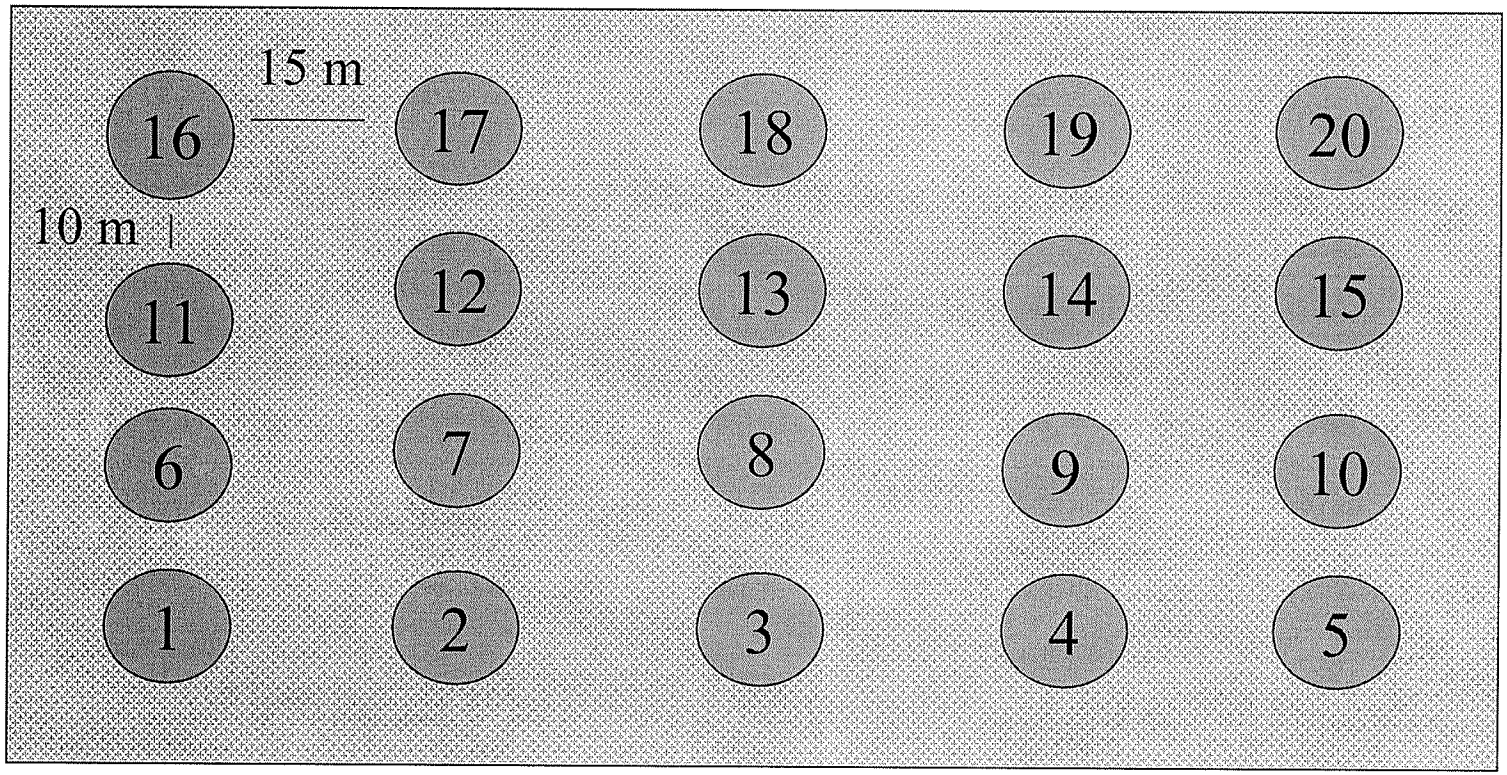
Fungal isolates

The *M. graminicola* isolates were obtained in the summer of 2000 from two wheat fields in Manitoba, separated by more than 200 km. The first field was in Carman with 7 years of wheat monoculture and the second field was located southeast of Minnedosa. The fields were heavily infested with speckled leaf blotch from naturally occurring inoculum. Leaves with typical symptoms were sampled using a hierarchical strategy. Twenty stations were located at regular intervals of 10 m by 15 m within each field (Fig. 2). At each station, 10 flag leaves were collected, placed in paper envelopes and immediately put on ice in a cooler. The samples were taken back to the laboratory, dried at room temperature, and stored at 5^0C until processed.

Forty-four single spore *M. graminicola* isolates were used to compare different stations in two field locations and were designated Set I (Table 1). As shown in Table 1, a numbering system was used to identify the isolates. The letter represents the field location (Carman or Minnedosa), the first number represents the station number in the field (ranging from 1 to 20), the second number represents the leaf number at this station, and the third number represents the lesion number of a particular leaf. For example, C1-2-3 would indicate that the isolate originated from the Carman location, station 1, leaf 2, lesion 3. C1-2-4 would identify the same location, station and leaf, but lesion 4 on leaf 2.

Forty of these isolates represented stations at both Carman (20) and Minnedosa (20) and four isolates were added as controls. Two isolates (BS1, CS1) represented conidial cultures originating from *M. graminicola* ascospores that were isolated in the summer of 2001, and the two additional isolates represented race I (96-36) and race II

Figure 2. The field station layout in both Carman and Minnedosa, Manitoba. Susceptible leaves infected with *Mycosphaerella graminicola* were sampled at twenty stations using a hierarchical procedure. Pycnidial isolates used for AFLP analysis were selected from these leaves.


15 m
10 m
16
17
18
19
20
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1
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Table 2. Forty-four single spore isolates of *Mycosphaerella graminicola* taken from different stations in two common wheat field locations and used in an AFLP study.

Isolate No.	Isolate Name	Place of Origin
1	96-36	Manitoba
2	#2(SS55-3)	Manitoba
3	BS-1	Carman
4	CS-1	Carman
5	C1-2-1	Carman
6	C2-1-1	Carman
7	C4-2-2	Carman
8	C5-4-1	Carman
9	C6-1-2	Carman
10	C7-1-1	Carman
11	C8-2-2	Carman
12	C9-1-1	Carman
13	C10-1-1	Carman
14	C11-1-2	Carman
15	C12-1-1	Carman
16	C13-1-2	Carman
17	C14-1-2	Carman
18	C15-1-1	Carman
19	C16-3-1	Carman
20	C17-3-2	Carman
21	C18-2-1	Carman
22	C19-1-2	Carman
23	C20-1-1	Carman

24	C20-7-1	Carman
25	M1-1-1	Minnedosa
26	M2-1-1	Minnedosa
27	M4-1-2	Minnedosa
28	M5-2-1	Minnedosa
29	M6-1-2	Minnedosa
30	M7-2-2	Minnedosa
31	M8-2-1	Minnedosa
32	M9-1-1	Minnedosa
33	M10-1-2	Minnedosa
34	M11-2-2	Minnedosa
35	M12-1-1	Minnedosa
36	M13-2-2	Minnedosa
37	M14-1-2	Minnedosa
38	M15-1-1	Minnedosa
39	M15-4-2	Minnedosa
40	M15-6-2	Minnedosa
41	M17-1-1	Minnedosa
42	M18-1-1	Minnedosa
43	M19-9-1	Minnedosa
44	M20-1-2	Minnedosa

C=Carman; M=Minnedosa
Example:
C1-1-1
C1= station 1 in Carman
C1-1 = leaf 1 of station 1 in Carman
C1-1-1 = lesion 1 of leaf 1 of station 1 in Carman

-all isolates originate from hexaploid wheat

(#2(SS55-3)) from a speckled leaf blotch collection (L. Lamari).

The forty-two *M. graminicola* isolates representing a single station in Carman and a single station in Minnedosa were designated Set II (Table 2) and were used in a separate AFLP analysis. At station 1 in both locations, eight leaves were used as the source of pycnidial isolates. Pycnidia were selected from lesions of different leaves and from different lesions of the same leaf. This was done in order to observe the genetic similarity of isolates due to the location of the lesions. Different lesions of the same leaf were isolated from two to three randomly selected leaves from both field populations. From each of these pycnidia, a single spore was isolated. Eleven single pycnidia and 11 single spores (one spore from each pycnidium) represented Carman and ten pycnidia and their corresponding 10 single spores represented Minnedosa.

Table 3. List of 22 single-pycnidium isolates of *Mycosphaerella graminicola* and their respective single-spore isolates obtained from leaves collected at two, 1 m^2 stations in Carman and Minnedosa, Manitoba.

Isolate No.	Isolate Name	Place of Origin
1	C1-3-1 sp	Carman
2	C1-3-1 ss	Carman
3	C1-3-2 sp	Carman
4	C1-3-2 ss	Carman
5	C1-4-1 sp	Carman
6	C1-4-1 ss	Carman
7	C1-4-2 sp	Carman
8	C1-4-2 ss	Carman
9	C1-5-3 sp	Carman
10	C1-5-3 ss	Carman
11	C1-6-2 sp	Carman
12	C1-6-2 ss	Carman
13	C1-7-2 ss	Carman
14	C1-7-2 sp	Carman
15	C1-8-1 sp	Carman
16	C1-8-1 ss	Carman
17	C1-8-3 sp	Carman
18	C1-8-3 ss	Carman
19	C1-9-2 sp	Carman
20	C1-9-2 ss	Carman
21	C1-10-1 sp	Carman
22	C1-10-1 ss	Carman

23	M1-2-1 sp	Minnedosa
24	M1-2-1 ss	Minnedosa
25	M1-3-1 sp	Minnedosa
26	M1-3-1 ss	Minnedosa
27	M1-3-2 sp	Minnedosa
28	M1-3-2 ss	Minnedosa
29	M1-5-1 sp	Minnedosa
30	M1-5-1 ss	Minnedosa
31	M1-6-1 sp	Minnedosa
32	M1-6-1 ss	Minnedosa
33	M1-6-2 sp	Minnedosa
34	M1-6-2 ss	Minnedosa
35	M1-7-1 sp	Minnedosa
36	M1-7-1 ss	Minnedosa
37	M1-8-1 sp	Minnedosa
38	M1-8-1 ss	Minnedosa
39	M1-9-1 sp	Minnedosa
40	M1-9-1 ss	Minnedosa
41	M1-10-2 sp	Minnedosa
42	M1-10-2 ss	Minnedosa

C=Carman; M=Minnedosa; sp=single pycnidium; ss=single spore
Example:
C1-1-1
C1= station 1 in Carman
C1-1 = leaf 1 of station 1 in Carman
C1-1-1 = lesion 1 of leaf 1 of station 1 in Carman

Single spore isolation

The leaves collected in Carman and Minnedosa were used to isolate single cirrhi from pycnidia. Five leaf segments, three centimeters in length, were placed on moist filter paper in glass Petri dishes under fluorescent light overnight to cause the cirrhi to ooze from the pycnidia. Single cirrhi were picked with a sterile needle and streaked onto YMA^+ media. The culture plates were sealed with parafilm and allowed to grow in an incubator at 20^0C in the dark for 3 to 4 days. The growing colony was then spread out with a wire loop and allowed to grow for 4 additional days. One single spore was isolated from each of these pycnidial cultures. Spores were spread across a new YMA^+ plate with a wire loop. Using a dissecting microscope (Wild, Heerbrugg), single spores were located and cut out with a sterile knife and transferred to a new YMA^+ plate. These single spores were allowed to grow as previously described for single cirrhi. The inoculum was increased for both the single pycnidia and single spore cultures by streaking the spores over YMA^+ plates and placing them under light for a week. Ten milliliters of autoclaved distilled water was poured into the plate and the spores were suspended with a wire loop. One milliliter of autoclaved glycerin and 4 ml of the spore suspension were pipetted into 500 µl tubes, vortexed and frozen immediately with dry ice. These tubes were stored at -70^0C until needed.

Fungal cultures for DNA extraction

A small amount of frozen inoculum was chipped onto a YMA^+ plate, spread and grown under light for a week. A loop-full of spores from each of the budding cultures was transferred into an autoclaved 250 ml Erlenmeyer flask containing 100 ml of a yeast:sugar:distilled water solution (Appendix 1b). The cultures were allowed to grow

for one week on a shaker (at 150 rpm) at room temperature (22-25^{0}C) to allow "budding" spore production. The cultures (spores) were then centrifuged for 15 minutes at 2000 x g, the supernatant poured off and the spore pellets placed in a sterile plastic bag and frozen immediately with liquid nitrogen. The spores were lyophilized and kept at -20^{0}C until needed for DNA extraction. Approximately 200 mg of freeze-dried spores were recovered from each isolate.

DNA extraction

The Promega Wizard Genomic DNA Purification Kit (Cat. #A1120) was used, with a few modifications, to isolate genomic DNA from *M. graminicola* fungal tissue. Fifty milligrams of freeze-dried spores were placed into 1.5 ml microcentrifuge tubes, and the outside of the tubes were pre-cooled with liquid nitrogen. The tube was then carefully dipped with long tweezers into the liquid nitrogen until the fungal contents were covered. Once covered, the frozen spores were ground with an Eppendorf pestle to a fine powder. Six hundred microliters of Nuclei Lysis Solution was added to the tube, vortexed for three seconds to wet the tissue and then placed in a 65^{0}C water bath for 15 minutes. Three microliters of RNase solution were added to the cell lysate and mixed well by shaking. The mixture was incubated for 15 minutes at 37^{0}C and then cooled to room temperature (22^{0}C). Two hundred microliters of protein precipitation solution were added to the tube, vortexed vigorously at high speed for 20 seconds and then centrifuged for 3 minutes at 15,000 x g. The supernatant, containing the DNA, was carefully removed and transferred to a clean 1.5 ml microcentrifuge tube. An equal volume (1:1) of phenol/chloroform:isoamyl alcohol (24:1) was added to the supernatant in the new tube and mixed by inverting. The tubes were spun at 13,000 rpm for 5 minutes at room

temperature. The aqueous layer was transferred to a new tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed by inversion. The tube was spun again at 13,000 rpm for 5 minutes at room temperature and this chloroform:isoamyl alcohol step was repeated again to ensure removal of all phenol. The aqueous layer was then transferred to a new tube and 1/10 volume of 3M sodium acetate and 2 volumes of 95% ethanol (-20^{0}C) were added to the tubes to precipitate the DNA. The tubes were stored at -20^{0}C overnight. The DNA was then pelleted by centrifugation at 3000 x g for 20 minutes at room temperature. The DNA pellets were washed twice with 500 µl of 70% ethanol (-20^{0}C) and spun at 3000 x g for 5 minutes after each wash. The samples were allowed to air-dry for 2-3 h and 200 µl of Tris (10mM, pH 7.5)-EDTA (1 mM, pH 8) (TE) buffer were added to each tube for re-hydration. The concentration of DNA was measured using a spectrophotometer at O.D. 260 nm and was adjusted to a final working concentration of 50 ng/µl. Five microliters of DNA of each sample were electrophoresed in 1% agarose gel with 1X Tris-acetate (0.04M)/EDTA (0.001M) (TAE) buffer (5 ml of 50x stock solution) and ethidium bromide (EtBr) (10 mg/ml) staining to check for DNA degradation, RNA contamination and to confirm the concentration by visual comparison with known concentrations of a standard gel electrophoresis marker. The DNA solution was stored at 4^{0}C until needed for AFLP analysis.

AFLP assays

Adaptor and primer oligonucleotide sequences are listed in Table 4. Three *Eco*RI:*Mse*I primer combinations were used for Set I and two *Eco*RI:*Mse*I primer combinations were used for Set II (Table 4).

Table 4. Adaptor sequences and primer sequences and combinations used in AFLP analysis of 86 *Mycosphaerella graminicola* isolates from Carman and Minnedosa, MB.

Oligonucleotide	**Sequence**
Adaptors	
*Eco*RI 1	CTC GTA GAC TGC GTA CC
2	AAT TGG TAC GCA GTC TAC
*Mse*I 1	GAC GAT GAG TCC TGA G
2	TAC TCA GGA CTC AT
Non selective Primers	
Set I	
*Eco*RI-C	5' GAC TGC GTA CCA ATT CC 3'
*Mse*I-A	5' GAT GAG TCC TGA GTA AA 3'
Set II	
*Eco*RI-A	5' GAC TGC GTA CCA ATT CA 3'
*Mse*I-C	5' GAT GAG TCC TGA GTA AC 3'
Selective Primers	
Set I	
*Eco*RI-CA	5' GAC TGC GTA CCA ATT CCA 3'
*Eco*RI-CT	5' GAC TGC GTA CCA ATT CCT 3'
*Mse*I-AG	5' GAT GAG TCC TGA GTA AAG 3'
*Mse*I-AT	5' GAT GAG TCC TGA GTA AAT 3'
Set I primer combinations	E-CA M-AG E-CT M-AT E-CA M-AT
Set II	
*Eco*RI-AT	5' GAC TGC GTA CCA ATT CAT 3'
*Eco*RI-AC	5' GAC TGC GTA CCA ATT CAC 3'
*Mse*I-CG	5' GAT GAG TCC TGA GTA ACG 3'
Set II primer combinations	E-AT M-CG E-AC M-CG

Restriction Enzyme Digestion

*Mse*I digestion was performed in a 20 µl volume consisting of 12.5 µl of sterile HPLC-grade water and the final concentrations of: 1X of 10X React I buffer, 0.125 U/µl of *Mse*I (5 U/µl) (Invitrogen) and 12.5 ng/µl of DNA (50 ng/µl). This digestion was incubated at 37^{0}C for 2 h (mixed and spun after the first hour). *Eco*RI digestion was performed in a 80 µl volume consisting of 51.75 µl HPLC-grade water and the final concentrations of: 1X of 10X React III buffer, 0.03125 U/µl of *Eco*RI (10 U/µl) (Invitrogen) and 3.125 ng/µl *Mse*I digestion solution. This digestion was incubated at 37^{0}C for 2 h (mixed and spun after the first hour).

Ligation Reaction

Adaptors were prepared by heating Ea1.1 (5 pmol/µl), Ea1.2 (5 pmol/µl), Ma1.1 (50 pmol/µl) and Ma1.2 (50 pmol/µl) (Invitrogen) separately to 65^{0}C at which time Ea1.1 was added to Ea1.2 and Ma1.1 was added to Ma1.2. Adaptors were annealed by incubating for 10 min at 65^{0}C, 10 min at 37^{0}C and 20 min at 22^{0}C. Forty microliters of the ligation mixture [19 µl HPLC-grade water, 16 µl of 5X ligation buffer, 0.0125 U/µl T4 DNA ligase, 1.25 pmol/µl M adaptor, 0.125 U/µl E adaptor] was added to 40 µl of digested DNA and incubated 2 h at 22^{0}C. Products were diluted 1:2 in HPLC-grade water (80 µl digested and ligated DNA:160 µl HPLC-grade water) to a final DNA concentration of 0.52 ng/µl.

Pre-amplification

Seven microliters of 1:3 diluted DNA samples (0.52 ng/µl) were added to a pre-amplification PCR mixture [1X PCR buffer (Invitrogen), 1.2 ng/µl *Eco*RI-C or A, 1.2 ng/µl *Mse*I-A or C (Invitrogen), 0.8 mM/µl dNTPs, 1.5 mM $MgCl_2$ (Invitrogen), 0.04

U/µl Taq DNA polymerase (Invitrogen), 10.55 µl of sterile HPLC-grade water; 25 µl volume]. Amplification was carried out in a thermal cycler PTC-100™ (MJ Research, Inc.) programmed for 2 min at 95°C, followed by 30 cycles of 0.5 min at 94°C, 1 min at 56°C and 1 min at 72°C and followed by 10 min at 72°C at the end. The preamplified products were diluted 1:10 in HPLC-grade water.

Selective amplification

The selective amplification was performed in a 20 µl volume with: 5 µl of the 1:10 diluted samples, 1X PCR buffer, 0.25 ng/µl *Eco*RI selective primer (Invitrogen), 1.5 ng/µl *Mse*I selective primer (Invitrogen), 0.8 mM/µl dNTPs and 1.5 mM MgCl2, 0.05 u/µl Taq DNA polymerase (Invitrogen). Primer combinations are listed in Table 4. The thermocycling program used was 2 min at 95°C followed by 10 cycles of 0.5 min at 94°C, 1 min at 65°C and 1.5 min at 72°C, followed by 23 cycles of 0.5 min at 94°C, 0.5 min at 56°C and 1 min at 72°C, and 10 min at 72°C at the end. An equal volume of dye (20 µl), which also stopped the reaction, was added to the amplified products and the sample tubes were stored in 4°C.

Preparation of Plates and Gel

The short plate and IPC plate were cleaned very carefully, rinsed with double distilled (dd) water and washed with 4 ml of 95% ethanol. A fresh binding solution of 1449 µl 95% ethanol, 7.5 µl glacial acetic acid and 3 µl bind silane (Promega) was spread on the short plate with Kim wipes. After 10 min, the short plate was gently washed twice with 3 ml of 95% ethanol. The IPC plate was treated with 1444 µl of Sigmacote (Sigma) and after 5 min wiped with Kim wipes. A 6% polyacrylamide gel was prepared with 50.2 g urea, 1X Tris-borate (0.045M)/EDTA (0.001M) (TBE) buffer (24 ml of 5X stock

solution), 6% acrylamide:bisacrylamide (19:1) (18 ml of 40% stock solution) and double distilled water added to a final volume of 120 ml. Immediately before pouring the gel, 420 µl of ammonium persulfate (APS) (10% stock solution) (Sigma) and 84 µl of TEMED (N,N,N,N-tetramethylethyl-enediamine) (Sigma) were added and swirled into the solution. The gel was allowed to polymerize for 2 h. During the 30 min warming time at 85 W (EC600-90, E-C Apparatus Corporation) of the 1X TBE running buffer, the DNA samples were denatured for 10 min at 95^{0}C and then placed immediately on ice for 10 min before loading the gel. Ten microliters of the PCR products were loaded into the gel and electrophoresis was carried out for 2.5 h at 85 W. After electrophoresis, one glass plate was removed and the plate with the gel was placed in stop solution (10% glacial acetic acid, 200 ml glacial acetic acid and 1800 ml double distilled (dd) water), agitated on a shaker for 20 min and then rinsed 3 times with dd water. The stop solution was saved for later use. The Silver Sequence ^{TM} DNA Staining Reagents kit (Promega, cat. #Q4132, Madison, WI) was used to stain and develop the gels. The gel was placed in a staining solution (2 g silver nitrate, 3 ml of 37% formaldehyde, 2000 ml dd water) and agitated for 30 min. The gel was then rinsed with chilled dd water (10^{0}C) and immediately placed into the first developing solution. To prepare the developing solution, 60 g sodium carbonate dissolved in 2000 ml dd water was chilled to 10^{0}C, and then immediately before use, 3 ml of 37% formaldehyde and 400 µl sodium thiosulfate (0.1g/ml) were added to the solution. The gel in the first developing tray was rocked back and forth until the first bands become visible. Then the gel was moved into the second developing tray and was rocked for 2 to 3 minutes or until all bands became visible. Extended developing time results in high background. The stop solution saved in one of

the previous steps was poured into the tray to stop the developing and the gel was shaken for 1 or 2 minutes. The gel was rinsed twice with dd water and allowed to dry at room temperature.

Data analysis

Polymorphic AFLP bands were scored as 1 (presence) or 0 (absence) for each individual and stored as a binary matrix. It is assumed that each AFLP band corresponds to a different locus with two alleles, presence or absence of the band, respectively. Eighty-two binary scores for Set I (three primer combinations) and 41 binary scores for Set II (two primer combinations) were used for analysis. Genetic similarity was calculated as a simple matching coefficient using NTSYSpc (version 2.1)(Exeter Software, Setauket NY), where similarity between individual pairs *i* and *j* was:

$$Sij = (a + b)/(a + b + c + d)$$

where a = number of 1-1 matches, b = number of 1-0 matches, c = number of 0-1 matches, and d = number of 0-0 matches.

The similarity matrix was used to perform cluster analysis using the unweighted pair group arithmetic mean method (UPGMA) in NTSYSpc (version 2.1) and dendrograms were constructed.

Arlequin (version 2.0, downloaded from URL http:// anthropologie unige.ch/arlequin/) was used to conduct AMOVA (Analysis of Molecular Variance). AMOVA was used to partition the marker variation into between- and within- population components and statistical significance was tested with 1000 permutations (Excoffier et al. 1992). The Minnedosa and Carman fields represented two populations.

4.1.3 RESULTS

AFLP banding patterns

AFLP profiles revealed an abundance of polymorphic bands for the Set I data. Figure 3 shows an example of one of the AFLP gels showing extensive polymorphism between individuals. The genotype differences generated by each primer pair combination were able to differentiate and fingerprint each of the 44 genotypes of the *M. graminicola* isolates. Three primer combinations yielded a total of 216 amplification products, of which 82 bands were polymorphic.

The two primer combinations used for Set II produced 121 amplification products and 41 scoreable polymorphic bands. Figure 4 is an example of an AFLP gel showing polymorphism between individuals from different lesions and leaves. There were different banding patterns among isolates, but not as many as revealed with Set I. In all cases, single spores taken from a single pycnidium had identical profiles, as can be seen as pairings in the AFLP gel (Fig. 4).

AMOVA analysis of genetic variation and genetic structure

The Set I Carman and Minnedosa populations were both found to have high within field genetic variation, which accounted for 99.02%, and the proportion among populations accounted for only 0.98% ($P \leq 0.05$). Set II showed the same trend, with 88.96% of genetic variation partitioned within populations and 11.04% partitioned among populations (Table 5).

Cluster analysis

In order to quantify and illustrate the relatedness between the 44 isolates of Set I and the 42 isolates Set II, the genetic similarity matrix was used to perform cluster

Figure 3. DNA banding pattern of *Mycosphaerella graminicola* isolates from different field stations in Carman and Minnedosa, Manitoba, obtained with selective primers *Eco*RI-CA and *Mse*I-AG. The isolates were loaded in the order: 4 control isolates (I), 20 Carman isolates (II) and 20 Minnedosa isolates (III). Note that each isolate has a unique AFLP profile.

I II III

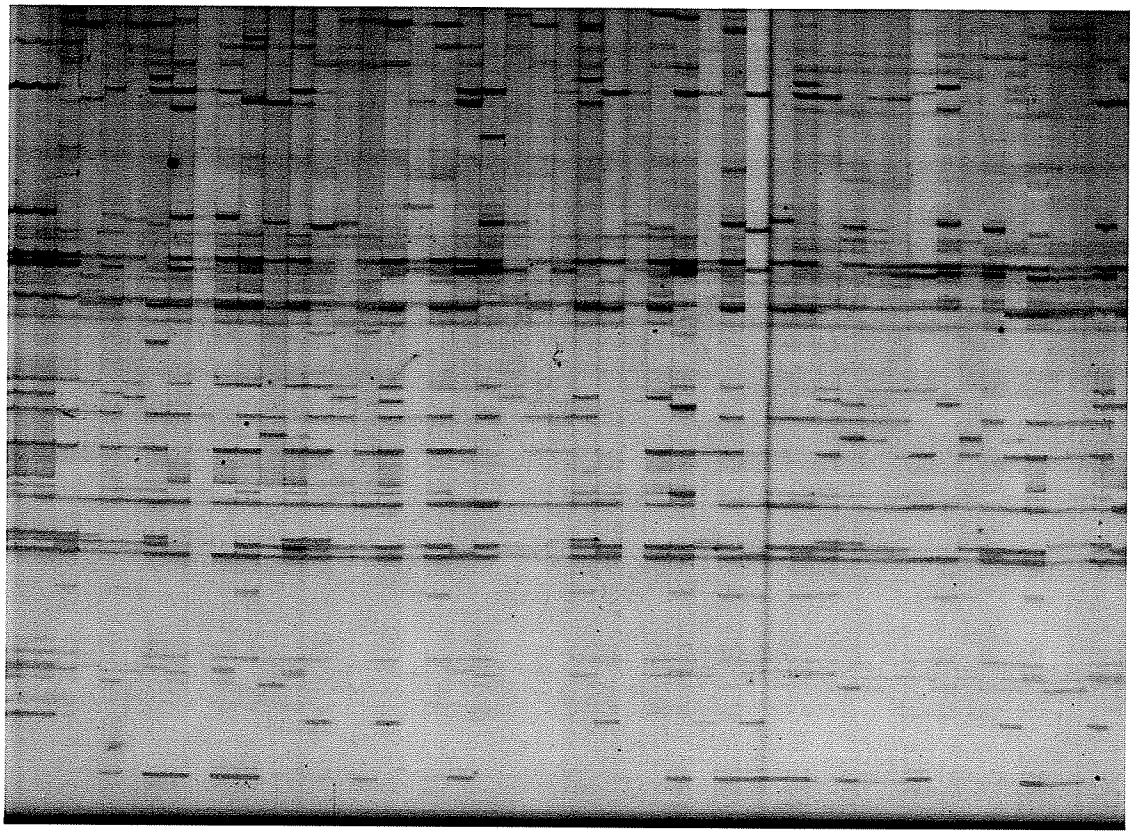


Figure 4. DNA banding pattern of *Mycosphaerella graminicola* isolates from a single field station in both Carman and Minnedosa, Manitoba, obtained with selective primers *Eco*RI-AT and *Mse*I-CG. The isolates were loaded in the order: 22 Carman isolates (a single pycnidium and its single spore in the adjacent lane) (I), 20 Minnedosa isolates (a single pycnidium and its single spore in the adjacent lane) (II). Note that all the single pycnidia and their respective single spores share identical AFLP profiles and show up as pairs in the gel.

I II

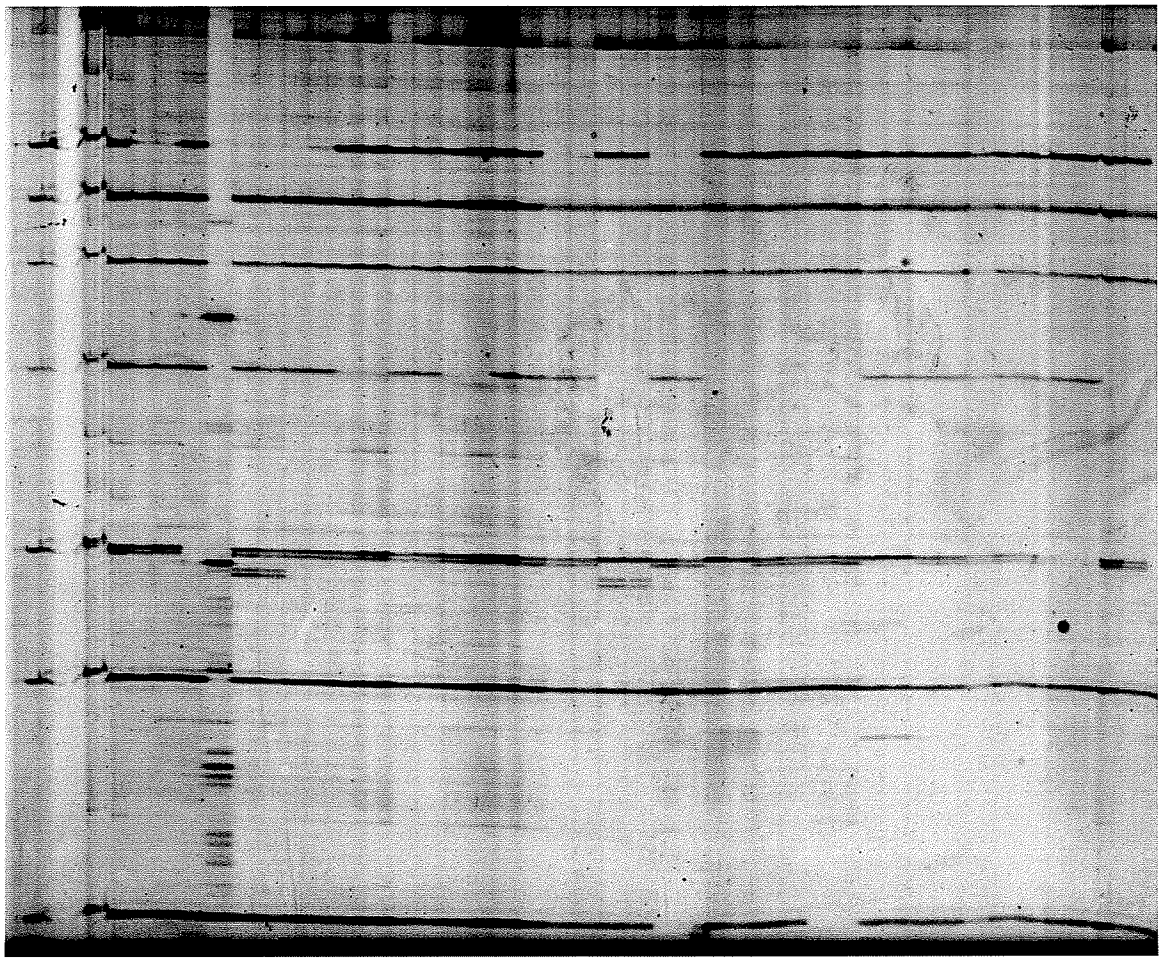


Table 5. Analysis of molecular variance (AMOVA) of 44 isolates of *Mycosphaerella graminicola* taken from different stations within two fields (Set I) and of 42 isolates of *M. graminicola* taken from a single station in each of two fields (Set II).

Source of variation	**Variance component**	**Percentage of variation**	**P[a]**
Set I			
Between populations	0.12682	0.98	<0.05
Within populations	12.79524	99.02	<0.05
Set II			
Between populations	0.72643	11.04	<0.05
Within populations	5.85409	88.96	<0.05

[a]Probability of having a more extreme variance component than the observed values by chance alone, computed from 1000 permutations.

analysis. Figure 5 shows the dendrogram of Set I, in which the Carman and Minnedosa isolates are generally interspersed throughout. An isolate from Carman was just as likely to appear next to an isolate from Minnedosa. There was no clear-cut separation of populations and genetic variation between populations was very small (0.98%). No distinct groupings were present between or within populations and all individuals could be distinguished. There was a substantial amount of variation within populations (99.02%), and the relatively long branches represented a large amount of genetic diversity between isolates (Fig. 5). Race 1 isolate 96-36 and race 2 isolate #2(ss3) were genetically distinct and part of different clusters, as were the controls BS-1 and CS-1 (Fig. 5).

Figure 6 shows the resulting clustering pattern for Set II, in which high levels of genetic diversity within populations are again evident (88.96%). There were no distinct groupings and isolates representing different leaves were always genetically unique. Generally, this was also true when comparing different lesions of the same leaf. Chosen randomly, Carman leaves 3, 4 and 8 and Minnedosa leaves 3 and 6 were each represented by two different lesions. Only lesions isolated from Carman leaf 3 (C1-3-1 and C1-3-2) were genetically identical and these were also the only isolates to form a unique cluster, separate from the rest. The genetic variation between populations was low at 11.04%, but there was more potential for distinction between Carman and Minnedosa isolates in Set II than there was for Set I. Most of the isolates from the same population were in close proximity on the dendrogram, but still not distinct enough to form separate population clusters (Fig. 6). Pycnidia and their corresponding single spores were always genetically identical.

Figure 5. UPGMA clustering of 44 *Mycosphaerella graminicola* isolates from different field stations in Carman and Minnedosa, Manitoba, based on 82 AFLP bands. **M** represents Minnedosa and **C** represents Carman. Note that each isolate is genetically distinct and that the Minnedosa and Carman isolates are randomly dispersed throughout the dendrogram.

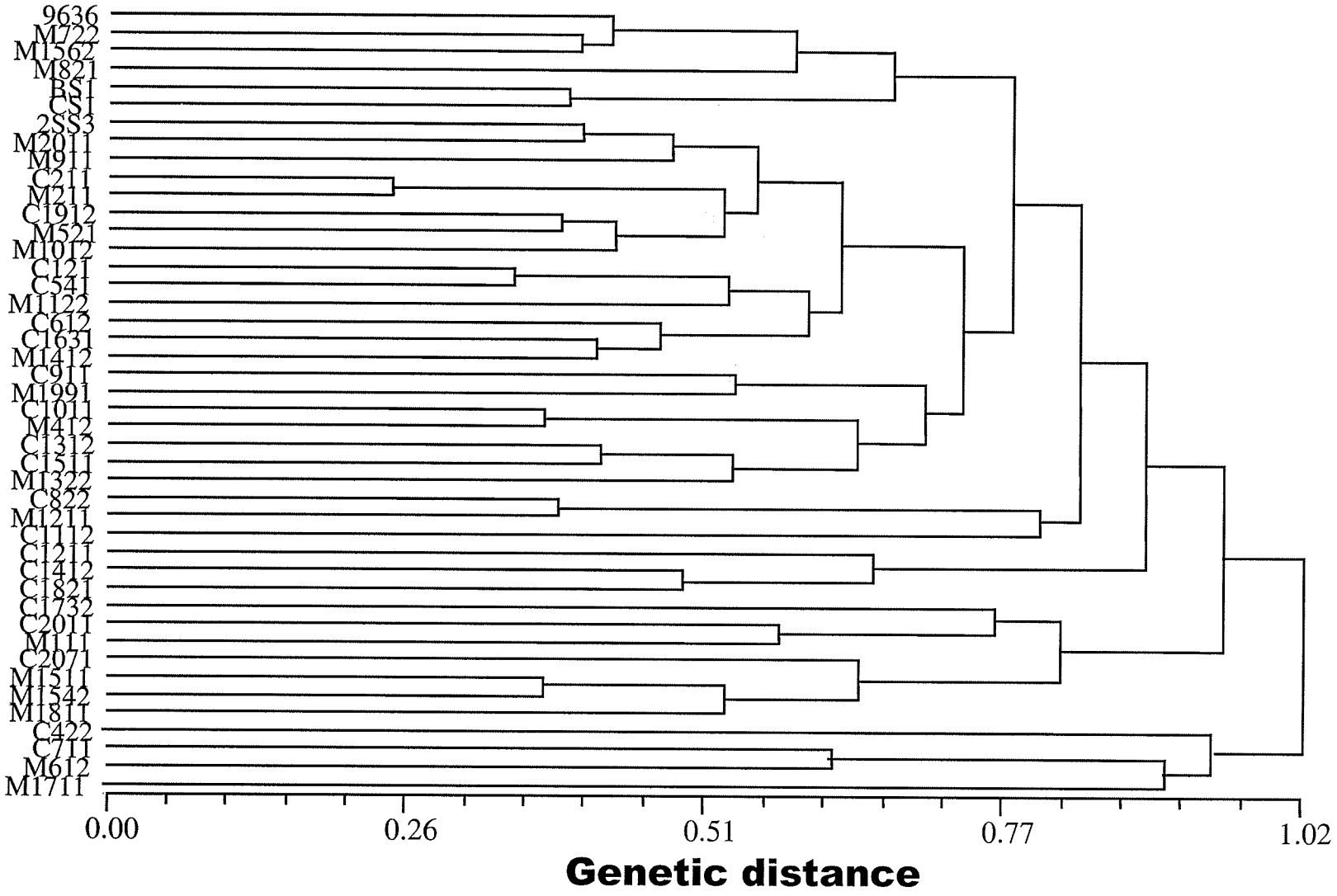
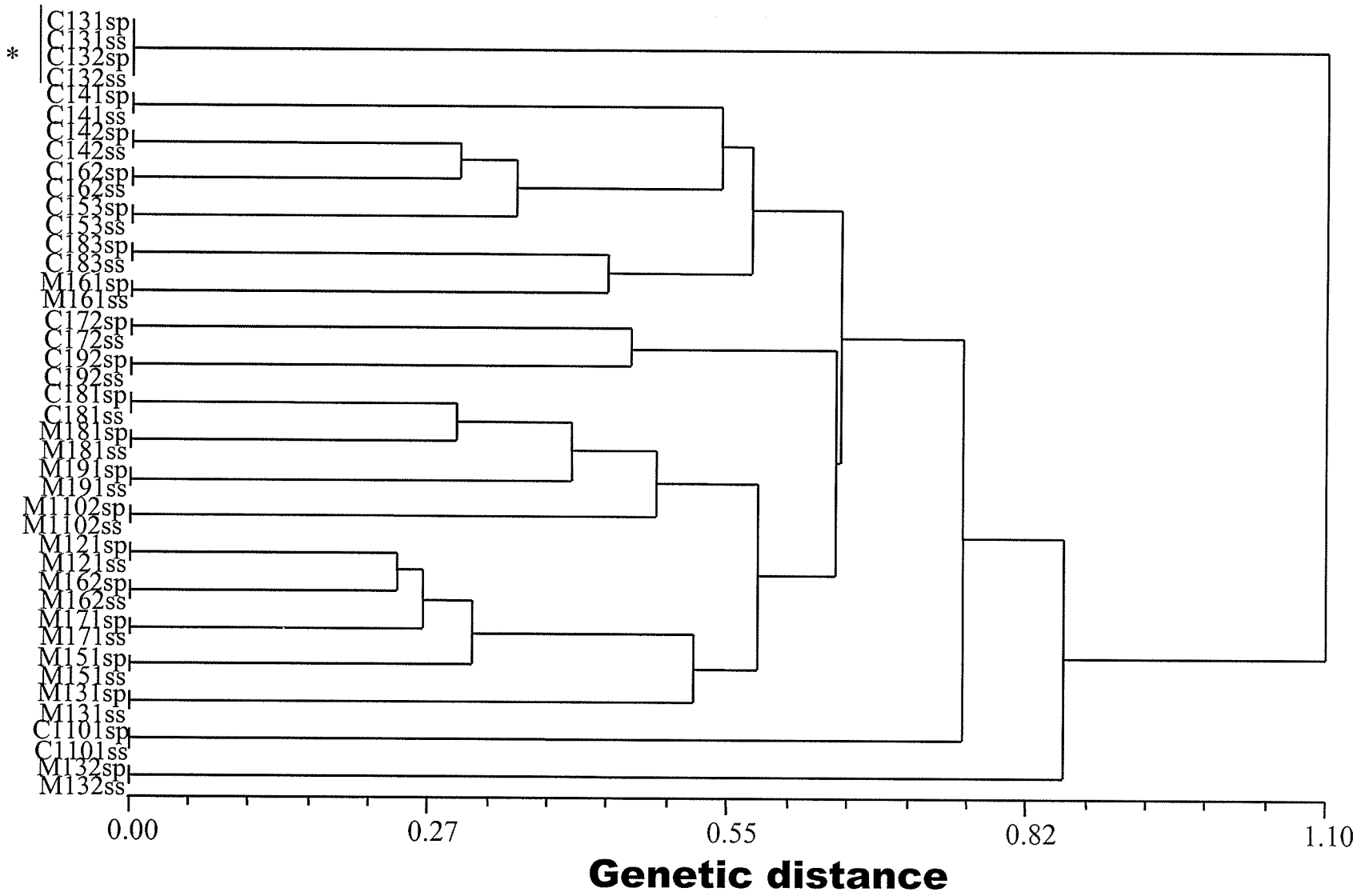

9636
M722
M1562
M821
BS1
CS1
2SS3
M2011
M911
C211
M211
C1912
M521
M1012
C121
C541
M1122
C612
C1631
M1412
C911
M1991
C1011
M412
C1312
C1511
M1322
C822
M1211
C1112
C1211
C1412
C1821
C1732
C2011
M111
C2071
M1511
M1542
M1811
C422
C711
M612
M1711
0.00
0.26
0.51
0.77
1.02
Genetic distance

Figure 6. UPGMA clustering of 42 *Mycosphaerella graminicola* isolates from a single field station at Carman and Minnedosa, Manitoba, based on 41 AFLP bands. **M** represents Minnedosa and **C** represents Carman. Note that single pycnidiospores and their respective pycnidia always share the same branch (sp=single pycnidia; ss=single spore). * Two single spores (and their respective pycnidia) from the same lesion shared an identical genotype and clustered together (C1-3-1/C1-3-2).


C131sp
C131ss
C132sp
C132ss
C141sp
C141ss
C142sp
C142ss
C162sp
C162ss
C153sp
C153ss
C183sp
C183ss
M161sp
M161ss
C172sp
C172ss
C192sp
C192ss
C181sp
C181ss
M181sp
M181ss
M191sp
M191ss
M1102sp
M1102ss
M121sp
M121ss
M162sp
M162ss
M171sp
M171ss
M151sp
M151ss
M131sp
M131ss
C1101sp
C1101ss
M132sp
M132ss
0.00
0.27
0.55
0.82
1.10
Genetic distance

4.1.4 DISCUSSION

A high level of polymorphism was detected in the AFLP analysis of two *M. graminicola* populations separated by over 200 km, suggesting widespread sexual recombination was occurring. Due to the lack of detection of clonal isolates, our findings suggest that the asexual state did not contribute significantly to inoculum in these two Manitoba fields, even on a small-scale (single station sampling). The high level of polymorphism is consistent and confirms the presence of the teleomorph *M. graminicola* in Carman, Manitoba as shown by direct observation (Hoorne et al. 2002).

As shown in the field-scale (Set I) AFLP gel and dendrogram, (Figs. 3 and 5), it was clear that each individual from Carman and Minnedosa had a unique genotype. In a collection of 711 isolates of *M. graminicola* from one field in Oregon, Chen et al. (1994) found that there were 654 genotypes and that this was due to a random-mating population. In our study, the observed clustering patterns of the two populations appeared to be independent of geographic location, as the individuals from both locations were randomly interspersed throughout the dendrogram (Fig. 5).

The 42 isolates of Set II, which represented a single station (1 m^2) from each field, displayed comparable results to those of Set I. Polymorphism was evident (Fig. 4) and no distinct clustering between isolates was observed in the dendrogram, except for single pycnidia and their respective single spores (Fig. 6). Single pycnidia and single spores always shared identical DNA fingerprints, and can be seen as pairs across the gel (Fig. 4). They also shared 100% genetic similarity in the dendrogram and shared identical branches (Fig. 6). In Set II, genetic variation was always detected when analyzing different leaves of the same station and 80% of the time when analyzing different lesions

of the same leaf (Carman leaves 4 and 8; Minnedosa leaves 3 and 6) (Fig. 6). However in one case, C1-3-1/C1-3-2, isolates from two lesions on the same leaf were genetically identical, suggesting that the lesions may have been caused by spores from the same pycnidium due to rain-splash dispersal, by multiple infections from the same spore, or by genetically related ascospores that could not be differentiated by the primers used in this study.

AMOVA analysis of Set I revealed that 99.02% of the genetic variation occurred within the Minnedosa and Carman *M. graminicola* populations and that 0.98% occurred between them (Table 5). This would result if the primary source of inoculum was air-borne ascospores that are dispersed evenly across the field (Razavi and Hughes 2001). A study done by Chen et al. (1994) in Oregon found that 99% of the total diversity was due to within field variation and that the most likely reason for the uniform level of genetic variation among the collections is the occurrence of ascospores originating from outside the field (Shaw and Royle 1987, 1989b, Schuh 1990b). Set II AMOVA analysis revealed that 88.96% of the genetic variation occurred within populations, which is also a very high percentage, but lower than that of Set I. However, this may reflect the nature of the population of Set II, where half of the isolates were replicates ie. single spores and their respective pycnidia. Also, some Set II isolates were taken from lesions on the same leaf, which have a higher probability of being the products of rain-splashed asexual spores. When these points are taken into consideration, the results of the two sets were very similar. Our findings suggest that a great deal of genetic variation existed within a relatively small sample of isolates collected from a single geographic area, and this in agreement with the findings of McDonald and Martinez (1990b).

AFLP analysis was able to clearly discriminate the clonal isolates from the unique isolates, indicating the reliability and accuracy of this marker system. Polymorphism was detected everywhere except between single pycnidia and their respective single spore isolates. We expected the pycnidia and their single spores to be clonal and this was demonstrated without exception. Pycnidiospores are generated through asexual reproduction and are, therefore, clonal (Kohli et al. 1992, Boeger et al. 1993, Chen and McDonald 1996). Another positive aspect of using AFLP analysis in this study was the abundance of polymorphic markers generated, which helped to discriminate the isolates in a fewer amount of gels than other techniques. These points suggest that the AFLP technique is a highly effective and efficient molecular tool in characterizing the genetic diversity of this pathogen without creating artifacts.

Due to the detection of a high degree of polymorphism among isolates, the results of this study suggest that the sexual state of *M. graminicola* exists in Manitoba and has widespread occurrence. This indirect proof is consistent with and confirms direct evidence previously reported in Manitoba by Hoorne et al. (2002). On a local and regional scale, it was demonstrated that the isolates selected in this study were all caused by ascospores and that clonal development of pycnidiospores appeared to be negligible. Hence, the speckled leaf blotch epidemics that we observe in Manitoba are mainly caused by ascospores. A high level of genetic variation in *M. graminicola* populations is a point of concern because they may contain sufficient variability to allow for rapid selection of clones that are resistant to fungicides or virulent to resistant varieties (McDonald and Martinez 1990a).

5. GENERAL DISCUSSION

This is the first report by direct observation that the teleomorph *M. graminicola* exists in Canada, even though other groups had already suggested the occurrence of the sexual state through molecular analysis (Razavi and Hughes 2001, McDonald et al. 1999). In this study, direct and indirect methods of observation were both used to provide evidence that the ascogenous state of the pathogen exists in Manitoba. AFLP analysis (indirect method) was used to determine the genetic structure of two *M. graminicola* populations, i.e. genetic variation within and between populations.

Ascospores were the predominant form of primary inoculum in the epidemiology of speckled leaf blotch in Manitoba, as indicated by the extensive polymorphism revealed by AFLP analysis. The failure to detect clonality (i.e. evidence of involvement of pycnidiospores) suggests that pycnidiospores had a negligible role in the disease in the year of study. Since the fields were sampled only once at the flag leaf stage, information about the secondary cycle of inoculum cannot confidently be derived from the present study. It would be interesting to carry out multiple surveys at different times and to compare polymorphism data. However, the sampling in the present study was carried out at the time the flag leaf was severely infected (late in the season, but before senescence). Under these conditions, lesions from secondary inoculum would have been sampled, if they occurred. The fact that all pycnidia sampled were of different genotypes suggests that most lesions originated from ascospore infections and not from pycnidiospores, raising the question of whether ascospores acted as secondary inoculum (i.e. produced on leaves during the same growing season). This hypothesis does not appear likely under our conditions, given the short growing season and the time it takes for mature

pseudothecia to be produced. It is more likely that staggered maturation dates of pseudothecia (on stubble) and release of ascospores were responsible for the infestation observed in the field in 2001. In England, Hunter et al. (1999) reported that the time from the first observed symptoms of speckled leaf blotch in the crop to first detection of ascocarps on the growing plants was 94-140 days. The growing season in Manitoba is 100 days or less and the first disease symptoms in our wheat crops are not noticeable until mid July. Harvest begins in late August, which would not leave sufficient time for the ascocarps to mature. In addition to having a longer growing season, the weather conditions in temperate regions (England) are more favourable to disease development for a longer period of time. Therefore, overwintering pseudothecia on stubble likely play a predominant role as the source of primary inoculum in Manitoba. In our study, pseudothecia were found on 1- and 2- year-old stubble. Control measures that reduce stubble, such as rotation and tillage, would probably be very beneficial in reducing speckled leaf blotch disease in our province. Two or more years of rotation, away from wheat, may be needed in Manitoba.

The sexual state and its contribution to the genetic variation in the *M. graminicola* population of Manitoba was investigated. It would be desirable to perform a more in-depth study of the genetic variation of the pathogen by sampling more field locations. Since the teleomorph exists in Manitoba and appears to be widespread, a high degree of polymorphism would still likely be detected in a larger collection than the one used in this study. Also, the analysis of Set II (single stations) should be expanded. In our study, only a single station was chosen from each field. An investigation using more stations and isolates/station should be carried out to obtain a more accurate picture of the genetic

structure on the smallest scale. It would be interesting to see if more clones would be detected in a larger collection of isolates originating from the same leaf, as this was the only case in which we found identical isolates. However, a high level of polymorphism will still likely predominate at the level of a single station, considering the overwhelmingly high level of genetic variation found in the two *M. graminicola* populations used in the present study.

In conclusion, the results of this study demonstrated the presence of the sexual state of the pathogen in Manitoba and the significant role of ascospores in the epidemics of speckled leaf blotch. Larger populations and different sampling dates are needed to elucidate the role of pycnidiospores in the epidemiology of the disease under Manitoba conditions.

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7. APPENDICES

Appendix 1. Culture media.
Appendix 1a. Yeast-malt agar with antibiotic (YMA^{+}) medium:

Yeast extract . . . . . 4.0 g
Malt extract . . . . . 4.0 g
Sucrose . . . . . 4.0 g
Agar . . . . . 15.0 g
Distilled water . . . . . 1000.0 ml
Chloramphenicol . . . . . 0.25 g (added after autoclaving treatment)

Reference: Eyal, Z., Scharen, A.L., Prescott, J.M. and van Ginkel, M. 1987. The Septoria Diseases of Wheat: Concepts and Methods of Disease Management. International Maize and Wheat Improvement Center (CIMMYT), Mexico, DF. 52 pp.

Appendix 1b. Yeast sucrose liquid medium

Sucrose . . . . . .10 g
Yeast extract . . . . . .10 g
Distilled water . . . . . 1000 ml

Reference: Eyal, Z., Scharen, A.L., Prescott, J.M. and van Ginkel, M. 1987. The Septoria Diseases of Wheat: Concepts and Methods of Disease Management. International Maize and Wheat Improvement Center (CIMMYT), Mexico, DF. 52 pp.

Appendix 2

Analyses of banding patterns of *Mycosphaerella graminicola* isolates of Set I & II generated by AFLP assays.

Marker	Genotypes	a	N	b	β	p	α	PIC	MI
AFLP	Set I								
	M-AG E-CA	1	82	82	0.44	36	36	0.35	5.54
	M-AT E-CT	1	68	68	0.22	15	15	0.28	0.92
	M-AT E-CA	1	66	66	0.47	31	31	0.28	4.08
	All Set I genotypes	3	216	72	0.38	27.3	27.3	0.31	3.22
	Set II								
	M-CG E-AT	1	57	57	0.37	21	21	0.35	2.72
	M-CG E-AC	1	64	64	0.31	20	20	0.24	1.49
	All Set II genotypes	2	121	60.5	0.34	20.5	20.5	0.30	2.09

a: Number of assay units; N: Total number of bands; b: Number of bands per assay units; β: proportion of polymorphic bands; p: number of polymorphic bands per assay unit; and α: number of loci per assay unit

$$\text{PIC (polymorphic information content)} = 1 - \sum_{i=1}^{n} fi^2$$

where fi is the frequency of the ith allele in the set of 44 isolates

$$\text{MI (marker index)} = \text{PIC} \times \beta \times \alpha$$

Appendix 3

AMOVA ANALYSIS for different field stations at Carman and Minnedosa, Manitoba (Set I).

AMOVA design and results :

Reference: Weir, B.S. and Cockerham, C.C. 1984.
Excoffier, L., Smouse, P., and Quattro, J. 1992.
Weir, B. S., 1996.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	1	15.140	0.12682 Va	0.98
Within populations	35	447.833	12.79524 Vb	99.02
Total	36	462.973	12.92206	

Fixation Index FST : 0.00981

Significance tests (1023 permutations)

Va and FST : P(rand. value > obs. value) = 0.21505
P(rand. value = obs. value) = 0.00000
P(rand. value >= obs. value) = 0.21505+-0.01220

== Comparisons of pairs of population samples

List of labels for population samples used below:

Label	Population name
1:	Carman
2:	Minnedosa

Population pairwise FSTs

Distance method: Pairwise difference

	1	2
1	0.00000	
2	0.00981	0.00000

FST P values

Number of permutations : 1023

	1	2
1	*	
2	0.17285+-0.0105	*

Matrix of significant Fst P values
Significance Level=0.0500

Number of permutations : 1023

	1	2
1		-
2	-	

The distance (ie. Distinction) between populations 1 and 2 are not significant

Appendix 4

AMOVA ANALYSIS for single stations at Carman and Minnedosa, Manitoba (Set II).

AMOVA design and results :

Reference: Weir, B.S. and Cockerham, C.C. 1984.
Excoffier, L., Smouse, P., and Quattro, J. 1992.
Weir, B. S., 1996.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	1	21.074	0.72643 Va	11.04
Within populations	40	234.164	5.85409 Vb	88.96
Total	41	255.238	6.58052	

Fixation Index FST : 0.11039

Significance tests (1023 permutations)

Va and FST : P(rand. value > obs. value) = 0.00000
P(rand. value = obs. value) = 0.00000
P(rand. value >= obs. value) = 0.00000+-0.00000

== Comparisons of pairs of population samples

List of labels for population samples used below:

Label	Population name
1:	Carman-1 station
2:	Minnedosa-1 station

Population pairwise FSTs

Distance method: Pairwise difference

	1	2
1	0.00000	
2	0.11039	0.00000

FST P values

Number of permutations : 1023

	1	2
1	*	
2	0.00000+-0.0000	*

Matrix of significant Fst P values
Significance Level=0.0500

Number of permutations : 1023

	1	2	
1		+	Difference between C & M is significant
2	+		

Appendix 5

AMOVA ANALYSIS for single spores only from the single stations (Set II) at Carman and Minnedosa, Manitoba.

AMOVA design and results :

Reference: Weir, B.S. and Cockerham, C.C. 1984.
Excoffier, L., Smouse, P., and Quattro, J. 1992.
Weir, B. S., 1996.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	1	10.537	0.41762 Va	6.35
Within populations	19	117.082	6.16220 Vb	93.65
Total	20	127.619	6.57982	

Fixation Index FST : 0.06347

Significance tests (1023 permutations)

Va and FST : P(rand. value > obs. value) = 0.01173
P(rand. value = obs. value) = 0.00098
P(rand. value >= obs. value) = 0.01271+-0.00338

== Comparisons of pairs of population samples

List of labels for population samples used below:

Label	Population name
1:	Carman- 1 station
2:	Minnedosa- 1 station

Population pairwise FSTs

FST P values

Number of permutations : 1023

	1	2
1	*	
2	0.00684+-0.0023	*

Matrix of significant Fst P values
Significance Level=0.0500

Number of permutations : 1023

	1	2
1		+
2	+	

Appendix 6 AFLP binary scores of twenty *Mycosphaerella graminicola* isolates collected at different field stations in Carman, Manitoba using three primer combinations.

Primers	Marker	9636	2S S3	BS 1	CS 1	C1 21	C2 11	C4 22	C5 41	C6 12	C7 11	C8 22	C9 11	C1 011	C1 112	C1 211	C1 312	C1 412	C1 511	C1 631	C1 732	C1 821	C1 912	C2 011	C2 071
AG/CA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0
AG/CA	2	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
AG/CA	3	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
AG/CA	4	0	1	0	0	1	0	0	1	1	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0
AG/CA	5	1	1	1	1	1	0	1	0	0	1	1	1	0	1	1	0	1	1	0	0	0	1	0	1
AG/CA	6	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AG/CA	7	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	1	0	1	1	1	0
AG/CA	8	1	1	1	1	1	1	0	1	1	0	0	1	1	0	0	1	1	1	1	0	1	1	1	1
AG/CA	9	1	1	1	1	0	0	1	1	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	1
AG/CA	10	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	1
AG/CA	11	0	1	0	0	1	1	1	1	0	1	1	0	1	0	0	0	1	0	1	0	0	1	1	0
AG/CA	12	1	0	0	0	1	0	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	1	0	0
AG/CA	13	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
AG/CA	14	1	1	0	0	1	0	1	0	0	1	0	1	1	0	1	0	1	1	0	1	1	0	1	0
AG/CA	15	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	0
AG/CA	16	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
AG/CA	17	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
AG/CA	18	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
AG/CA	19	0	1	0	0	0	1	1	1	0	1	0	0	0	0	1	0	1	1	0	1	0	0	1	0
AG/CA	20	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
AG/CA	21	1	0	1	1	1	0	0	0	0	0	0	0	1	1	0	1	0	0	1	0	1	1	0	1
AG/CA	22	0	1	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	1	0	1	0	0	0	0
AG/CA	23	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0
AG/CA	24	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
AG/CA	25	0	1	1	1	0	1	0	0	0	1	1	1	1	1	0	1	0	0	0	0	0	1	1	1
AG/CA	26	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
AG/CA	27	0	1	1	1	0	1	0	0	0	1	1	1	1	1	0	1	0	0	0	0	0	1	1	1
AG/CA	28	0	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
AG/CA	29	0	1	1	1	1	0	1	0	1	0	0	1	0	0	0	1	1	1	1	0	0	0	1	1
AG/CA	30	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AG/CA	31	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	0
AG/CA	32	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0
AG/CA	33	1	1	0	1	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	0	0	0	0	0
AG/CA	34	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0
AG/CA	35	0	0	1	1	0	0	0	0	1	0	1	0	1	1	1	1	0	1	0	0	0	0	0	0
AG/CA	36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CT	37	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0
AT/CT	38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0
AT/CT	39	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	1	0	0
AT/CT	40	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
AT/CT	41	0	1	0	1	1	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	1	0	1	0
AT/CT	42	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	1	0
AT/CT	43	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CT	44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0
AT/CT	45	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0

AT/CT	**46**	1	1	1	1	0	1	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	0	0	1
AT/CT	**47**	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	0	0
AT/CT	**48**	0	1	0	0	0	1	0	0	1	1	0	1	1	1	0	1	0	1	1	1	0	1	0	1
AT/CT	**49**	1	1	1	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0
AT/CT	**50**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CT	**51**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
AT/CA	**52**	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
AT/CA	**53**	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CA	**54**	0	1	0	1	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	0	0	0	0	0
AT/CA	**55**	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0
AT/CA	**56**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
AT/CA	**57**	0	0	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
AT/CA	**58**	1	1	0	0	1	1	0	1	1	0	0	1	1	1	0	1	0	0	1	1	1	1	1	1
AT/CA	**59**	1	1	0	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	1	0	1	1	0	0
AT/CA	**60**	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
AT/CA	**61**	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	0	1	0	0	0
AT/CA	**62**	0	0	0	0	1	1	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0
AT/CA	**63**	1	1	0	0	1	1	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0
AT/CA	**64**	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0
AT/CA	**65**	1	1	0	1	1	1	0	1	1	0	1	0	0	1	1	1	1	1	1	0	1	1	0	0
AT/CA	**66**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
AT/CA	**67**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
AT/CA	**68**	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
AT/CA	**69**	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CA	**70**	0	0	1	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0	1
AT/CA	**71**	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0
AT/CA	**72**	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1
AT/CA	**73**	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CA	**74**	0	1	0	0	1	1	1	1	1	0	0	1	0	0	1	1	1	1	0	1	1	1	1	1
AT/CA	**75**	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
AT/CA	**76**	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0
AT/CA	**77**	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
AT/CA	**78**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
AT/CA	**79**	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
AT/CA	**80**	0	0	1	1	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0
AT/CA	**81**	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
AT/CA	**82**	1	1	0	1	1	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0

Appendix 7 AFLP binary scores of twenty *Mycosphaerella graminicola* isolates collected at different field stations in Minnedosa, Manitoba using three primer combinations.

Primers	Marker	M1 11	M2 11	M4 12	M5 21	M6 12	M7 22	M8 21	M9 11	M1 012	M1 122	M1 211	M1 322	M1 412	M1 511	M1 542	M1 562	M1 711	M1 811	M19 91	M20 11
AG/CA	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1
AG/CA	2	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0
AG/CA	3	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
AG/CA	4	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0
AG/CA	5	1	1	0	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	1
AG/CA	6	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1
AG/CA	7	1	0	1	0	0	1	0	0	1	0	1	0	1	0	0	1	0	0	1	0
AG/CA	8	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	0	1	1	1	1
AG/CA	9	0	0	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	1	0	1
AG/CA	10	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	1	1	0	1	1
AG/CA	11	1	0	1	1	0	0	0	1	0	1	1	1	1	0	0	0	1	1	0	1
AG/CA	12	0	0	1	0	1	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1
AG/CA	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
AG/CA	14	0	1	1	0	0	0	0	0	1	1	0	0	1	1	1	1	0	1	0	1
AG/CA	15	0	0	1	0	1	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0
AG/CA	16	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0	0
AG/CA	17	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0
AG/CA	18	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
AG/CA	19	1	1	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1
AG/CA	20	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
AG/CA	21	0	0	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	1	1	0
AG/CA	22	1	1	0	1	0	1	0	1	1	1	0	1	1	0	1	0	1	0	1	1
AG/CA	23	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
AG/CA	24	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0
AG/CA	25	0	1	0	1	1	1	0	1	1	0	1	1	0	1	1	1	0	1	1	1
AG/CA	26	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
AG/CA	27	0	1	0	1	1	1	0	1	1	0	1	1	0	1	1	1	0	1	1	1
AG/CA	28	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
AG/CA	29	0	0	1	1	0	1	1	1	1	1	0	1	1	0	0	1	1	1	0	1
AG/CA	30	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	1	0	0	0	0
AG/CA	31	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	1	1
AG/CA	32	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1
AG/CA	33	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0
AG/CA	34	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
AG/CA	35	0	0	1	0	0	0	1	0	0	0	0	1	1	0	1	1	0	1	0	0
AG/CA	36	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
AT/CT	37	0	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1
AT/CT	38	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CT	39	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
AT/CT	40	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
AT/CT	41	1	0	0	0	1	0	0	1	0	0	0	0	1	1	1	0	1	1	1	1
AT/CT	42	1	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
AT/CT	43	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
AT/CT	44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

AT/CT	45	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CT	46	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	0	0	1
AT/CT	47	0	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	0	0	0	1
AT/CT	48	1	1	1	1	0	0	0	1	1	1	0	1	1	1	1	0	0	1	1	1
AT/CT	49	0	0	0	1	0	1	1	0	0	0	0	0	1	1	1	1	0	0	1	1
AT/CT	50	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CT	51	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CA	52	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CA	53	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
AT/CA	54	0	0	0	0	0	1	0	0	1	0	1	1	0	0	0	1	0	0	0	0
AT/CA	55	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0
AT/CA	56	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CA	57	0	1	0	0	0	0	0	1	1	0	1	1	1	0	0	0	0	0	1	0
AT/CA	58	1	0	1	1	0	0	1	0	0	1	0	0	0	1	1	1	1	0	1	1
AT/CA	59	0	1	0	1	0	1	0	1	1	1	1	1	1	0	0	1	0	0	0	1
AT/CA	60	0	1	0	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0
AT/CA	61	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	1
AT/CA	62	0	1	0	1	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	0
AT/CA	63	0	1	0	1	0	1	0	0	1	1	0	0	1	0	0	1	0	0	0	0
AT/CA	64	0	1	0	1	0	0	1	0	1	0	0	0	1	0	1	1	0	0	0	0
AT/CA	65	0	1	0	1	0	1	1	1	1	1	1	1	1	0	0	1	0	0	0	1
AT/CA	66	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CA	67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
AT/CA	68	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
AT/CA	69	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0	1	1
AT/CA	70	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
AT/CA	71	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
AT/CA	72	0	1	1	0	1	1	1	0	0	0	0	0	0	1	0	1	0	0	0	0
AT/CA	73	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AT/CA	74	1	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1	1	1
AT/CA	75	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
AT/CA	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
AT/CA	77	0	0	1	0	0	1	1	0	0	0	0	1	1	1	1	1	1	0	0	1
AT/CA	78	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
AT/CA	79	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1	0	0	0	0	0
AT/CA	80	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1
AT/CA	81	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
AT/CA	82	0	0	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0

Appendix 8 AFLP binary scores of 22 *Mycosphaerella graminicola* isolates collected at a single field station in Carman, Manitoba using two primer combinations.

Primers	Marker	C131sp	C131ss	C132sp	C132ss	C141sp	C141ss	C142sp	C142ss	C153sp	C153ss	C162sp	C162ss	C172sp	C172ss	C181sp	C181ss	C183sp	C183ss	C192sp	C192ss	C1101sp	C1101ss
CG/AT	1	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1
CG/AT	2	0	0	0	0	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1
CG/AT	3	1	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	0	0	0	0
CG/AT	4	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0
CG/AT	5	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	8	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	9	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	12	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	13	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	14	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	15	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
CG/AT	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
CG/AT	18	0	0	0	0	1	1	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	1
CG/AT	19	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
CG/AT	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
CG/AT	21	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
CG/AC	22	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
CG/AC	23	1	1	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0
CG/AC	24	1	1	0	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	1
CG/AC	25	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
CG/AC	26	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0
CG/AC	27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
CG/AC	28	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	1
CG/AC	29	0	0	0	0	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0	1	1
CG/AC	30	1	1	0	0	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0
CG/AC	31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
CG/AC	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AC	33	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	0	0	0	1	1	0	0
CG/AC	34	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	1	1	1
CG/AC	35	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
CG/AC	36	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	1	0	0
CG/AC	37	0	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CG/AC	38	0	0	1	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	1	1	0	0
CG/AC	39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
CG/AC	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
CG/AC	41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	1

Appendix 9 - AFLP binary scores of twenty *Mycosphaerella graminicola* isolates collected at a single field station in Minnedosa, Manitoba using two primer combinations.

Primers	Marker	M1 21s p	M1 21s s	M1 31s p	M1 31s s	M1 32s p	M1 32s s	M1 51s p	M1 51s s	M1 61s p	M1 61s s	M1 62s p	M1 62s s	M1 71s p	M1 71s s	M1 81s p	M1 81s s	M1 91s p	M1 91s s	M1 102 sp	M1 102 ss
CG/AT	1	1	1	1	1	0	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1
CG/AT	2	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0
CG/AT	3	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
CG/AT	4	0	0	1	1	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0
CG/AT	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
CG/AT	7	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	8	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1
CG/AT	9	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1
CG/AT	10	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
CG/AT	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
CG/AT	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0
CG/AT	15	0	0	1	1	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0
CG/AT	16	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AT	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
CG/AT	19	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CG/AT	20	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0
CG/AT	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
CG/AC	22	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	1	1	1
CG/AC	23	1	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0
CG/AC	24	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1
CG/AC	25	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
CG/AC	26	1	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0
CG/AC	27	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CG/AC	28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
CG/AC	29	1	1	1	1	0	0	1	1	0	0	1	1	1	1	1	1	1	1	0	0
CG/AC	30	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0
CG/AC	31	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
CG/AC	32	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
CG/AC	33	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	0	0
CG/AC	34	0	0	1	1	1	1	0	0	1	1	0	0	1	1	1	1	1	1	1	1
CG/AC	35	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	1	1	1
CG/AC	36	1	1	1	1	0	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1
CG/AC	37	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
CG/AC	38	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CG/AC	39	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
CG/AC	40	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
CG/AC	41	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1