PATHOGENIC AND MOLECULAR VARIABILITY IN A POPULATION OF *MYCOSPHAERELLA GRAMINICOLA*, CAUSE OF SEPTORIA TRITICI LEAF BLOTCH OF WHEAT

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ABSTRACT

Mycosphaerella graminicola, cause of Septoria tritici leaf blotch of wheat, is an important disease throughout the world. Information about the population structure of the pathogen is useful to improve control strategies. Molecular studies have shown that there was a high level of genetic variability within populations of *M. graminicola*, but no attempt was made to relate this variability to pathogenicity. The objectives of this research were to study a population of *M. graminicola* for both variability at the molecular level and pathogenicity and to determine whether any relationship between these two types of variability exists.

Ninety isolates of *M. graminicola* were collected using hierarchical sampling of leaves and lesions from 10 locations within a wheat field near Saskatoon. The isolates were tested for the degree of pathogenicity (aggressiveness) on a single susceptible cultivar and the components of pathogenicity, incubation period, latent period and disease severity, were evaluated. There were significant differences among isolates for all components at the lesion sampling level only but not at the leaf and location level. A subsample of 40 isolates was tested for variability for virulence on a set of six differential cultivars. A significant isolate x cultivar interaction was detected, but since the magnitude of the variability was low no attempt was made to classify the isolates into races.

Using 15 RAPD primers the percentage of polymorphic loci, number of molecular phenotypes (haplotypes) and gene diversity of 90 isolates were estimated. A high level of genetic variability was found within the population. Partitioning this variability into different components showed that most of the variability was distributed within

locations. This type of distribution suggested that air-borne ascospores were the primary source of inoculum in the field. A similar type of distribution of genetic variability was detected using eight microsatellite markers.

Little relationship between molecular and pathogenic variability was found, suggesting that DNA fingerprinting has little value for monitoring the development of new virulent genotypes of the pathogen.

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LIST OF ABREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic acid
NTSYS	Numerical Taxonomy and Multivariate Analysis System
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SCAR	Sequence Characterized Amplified Region
SIMQUAL	Similarity of Qualitative data
SSRs	Simple Sequence Repeats
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
UBC	University of British Columbia
UPGMA	Unweighted Pair Group Method with arithmetic Average
WA	Water Agar
YMDA	Yeast Malt Dextrose Agar

CHAPTER 1

INTRODUCTION

Mycosphaerella graminicola (Fückl) J. Schröt. in Cohn (anamorph *Septoria tritici* Roberge in Desmaz.) causes Septoria tritici blotch, an important disease of wheat with worldwide distribution. It is particularly important in some European countries and in North America. This disease has the potential of reducing yields by 30-40% (Eyal et al. 1987) and in 1998 in the UK alone, losses were estimated to be £35.5 million (Hardwick et al. 2001). The annual yield reduction caused by Septoria tritici blotch (STB) and Septoria nodorum blotch (SNB) averages 1% in the United States (Coakley et al. 1985), and was estimated to be approximately nine million tonnes worldwide in 1982, a loss valued at over $1 billion (Scharen and Sanderson 1985; Eyal and Levy 1987). In the northern cropping areas of Saskatchewan, the leaf spotting complex, of which *M. graminicola* is an important component, may cause up to a 15% yield loss (G. R. Hughes, personal communication).

M. graminicola reproduces both sexually and asexually. The sexual spores are ascospores, which are air-borne and are the primary source of inoculum where the sexual stage occurs. The sexual stage of this fungus was first discovered in New Zealand (Sanderson 1972) and since then has been reported in Australia (Brown et al.1978), United Kingdom (Scott et al. 1988), United States (Garcia and Marshall 1992), France (Halama 1996) and Canada (Hoorne et al. 2002). The asexual spores are pycnidiospores which are dispersed over short distances by rain splash.

Septoria tritici blotch can be controlled by cultural practices, foliar fungicides and resistant varieties. Information about the genetic structure of the pathogen population is useful for development of disease-management strategies. Genetic structure refers to the amount and distribution of genetic variation within and among pathogen populations.

Early studies evaluated the genetic variation among isolates collected from a wide geographical area (Eyal et al. 1985; Eyal and Levy 1987). Such studies provide insight into the range of variability existing over a large geographical area, and probably among isolates representing different populations. However, to study the genetic structure of a single pathogen population, isolates of the pathogen have to be collected from a more restricted geographical area, e. g., a single field.

McDonald and Martinez (1990b, 1991) studied the genetic structure of *M. graminicola* using RFLP markers and showed that there was a high level of genetic variability among different locations within a field, among lesions of one leaf and even among the pycnidia of a single lesion. However, no attempt was made to relate this variability at the molecular level to variability for pathogenicity. From an epidemiological perspective, it is important to know whether variability at the molecular level is just neutral variability or whether it is related to pathogenicity.

Currently, two views exist regarding the pathogenicity of *M. graminicola.* Most studies suggest that there are true virulence differences among isolates of the pathogen (Eyal et al. 1985; Eyal and Levy 1987; Kema et al. 1996a, 1996b). However, others believe that differences in virulence do not exist; the only difference among isolates is based on the degree of pathogenicity (Marshall 1985; van Ginkel and Scharen 1988; van Ginkel and Rajaram 1995). Since the type of pathogenic variability present in the

population will influence the resistance breeding strategy used, it is necessary to determine whether differences in pathogenicity within the pathogen population are due to virulence or to degrees of pathogenicity (aggressiveness), or to possibly both.

This study tested the hypothesis that that there is a relationship between variability at the molecular level and variability for pathogenicity within a population of *M. graminicola*. Therefore, the objectives of this study were:

1. to investigate the variability for degree of pathogenicity (aggressiveness) within a population of isolates of *M. graminicola* collected at different sampling levels;
2. to investigate the variability for virulence among these isolates;
3. to study the molecular genetic variability of the population using DNA markers; and
4. to examine the relationship between pathogenic and molecular variability.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy and nomenclature

The genus *Septoria* is classified in the class Deuteromycetes (imperfect fungi) and the order Sphaeropsidales, which comprises more than 2000 form-species. Many of these cause economically important leaf spot diseases on cereals, grasses and other crops (Sprague 1950). Some important pathogens of cereals, which originally had been placed in *Septoria,* have been transferred to the genus *Stagonospora* (Cunfer and Ueng 1999).

Septoria tritici Roberge in Desmaz., the cause of Septoria tritici blotch of wheat is the most economically important species of the genus *Septoria* on cereals. Two other species, *Septoria passerinii* Sacc., the cause of speckled leaf blotch of barley, and *Septoria secalis* Prill&Delacr., the cause of leaf spot of rye, are not economically important and are considered minor pathogens of cereals (Sprague 1944, Sprague 1950). The sexual state of *S. tritici* is *Mycosphaerella graminicola* (Fückl) J. Schröt. in Cohn, which is classified in the class Loculoascomycetes (filamentous ascomycetes), order Dothideales, and family Dothideaceae (Palmer and Skinner 2002).

In the sexual stage, *M. graminicola* produces pseudothecia which are subepidermal, globose, dark brown in color and 70-100 μm in diameter. Ascospores are hyaline, eliptical and 10-15 x 2-3 μm with two unequal-sized cells, (Eyal et al. 1987). The anamorph *Septoria tritici* produces slender and elongated pycnidiospores, which are

enclosed within a pycnidium. Pycnidia are embedded in the epidermal and mesophyll tissue of the leaf and can be detected visually. *S. tritici* produces two forms of pycnidiospores, macropycnidiospores (35-98 x 1-3 μm) with 3-5 septa and micropycnidiospores (8-10.5 x 0.8-1 μm) with no septa. Both of these pycnidiospores are pathogenic on wheat (Shipton et al. 1971). Shearer and Wilcoxson (1978) reported that spore size was influenced by external factors such as temperature, leaf wetness and possibly age of host tissue. They found that spore size diminished with increased temperature above 18°C and with increased wetness.

2.2 Disease Symptoms

Symptoms of Septoria tritici blotch on wheat usually appear on leaves and culms, but may occasionally occur on the rachis and glumes. Generally, lesions develop first on the tips of lower leaves as small chlorotic areas, which later enlarge into elongated light-brown necrotic spots. Under favorable environmental conditions, lesions coalesce and the leaf tissue takes on an ash-gray to light gray color (Pedersen 1989).

Pycnidia develop in necrotic lesions and their color ranges from light to dark brown. They are scattered within the lesion and can be detected on both the upper and lower surfaces of the leaf (Eyal et al. 1987). The size of pycnidia may vary among different cultivars and may also be affected by the density of pycnidia in the infected tissue. Eyal and Brown (1976) reported that as the number of pycnidia on the leaf increased their size decreased.

2.3 Infection process and histology of pathogenesis

Under favorable environmental conditions both the sexual ascospores and the asexual pycnidiospores can germinate and penetrate plant tissues after landing on a host leaf

(Palmer and Skinner 2002). Penetration of the leaf surface is almost exclusively through stomata (Cohen and Eyal 1993; Duncan and Howard 2000; Hilu and Bever 1957; Kema et al. 1996d). Kema et al. (1996d) reported that infection was a random process, since many germ tubes crossed stomata without penetrating them. After penetration, the fungus colonizes the mesophyll tissue of the leaf by growing intercellularly, but does not produce any feeding structures such as haustoria (Palmer and Skinner 2002).

Kema et al. (1996d) found that fungal hyphae grow prolifically in a susceptible cultivar, while in a resistant cultivar fungal colonization is restricted. They observed condensation of chloroplasts followed by intense swelling of cells in the susceptible cultivar, believed to be a response to soluble compounds produced by the fungus. In addition to chloroplast alteration, starch granules were also released from the chloroplasts (Kema et al. 1996d). The role of starch release is not known, but it may be a response by the plant to prevent further colonization of the fungus (Kema et al. 1996d). In incompatible reactions the fungus was restricted to substomatal chambers and no visible detrimental effect in the surrounding mesophyll cells was observed. These authors also suggested that soluble toxic compounds and pectin-degrading enzymes may be involved in pathogenicity of *M. graminicola*. They observed that in compatible reactions, mesophyll cells were severely affected in advance of the invasion by the fungus and cell collapse occurred within a short period of time.

Kema et al. (1996d) did not observe papilla formation or thickening of the walls of the mesophyll cells of the resistant cultivar. They suggested that resistance does not rely on defense responses to fungal cell wall-degrading enzymes, but rather on the production of compounds that prevent fungal colonization and hence, pycnidium

formation. Since the growth of the fungus is strictly intercellular, it has been suggested that intercellular washing fluids need to be analyzed to help understand the biochemical basis of resistance in this pathosystem (Kema et al. 1996d).

2.4 Epidemiology of the disease

Infected crop residue and volunteer wheat plants are important sources of primary inoculum. Pycnidiospores survive in pycnidia on infected stubble for several months. In some countries ascospores released from pseudothecia are important sources of primary inoculum (Sanderson and Hampton 1978; Shaw and Royle 1989).

Recent studies indicate that ascospores do not originate only from overwintering stubble or volunteer plants as previously thought, but are also released from ascocarps produced on infected leaves of the current wheat crop (Hunter et al. 1999). This agrees with observation of Kema et al. (1996c) who showed that under laboratory conditions the pathogen is able to complete a sexual cycle within five weeks. Analysis of field samples also showed that ascospores were released from wheat debris and from young wheat plants during the fall, spring and summer seasons. They concluded that *M. graminicola* is able to complete several sexual cycles per season. Therefore, they suggested that after establishment of the disease, progress of Septoria tritici blotch does not merely depend on splash-dispersed pycnidiospores (Shaw and Royle 1993; Royle 1994), but also on air-borne ascospores produced during the growing season (Kema et al. 1996c).

Pycnidiospores are the most common secondary inoculum source and can remain viable for several months at temperatures between 2 °C and 10 °C. The slime (cirrhus), in which the spores are exuded, protects them from radiation and desiccation and

stimulates their germination. Conidia produced during wet periods are disseminated by rain splash from debris or the lower leaves of a crop to the upper leaves of the canopy (vertical transport) or to the surrounding leaves (horizontal transport). Horizontal transport may also occur in the absence of rain splash through leaf contact (Royle et al. 1995).

The optimum temperature for the development of Septoria tritici blotch is between 15 and 20°C (Wiese 1987). Magboul et al. (1992) reported that at a fixed leaf wetness period (96h), the maximum lesion unit per square centimeter of leaf area was obtained at 20°C but, at higher or lower temperatures, the number of lesions per leaf area was reduced.

A second important factor for dissemination and development of *M. graminicola* is moisture (Shaner and Finney 1976). Hess and Shaner (1987) reported that disease severity on the flag leaves under controlled conditions increased as the moist period increased from 24 to 96 h. Similarly, Magboul et al. (1992) showed that there was a linear relationship between leaf wetness period and the rate of disease development. Pycnidiospores are released from pycnidia when the relative humidity is near 100% (Eyal 1971).

Recently, Chungu et al. (2001) studied the effects of incubation temperature, leaf wetness duration, inoculum concentration and the interaction between leaf-wetness duration and inoculum concentration on the development of Septoria tritici blotch at the seedling stage in two bread and two durum wheats. They found that pycnidia were observed four days earlier when the incubation temperature increased from 18°C day/15°C night to 22°C day/15°C night or when the inoculum concentration increased

from $1x10^6$ spores/ml to $1x10^7$ spores/ml. They also found that there were more pycnidia when the duration of leaf wetness was 72h rather than 48 or 60h.

Shaner and Buechley (1995) reported that initial symptoms in winter wheat in Indiana usually appeared early in the spring on leaves near or touching the soil surface. This suggests that primary infection takes place during the fall before the onset of cold weather and that *M. graminicola* overwinters as asymptomatic infections. They showed that disease severity is generally low until after all of the leaves of the plant have expanded, however, once the flag leaf has emerged disease severity increases rapidly. They found that under favorable environmental conditions, all of the foliage can be blotched within 25 to 35 days after spike emergence.

2.5 Control Methods

The disease can be controlled by application of cultural practices, resistant cultivars and foliar fungicides.

2.5.1 Disease assessment

Assessment of the disease is essential to timely implement disease control methods, for the study of the genetic variability of the pathogen or for screening germplasm for resistance. Eyal et al. (1987) reviewed methods of assessing the severity of Septoria diseases. Disease rating is usually based on necrotic leaf area, the density of pycnidia or a combination of these two. James (1971) used an electronic scanner to develop standard diagrams for estimating percentage affected area and Eyal and Brown (1976) used a television scanner to develop a scale to quantify pycnidial coverage on leaves. Although these methods provide a more accurate estimate of disease severity, the preparation of samples for scanning is extremely labor intensive, time consuming and

expensive. Adequate estimates of disease severity have been obtained from visual ratings, which require less time and no equipment.

Different types of visual rating scales have been used. Saari and Prescott (1975) developed a 0-9 scale for evaluating the severity of foliar diseases other than rusts in wheat, barley and triticale. This method was later improved to a double-digit (00-99) scale (Eyal et al. 1987). The first digit gives the relative height of the disease on the plant using the original 0-9 Saari-Prescott scale as a measure and the second digit shows disease severity as a percentage where, 0 = 0% and 9 = 90% (Eyal et al. 1987). This method has been useful for plant breeders who require a method suitable for rapid screening of breeding lines in the field. Rosielle (1972) developed a scale which combined pycnidial density and the amount of necrosis. This method or a variation of it has been widely used for the classification of plants into resistant or susceptible categories. Brokenshire (1976) used different parameters including incubation period, latent period, disease severity and sporulation level to classify the reaction of wheat accessions to *M. graminicola*.

There are also other techniques that can be used to assess the severity of the disease under laboratory conditions. Arraiano et al. (2001b) used a detached seedling leaf technique to study resistance to *M. graminicola* in wheat. They found that there was a close relationship between the results of field and seedling trials to those of detached leaf tests under a wide range of environmental conditions. An important difference between the method of Arraiano et al. (2001b) and those of previous reports was that the entire seedling was sprayed evenly before cutting sections from the leaves, whereas in earlier studies a drop of inoculum was placed on the surface of the detached leaf.

They also kept the leaves in darkness for 48 h after inoculation to promote penetration and infection efficiency of *M. graminicola* isolates. In previous tests, failure was often due to senescence of the leaf segments during the long incubation time, which was needed for the development of disease symptoms. Arraiano et al. (2001b) used an agar medium containing benzimidazole and inserted the cut end of the leaf segment into the agar, which resulted in the segment remaining green for about 30 days.

2.5.2 Resistant cultivars

One of the most important, economical and environmentally safe control methods is to use resistant cultivars. Several reviews of the genetics of resistance to Septoria tritici blotch of wheat and of breeding for resistance are available (Shipton et al. 1971; King et al. 1983; Eyal 1999).

In most breeding programs, finding sources of resistance and utilizing them have the highest priorities. The best sources of resistance to Septoria tritici blotch are reported to be Russian winter wheats (Aurora, Bezostaya 1, Kavkaz and others), wheat lines from Argentina, Brazil and Urguay and, to a lesser extent, lines from the United States (Mann et al. 1985). Recently, by crossing durum wheat with accessions of *Aegilops squarrosa* (syn: *Triticum tauschii* and *Ae. tauschii*) new synthetic hexaploid sources of resistance have been developed. Some of these accessions have shown a high level of resistance to *M. graminicola* (van Ginkel and Rajaram 1999).

Inheritance of resistance to *M. graminicola* has been reported to be monogenic, oligogenic or polygenic. For example, resistance in the spring wheat accessions Bobwhite 'S' (CIMMYT, Mexico) and Kavkaz/K4500 L.6.A.4 (CIMMYT, Mexico) was controlled by several genes with additive effects (Gilchrist and Velazquez, 1994;

Jlibene et al. 1994; Dubin and Rajaram 1996). Resistance in cultivars Colotana and Klein Titan derived from Frontana, and was controlled by at least two recessive genes (Danon and Eyal, 1990). Resistance in Tadinia, Bulgaria 88 (Rillo and Caldwell 1966), Israel 493 and Veranopolis (Wilson 1979; Ballantyne and Thompson 1995) was controlled by a single dominant gene. The gene for resistance in Bulgaria 88, Oasis and Sulivan has been designated as *Stb1*, in Veranopolis and Nova Prata as *Stb2*, in Israel 493 as *Stb3* and in Cleo, Tadinia and Tadorna as *Stb4* (McIntosh et al. 1998).

Arraiano et al. (2001a) reported a new gene (*Stb5*) for resistance to Septoria tritici blotch in Sears's "synthetic 6X" hexaploid wheat. *Stb5* was mapped to the short arm of chromosome 7D near the centromere. A high level of resistance to *M. graminicola* has also been reported in *Triticum dicoccum*, *T. carthicum*, *T. polonicum*, *T. pyramidale*, *T. durum* and *Triticale* (Rosielle 1972; King et al. 1983).

Recently, McCartney et al. (2002) studied the inheritance of resistance in intra-specific reciprocal crosses between hexaploid wheat lines Salamouni, ST6, Katepwa, and Eric, and the durum wheat lines Coulter and 4B1149 to two isolates of *M. graminicola* under controlled environment experiments. They found that resistance was controlled by incompletely dominant genes in all cases. The cultivar Salamouni had three independent resistance genes effective against isolate MG 2, two of which also controlled resistance to isolate MG 96-36. The cultivar ST6 had a single resistance gene that conferred resistance to isolate MG 2 and no gene for resistance to isolate MG 96-36. The resistance genes in Salamouni and ST6 were not allelic. Two independent genes controlled resistance to isolate MG 2 in Coulter, one of which also controlled resistance

to isolate MG 96-36. They concluded that a gene-for-gene interaction is operating in the wheat-*M. graminicola* pathosystem (McCartney et al. 2002).

Brading et al. (2002) investigated the inheritance of resistance in two winter wheat cultivars Flame and Hereward. The same single, semidominant gene controlled resistance to isolate IPO 323 in each of the resistant cultivars. Using microsatellite markers, they mapped the resistance gene in the cultivar Flame to the short arm of chromosome 3A and designated it *Stb6*. They also studied the inheritance of avirulence by crossing isolate IPO 323 with a virulent one. Their results also suggested that isolate-specific resistance of wheat to Septoria tritici blotch follows a gene-for-gene relationship.

2.5.3 Cultural practices

One of the important means of carryover of the pathogen from one growing season to the next is survival on plant residue. To reduce the severity of the disease, crop rotation in combination with other cultural practices has been recommended (Krupinsky 1999). The use of crop rotation provides an opportunity for decomposition of infected plant residue while non-host crops are being grown (Krupinsky 1999). Using crop rotation may not eliminate the disease but will cause a reduction in the inoculum level of the pathogen.

Pedersen and Hughes (1992) found that crop rotation was effective in reducing the severity of the Septoria disease complex in north-central Saskatchewan. Under high disease pressure, a rotation of two years between wheat crops delayed the development of an epidemic and gave greatest reduction in disease severity. Eyal (1981) reported that a 3-5 year rotation decreased the incidence of Septoria tritici blotch in Israel, but even a

6-8 year interval between crops did not completely eliminate outbreaks of the disease. For southeastern Saskatchewan, Fernandez et al. (1998) recommended two years of spring wheat followed by two years of a non-cereal crop or a non-cereal crop and summer fallow to reduce the severity of leaf spotting disease.

In addition to crop rotation, claims have been made that inter-cropping can be effective in reducing inoculum movement in the field. Bannon and Cooke (1998) reported that the dispersal of pycnidiospores of *S. tritici* was reduced 33% horizontally and 63% vertically in a wheat-clover intercrop system.

The use of cultivar mixtures has also reduced disease severity. Mundt et al. (1995) reported that, on average, cultivar mixtures reduced severity of Septoria tritici blotch by 27, 9 and 15% in three successive seasons compared to the mean severity on pure stands of the components of the mixtures. In another study, Mundt et al. (1999) reported that progression of the epidemic was reduced in mixtures of a susceptible and moderately resistant cultivars, sometimes to below the level of the more resistant cultivar grown in pure stand.

Reports of the effect of nitrogen fertilizer on the severity of Septoria tritici blotch on wheat are contradictory. Shipton et al. (1971) reported that high rates of nitrogen fertilizer increased severity, while Tompkins et al. (1993) in Saskatchewan found that greater disease severity was associated with low nitrogen fertility and suggested that lesion development might be promoted by nitrogen deficiency. Similar results were reported by Fernandez et al. (1998) who found that in dry years, nitrogen deficiency increased the severity of the leaf-spotting disease complex.

Other cultural practices including burning stubble and deep ploughing have been reported to be effective in reducing the severity of the disease (Brown and Rosielle 1980). However, because of environmental concerns, these practices are not recommended.

2.5.4 Chemical control

Where the disease causes substantial yield loss, use of foliar fungicides is generally economic. In the United Kingdom, over 95% of the wheat crop is sprayed annually (Cook 1999).

A range of fungicides is now available to control this disease. Protectant fungicides such as the dithiocarbamates (Maneb, Manzate, Mancozeb, Zineb) have been reported to be effective in controlling Septoria tritici blotch, but they require repeated application at 10-14 day intervals (Eyal et al. 1987).

Systemic fungicides with curative properties such as benomyl (Benlate), prochloraz (Sportak), triadimefon (Bayleton) and propiconazole (Tilt) provide a longer time period of protection than do dithiocarbamates (Eyal et al. 1987). In New Zealand, a single spray of benomyl at the 4-5 leaf growth stage was sufficient to control Septoria tritici blotch of wheat (Sanderson and Gaunt 1980). However, there is evidence that isolates of the pathogen have developed resistance to this group of fungicides (Zelikovitch et al. 1986) and combining protectant and systemic fungicides provides better control of the disease. The systemic fungicides lengthen the period of protection and the protectant fungicide reduces the selection pressure on the pathogen exerted by the systemic fungicides and expands the control spectrum and longevity of control (Eyal et al. 1987).

2.6 Pathogenic specialization

There have been conflicting reports regarding the existence of specific virulence in the wheat-*M. graminicola* pathosystem. Some researchers believe that there are no true differences in the virulence of isolates, the only difference is in their degree of pathogenicity (aggressiveness). Marshall (1985) studied genetic variability of *M. graminicola* on spring and winter wheat cultivars at three locations in California and 13 locations in nine other states in both field and greenhouse conditions. He found that populations of *M. graminicola* tend to be more aggressive in central and north central California as well as northern regions of Indiana and Ohio, whereas populations from northern Alabama, Mississippi, Eastern Arkansas, southern Nebraska and central Kansas were less aggressive. van Ginkel and Scharen (1988) tested 34 isolates of *M. graminicola* from Tunisia, Turkey, Israel, Syria, Portugal, Italy and Spain on 13 durum wheat and one bread wheat accessions. Significant differences were found among cultivars and among isolates for disease severity. However, the cultivar x isolate interaction component was small (less than 0.3%) and not significant. They concluded that the isolates varied in aggressiveness and the cultivars varied in race non-specific resistance. van Ginkel and Rajaram (1995) believed that under natural field conditions, differences within the pathogen population are mainly due to aggressiveness rather than virulence. However, numerous reports indicate differences in virulence among *M. graminicola* isolates (Eyal et al. 1973; Saadaoui 1987; Eyal and Levy 1987; Ballantyne 1989; Kema et al. 1995; Ackerman et al. 1995; Jlibene et al. 1995; Kema et al. 1996a, 1996b; Kema and van Silfhout 1997; Grieger 2001; and Brading et al. 2002).

Differences in virulence were first reported in Israel by Eyal et al. (1973). Later, Eyal et al. (1985) evaluated the virulence patterns of 97 isolates of *M. graminicola* from 22 countries on seedlings of 35 wheat and triticale cultivars. They found significant isolate x cultivar interactions, indicating the existence of cultivar-specific virulence genes among isolates. Similar results were reported by Eyal and Levy (1987). Saadaoui (1987) tested 19 isolates of *M. graminicola* collected from all wheat growing areas in Morocco on a set of seven differential cultivars. Based on the differential interaction between cultivars and isolates, he reported three distinct physiological races. Ballantyne (1989) reported that there were true physiological races among isolates in Australia, and that there were also differences in the degree of pathogenicity (aggressiveness).

Later, Kema et al. (1995) studied the variation for virulence among 78 isolates of *M. graminicola* collected from 16 countries. The isolates were inoculated onto 22 differential cultivars at the seedling and adult plant stages. Significant cultivar x isolate interactions at both growth stages indicated cultivar specificity and variation for virulence among isolates. Ackerman et al. (1995) tested the pathogenic variability of 10 *M. graminicola* isolates from South America and showed that there was interaction between isolates and tester cultivars.

In Morocco, Jlibene et al. (1995) conducted two experiments to study host-pathogen interaction of wheat and *M. graminicola*. The first experiment consisted of inoculating both bread and durum wheats with isolates of *M. graminicola* collected from bread and durum wheat fields. In the second experiment, a differential set of bread wheat genotypes was inoculated with isolates collected from bread wheat fields only. Some degree of specialization at the species level was found in *M. graminicola*. Durum wheat

isolates tended to be specific to durum wheat and bread wheat isolates to bread wheat. As well, isolates from bread wheat showed a differential virulence reaction on the bread wheat genotypes. They grouped 27 isolates into 8 classes.

Kema et al. (1996a) conducted a larger study to examine genetic variation for virulence in the wheat-*M. graminicola* pathosystem. They studied genetic variation for virulence among 63 isolates of *M. graminicola* originating from 13 countries in two seedling experiments. The first experiment involved *M. graminicola* isolates from bread wheat and the second experiment *M. graminicola* isolates from durum wheat. There was a significant cultivar x isolate interaction in both experiments. This finding was in contrast to those of van Ginkel and Scharen (1988) who failed to find any interaction between host and pathogen genotypes. Kema and van Silfhout (1997) showed that there was significant interaction between host cultivars and pathogen isolates in tests of seedling and adult plants. They concluded that specificity exists in the wheat- *M. graminicola* pathosystem.

Grieger (2001) differentiated isolates of *M. graminicola* from western Canada into two races based on the reaction of hexaploid wheat line ST6. Race 1 was virulent on ST6, whereas race 2 was avirulent on ST6.

Based on the occurrence of significant isolate x cultivar interactions, the existence of a gene-for-gene relationship has been suggested in the wheat-*M. graminicola* pathosystem (Eyal et al. 1985; Kema et al. 1996a, 1996b). Recently, Kema et al. (2000) showed that avirulence in the pathogen was controlled by a single locus; this is consistent with a gene-for-gene interaction. Brading et al. (2002) and McCartney et al.

(2002) also proposed that isolate-specific resistance of wheat to Septoria tritici blotch follows a gene-for-gene relationship.

2.7 Genetic structure of populations

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

Knowledge of the distribution of genetic variability within the pathogen populations is useful for designing the best strategy for the deployment of resistance genes. For example, if a population has a high level of pathogenic variation within local populations, these populations would have the potential to adapt rapidly to resistance genes. Therefore, using single major resistance genes will not be effective for controlling the disease (Goodwin et al. 1992).

Historically, genetic structures of pathogen populations have been characterized by phenotypic markers such as virulence analysis on a set of differential cultivars carrying different resistance genes. However, studying virulence analysis alone provides limited insight into the true genetic diversity of the population (Leung et al. 1993). Because virulence genes represent only a small fraction of the genome and because they are subject to host selection, they do not necessarily represent a random sample of genes. To avoid a biased estimate of genetic diversity, population structure should be determined by use of neutral genetic markers distributed randomly throughout the genome.

2.7.1 Molecular tools for studying genetic variability

Advances in molecular biology in recent years have provided different types of protein- or DNA-based markers for the characterization of pathogen populations. These techniques include isozyme analysis, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple-sequence-repeat polymorphism (SSR) or microsatellites, and other techniques.

Over the past few years, RAPD markers have been used in breeding programs, genetic mapping, population genetics or epidemiological studies. RAPD has been a method of choice in studying fungal systematics because of its potential for detecting polymorphism among individuals at various taxonomic levels including species, sub-species, strains and isolates (Tingey and del Tufo 1993). Czembor and Arseniuk (1996) studied the genetic similarity of three species, *Septoria tritici*, *Stagonospora nodorum* and *Stagonospora avenae* f. sp. *triticea* by RAPD assay. The results showed that there was a high level of variation among *S. tritici* isolates and a slightly lower variation among those of *S. nodorum*. However, the isolates of *S. avenae* f. sp. *triticea* were more homogeneous which enabled this species to be distinguished from the others.

Recently, Kema et al. (2002) used a combination of RAPD and AFLP markers to generate a genetic linkage map of *M. graminicola*. They found that some of these markers were tightly linked to genes controlling biologically important traits such as mating type and avirulence. In addition, RAPD markers have been used to estimate the genetic variability of the pathogens causing net blotch of barley (Peever and Milgroom 1994; Peltonen et al. 1996; Jonsson et al. 2002), ascochyta blight of chickpea (Udupa et

al. 1998; Santra et al. 2001), brown spot disease of pine (Huang et al. 1995), dieback of grapevine (Péros et al. 1997) and powdery mildew of barley (McDermott et al. 1994).

RAPD markers can be converted to sequence characterized amplified region (SCAR) markers which will be more reliable and reproducible (Paran and Michelmore 1993). Schilling et al. (1996) developed SCAR markers for detection of different species of *Fusarium*. Recently, Goodwin and Kema (1998) developed SCAR markers for *M. graminicola*, which would be useful in detection of genetic variability within a population of this fungus.

Microsatellites are PCR-based markers, which are codominant and highly reproducible. This makes them an ideal tool for the study of genetic diversity. Microsatellites, also known as simple-sequence repeats (SSRs), consist of tandemly repeated units of mono-, di-, tri- or tetranucleotide repeats of varying length (Tautz 1989). They are widely dispersed in the genomes of eukaryotes (Tautz 1989; Hamada et al. 1982), including those of fungi (Rosewich and McDonald 1994). Polymorphism among microsatellite loci is mainly due to variation in the number of units. Microsatellites commonly occur in the non-coding regions of the genome but they may also be found within the coding regions (Tautz et al. 1986). The DNA sequences flanking microsatellites usually are conserved, allowing the development of PCR primers. These primers will amplify the microsatellite locus and enable the detection of different alleles of a locus. Mutation in the flanking regions of microsatellites is important and may produce null alleles, because the primers will not be able to bind and amplify the microsatellite region (Jarne and Lagoda 1996).

It has been reported that SSRs have the highest power of detecting intraspecific variation. Powell et al. (1996b) compared four genetic marker systems including RFLP, AFLP, RAPD and SSR in an analysis of soybean germplasm. The results showed that SSR markers had the highest expected heterozygosity (0.60). Teulat et al. (2000) studied genetic diversity in 14 coconut (*Cocos nucifera*) populations using simple-sequence repeats (SSRs) and amplified fragment length polymorphism (AFLP). The results showed that both AFLP and SSRs were informative in evaluating genetic diversity in coconut. However, SSRs had a greater ability to separate closely related individuals within a population. In another study, Fahima et al. (1998) used 23 wheat microsatellite markers to detect DNA polymorphism among 21 accessions of *Triticum dicoccoides* collected from Israel. They clustered the accessions based on their genetic similarity value and found that the genetic diversity of *T. dicoccoides* was correlated with their geographic distribution.

Recently, Owen et al. (1998) isolated and characterized nine single-locus microsatellite markers in *M. graminicola*. They also developed specific primers for the flanking regions of these loci. They used these primers to study genetic diversity among 12 isolates of the pathogen collected from a single field at Long Ashton Research Station, England. The number of alleles ranged from two to four with a genetic diversity value between 0.278 and 0.736.

2.7.2 Measuring genetic variability of a population

The genetic variability of a population can be estimated by measuring gene and genotypic frequencies. Nei (1973) introduced the concept of gene diversity to describe genetic variability in both sexually and asexually reproducing populations. Gene

diversity (H) is defined as the probability of obtaining two different alleles at a locus when two haploid individuals are sampled randomly from a population. This can be calculated by:

$H = 1-\Sigma X_i^2$

where X_i is the frequency of the i^{th} allele at a particular locus. In RAPD markers the range of gene diversity is between 0 to 0.5 and the maximum value is obtained when the frequencies of two alleles of a particular locus are equal. In multi-allelic markers, the maximum value for gene diversity would increase with increasing number of alleles per locus. For example, the maximum gene diversity for loci with three and four alleles would be 0.67 and 0.75, respectively. At the other extreme, a genetically uniform population (with no allelic variation at the loci sampled) will have a diversity of 0 because there is only one allele (Leung et al. 1993).

After estimating the genetic diversity of a population, it is important to determine how this variability is distributed among and within different sub-populations. Nei (1973) proposed a method for partitioning the genetic diversity of a population into different components. He suggested that genetic differentiation between sub-populations can be estimated as:

$G_{ST} = (H_T - H_S) / H_T$

where H_T is the total genetic diversity of a population and H_S is the average genetic diversity within sub populations. G_{ST} is the proportion of the total genetic variation accounted for by variation among sub populations. When the average genetic diversity within sub populations is small relative to the total population, the G_{ST} value will be large indicating substantial genetic variability among sub populations. When the

average genetic diversity within sub populations is large relative to the total variability of the population, the G_{ST} value will be small indicating little genetic variability among sub populations (Nei 1973).

2.8 Relationship between pathogenic and molecular variability

Knowledge of the relationship between the molecular and pathogenic variability of a pathogen population would be of practical interest. A close association between molecular and pathogenic variability means that DNA fingerprinting could be used for rapid pathotype diagnosis and monitoring the development of new virulent genotypes.

The literature indicates that the association between molecular and pathogenic variability ranges from perfect to random in different pathogen populations (Leung et al. 1993). Burdon and Roelfs (1985) tested isozyme polymorphism among and between isolates of *P. graminis* f. sp. *tritici* belonging to different virulence groups. They found that isolates from the same virulence group had identical isozyme genotypes, whereas isolates from different virulence groups had different isozyme genotypes. They concluded that in *P. graminis* f. sp. *tritici*, variability estimated by isozyme and by virulence analysis were highly correlated. Similarly, Levy et al. (1991) tested a collection of isolates from an asexual population of *Magnaporthe grisea* for molecular and virulence polymorphism. A perfect correlation existed between these two types of variability.

In another study, Chen et al. (1993) examined the relationship between molecular variation and virulence of the wheat stripe rust fungus (*Puccinia striiformis*) and found a high degree of molecular polymorphism among isolates that had the same virulence phenotype. When they compared similarity matrices based on RAPD and virulence

data, there was a very poor correlation (r = 0.17) between the two types of polymorphism. They concluded that the molecular polymorphism observed in *P. striiformis* was largely independent of virulence polymorphism. In a similar study, Kolmer et al. (1995) examined the correlation between virulence and molecular polymorphism in *Puccinia recondita* f. sp. *tritici* in Canada and found a weak relationship (r = 0.58) between these two types of variability. Likewise, Adhikari et al. (1999) found a weak correlation (r = 0.52) between molecular haplotypes and virulence phenotypes in *Xanthomonas oryzae* pv. *oryzae*. It appears that in most pathosystems, particularly those that reproduce sexually, the relationship between pathogenic and molecular variability is very low.

CHAPTER 3

VARIABILITY FOR PATHOGENICITY WITHIN A POPULATION OF *MYCOSPHAERELLA GRAMINICOLA* COLLECTED AT DIFFERENT SAMPLING LEVELS

3.1 Introduction

Septoria tritici blotch is one of the most important leaf spotting diseases of wheat and is distributed worldwide (Eyal et al. 1987). The causal fungus, *Mycosphaerella graminicola*, can reproduce sexually and has a bipolar, heterothallic mating system (Kema et al. 1996c). Since sexual reproduction is an important way to create genotypic variability within the population, knowledge about the genetic structure of populations of this pathogen may be valuable for designing an effective control strategy.

The earliest studies on genetic variability of *M. graminicola* involved isolates collected from different geographical areas (Eyal et al. 1985; Eyal and Levy 1987). Such studies would indicate the range of genetic variability present over a large geographical area, since isolates may have been sampled from different populations. The results of those studies provide little information on the genetic variability within a local population. To study the genetic structure of a pathogen population at local level, isolates of the pathogen have to be collected from a single field.

McDonald and Martinez (1990b, 1991) used RFLP markers to study the genetic structure of *M. graminicola* and reported that there was a high level of genetic variability among locations, leaves, lesions, and even among pycnidia from a single

lesion. However, they did not show whether the same degree of variability occurred when isolates were tested for pathogenicity. Therefore, the objectives of this study were to examine the variability for pathogenicity within a population of *M. graminicola* and to determine the relative distribution of this variability at different sampling levels of the population.

3.2 Materials and Methods

3.2.1 Collection of isolates of the fungus

Ninety isolates of *M. graminicola* were collected using a hierarchical sampling procedure (Fig 3.1) from a single wheat field seeded to the cultivar CDC Teal (Hughes and Hucl 1993) at the Kernen Research Farm, University of Saskatchewan, Saskatoon in 1998. Within this field, 10 locations (sites), approximately 100 m apart from each other, were randomly selected. At each location, three flag leaves from different plants were chosen from an area about 1 m^2 and one pycnidium from each of three distinct lesions was selected from each flag leaf.

3.2.2 Isolation of the pathogen and inoculum preparation

To isolate the fungus, infected leaves were surface sterilized with 0.6 % sodium hypochlorite solution for two minutes, rinsed three times in sterile distilled water and transferred to Petri plates containing water agar (WA) (15g agar / L H_2O). The plates were incubated at 21°C under a 12 h photoperiod. After 24 h, the cirrhus from a single pycnidium from each lesion was transferred using a sterile needle to a Petri plate containing yeast malt dextrose agar (YMDA) (yeast extract 4 g, malt extract 4 g, dextrose 4 g and agar 15 g , plus 200 ppm streptomycin in 1L H_2O) for multiplication.

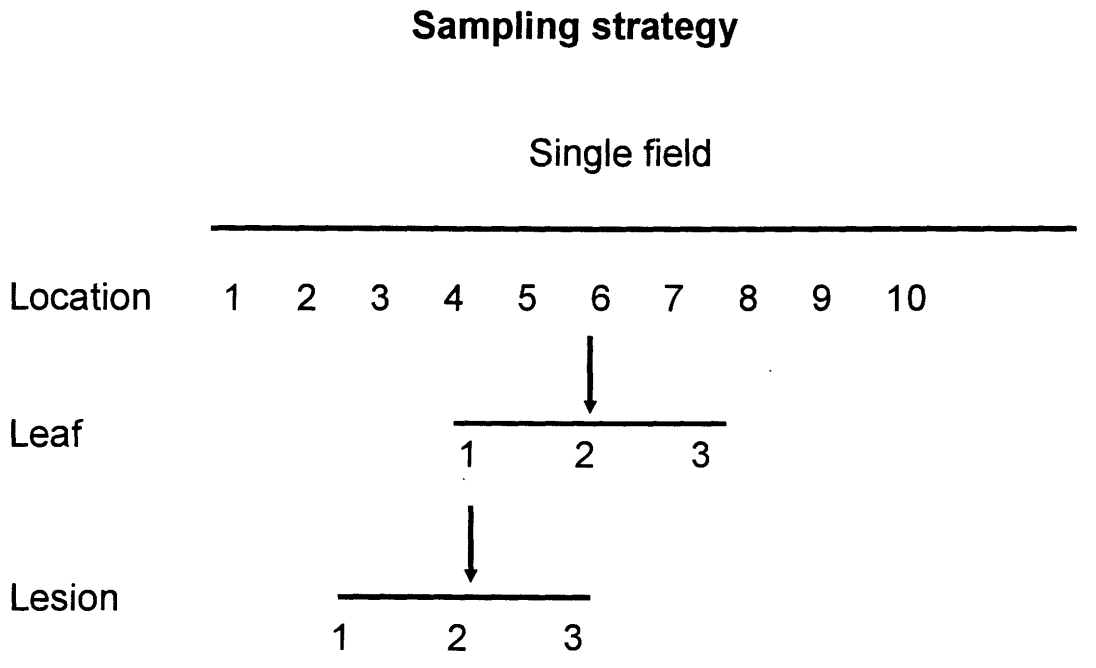


Figure 3.1 Hierarchical sampling strategy to provide 90 isolates of *Mycosphaerella graminicola* from a single wheat field in Saskatoon sampled in 1998.

Each single-pycnidial isolate was transferred to a sterilized sand culture (small vials with screw caps were filled with 4 g of sand, autoclaved and 0.5 ml of turbid liquid culture (5 days old) was added aseptically and sealed with parafilm) and stored at 4°C in a refrigerator for long-term storage and future use. Inoculum was produced by transferring a small amount of the sand culture to 150 ml glass bottles containing 100 ml of yeast malt (YM) (4 g yeast extract, 0.5 g malt extract per liter) liquid medium which were shaken by hand twice daily for five days (Fig 3.2). Before use, the inoculum was filtered through two layers of cheesecloth and the spore concentration was adjusted to 10^6 spores / ml using a hemacytometer.

3.2.3 Testing variability for components of pathogenicity

Isolates were tested for pathogenicity on the susceptible bread wheat cultivar Conway (Hughes and Hucl 1992). Because of the limitations of mist chamber space and inoculum preparation, all 90 isolates could not be tested at the same time, so an incomplete block design (9 x 10 rectangular lattice) was used with eight replications. Each replication was composed of 10 blocks with nine isolates within each block. Randomization was done among and within blocks of each replication according to Cochran and Cox (1992). Within each replication, the isolates were divided into three groups. Groups 1 and 2 were each composed of 27 isolates in three blocks; group 3 contained 36 isolates in four blocks. The isolates of each group were tested at the same time.

Seeds of cv. Conway were germinated on a filter paper using 10 ppm GA_3 solution and five seeds were planted in each 15.5 cm plastic pot. Plants in each pot were fertilized at the two-leaf stage, with 10 g controlled release fertilizer Nutricote® (Chisso-

Figure 3.2 Multiplication of isolates in YM liquid media for inoculum preparation.

Figure 3.3 Covering of the plants after inoculation to prevent cross contamination of *M. graminicola* isolates.

Asahi Co. Ltd., Tokyo, Japan). Seedlings in each pot were inoculated at the third leaf stage with 20 mL of spore suspension and after inoculation, each pot was covered with a plastic bag to prevent cross contamination (Fig 3.3). The inoculated plants were kept in a mist chamber for 72 h and then returned to the growth chamber bench at 21°C (day) / 16°C (night) temperatures with 16h photoperiod.

After inoculation, the third leaf of each plant was checked daily and the incubation period (time between inoculation and appearance of symptoms) and latent period (time between inoculation and pycnidial formation) were recorded for each plant. Five days after pycnidia developed, disease severity was estimated using the 0-6 scale of Brokenshire (1976) (Fig 3.4). The data were analyzed using PROC GLM of the Statistical Analysis System (SAS), ver 6.

3.3 Results

After 13-14 days, the inoculated plants started to show symptoms of the disease. The symptoms first appeared as necrotic lesions starting at the leaf tips; pycnidia developed later in the necrotic areas.

Analysis of the components of pathogenicity showed that there were significant differences among the 90 isolates for incubation period, latent period and disease severity at the lesion sampling level only. There were no significant differences for these components at the leaf and location sampling levels (Table 3.1). Although these significant differences existed, the magnitude of the mean squares was very low. Appendix A shows the adjusted means for incubation period, latent period and disease severity of the 90 isolates. The difference between the maximum and minimum values

0 1 2 3 4 5

for incubation period was 1.34 days, for latent period 2.29 days and for disease severity 1.32 (Table 3.2), suggesting that variability was low. In addition, the expression of these components of pathogenicity was continuous and their frequency distributions appeared to be normal distributions (Fig 3.5), suggesting that pathogenicity traits are quantitative. The incubation period of most isolates was approximately 14 days, but there were a few isolates with a 13- and a 15-day incubation period. Similarly, most isolates had a latent period of approximately 17 days, although there were a few isolates with latent periods of 16 and 18 days. Similar variability was observed for disease severity. The disease severity caused by the majority of the isolates was approximately 3.35 (based on the 0-6 scale), but there were a few isolates which caused a higher or lower disease severity.

To determine how the variability of the components of pathogenicity was distributed at different sampling levels, components of variance were estimated for each source of variation using mean-square expectations (Table 3.3). Standard errors of the estimated variance components were estimated by:

$\text{var}(M) = 2M^2 / df+2$

where M is the mean square for which the standard error is being estimated and df is the degrees of freedom associated with that mean square (R. J. Baker, personal communication). For all components of pathogenicity, there was significant variation among isolates only at the lesion sampling level (Table 3.4). Variation among isolates at the leaf and location levels was not significant.

Table 3.1 Analysis of variance of incubation period, latent period and disease severity of 90 isolates of *Mycosphaerella graminicola* tested on the susceptible wheat cv. Conway

Source of variation	df	Mean square		
		Incubation period	Latent period	Disease severity
Replication	7	21.21	25.88	11.74
Block (rep)	72	1.50	3.18	1.65
Location	9	0.28	0.60	0.45
Leaf (location)	20	0.35	1.27	0.72
Lesion (leaf loc)	60	0.75***	2.09***	0.81***
Error	549	0.32	0.66	0.46
C.V		4.09	4.8	20.32

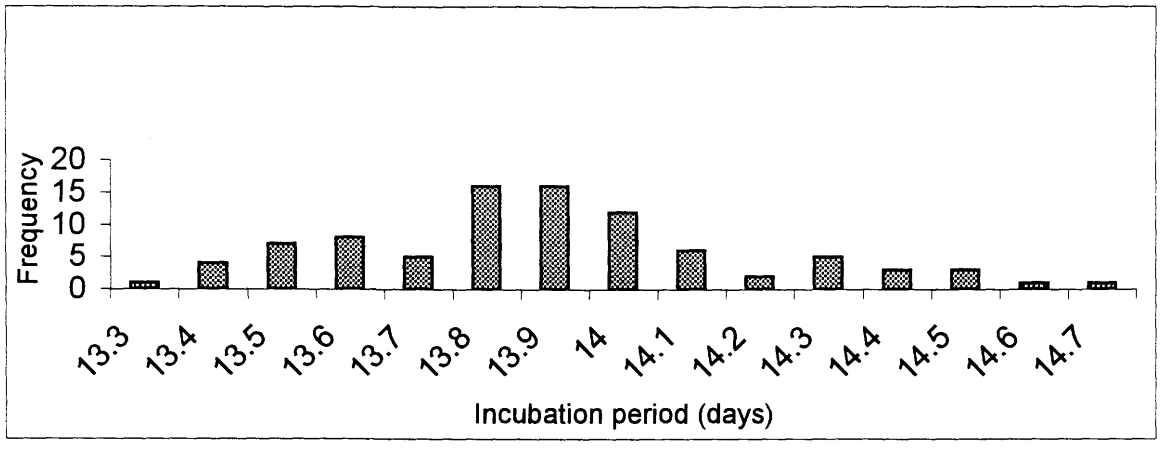
*** significant at the 0.1% probability level.

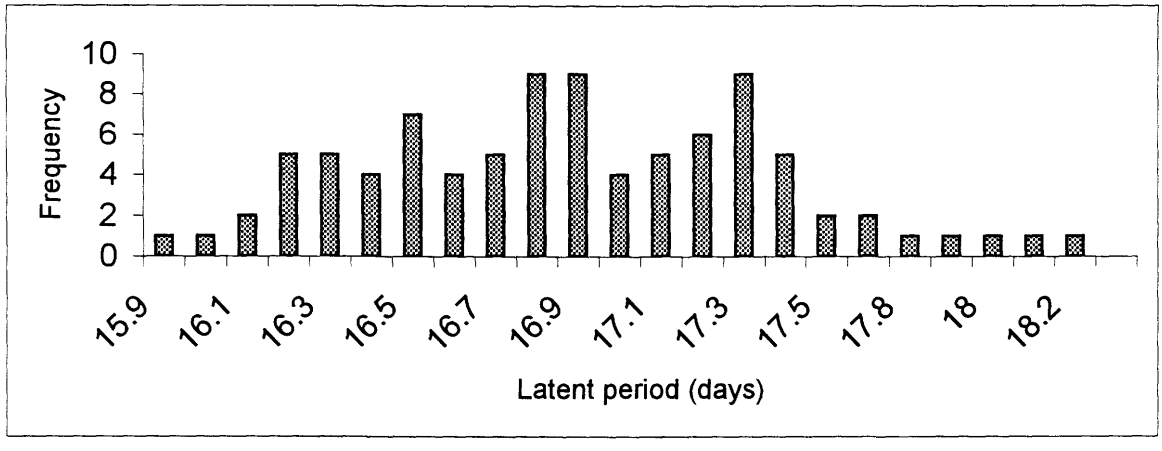
Table 3.2 The mean, standard deviation and range for incubation period, latent period and disease severity of 90 isolates of *Mycosphaerella graminicola* tested on the susceptible wheat cv. Conway

Parameter	Incubation period	Latent period	Disease severity
Mean	13.88 ± 0.29[a]	16.89 ± 0.49	3.35 ± 0.33
Maximum	14.65	18.17	4.05
Minimum	13.31	15.88	2.73

[a] Standard deviation.

Figure 3.5 Frequency distributions for incubation period, latent period and disease severity of 90 isolates of *Mycosphaerella graminicola* tested on the susceptible bread wheat cultivar Conway. The disease severity scores are based on the 0-6 scale of Brokenshire (1976).


Frequency
20
15
10
5
0
13.3
13.4
13.5
13.6
13.7
13.8
13.9
14
14.1
14.2
14.3
14.4
14.5
14.6
14.7
Incubation period (days)


Frequency
10
8
6
4
2
0
15.9
16.1
16.3
16.5
16.7
16.9
17.1
17.3
17.5
17.8
18
18.2
Latent period (days)

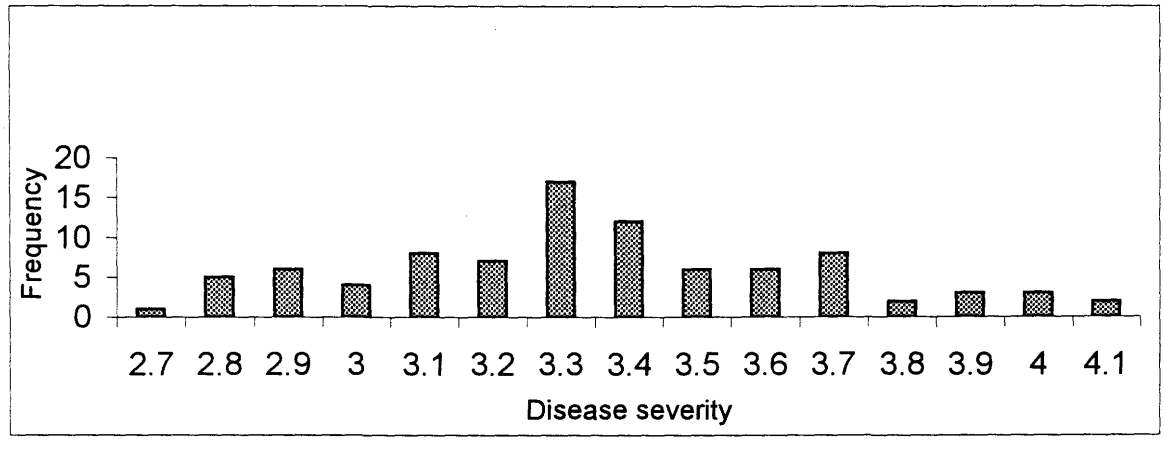

Frequency
20
15
10
5
0
2.7
2.8
2.9
3
3.1
3.2
3.3
3.4
3.5
3.6
3.7
3.8
3.9
4
4.1
Disease severity

Table 3.3 Adjusted expectations of mean square for the sources of variation in the incomplete block design used in testing variability of pathogenicity of *Mycosphaerella graminicola* isolates

Source	Expected Mean Square
Replication	Var (Error) + 8.97 Var (Block (Rep))+ 89.68 Var (Rep)
Block (Rep)	Var (Error) + 7.85 Var (Block (Rep))
Location	Var (Error) + 3.88 Var (Lesion (Loc Leaf))+ 11.63 Var (Leaf (Loc)) + 34.88 Var (Loc)
Leaf (Loc)	Var (Error) + 7.44 Var (Lesion (Loc Leaf)) + 22.31 Var (leaf (Loc))
Lesion (Loc Leaf)	Var (Error) + 7.50 Var (Lesion (Loc Leaf))

Table 3.4 Estimates and standard errors of variance components in the study of variability of pathogenicity of the *Mycosphaerella graminicola* isolates

Source of variation [a]	Incubation period	Latent period	Disease severity
V(l)	0.00	0.00	0.00
V(f)	0.00	0.00	0.00
V(z)	0.06 ± 0.02[b]	0.19 ± 0.05	0.05 ± 0.02
V(e)	0.32 ± 0.02	0.66 ± 0.04	0.46 ± 0.03

[a] V(l) = variance among locations; V(f) = variance among leaves within locations; V(z) = variance among lesions within leaves within locations; V(e) = error variance.
[b] Standard error.

3.4 Discussion

Since the cultivar Conway was susceptible to all 90 isolates, it provided a precise measurement of the degree of pathogenicity of the isolates. Significant variation for the components of pathogenicity, incubation period, latent period and disease severity, existed among isolates collected from a single pathogen population. This finding agrees with van Ginkel and Scharen (1988), Marshall (1985), and van Ginkel and Rajaram (1995) who reported that there was variability in the degree of pathogenicity (aggressiveness) among isolates of *M. graminicola*.

Variability for components of pathogenicity implies that some isolates have short incubation periods, short latent periods and the ability to cause severe disease on the host plant. Similarly, there are some isolates of the pathogen, which have long incubation periods, long latent periods and cause less disease. If these isolates have identical capacities to produce spores, the isolates with short incubation and latent periods will have a much greater impact on the development of an epidemic. Since isolates with short incubation period and short latent period will have a shorter generation time than others, the contribution of these genotypes in providing inoculum during a growing season will be larger than others, therefore, there will be selection pressure for those isolates in the population.

Differences in the degree of pathogenicity (aggressiveness) might be related to variation in the production of cell-wall-degrading enzymes. Nicholson et al. (1976) found that variation in the production of pectic enzymes was the reason for the variability in aggressiveness among isolates of *Colletotrichum graminicola*. Similarly, Lalaoui et al. (2000) found that a highly aggressive isolate of *Phaeosphaeria nodorum*

produced more of the cell-wall-degrading enzymes xylanase, cellulase, polygalacturonase and butyrate esterase than two weakly aggressive isolates. Le Cam et al. (1994) showed that in *Mycocentrospora acerina,* the casual agent of liquorice rot of carrots, highly aggressive isolates produced polygalacturonase and pectin methyl-esterase more rapidly than less aggressive isolates.

Recent studies suggest that variability in the degree of pathogenicity might be related to mutation of avirulence genes. Bai et al. (2000) reported that aggressiveness of *Xanthomonas oryzae* pv. *oryzae* is controlled by the *avrBs3* gene family. Some of these genes such as *avrXa7* made a major contribution to pathogen aggressiveness, but others such as *avrXa10* made little contribution. They showed that mutation of *avrXa7* reduced aggressiveness, whereas mutation of *avrXa10* had little effect.

Variability in the degree of pathogenicity has been reported in other phytopathogenic fungi. Krupinsky (1997a) showed that when isolates of *Stagonospora nodorum* were chosen at random, significant differences in aggressiveness were found. However, when only isolates that produced severe symptoms and isolates that produced mild symptoms were tested, it was found that the highly aggressive isolates consistently produced more severe symptoms on wheat seedlings than the weakly aggressive ones. No isolate x cultivar interaction was detected in either of these tests, but when highly and weakly aggressive isolates were compared, there was a significant cultivar x isolate interaction. However, since the magnitude of the interaction mean square was low compared to those of the main effects, Krupinsky (1997a) speculated that specificity is detected only when highly aggressive and weakly aggressive isolates are tested at the same time.

Similar results were obtained when the aggressiveness of *Stagonospora nodorum* isolates from perennial grasses were studied on wheat (Krupinsky 1997b).

In the present study, partitioning of the variance into location, leaf and lesion sources showed that the majority of variation occurred among lesions and that there was no significant difference among leaves and among locations. At the lesion sampling level, incubation period, latent period and disease severity of individual isolates within each leaf are being compared. However, at the leaf sampling level, comparisons are based on the mean of three isolates, and at the location sampling level, on the mean of nine isolates originating from three lesions.

The results of this analysis indicate that there is no hierarchical structure for components of pathogenicity within the population of *M. graminicola*, suggesting no differentiation among leaves and among locations. If there was a hierarchical structure within the population, significant differences for components of pathogenicity among leaves and among locations would be expected. However, the only significant variation was detected among lesions. This agrees partially with McDonald and Martinez (1990b, 1991) who reported that the majority of molecular variability of *M. graminicola* was distributed at the lesion sampling level.

A high degree of variability at the lesion sampling level and a low degree of variability at the leaf and location sampling levels suggest that the primary source of inoculum was most likely air-borne ascospores, which would have been dispersed evenly across the field. Since ascospores are haploid and are produced through sexual reproduction, isolates from lesions on a single leaf originating from infection by air-borne ascospores, would be genetically different. If the primary source of inoculum was

pycnidiospores, all the pycnidiospores originating from a single pycnidium would be genetically identical, since they are produced through asexual reproduction. Since pycnidiospores are dispersed only short distances by rain splash, a clonal structure within the population would be expected and would result in low variability among lesions and high variability among leaves and among locations. However, the results of this experiment do not indicate clonal structure. Shaw and Royle (1989) reported that epidemics of *M. graminicola* in the United Kingdom are initiated by air-borne ascospores. Kema et al. (1996c), showed that *M. graminicola* is able to complete several sexual cycles per season. Recently, this was confirmed by Hunter et al. (1999) who demonstrated that ascospores are released throughout the year in the United Kingdom.

CHAPTER 4

MOLECULAR VARIABILITY OF *MYCOSPHAERELLA GRAMINICOLA* AS DETERMINED BY THE USE OF RAPD MARKERS

4.1 Introduction

The pathogen *Mycosphaerella graminicola* (anamorph *Septoria tritici*), cause of Septoria tritici blotch, is an important leaf-spotting disease of wheat. Under conditions favorable for disease development, Septoria tritici blotch has the potential to cause substantial yield loss. The disease can be controlled by cultural practices, application of foliar fungicides and resistant cultivars.

Knowledge about the genetic structure of pathogens may be useful for improving control strategies, since the amount of genetic variation present within a population indicates how rapidly a pathogen can evolve. This information may eventually be used to predict how long control measures such as fungicides and resistant cultivars are likely to be effective (McDonald and McDermott 1993; McDonald and Linde 2002).

Traditionally, virulence analysis has been used to investigate the genetic structure of plant pathogens. However, virulence analysis has some drawbacks which preclude its routine application. The main disadvantage of using virulence data to study the genetic structure is that the genes involved in host specificity represent only a small fraction of the pathogen genome and may be subjected to strong selection by the host. Therefore, information inferred from virulence data may not reflect the true genetic diversity and evolutionary history of the isolates examined (Leung et al. 1993). Another problem is

that scoring of disease phenotypes is influenced by environmental variation and the judgement of the evaluator.

Over the past decade genetic markers based on the electrophoretic separation of protein or DNA molecules have provided useful information for the study of the genetic structure of pathogen populations. Genetic markers are ideal tools for those pathogens which lack well characterized virulence interactions with their hosts. These markers, unlike traditional markers, are insensitive to environmental effects and are not subject to natural selection (McDermott and McDonald 1993).

Different types of molecular markers have been used to study the genetic variability of plant pathogenic fungi. Each offers a special advantage in terms of ability to detect polymorphisms, and ease and economy of use (Michelmore and Hulbert, 1987). Use of random amplified polymorphic DNA (RAPD) involves the polymerase chain reaction (PCR) to detect genetic variability and has rapidly gained popularity because of its simplicity and potential to quickly screen a large number of individuals with a minimal amount of DNA (Schnieder et al. 1998). The objective of this study was to investigate the amount of genetic variability within a population of *M. graminicola* using RAPD markers and to determine how this variability was distributed at the different sampling levels.

4.2 Materials and Methods

4.2.1 Collection and multiplication of isolates

Ninety isolates of *M. graminicola* were collected using the hierarchical sampling procedure from a single field in Saskatoon described in Section 3.2.1. In addition, a

group of "outliers" consisting of one isolate each of *Stagonospora nodorum* and *Septoria triseti* (isolated from canary seed) and three isolates of *M. graminicola* collected from different areas of Saskatchewan (St124, St127 and St130 from Outlook, Nipawin and Prince Albert, respectively) were included in the study.

The isolates were multiplied in liquid YM media (4 g yeast extract, 0.5 g malt extract per liter) for 5 days. Then, 1 ml of liquid medium containing pycnidiospores was transferred to a YMDA (4 g yeast extract, 4 g malt extract, 4 g dextrose, 15 g agar, plus 200 ppm streptomycin per liter) plate which was kept at 21 ± 1°C under continuous fluorescent light for four days. By this time pycnidiospores had multiplied and covered the surface of the medium. The pycnidiospores on the surface of the YMDA culture were scraped off using a spatula, collected in sterile Petri plates and stored at -20°C overnight. The harvested pycnidiospores were freeze dried for approximately 24 h before DNA extraction.

4.2.2 Optimization and extraction of DNA

To optimize extraction of genomic DNA two extraction methods were tested, the protocol of Raeder and Broda (1985) and a modification of the rapid extraction protocol of Cenis (1992). The protocol of Cenis (1992) was discussed in detail by Ganeshan (1997) and the protocol of Raeder and Broda (1985) follows.

The freeze-dried fungal pycnidiospores were ground to a fine powder using a pre-chilled mortar and pestle and 100 mg was placed in a 1.5 ml centrifuge tube. The ground pycnidiospores were homogenized in 500 µl of extraction buffer (200 mM Tris-HCl (pH 8.5), 250 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulphate) using a micropipette tip, 350 µl of phenol and 150 µl of chloroform were added and the

suspension mixed gently with the pipette tip. The tubes were then centrifuged at 13,000 rpm at 4°C for 1 h and the upper aqueous layer was removed with a cut-off pipette tip and transferred to a clean 1.5 ml tube. RNA was precipitated by adding 25 µl of ribonuclease solution (20 mg of ribonuclease A plus 1 ml of TE buffer (Appendix E) boiled for 5 minutes) and incubating at 37°C for 10 min. An equal volume of chloroform was added, the solution was mixed gently and then centrifuged at 13,000 rpm for 10 min. After centrifugation, the upper aqueous layer was transferred to a new tube with a cut-off pipette tip and a 0.54 volume of cold isopropyl alcohol was added to precipitate the DNA. The DNA pellet was formed by a quick (5-10 second) centrifugation and the supernatant was removed. The DNA pellet was washed in 70% ethanol, dried at room temperature for 2-6 h and then resuspended in 100 µl of TE buffer. The quality and concentration of DNA was measured with a Gene Quanta RNA/DNA calculator (Pharmacia Biotech, Canada), and a stock solution of DNA (25 ng / µl concentration) was prepared for each isolate and stored at -20°C.

The protocol of Raeder & Broda (1985) yielded high concentrations of DNA (50-75 µg / ml) with a spectrophotometric ratio between 1.5-2. When compared to λ DNA in agarose gel electrophoresis, the extracted DNA had a high molecular weight without RNA contamination. The method of Cenis (1992) yielded a low concentration of DNA (1-5 µg / ml) with a spectrophotometric ratio between 1.1- 1.5. Drying fungal mycelium before extraction or adding sterile sand during grinding failed to change the results. Based on these results the protocol of Raeder & Broda (1985) was used for extraction of DNA of all isolates.

4.2.3 DNA amplification

DNA was amplified based on standard protocols with some modification. Each 25 µl reaction mixture contained 2.5 µl of 10X Taq polymerase reaction buffer (Gibco BRL, Life Technologies, Canada), 3.0 mM $MgCl_2$, 200 µm each of dATP, dTTP, dCTP, and dGTP, 0.3 µm primer, 37.5 ng of template DNA, 1.0 unit of Taq DNA polymerase (Gibco BRL) and 16.25 µl of double-distilled water. Amplification was carried out in a thermocycler (TECHNE GENIUS, Techne (Cambridge) Ltd, UK) programmed for 1 minute at 94°C for initial denaturation, followed by 34 cycles involving a denaturation temperature of 94°C for 20 seconds, an annealing temperature of 36°C for 45 seconds, and an extension temperature of 72°C for 1 minute. The final extension cycle was for 5 minutes at 72°C. After amplification, 5 µl of loading buffer (Appendix E) was added to each reaction tube and 20 µl of the product was used for electrophoresis.

4.2.4 Electrophoresis

PCR products were separated on 1.5 % agarose (w/v) gels in 1X Tris-acetate (TAE) buffer (Appendix E) at 100 V for 2 h. A 1kb DNA ladder was included as the molecular weight marker on each gel. In addition to two lanes of molecular weight markers, a standard isolate of *M. graminicola* (St6) was included on each gel to insure accurate comparison among the isolates. The products were stained with ethidium bromide (0.1 µg /ml) and DNA banding patterns were visualized under UV light and photographed.

4.2.5 Screening primers

To select appropriate primers, primer sets UBC 601-799 from the University of British Columbia (Vancouver, Canada) and primer sets F, G, H, I and J from Operon Technologies, Inc. (Alameda, California, USA) were screened using DNA of a single

isolate of *M. graminicola*. While some primers did not produce any amplified bands at all and some produced bands which were not reproducible, certain primers produced sharp and reproducible bands. From these, 15 reproducible primers were chosen to test for DNA polymorphism among the selected isolates (Fig. 4.1).

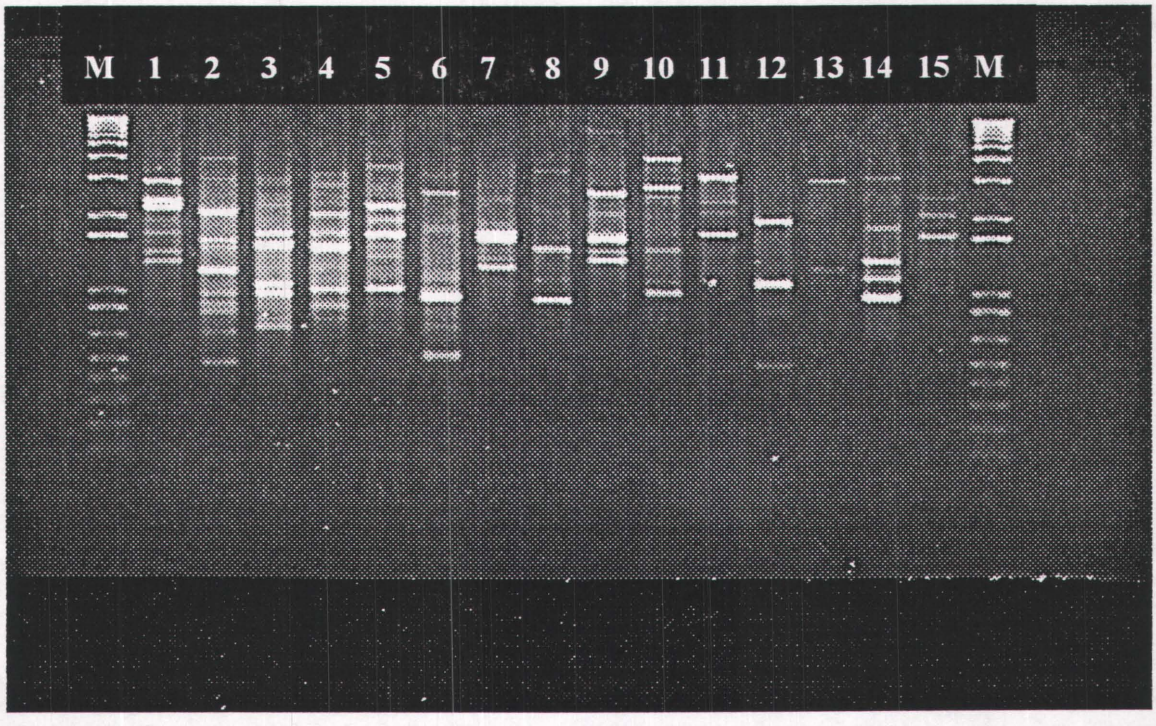


Figure 4.1 DNA banding pattern for the isolate St1 of *Mycosphaerella graminicola* produced by fifteen different random primers: UBC 648, UBC 726, UBC 736, UBC 737, UBC 757, UBC 758, UBC 763, UBC 767, UBC 772, OP F6, OP G12, OP G13, OP H8, OP H13, OP I10.

4.2.6 Analysis of data

To determine how much molecular variability existed within the population and how it was distributed at different sampling levels, gene diversity of the population was calculated. By considering the presence of a band at a particular locus as a positive allele and the absence of a band as a null allele, the frequency of positive alleles at each RAPD locus in the population was calculated. Gene diversity was then estimated using Nei's (1973) formula:

$H = 1 - \Sigma X_i^2$

where H is the gene diversity of the population and X_i is the frequency of the i^{th} allele of a particular locus.

To determine genetic variability at different sampling levels, the total gene diversity was partitioned into among- and within-location components using Nei's (1973) hierarchical gene diversity analysis as described by Goodwin et al (1993). In this analysis $H_T = H_S + D_{ST}$, where H_T is the total gene diversity of the population, H_S is the average gene diversity within subpopulations and D_{ST} is gene differentiation among subpopulations. The proportion of gene differentiation to total gene diversity of the population is calculated by

$G_{ST} = D_{ST} / H_T$.

If the amount of gene diversity within subpopulations is high, but among subpopulations is low, the G_{ST} value will be small. However, if the amount of gene diversity within subpopulations is low, but high among subpopulations, the G_{ST} value will be large.

A second analysis was conducted to determine how many molecular phenotypes (haplotypes) were present in the population to determine if there was any clonal structure in the pathogen population. To conduct this analysis, the nomenclature described by Kolmer et al. (1995) was used. According to this nomenclature, each isolate of *M. graminicola* was assigned a 15-digit molecular phenotype based on the banding pattern for each of the 15 selected primers. Each digit corresponds to the most common band produced by each primer for that isolate. For example, molecular phenotype 111112111211111 indicates that the most common band was produced by primers 1, 2, 3, 4, 5, 7, 8, 9, 11, 12, 13, 14, and 15 and the second most common band by primers 6 and 10. Similarly, molecular phenotype 111211311111111 indicates that the most common band was produced by primers 1, 2, 3, 5, 6, 8, 9, 10, 11, 12, 13, 14, and 15 and the second and the third most common bands by primers 4 and 7, respectively. Based on this nomenclature it was possible to detect the number of different haplotypes within the population and how they were distributed at the different sampling levels.

To investigate the relationship between different isolates, the RAPD banding pattern was scored for the presence (1) or absence (0) of bands for each primer-isolate combination and the data were entered in a binary matrix. Using the SIMQUAL (Similarity of Qualitative Data) program of NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) (Rohlf 1998), a similarity matrix was calculated for all possible pairs of isolates using the coefficient of Jaccard. Based on Jaccard's coefficient, the similarity of isolates is calculated by the formula $a / a + b + c$, where

'a' is the number of bands shared by two isolates, 'b' is the number of bands present only in one isolate, and 'c' is the number of bands present only in the other isolate. The similarity matrix was then used to construct a dendrogram using the UPGMA (unweighted pair group method of arithmetic average) method of the SAHN program of NTSYS-pc.

4.3 Results

4.3.1 DNA polymorphism among isolates

The 15 primers produced 131 reproducible fragments. On average, 8.8 fragments were produced per primer with a range of two (UBC 758) to thirteen fragments (UBC 757) per primer. From a total of 131 fragments, 126 fragments (96%) were polymorphic among the 90 isolates of *M. graminicola* (Table 4.1).

Representative DNA polymorphism among 12 isolates of *M. graminicola* produced with primers UBC 726, UBC 772, UBC 736, and OP H8 is shown in Fig. 4.2. Primers UBC 726 and OP F6 amplified a fragment which was specific to *M. graminicola* isolates and primers UBC 772, UBC 763 and UBC 758 amplified a fragment which was specific to both *M. graminicola* and *S. triseti* isolates. Fig. 4.3 shows a specific fragment, which was present in all of the isolates of *M. graminicola*. These fragments have the potential to be used as species-specific markers to identify *M. graminicola* from other related species.

Table 4.1 Sequence of 15 random decamer primers and the number of polymorphic and monomorphic fragments produced by each primer when tested on isolates of *Mycosphaerella graminicola*

Primer no.	Sequence	No of polymorphic bands	No of monomorphic bands	Total number of bands
UBC 648	GCACGCGAGA	4	0	4
UBC 726	GGTGTGGGTG	9	1	10
UBC 736	GAGGGAGGAG	8	0	8
UBC 737	GGTGGGTGTG	12	0	12
UBC 757	GGAAGGGAGG	13	0	13
UBC 758	GGTTGGGTGG	1	1	2
UBC 763	CACACCACCC	6	1	7
UBC 767	ACCCACCACC	11	0	11
UBC 772	CCCACCACCC	10	1	11
OP F6	GGGAATTCGG	11	1	12
OP G12	CAGCTCACGA	12	0	12
OP G13	CTCTCCGCCA	7	0	7
OP H8	GAAACACCCC	4	0	4
OP H13	GACGCCACAC	7	0	7
OP I10	ACAACGCGAG	11	0	11

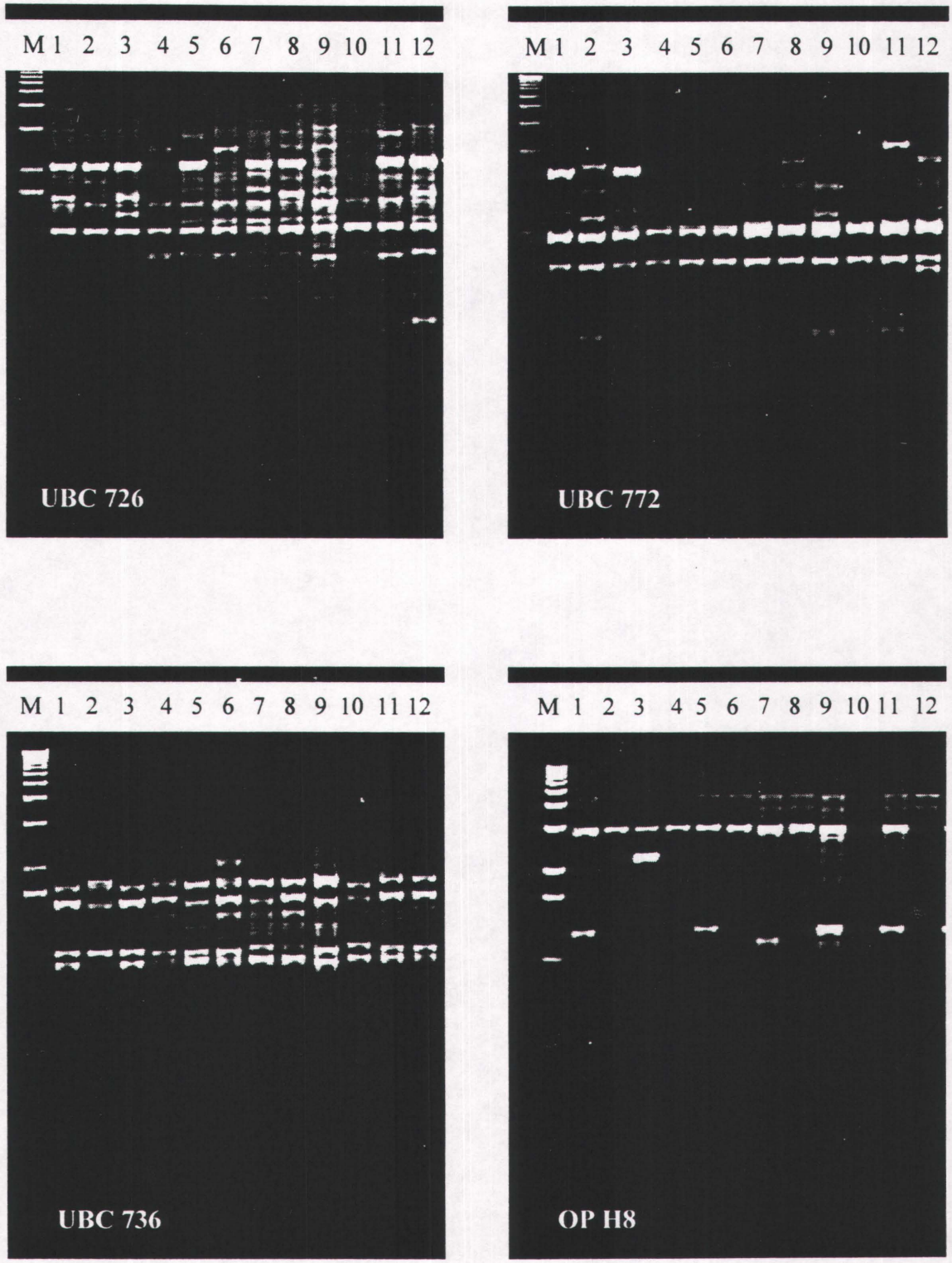


Figure 4.2 DNA polymorphism among 12 isolates of *Mycosphaerella graminicola* (St1-St12 in lanes 2-13) generated by primers UBC 726, UBC 772, UBC 736, and OP H8.

Figure 4.3 RAPD amplification profiles generated by primer UBC 763 for 90 isolates of *Mycosphaerella graminicola* (St1-90), and each of *Stagonospora nodorum* and *Septoria triseti,* and three isolates of *M. graminicola* (St124, St127 and St130) collected from different areas of Saskatchewan.

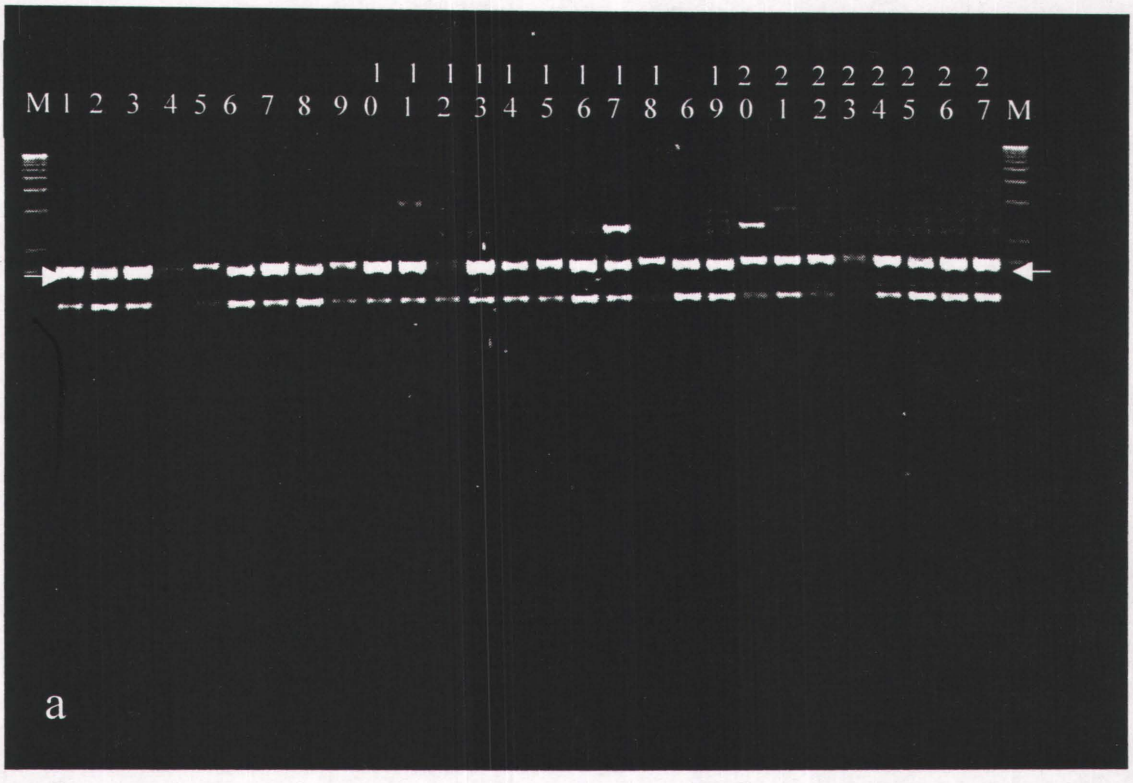
(a) lanes 1 and 30 = molecular weight marker, lanes 2-29 = isolates St1-St27 collected from locations 1,2 and 3.

(b) lanes 1 and 30 = molecular weight marker, lanes 2-29 = isolates St28-St54 collected from locations 4, 5 and 6.

(c) lanes 1 and 30 = molecular weight marker, lanes 2-29 = isolates St55-St72 collected from locations 7,8 and 9.

(d) lanes 1 and 15 = molecular weight marker, lanes 2-11 = isolates St73-St90 collected from location 10, lanes 12 = St6, lane 13 = *S. nodorum*, lane 14 = *S. triseti*, lane 15-17 = St124, St127 and St130.

The isolate St6 has been repeated in every gel for precise comparison of isolates. The arrow shows the fragment, which was specific to the *M. graminicola* and *S. triseti* isolates, but was not present in *S. nodorum*.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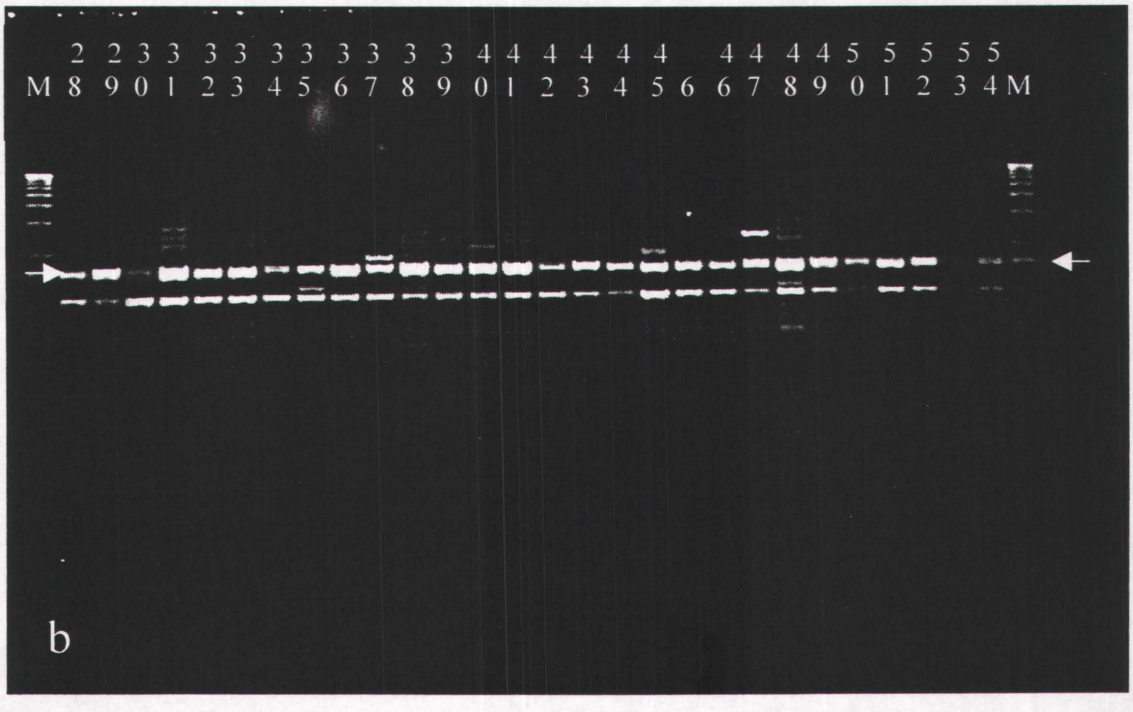
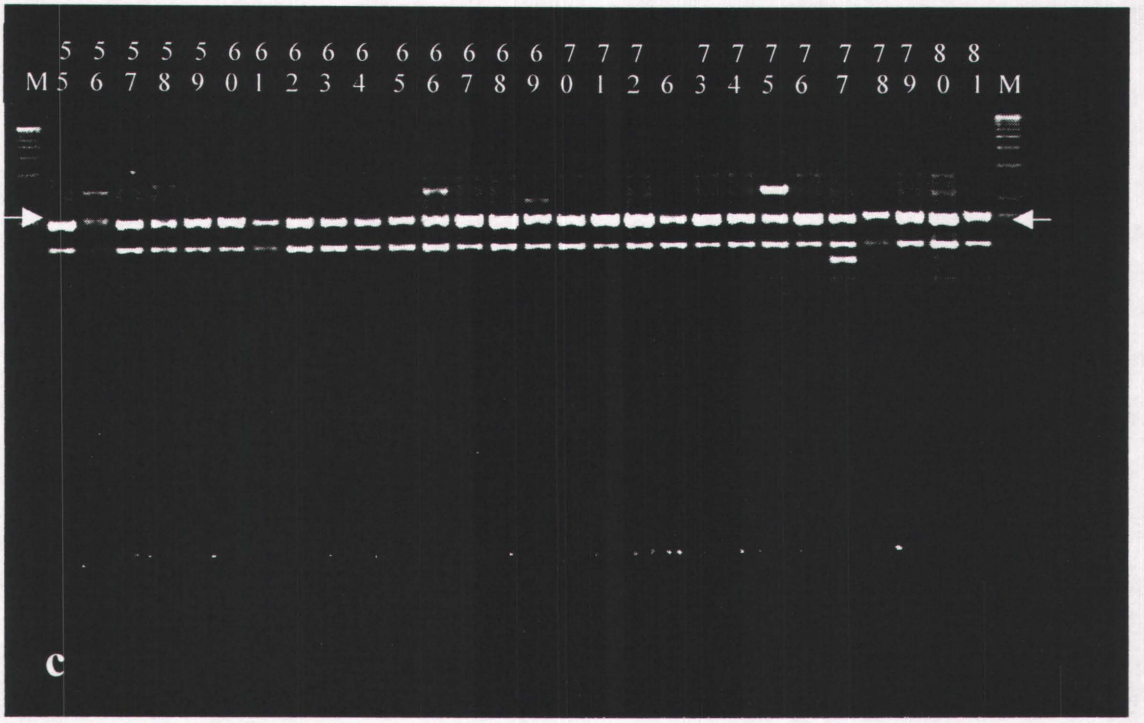
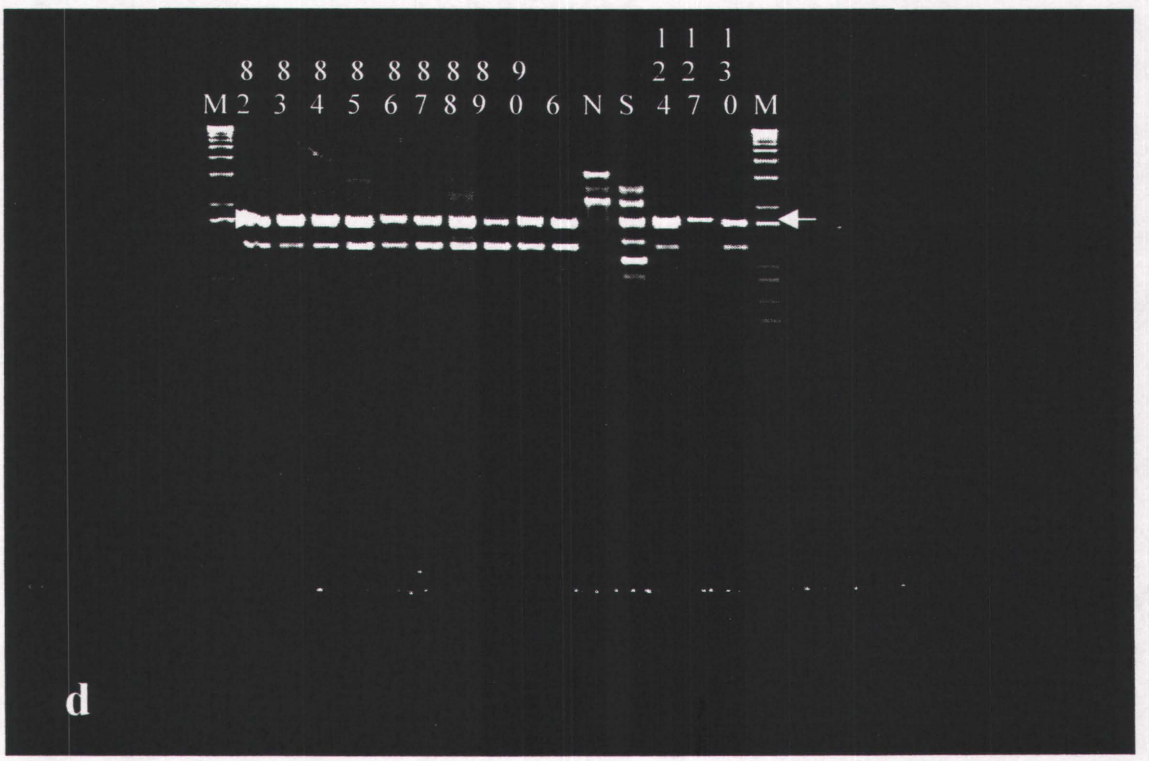
a


M 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 6 7 8 9 0 1 2 3 4 M
b


M 5 5 6 5 5 7 5 5 8 5 5 9 6 6 0 6 6 1 6 6 2 6 6 3 6 6 4 6 6 5 6 6 6 6 6 7 6 6 8 6 6 9 7 7 0 7 7 1 7 7 2 7 6 3 7 7 4 7 7 5 7 7 6 7 7 7 7 7 8 7 8 9 8 8 0 8 8 1 M
c


M 8 2 8 3 8 4 8 5 8 6 8 7 8 8 8 9 9 0 6 N S 1 2 4 1 2 7 1 3 0 M
d

4.3.2 Genetic diversity of the population

The frequency of the positive allele at each RAPD locus within each location is summarized in Appendix B and the gene diversity values are listed in Appendix C. Gene diversity among 90 isolates of *M. graminicola* ranged from 0.000 to 0.498. The average gene diversity of the population over the 131 RAPD loci was estimated to be 0.179.

When the total gene diversity was partitioned into within- and among-location components, the average gene diversity within locations was 0.154, among locations was 0.025 and the G_{ST} value was 0.140 (Table 4.2). This means that of the total genetic variability of the population of *M. graminicola*, approximately 14% was distributed among locations and 86% within locations.

Table 4.2 Partitioning of the total gene diversity into within- and among-location components

Source	Gene diversity	Proportion
H_T	0.179	
H_S	0.154	86 %
D_{ST}	0.025	14 %
G_{ST}	0.140	

H_T = Total gene diversity;
H_S = Gene diversity within locations;
D_{ST} = Gene diversity among locations;
G_{ST} = Proportion of gene diversity among locations to total diversity of the population.

In total, 40 molecular phenotypes (haplotypes) were detected (Table 4.3). Since isolates St 28, St 77 and St 84 did not produce any amplification product with primers OP I10, OP H8 and OP G13, respectively, these were not included in the molecular phenotype analysis. Molecular phenotype 1 had the highest frequency (0.20) in the population and was present in isolates from most locations in the field. Twenty seven molecular phenotypes occurred only once in the population. These molecular phenotypes were randomly distributed across locations. For example, locations 2 and 5 each had nine haplotypes indicating that every isolate at these locations was a unique genotype. Similarly, each of locations 3, 8 and 9 had eight haplotypes (Table 4.3). This type of distribution of haplotypes among locations indicated that there was no clonal structure in the population and suggests that asexual reproduction most likely plays a minor role in determining the population structure of *M. graminicola*.

The dendrogram generated from the similarity matrix based on Jaccard's coefficient and the UPGMA clustering algorithm is shown in Fig. 4.4. This analysis showed that, at the 0.20 similarity level, three distinct cluster groups were detected. As expected, the isolate of *S. nodorum* and the isolate of *S. triseti,* were outliers in the dendrogram. However, all 90 isolates of *M. graminicola* collected from the single wheat field in Saskatoon and the three *M. graminicola* isolates St124 (Outlook), St127 (Prince Albert), and St130 (Nipawin), grouped into a single major cluster.

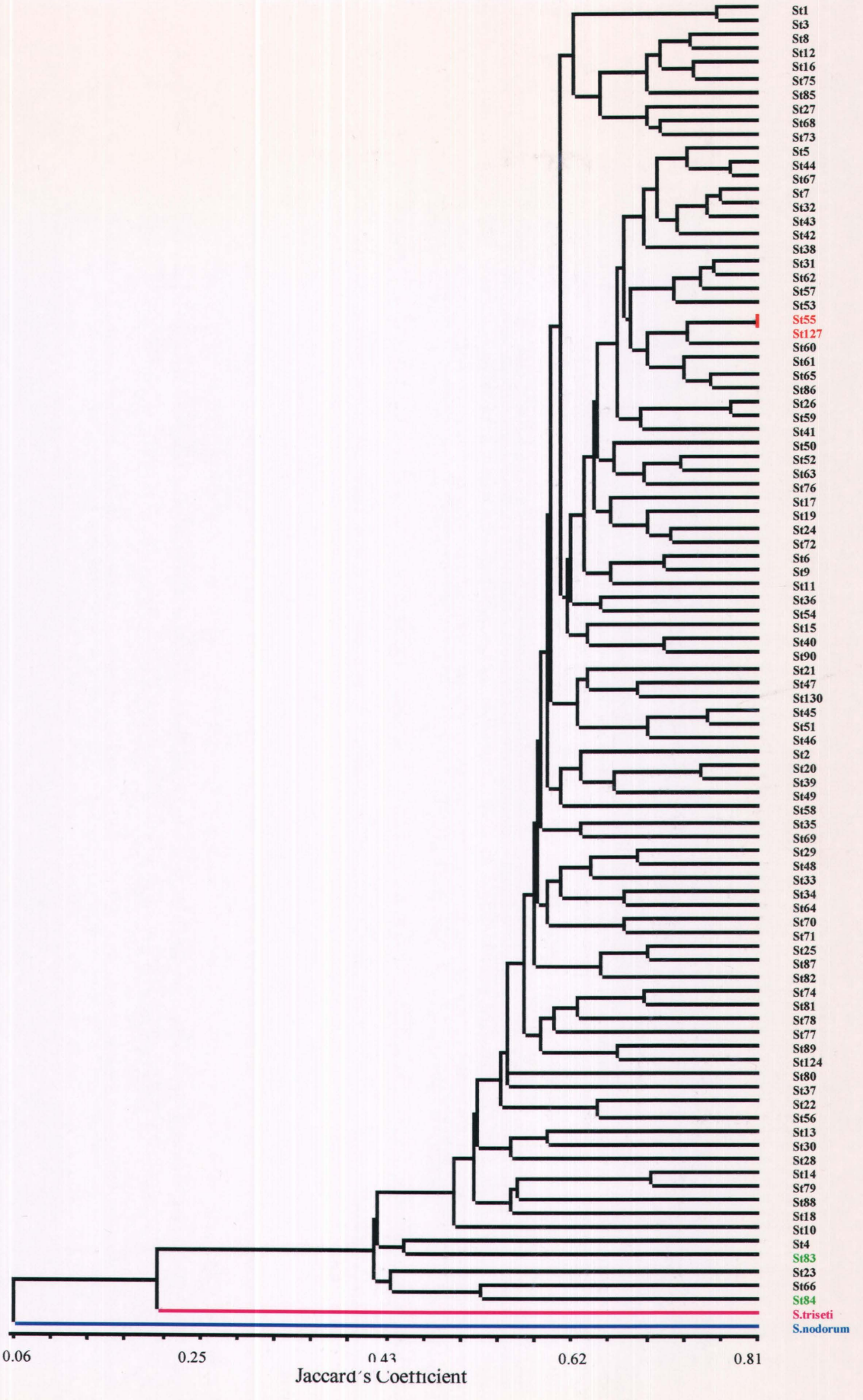
Clustering of the three geographically diverse isolates of *M. graminicola* with the 90 isolates collected from a single field in Saskatoon indicated that similarity or dissimilarity among isolates of *M. graminicola* is independent of their geographical

Table 4.3 Designated molecular phenotypes (haplotypes) of isolates of *Mycosphaerella graminicola* and their distribution within 10 locations of a single field based on 15 random primers

No.	Molecular phenotype (haplotype)	Number within population	Locations [a]
HP 1	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	18	1(4), 3(2), 4(3), 5(1), 6(3), 7(2), 8(1), 9(1), 10(1)
HP 2	1 1 2 1 1 1 1 1 1 1 1 1 1 1 1	1	2(1)
HP 3	1 1 1 2 1 1 1 1 1 1 1 1 1 1 1	2	7(1), 9(1)
HP 4	1 1 1 1 1 1 1 2 1 1 1 1 1 1 1	1	7(1)
HP 5	1 1 1 1 1 1 1 1 1 1 2 1 1 1 1	5	2(1), 3(1), 4(1), 6(1), 7(1)
HP 6	1 1 1 1 1 1 1 1 1 1 1 1 2 1 1 1	6	1(3), 3(1), 4(1), 10(1)
HP 7	1 1 1 1 1 1 1 1 1 1 1 1 1 2 1 1	6	2(1), 5(1), 7(2), 8(2)
HP 8	1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 1	1	1(1)
HP 9	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2	3	3(1), 5(1), 9(1)
HP 10	1 1 3 1 1 1 1 1 1 1 1 1 1 1 1 1	1	5(1)
HP 11	1 1 1 3 1 1 1 1 1 1 1 1 1 1 1 1	1	6(1)
HP 12	1 1 1 1 3 1 1 1 1 1 1 1 1 1 1 1	1	9(1)
HP 13	1 1 1 1 1 1 1 1 1 1 1 3 1 1 1 1	4	4(1), 6(1), 8(1), 9(1)
HP 14	1 1 1 1 1 1 1 1 1 1 1 1 3 1 1 1	2	5(1), 8(1)
HP 15	1 1 1 1 1 1 1 1 1 1 1 1 4 1 1 1	1	2(1)
HP 16	2 1 1 1 1 1 1 1 1 1 1 1 1 2 1 1	1	3(1)
HP 17	1 1 2 1 1 1 1 2 1 1 1 1 1 1 1 1	1	1(1)
HP 18	1 1 2 1 1 1 1 1 1 1 1 1 1 2 1 1	2	3(1), 10(1)
HP 19	1 1 2 1 1 1 1 1 1 1 2 1 1 1 1 1	1	7(1)
HP 20	1 1 1 2 1 1 1 1 1 1 1 1 2 1 1 1	1	4(1)
HP 21	1 1 1 1 1 1 1 1 1 1 1 1 2 2 1 1	5	2(1), 4(1), 5(1), 8(1), 9(1)
HP 22	1 1 1 1 1 1 1 1 1 1 1 1 2 1 2 1	2	5(1), 8(1)
HP 23	1 1 2 1 1 1 1 1 1 1 1 1 3 1 1 1	1	10(1)
HP 24	1 1 1 2 1 1 1 1 1 1 1 1 3 1 1 1	1	7(1)
HP 25	1 1 1 1 1 1 1 1 1 1 2 1 3 1 1 1	1	6(1)
HP 26	1 1 1 1 1 1 1 1 1 1 1 2 3 1 1 1	1	8(1)
HP 27	1 1 2 1 1 1 1 1 1 1 1 4 1 1 1 1	1	9(1)
HP 28	1 1 1 1 1 1 1 1 1 1 1 1 2 4 1 1	3	5(1), 6(2)
HP 29	1 1 1 1 1 1 1 1 1 1 1 1 3 2 1 1	1	2(1)
HP 30	1 1 1 1 1 1 1 1 1 1 1 1 3 1 1 2	1	10(1)
HP 31	1 1 1 1 1 1 1 1 1 1 1 1 4 2 1 1	2	10(2)
HP 32	1 1 1 1 1 1 1 1 1 1 1 1 4 3 1 1	1	2(1)
HP 33	1 1 1 5 1 1 1 1 1 1 2 1 1 1 1 1	1	2(1)
HP 34	1 1 2 1 1 1 1 1 1 1 2 2 1 1 1 1	1	9(1)
HP 35	2 1 2 1 1 1 1 1 1 1 1 1 1 3 1 1	1	10(1)
HP 36	1 1 3 1 1 1 1 1 1 1 1 3 2 1 1 1	1	5(1)
HP 37	1 1 2 2 1 1 1 1 1 1 1 2 1 1 2 1	1	3(1)
HP 38	2 1 1 1 4 1 1 1 1 1 1 4 3 1 1 1	1	3(1)
HP 39	4 1 2 1 1 1 1 1 1 1 1 4 2 1 1 1	1	2(1)
HP 40	1 1 1 1 2 1 1 1 1 1 1 4 5 1 2 1	1	8(1)

[a] Numbers within brackets shows the number of molecular phenotypes within that location.

Figure 4.4 Dendrogram of similarity of 90 isolates of *Mycosphaerella graminicola* from a single field and outlier species including *Stagonospora nodorum*, *Septoria triseti* and three isolates of *M. graminicola* (St124, St127 and St130 collected from Outlook, Nipawin and Prince Albert of Saskatchewan, respectively) based on random amplified polymorphic DNA (RAPD). The UPGMA algorithm of the SAHN program of NTSYS-pc and the similarity coefficient of Jaccard was used for cluster analysis. Isolates bolded in red were from different geographical areas but had the highest genetic similarity. Isolates in green bold font were from the same leaf and had very low genetic similarity.


St1
St3
St8
St12
St16
St75
St85
St27
St68
St73
St5
St44
St67
St7
St32
St43
St42
St38
St31
St62
St57
St53
St55
St127
St60
St61
St65
St86
St26
St59
St41
St50
St52
St63
St76
St17
St19
St24
St72
St6
St9
St11
St36
St54
St15
St40
St90
St21
St47
St130
St45
St51
St46
St2
St20
St39
St49
St58
St35
St69
St29
St48
St33
St34
St64
St70
St71
St25
St87
St82
St74
St81
St78
St77
St89
St124
St80
St37
St22
St56
St13
St30
St28
St14
St79
St88
St18
St10
St4
St83
St23
St66
St84
S.triseti
S.nodorum
0.06
0.25
0.43
0.62
0.81
Jaccard's Coefficient

distribution. Within the major cluster of *M. graminicola* isolates, the maximum similarity was about 81% between isolates St55 from Saskatoon and St127 from the Prince Albert area. Isolates collected from different lesions of the same leaf, such as St83 and St84 frequently had a very low (43%) similarity.

4.4 Discussion

These results indicated that there was a high level of genetic variability within the population of *M. graminicola* and that the population was composed of many different genotypes.

Partitioning the total gene diversity into among- and within-location components showed that the majority of the genetic variability (about 86%) occurred at the lowest sampling level within locations, an area about 1 m^2, a finding which agreed with McDonald and Martinez (1990b, 1991). Similar patterns for the amount and distribution of genetic variability have been reported for *Rhynchosporium secalis* (Goodwin et al. 1992; McDonald et al. 1999; Salamati et al. 2000) and *Stagonospora nodorum* (McDonald et al. 1994). This type of distribution of genetic variability would result if the primary source of inoculum was air-borne ascospores which would be dispersed randomly across the field. If the population of *M. graminicola* was derived from asexual reproduction, it would be expected to have a clonal structure, since pycnidiospores which originate from the same pycnidium are genetically identical and do not have the potential to be dispersed over a long distance. In this case, low genetic variability within locations and high variability among locations would be expected. This was not observed.

Multilocus analysis of RAPD data, which resulted in the detection of 40 molecular phenotypes (haplotypes), also showed that similar haplotypes were randomly distributed among locations and that there was little indication of clonal structure in the population. However, McDonald and Martinez (1990b) reported that similar haplotypes always originated from the same sampling site (location) and concluded that the population of *M. graminicola* was composed of a cluster of overlapping clones. The results of this study do not agree with their conclusion. This discrepancy is related to differences in sampling strategy. They collected 93 isolates from seven locations, approximately 10 m apart in a field in California, and at each location a single infected leaf was sampled from each of three or four plants. A total of 35 distinct lesions from 19 leaves was randomly chosen and from each lesion three different pycnidia were sampled. However, in the current study only a single pycnidium was isolated from each lesion. McDonald and Martinez (1990b) reported that unlike haplotypes were produced from different pycnidia of the same lesion in only 26% of the comparisons, whereas pycnidia from the same lesion were identical in 74% of the comparisons. Similarly, Schnieder et al. (1998) reported that isolates from the same pycnidium or from the same lesion had identical banding patterns, suggesting that these isolates originated from the same ancestor. Therefore, detection of a clonal structure in the population of *M. graminicola* in California (McDonald and Martinez 1990b) might be a result of the different sampling strategy.

Results of the cluster analysis also showed that the maximum similarity among isolates of *M. graminicola* was approximately 81%. Interestingly, some of the isolates, which had been collected from different lesions of the same leaf, showed maximum

dissimilarity suggesting that each lesion was the result of infection by a genetically different spore. This finding supports the hypothesis that a genetically diverse population (ascospores) provided the initial inoculum (Chen and McDonald 1996).

In addition, the cluster analysis showed that all isolates of *M. graminicola* grouped in the same cluster and that this cluster was distinct from both the *S. nodorum* and *S. triseti* isolates. Similar results were reported by Czembor and Arseniuk (1996) who used a random amplified polymorphic DNA (RAPD) assay to differentiate among isolates of *Stagonospora nodorum, S. avenae* f. sp. *triticea* and *Septoria tritici.* They found that isolates of *S. tritici* were distinct from isolates of *S. nodorum* and *S. avenae* f. sp. *triticea.*

The species-specific banding patterns that were revealed in the present study can be used to develop suitable SCAR markers to distinguish *M. graminicola* from other related species. Beck and Ligon (1995) developed specific PCR primers to detect *S. nodorum* and *S. tritici* in wheat leaf tissue before the development of obvious disease symptoms.

Overall, the results of this study showed that there were high levels of genetic variability within the population of *M. graminicola* studied. Most of the genetic variability was distributed at the lowest sampling level (within locations), a result in agreement with the pathogenicity data (Section 3.4). Low variability among locations and a high degree of variability within locations suggest that the primary source of inoculum was air-borne ascospores, which could be dispersed evenly across the field. This hypothesis was supported by a recent report (Hoorne et al. 2002) that the sexual stage of *M. graminicola* exists in Canada.

A high degree of genetic variability within a small sampling site (an area about $1m^2$) implies that populations of the pathogen are composed of a large number of genetically different isolates. This provides the opportunity for rapid selection of genotypes that are virulent on wheat cultivars with race-specific resistance. Consequently, conventional breeding methods using single major resistance genes may not be effective to control the disease (Goodwin et al. 1992). Therefore, in breeding for resistance to *M. graminicola,* emphasis should be given to using non-race specific resistance, pyramiding of major resistance genes or using cultivar mixtures. Boeger et al. (1993) suggested that plant breeders should use a wide spectrum of *M. graminicola* genotypes when testing wheat cultivars to this pathogen in any location.

CHAPTER 5

STUDY ON THE MOLECULAR VARIABILITY IN THE POPULATION OF *MYCOSPHAERELLA GRAMINICOLA* USING MICROSATELLITE MARKERS

5.1 Introduction

Microsatellites, also known as simple-sequence repeats (SSRs) (Jacob et al. 1991) or short tandem repeats (Edwards et al. 1991), are a class of molecular marker based on tandem repeats of short (2-6 bp) DNA sequences (Litt and Luty 1989), and can be distributed throughout the genome (Tautz 1989; Hamada et al. 1982). Microsatellites are classified into three families: pure, compound and interrupted repeats (Weber 1990). DNA polymorphism due to microsatellites is based on the number of repeat units in a defined region of the genome (Litt and Luty 1989; Weber and May 1989).

The number and composition of microsatellite repeats differ in plants and animals. In general, plants have about 10 times fewer SSRs than humans (Powell et al. 1996a). The function of SSRs in the genome is not clear, but it has been suggested that they are involved in providing hot spots for recombination (Tautz and Renz 1984). Microsatellite repeats occur in both coding and non-coding regions of eukaryotic genomes (Tautz et al. 1986). The DNA sequences flanking microsatellites are conserved and are used to design specific PCR primers, which will amplify the microsatellite locus and enable the detection of different alleles of a locus (Sun et al. 1998). SSRs are co-dominant markers, highly reproducible and inherited in a Mendelian

fashion, which makes them ideal tools for studies of genetic diversity (Morgante and Olivieri 1993).

Owen et al. (1998) characterized nine single locus microsatellite markers ST1A2, ST1A4, ST1B3, ST2E4, ST1E3, ST2C10, ST1G7, ST1E7 and ST1D7 in *Mycosphaerella graminicola* and developed specific primers for the flanking regions of these loci. The objective of this study was to use these primers to estimate genetic variability in this pathogen and to compare the results with those of the previous study based on RAPD markers (Chapter 4).

5.2 Materials and Methods

5.2.1 DNA extraction and amplification

Ninety isolates of *M. graminicola* were collected using a hierarchical sampling procedure from 10 different locations, leaves and lesions within a wheat field near Saskatoon as described in section 3.2.1. The isolates were multiplied in liquid medium YM (4 g yeast extract, 0.5 g malt extract per liter) for one week and genomic DNA extracted using the protocol of Raeder and Broda (1985).

Nine pairs of single locus microsatellite primers (Owen et al. 1998), synthesized by Gibco BRL (Canadian Life Technologies Inc.), were used to study the genetic variability of these isolates (Table 5.1). PCR was conducted based on the protocol of Owen et al. (1998). Each 25 µl reaction contained 1X PCR buffer, 0.2 mM each of the dNTP, 30 pmol forward primer, 30 pmol reverse primer, 1.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase (Gibco BRL), 10 ng of genomic DNA and 16.55 µl of distilled water. Amplification was carried out in a thermocycler (TECHNE GENIUS, Techne

Table 5.1 Characteristics of nine pairs of single locus microsatellite primers used to study genetic variability in *Mycosphaerella graminicola*

Locus	Reported repeat Motif	Forward (F) and reverse (R) primers sequences (5′ to 3′)
ST1A2 (MGR 7031)	$(GGC)_7/(GGT)_2$	F- CTCTCTCCCGTGCTGTGT TT R- CAGACCACCTGCACAGCAT
ST1A4 (MGR 7032)	$(CCG)_7$	F- GGTTCGATGGAGAGATTT R- TCACCTCCTCATCGCAGA
ST1B3 (MGR 7033)	$(CGG)_8$	F- CGCGCACTAGTAGACGCTCT R- TCTACCTTAATCCTCACCGCC
ST2E4 (MGR 7034)	$(GGC)_5$	F- GAAGATCAACAGCATGGGCGG R- CTCCAGAGGGATCACAAAGGC
ST1E3 (MGR 7035)	$(CGG)_5$	F- GTTCCGCCGGTCGAAGTCG R- GCCAAGGCACTGCTGCTCC
ST2C10 (MGR 7036)	$(AGCGG)_4$	F- AGGCGAGAAACTTGCTTGCAG R- AATGAACGTCCCATGGACGTG
ST1G7 (MGR 7037)	$(TG)_9$	F- ATGCTGAGAAGTTCGGTGAGG R- CGTTCTTCCACCTCCAACACT
ST1E7 (MGR 7038)	$(CGG)_5$	F- GATCTCGAGCAGGGCGGAAGT R- TCACACGCTGGTCTGTGAATC
ST1D7 (MGR 7039)	$(AC)_{22}$	F- TTGAAGTGGCATCCTCCATT R- AACTCGGCTGGTGGAACA

(Cambridge) Ltd, UK) programmed for 60 seconds at 95°C for initial denaturation, followed by 30 cycles involving a denaturation step at 95°C for 40 seconds, an annealing step at 58°C for 60 seconds, and extension at 72°C for 60 seconds. The final extension step was for 10 minutes at 72°C. Initially, an annealing temperature of 58°C was used for all of the primers, however, to get a specific single band, the annealing temperature was optimized separately for each pair of primers. The PCR products were resolved on 1.5% agarose (w/v) gels in 1X Tris-acetate buffer (Appendix E) at 100 V for 30 minutes, stained with ethidium bromide (0.1 µg/ml) and visualized under UV light. The results of optimization of PCR with different annealing temperatures for each pair of primers showed that the optimum temperature ranged between 58 to 65°C for the nine pairs of primers (Table 5.2). Figure 5.1 shows the result of optimization of PCR for

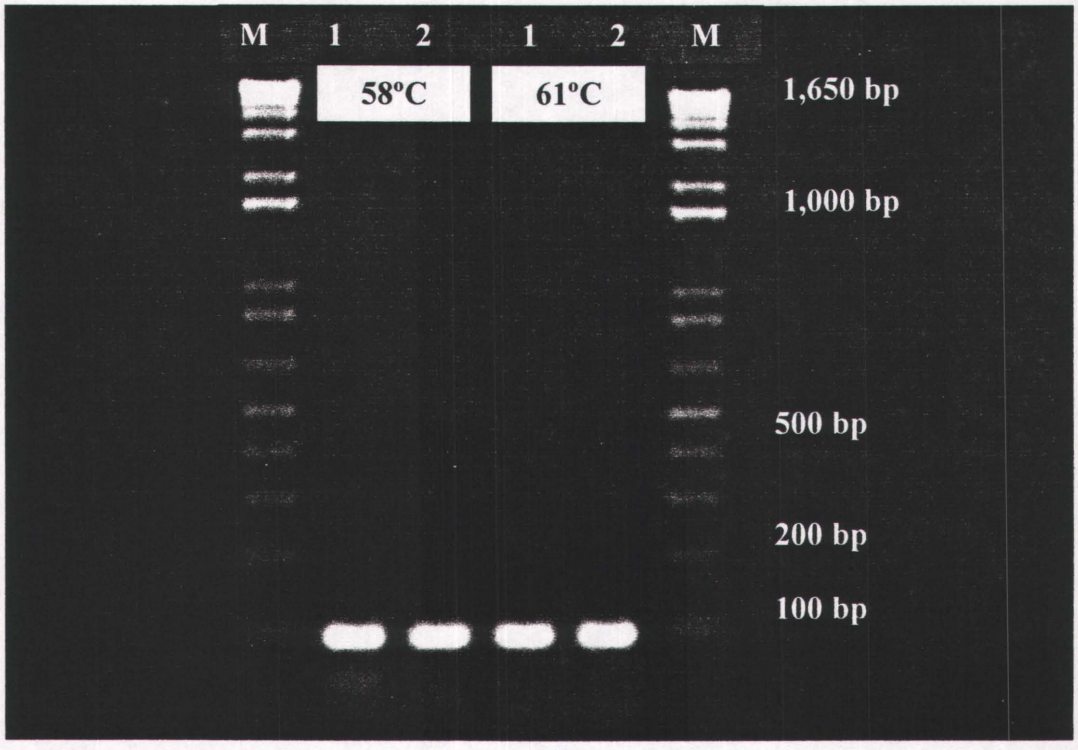


Figure 5.1 Optimization of PCR conditions for primer pair ST1D7 (MGR 7039) using different annealing temperatures (58 and 61°C) to amplify a single fragment from isolates of *Mycosphaerella graminicola* St1 and St2.

primer pair ST1D7. This primer pair amplified only a single fragment from *M. graminicola* isolates St1 and St2 at annealing temperatures of 58 and 61°C.

All 90 isolates were screened with each of the primer pairs using an optimal annealing temperature. After amplification, 5 µl of loading buffer (10% 6x blue/orange dye (Promega), 90% deionized formamide (Gibco BRL)) was added to each reaction tube, and after denaturing at 90°C for 5 minutes, 5 µl was used for electrophoresis.

For screening, electrophoresis of the PCR products was performed on a 6% urea-polyacrylamide gel (Appendix E) at a constant 85 W for 2 h and 5 µl of pGEM DNA ladder (Promega) was included as the molecular weight marker. After electrophoresis the gels were fixed in a 10% glacial acetic acid solution for 20-30 minutes followed by three washings in double distilled water with agitation. The gels were then placed in the staining solution (2 g $AgNO_3$, 3 ml 37% formaldehyde in 2 L double-distilled water) for 30 minutes. After staining, the gels were quickly rinsed (5-10 seconds) in double-distilled water and transferred to a pre-chilled developing solution (Appendix E). The gels were agitated in the developing solution until the DNA banding patterns were developed and then 2 L of 10% glacial acetic acid was added to the solution to stop over development of the gels. The gels were washed twice with double-distilled water and dried overnight. The dried gels were scanned using a Scanjet 6100 C (Hewlett Packard) and the DNA banding patterns were scored.

The size of the most intense band amplified by each primer pair was estimated using the program GEL ver. 2/17/89 (Thompson 1989). The program takes a set of standard DNA (molecular weight marker) fragment sizes and mobilities and predicts the sizes of unknown fragments based on least-square analysis developed by Southern (1979) and

Schaffer and Sederoff (1981). Fragments with different molecular weights were considered to be different alleles of a particular locus. Null alleles were assigned to isolates for which no amplification product was produced.

5.2.2 Data analysis

The frequencies of different alleles at each locus were calculated within each sampling location and within the total population (Appendix D1). The gene diversity of the population was estimated using Nei's formula (Nei 1973):

$H = 1 - \Sigma X_i^2$

where H is the gene diversity of the population and X_i is the frequency of different alleles at a particular locus. In estimating gene diversity of each locus, "nulls" were considered as a separate allele. Gene diversity was partitioned into within- and among-location components as described in section 4.2.6. In addition, multilocus analysis was conducted to detect the number of different molecular phenotypes (haplotypes) in the population using the procedure described by Kolmer et al. (1995).

To understand more about the biology of the pathogen and the roles of sexual and asexual reproduction in determining the population structure of *M. graminicola* in Saskatchewan, gametic disequilibrium analysis was conducted using Arlequin software ver. 2.000 (http://anthro.unige.ch/arlequin). Calculation of the coefficient of gametic disequilibrium (D) for loci with multiple alleles was based on the method suggested by Lewontin and Kojima (1960) as

$D = p_{ab} - p_a * p_b$

where, p_{ab} is the observed gametic frequency and $p_a * p_b$ is the product of observed frequencies of alleles a and b for any two loci. Chi-square analysis was done to test the

hypothesis of gametic equilibrium between each pair of alleles at two loci (allele by allele comparisons).

The chi-square test is very sensitive to low allelic frequencies. Since the expected allelic frequencies occur in the denominator of the chi-square formula, they can inflate the chi-square value when they are small (Weir 1990) and tests based on pairs of rare alleles at two loci may lead to a biased rejection of the null hypothesis of independence of the two loci. To avoid this problem, Chen and McDonald (1996) suggested that for chi-square analysis, alleles with frequencies less than 0.1 should be pooled into a single category. Therefore, alleles with frequencies of less than 0.1 in this study were pooled into a single category (Appendix D2). Since locus ST1G7 had only two alleles and the frequency of one of them was 0.02, this locus was not included in the analysis.

Tests for disequilibrium between two loci having multiple alleles (locus by locus comparison) were conducted using the procedures of Slatkin (1994) and Slatkin and Excoffier (1996).

5.3 Results

The number of amplified alleles per locus ranged from 1 to 5 with an average of 3, and the size of the fragments ranged between approximately 67 and 116 base pairs (Table 5.2). Primer pair ST1B3 did not produce a clear banding pattern, so the data for this locus were not included in the analysis. During screening, two primer pairs, ST2E4 and ST2C10 failed to amplify fragments from DNA of a number of isolates at the optimized annealing temperature. Absence of the band was scored as a null allele. The number of isolates exhibiting a null allele with primer pairs ST2E4 and ST2C10 were

Table 5.2 The optimum annealing temperatures, number of alleles and their range of molecular weight detected by eight SSR primers in the population of *Mycosphaerella graminicola*

Locus	Optimum annealing temperature	Range of fragment size (bp)	No. of alleles amplified	No. of isolates with null allele
ST1A2	65°C	67-76	5	0
ST1A4	58°C	98-116	3	0
ST2E4	58°C	75	1	11
ST1E3	65°C	67-70	3	0
ST2C10	58°C	75-87	3	15
ST1G7	65°C	90-96	2	0
ST1E7	58°C	85-91	3	0
ST1D7	61°C	85-105	4	0

11 and 15, respectively (Table 5.2). All primer pairs produced polymorphisms in the population. For SSR loci ST1A4, ST1G7 and ST1D7, polymorphism among alleles of each locus seemed to be due to the number of repeats. However, for some SSR loci differences among alleles seemed to be due to one or two nucleotides and/or the number of repeats. For example, for the locus ST1A2, five alleles (1, 2, 3, 4 and 5) were detected with molecular weights of approximately 67, 68, 70, 73 and 76 bp, respectively. The difference between alleles 1 and 2 was only one nucleotide, however, differences among alleles 1, 3, 4, and 5 were likely due to the number of repeats. A similar type of polymorphism was detected among alleles at the loci ST1E3 and ST1E7. Figure 5.2 illustrates polymorphism among alleles of the locus ST1E7. For the locus ST2C10 three alleles (1, 2 and 3) were amplified with molecular weights of

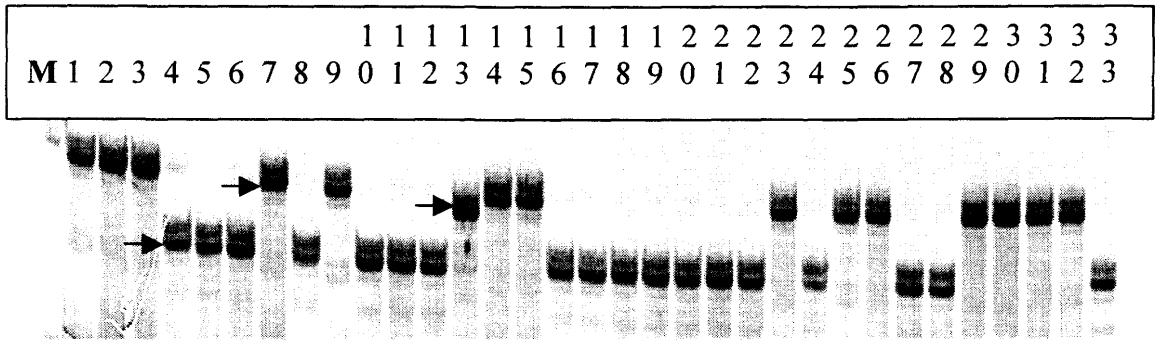


Figure 5.2 Representative DNA polymorphism among 33 isolates of *Mycosphaerella graminicola* detected by microsatellite primer pair ST1E7. Lane 1 was the molecular weight marker pGEM (Promega) and lanes 2-34 were *M. graminicola* isolates St1-St33. The arrows show three alleles of the locus ST1E7 with molecular weights of approximately 85, 91 and 90 bp (from left to right respectively).

approximately 75, 80 and 87 bp respectively. The difference between alleles 1 and 2 was due to the number of repeats but between 2 and 3 was likely due to two nucleotides.

The frequency of different alleles of each locus within each location and also within the total population used to estimate gene diversity is shown in Appendix D1. The gene diversity of the population ranged from 0.04 to 0.67 (Table 5.3). When the gene diversity of the population at each locus was partitioned into within- and among-location components, most variability for each locus was distributed within rather than among locations (Table 5.3). The average gene diversity of the population (H_T) was 0.441, of which approximately 0.386 (88%) occurred within locations (H_S).

Multilocus analysis based on eight SSR loci showed that 77 haplotypes were present in the population (data not shown). Only isolates St29, St48 and St56 each had one and isolates St22, St25 and St4 had two, three and five identical haplotypes, respectively, in the population. This indicated that only a small proportion (about 14%) of the population was clonal, suggesting that asexual reproduction plays a minor role in the genetic structure of the population. A large proportion (about 86%) of the population had genetically unique haplotypes.

Tests of gametic disequilibrium among individual alleles of microsatellite loci showed that, of the possible 206 allele-by-allele comparisons, only 12 tests (about 6%) were significant (P= 0.05) and in locus-by-locus comparisons none of the 21 pairs was significant (Table 5.4) indicating that the majority of alleles (94%) at the microsatellite loci were randomly associated.

Table 5.3 Gene diversity within and among locations of a population of *Mycosphaerella graminicola* tested with eight pairs of single-locus microsatellite primers

Locus	Total allelic diversity [a]	Allelic diversity among locations [b]	Allelic diversity within locations [c]
ST1A2	0.67	0.09	0.58
ST1A4	0.56	0.06	0.50
ST2E4	0.22	0.02	0.20
ST1E3	0.45	0.05	0.40
ST2C10	0.62	0.05	0.57
ST1G7	0.04	0.01	0.03
ST1E7	0.52	0.09	0.43
ST1D7	0.45	0.07	0.38
Mean	0.441	0.055	0.386

[a] Total allelic diversity for each locus in the population.
[b] Allelic diversity among locations for each locus.
[c] Average allelic diversity within locations for each locus.

5.4 Discussion

Eight single-locus microsatellite markers were used to study genetic variability in a Saskatchewan population of *M. graminicola*. The results showed that there was a high level of genetic variability within the population and that all eight loci were polymorphic among the 90 isolates tested. The number of alleles per locus ranged from 1 to 5 with an average of 3 per locus. It was noted that polymorphism among different alleles of a particular SSR locus does not merely depend on the number of repeat units.

Table 5.4 Measures of gametic disequilibrium among seven microsatellite loci in a population of *Mycosphaerella graminicola* in Saskatchewan

	ST1D7	ST1E7	ST1E3	ST2E4	ST1A4	ST1A2
ST1E7	1/9					
ST1E3	4/9	0/9				
ST2E4	0/6	0/6	0/6			
ST1A4	0/9	0/9	0/9	0/6		
ST1A2	1/12	1/12	1/12	0/8	1/12	
ST2C10	1/12	0/12	0/12	0/8	0/12	2/16

The numerators indicate the number of the tests that showed significant disequilibrium (P = 0.05) in relation to the total number of tests between individual alleles at two SSR loci. All tests of locus by locus comparisons were not significant.

In SSR loci ST1A2, ST1E3, ST1E7 and ST2C10 polymorphism among some alleles was thought to be due to one or two nucleotides. This hypothesis was based on the molecular weight of the alleles. DNA sequencing is the preferred means of detecting base-pair differences among alleles, but was not possible for this study because of financial limitations. Owen et al. (1998) reported similar results. One or two base-pair difference among alleles may be due to insertion or deletion events during DNA replication. Weber and May (1989) in screening human DNA sequences within Genbank for the presence of microsatellite sequences, found that about half of the repeat blocks contained one or more extra nucleotides in the run of repeats. Similarly, a single nucleotide deletion was reported in some SSR loci of *Arabidopsis thaliana* (Loridon et al. 1998).

Some primer pairs did not produce any amplification products in some isolates. It has been reported that because of the mutation in the primer binding sites of the genome, the expected DNA fragments are not amplified (Owen et al. 1998). The range of gene diversity of the population was between 0.04 to 0.67 with an average of 0.44. Similar results were reported by Owen et al. (1998) who studied 12 U.K. isolates of *M. graminicola* and determined that the average gene diversity was 0.49.

The average gene diversity estimated by microsatellite markers was higher than that estimated by RAPD markers (Chapter 4). Since the measurement of gene diversity is affected by the number of alleles per locus, this discrepancy may be related to the type of markers used. RAPDs are dominant markers and since the presence of a band is considered to represent one allele and the absence of band a second allele, only two alleles can be detected per locus. However, microsatellites are codominant markers and

have the potential to reveal an unlimited number of alleles per locus; therefore, the estimate of gene diversity would be higher. Powell et al. (1996b) compared RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis of soybean, and showed that SSR markers had the highest expected heterozygosity. Likewise, Sun et al. (1999) compared isozymes, RAPD and microsatellite markers in studying genetic diversity of *Elymus caninus* and found that the highest level of polymorphism was detected by SSR markers.

High levels of polymorphism have been reported in other studies of genetic variability with microsatellite markers. Borer et al. (1999) identified six polymorphic loci in the walleye, *Stizostedion vitreum*. These polymorphic loci had between 4 to 10 alleles per locus and the expected heterozygosity ranged from 0.40 to 0.70. Teulat et al. (2000) found 37 polymorphic microsatellite loci in coconut (*Cocos nucifera*), with the number of alleles per locus ranging between 2 to 16 and gene diversity ranging from 0.47 to 0.90.

Partitioning total gene diversity of the population into within- and among-location components showed that 88% of the variability was distributed within locations and only 12% occurred among locations. This agrees with the results of the analysis using RAPD markers where 86% of the variability occurred within locations and 14% among locations. A high degree of variability within location sampling level (an area about 1 m^2) indicates that the population originated from a genetically heterogeneous source of inoculum (ascospores). This was confirmed by multilocus analysis of eight SSR loci, in which 77 genotypes (haplotypes) were detected among 90 isolates of the pathogen. Multilocus analysis and DNA fingerprinting are considered to be direct measures of the

relative contribution of sexual or asexual reproduction to the genetic structure of fungal populations (Chen and McDonald 1996). These authors detected 617 genotypes among 673 isolates of *M. graminicola* sampled from a single field in Oregon.

The presence of a large number of isolates with different genotypes (haplotypes) and the pattern of distribution of genetic variability suggests that sexual reproduction occurs in the population. This conclusion is supported indirectly by the results of the gametic disequilibrium analysis. In the allele-by-allele comparisons only about 6% of allele pairs were non-randomly associated. In the locus-by-locus comparison, all loci were in gametic equilibrium. Chen and McDonald (1996) reported that sexual reproduction played a major role in the genetic structure of populations of *M. graminicola* in the United States.

The results of this study strongly suggest that the sexual state of *M. graminicola* occurs commonly in Saskatchewan. This hypothesis is supported by the recent discovery of the sexual state of the pathogen in Manitoba (Hoorne et al. 2002). The sexual state of *M. graminicola* has been reported in several countries around world including New Zealand (Sanderson 1972), Australia (Brown et al. 1978), United Kingdom (Scott et al. 1988), and the United States (Garcia and Marshall 1992).

Sexual reproduction can create new combinations of virulence genes in the population, providing the pathogen with the potential to defeat host resistance based on single resistance genes. Therefore, to maximize the life of resistant cultivars, cultivars with non-race specific resistance should be developed.

CHAPTER 6

VARIABILITY FOR VIRULENCE IN *MYCOSPHAERELLA GRAMINICOLA*

6.1 Introduction

Septoria tritici leaf blotch of wheat, caused by *Mycosphaerella graminicola*, is an important disease of wheat worldwide and in favorable environmental conditions has the potential to cause severe yield loss. Molecular studies have shown that there is a high level of genetic variability within populations of this fungus (McDonald and Martinez 1990a, 1990b, 1991; McDonald et al. 1995; Owen et al. 1998), but no attempt was made by these authors to test whether there was also variability for pathogenicity.

Two different views about the pathogenic variability of *M. graminicola* are held. Some researchers (van Ginkel and Scharen 1988; van Ginkel and Rajaram 1995; Marshall 1985) believe that there are no differences in virulence among isolates; the only difference is in their degree of pathogenicity (aggressiveness). However, others (Eyal et al. 1973; Saadaoui 1987; Eyal and Levy 1987; Ballantyne 1989; Kema et al. 1996a, 1996b; Kema and van Silfhout 1997; Brading et al. 2002; McCartney et al. 2002) believe that differences in virulence do exist among isolates. The study of pathogenicity (Chapter 3) showed that there were significant differences among isolates for incubation period, latent period and disease severity. The molecular study using RAPD markers (Chapter 4) also showed a high level of genetic variability within the population and 40 molecular phenotypes were detected among the 90 isolates tested.

The objective of this study was to determine whether there was also variability for virulence within the population of *M. graminicola* used in these studies.

6.2 Materials and Methods

6.2.1 Isolates and inoculum preparation

Forty isolates of *M. graminicola*, representing the 40 molecular phenotypes detected by RAPD analysis (Chapter 4), were selected from the population. These isolates were multiplied in YM liquid media (4 g yeast extract, 0.5 g malt extract per liter) and inoculum was produced as described earlier (Section 3.2.2). The spore concentration of the inoculum was adjusted to 2×10^6 spore/ml before use.

6.2.2 Selection of differential cultivars

To select appropriate differential cultivars, 80 bread wheat lines from the International Maize and Wheat Improvement Center (CIMMYT), Mexico, and 20 bread wheat and durum wheat cultivars from the USDA National Small Grains Collection, Aberdeen, Idaho, were screened in a preliminary test with two replications. Seeds of each cultivar were germinated on a filter paper using 10 ppm GA_3 and three seeds were planted in each 10 cm plastic pot. For each isolate-cultivar combination two pots (6 plants) were tested. All cultivars were inoculated 21 days after seeding (at the third-leaf stage) with each of six different isolates of *M. graminicola*. After inoculation, the plants were covered with plastic bags to prevent cross contamination, kept in a mist chamber for 72 h and then returned to the growth chamber bench at 21°C (day) / 16°C (night) temperature with 16h photoperiod. Three weeks after inoculation, the disease reactions of the genotypes were scored using a 0 to 5 rating scale (Fig 6.1) where 0 = immune, no

Figure 6.1 The rating scale (0-5) used for evaluation of host reaction to *Mycosphaerella graminicola* modified from that of Rosielle (1972). On this scale 0 = immune, no visible symptoms, no pycnidial formation; 1 = resistant, hypersensitive flecking in younger leaf tissue, no pycnidial formation; 2 = moderately resistant, very light pycnidial formation, some coalescence of lesions, mainly towards the leaf tip; 3 = moderately susceptible, light pycnidial formation, coalescence of lesions normally evident towards the leaf tips and elsewhere on the leaf blade; 4 = susceptible, moderate pycnidial formation, lesions much coalesced; and 5 = very susceptible, large, abundant pycnidia, lesions extensively coalesced.

0 1 2 3 4 5

visible symptoms, no pycnidial formation; 1 = resistant, hypersensitive flecking in younger leaf tissue, no pycnidial formation; 2 = moderately resistant, very light pycnidial formation (1-2 pycnidia/mm^2), some coalescence of lesions, mainly towards the leaf tip; 3 = moderately susceptible, light pycnidial formation (3-5 pycnidia/mm^2), coalescence of lesions normally evident towards the leaf tips and elsewhere on the leaf blade; 4 = susceptible, moderate pycnidial formation (>5 pycnidia/mm^2), lesions much coalesced; 5 = very susceptible, large, abundant pycnidia, lesions extensively coalesced. This scale is a slight modification of that of Rosielle (1972), where reaction type 1 had no or only occasional pycnidia on leaf tissues. Based on their reaction, six cultivars, Conway (susceptible), Anza (moderately susceptible), Veranopolis (moderately resistant), Colotana (resistant), Klein Titan (resistant) and Etit Hazera 38 (very resistant), were chosen as the differential set. The characteristics of these cultivars are shown in Table 6.1. Seed of these cultivars used in the virulence study was produced in the greenhouse at Saskatoon.

6.2.3 Testing of selected isolates on differential cultivars

Due to size limitation of the mist chamber, it was not possible to test all 40 isolates on the six differential cultivars at the same time. Therefore, an incomplete block design with four replications was used to conduct the study. The isolates were randomized in each replication and divided into two groups, each composed of 20 isolates. The isolates of each group were tested on the six differential cultivars at the same time.

Seeds of each differential cultivar were pre-germinated in a Petri plate on filter paper moistened with 10 ppm GA_3 solution and then planted in a 10 cm plastic pot (three seeds per pot). Plants in each differential set (six pots representing six differentials)

Table 6.1 Characteristics of the differential cultivars used in the virulence study of *Mycosphaerella graminicola* isolates

Cultivar	Accession number	Species	Number of resistance genes	Country of origin
Conway		*Triticum aestivum*	-	Canada
Anza	CI 15284	*Triticum aestivum*	?	USA
Veranopolis		*Triticum aestivum*	1[a]	Brazil
Colotana	PI 214392	*Triticum aestivum*	2[b]	Brazil
Klein Titan	CI 12615	*Triticum aestivum*	2[b]	Argentina
Etit Hazera 38	PI 295965	*Triticum durum*	?	Israel

[a]Wilson , 1979.
[b]Danon and Eyal, 1990.

were inoculated at the three-leaf stage with 50 ml of inoculum as described earlier (Section 6.2.2). After three weeks, the reaction of each cultivar was evaluated using the 0 to 5 rating scale modified from that of Rosielle (1972). Reaction types 0-1 (no pycnidial formation) were considered to be resistant and reaction types 2-5 with different levels of pycnidial density were considered to be susceptible.

The rating scale data were transformed by square root transformation to remove variance heterogeneity and the data were analyzed using PROC GLM of the Statistical Analysis System (SAS), ver. 6. Both transformed and untransformed data were subjected to analysis of variance. Plots of residuals versus fitted values showed that variances were heterogeneous, but transformation of the data did not remove heterogeneity and had no effect on the conclusions of the experiment. Therefore, the results of the analysis of untransformed data are presented.

6.3 Results

No immune reaction was observed among the cultivars inoculated with the 40 isolates of *M. graminicola*. The most resistant cultivar showed a few isolated necrotic lesions with no pycnidia, so reaction type 0 was not used and all reactions with no pycnidia were scored as reaction type 1. Analysis of variance showed that the main effect of cultivars and the isolate x cultivar interaction were highly significant (Table 6.2). This analysis of variance was based on three replications only because the data for replication 4 were not considered reliable due to a growth chamber malfunction.

The presence of an isolate x cultivar interaction suggested that there were distinct virulence factors in some isolates, which enabled them to attack certain differential

Table 6.2 Analysis of variance of disease reaction of six differential cultivars tested with 40 molecular phenotypes of *Mycosphaerella graminicola*

Source of variation	DF	MS
Replication	2	1.30
Block (rep)	3	0.24
Isolate	39	0.38
Cultivar	5	157.39***
Isolate X Cultivar	195	0.31***
Error	474	0.19

*** Significant at the 0.1% probability level.

cultivars but not others (Table 6.3). All isolates of *M. graminicola* were virulent on the cultivar Conway, indicating absence of resistance to the selected isolates. In contrast, cultivars Etit Hazera 38, Colotana and Klein Titan were uniformly resistant to all isolates, suggesting that the isolates did not possess virulence factor(s) effective against these sources of resistance. Cultivar Veranopolis was susceptible to isolates St10, 12, 78, 80, 81 and 89 and cultivar Anza was susceptible to St1, 2, 4, 10, 14, 39, 49, 54, 57, 58, 60, 67, 71, 73, 78, 79, 82 and 89.

Although the isolate x cultivar interaction was significant, the magnitude of this source of variation was very low with 2.67% of the total variance and, therefore, no attempt was made to classify isolates into different pathotype groups. The majority of the variation was related to differences among cultivars (Table 6.2). To reduce this

Table 6.3 Adjusted mean disease reaction of 40 isolates of *Mycosphaerella graminicola* tested on six differential cultivars in growth chamber experiments

Isolate	Cultivars						Mean
	Conway	Veranopolis	Anza	Colotana	Klein Titan	Etit Hazera 38	
St 1	**4.0** [a]	1.1	**2.8**	1.1	1.0	1.0	1.8
St 2	**4.0**	1.4	**2.4**	1.2	1.0	1.0	1.8
St 4	**4.0**	1.2	**2.0**	1.0	1.0	1.0	1.7
St 5	**4.0**	1.4	1.9	1.0	1.0	1.0	1.7
St 10	**4.0**	**2.2**	**2.0**	1.1	1.0	1.0	1.9
St 11	**4.0**	1.1	1.1	1.0	1.0	1.0	1.5
St 12	**4.0**	**2.1**	1.0	1.8	1.0	1.0	1.8
St 14	**4.0**	1.7	**2.2**	1.0	1.0	1.0	1.8
St 15	**4.0**	1.1	1.0	1.0	1.0	1.0	1.5
St 17	**4.2**	1.1	1.7	1.3	1.0	1.0	1.7
St 18	**3.9**	1.7	1.2	1.5	1.0	1.2	1.8
St 22	**4.0**	1.0	1.0	1.0	1.0	1.0	1.5
St 23	**3.9**	1.8	1.5	1.1	1.0	1.0	1.7
St 27	**3.8**	1.0	1.7	1.0	1.0	1.4	1.7
St 33	**4.0**	1.4	1.8	1.0	1.0	1.0	1.7
St 34	**3.9**	1.0	1.0	1.0	1.0	1.1	1.5
St 36	**4.0**	1.0	1.5	1.0	1.0	1.0	1.6
St 38	**3.9**	1.0	1.1	1.0	1.0	1.0	1.5
St 39	**4.0**	1.1	**2.3**	1.0	1.0	1.0	1.7
St 46	**4.1**	1.7	1.6	1.0	1.0	1.0	1.7
St 49	**4.0**	1.1	**2.1**	1.0	1.0	1.0	1.7
St 54	**4.0**	1.0	**2.1**	1.0	1.0	1.0	1.7
St 57	**4.0**	1.3	**2.0**	1.0	1.0	1.0	1.7
St 58	**4.0**	1.1	**3.0**	1.0	1.0	1.0	1.8
St 60	**4.1**	1.2	**2.8**	1.1	1.0	1.0	1.9
St 63	**4.0**	1.6	1.5	1.1	1.0	1.0	1.7
St 64	**4.0**	1.9	1.9	1.0	1.0	1.0	1.8
St 66	**4.0**	1.6	1.0	1.0	1.0	1.0	1.6
St 67	**3.9**	1.0	**2.1**	1.0	1.0	1.0	1.7
St 71	**3.8**	1.4	**2.9**	1.4	1.0	1.0	1.9
St 73	**3.8**	1.2	**2.0**	1.0	1.0	1.0	1.7
St 78	**4.0**	**2.4**	**3.1**	1.4	1.0	1.0	2.1
St 79	**4.1**	1.7	**2.8**	1.2	1.0	1.0	2.0
St 80	**4.0**	**2.5**	1.7	1.0	1.0	1.0	1.9
St 81	**4.0**	**2.1**	1.8	1.0	1.0	1.0	1.8
St 82	**3.8**	1.1	**2.7**	1.0	1.0	1.0	1.8
St 83	**4.0**	1.4	1.0	1.0	1.0	1.0	1.6
St 86	**4.0**	1.3	1.6	1.1	1.0	1.0	1.7
St 88	**3.7**	1.8	1.0	1.0	1.0	1.0	1.6
St 89	**4.0**	**2.0**	**3.0**	1.0	1.6	1.0	2.1
Mean	**4.0**	1.4	1.9	1.1	1.0	1.0	

[a] The disease reaction types are based on a 0-5 rating scale modified from that of Rosielle (1972) where 0 = immune, 1 = resistant, 2 = moderately resistant, 3 = moderately susceptible, 4 = susceptible and 5 = very susceptible. In this study, only reaction types < 2 were considered to be resistant, all others were considered susceptible. Bold font represents a susceptible reaction.

source of variation, the data for cultivars Etit Hazera 38, Klein Titan and Colotana, all uniformly resistant, and for cultivar Conway, uniformly susceptible to all of the isolates, were removed from the analysis. The analysis of variance of the data for the remaining two differential cultivars, Veranopolis and Anza, showed that the main effect of cultivars and the isolate x cultivar interaction were highly significant (Table 6.4).

Table 6.4 Analysis of variance of disease reaction of two differential cultivars tested with 40 molecular phenotypes of *Mycosphaerella graminicola*

Source of variation	DF	MS
Replication	2	4.04
Block (rep)	3	0.64
Isolate	39	0.89
Cultivar	1	10.63***
Isolate X Cultivar	39	0.85**
Error	154	0.45

** Significant at the 1% probability level.
*** Significant at the 0.1% probability level.

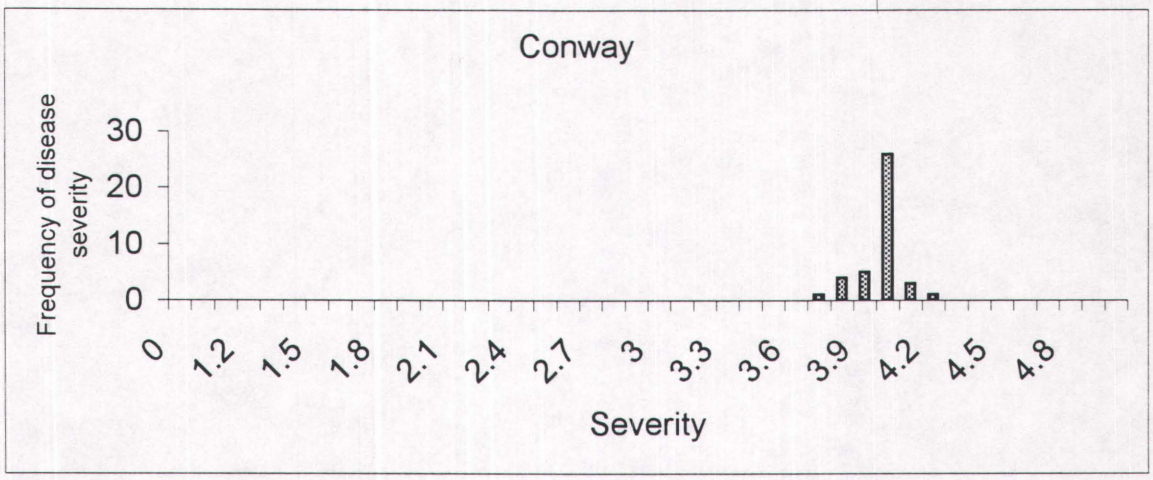
Removing the data for the uniformly resistant and susceptible cultivars from the analysis increased the relative magnitude of the isolate x cultivar interaction as compared to the analysis where the data for all cultivars were included. However, the magnitude of this component of variance was still low (19.12%). Parlevliet and Zadoks

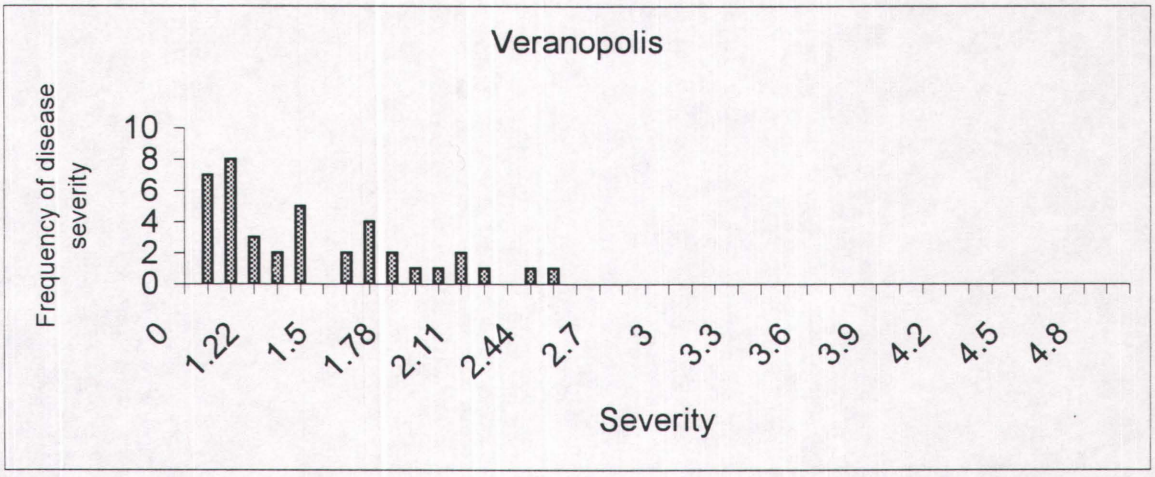
(1977) showed that a small isolate x cultivar interaction is more common in host-pathogen systems where virulence in the pathogen and resistance in the host are expressed quantitatively. A similar pattern of quantitative expression was observed in this study.

Figure 6.2 shows the distribution of the mean disease scores for cultivars Conway, Veranopolis and Anza. The distributions were continuous for all three cultivars, however, there were differences in the pattern of the distributions. In Conway, a highly susceptible cultivar, all isolates were virulent and differed only in their degree of pathogenicity, as was shown in Chapter 3. Distribution of responses of Conway to the isolates was normal suggesting the existence of highly pathogenic and less pathogenic isolates. Cultivars Veranopolis and Anza were resistant to some isolates and susceptible to others. Veranopolis showed resistance to the majority of isolates and consequently its distribution was skewed towards the resistant end of the scale. Anza showed more susceptibility and gave a wider range of responses than Veranopolis. Differences in virulence on both cultivars and in the degree of pathogenicity were evident on Anza, but for Veranopolis, differences in pathogenicity were less evident. This type of distribution suggests that variability for both virulence and degree of pathogenicity (aggressiveness) exists in the wheat-*M. graminicola* pathosystem.

These results also showed that different molecular phenotypes, which had been collected from the same leaf, caused different reactions on different cultivars. For example, the three different molecular phenotypes HP 7 (St10), HP 2 (St11) and HP 29 (St12) were all collected from the same leaf and differed in virulence. Molecular phenotype HP 2 (St11) was virulent on the susceptible cultivar Conway, but was

Figure 6.2 Distribution of the mean disease severity of the three differential cultivars Conway, Veranopolis and Anza tested with 40 molecular phenotypes of *Mycosphaerella graminicola*. Disease reaction was based on a 0-5 scale where reaction type 1 represents a resistant reaction and reaction types 2-5 represent increasing levels of susceptibility.


Conway
Frequency of disease severity
30
20
10
0
0
1.2
1.5
1.8
2.1
2.4
2.7
3
3.3
3.6
3.9
4.2
4.5
4.8
Severity


Veranopolis
Frequency of disease severity
10
8
6
4
2
0
0
1.22
1.5
1.78
2.11
2.44
2.7
3
3.3
3.6
3.9
4.2
4.5
4.8
Severity


Anza
Frequency of disease severity
8
6
4
2
0
0
1.22
1.5
1.78
2.11
2.44
2.67
3
3.3
3.6
3.9
4.2
4.5
4.8
Severity

avirulent on the other differential cultivars. Molecular phenotype HP 7 (St10) was virulent on cultivars Conway, Veranopolis and Anza, but avirulent on cultivars Colotana, Klein Titan and Etit Hazera 38. Molecular phenotype HP 29 (St12) was virulent on cultivars Conway and Veranopolis, but avirulent on the other cultivars. This indicated that different molecular phenotypes sampled from the same leaf had different combinations of virulence factors. However, other isolates collected from the same leaf, but with different molecular phenotypes, had similar reactions on the differential cultivars. For example both HP 6 (St1) and HP 17 (St2) were virulent on Conway and Anza, but avirulent on the other cultivars.

6.4 Discussion

The results of this experiment showed that the isolate x cultivar interaction was highly significant, but the magnitude of the variance of this interaction was small and thus did not suggest the existence of pathotypes/races among the isolates. The small magnitude of this variance may be due to the incomplete expression of resistance genes in the host plant. Jenns and Leonard (1985) found that a small interaction variance is common in those host-pathogen systems in which genes for race non-specific and race-specific resistance and genes for virulence and aggressiveness are operating. Using modeling, they showed that, as the number of randomly chosen isolates or cultivars was reduced, the magnitude of the variance attributable to the cultivar x isolate interaction increased. This variance was greatest when only the most cultivar and the most virulent isolate were included in the analysis. Similar results were obtained in this study. When

only the data of cultivars Veranopolis and Anza were included in the analysis, the magnitude of the interaction variance increased.

van Silfhout et al. (1989) tested 48 isolates of *M. graminicola* collected from *Triticum aestivum* and *T. durum* on 15 differential cultivars at the seedling stage and reported similar results. They found significant differences among cultivars and isolates, and a significant isolate x cultivar interaction, but this interaction accounted for only about 2% of the total variance. Although the magnitude of the interaction variance was low, they did find that certain isolates were able to overcome the resistance of some differential cultivars. Therefore, they suggested that each of these isolates must have one or more genes for specific virulence, which did not occur in the other isolates, implying that these isolates belonged to different races.

Cultivar Conway was highly susceptible to all selected isolates indicating that this cultivar does not carry any resistance genes effective against the tested isolates of *M. graminicola*. Another possibility is that since this cultivar has been grown commercially in Saskatchewan and Alberta, the pathogen may have adapted to this genotype. No such adaptation to the other differentials would have occurred since they have not been grown commercially in this province. Ahmed et al. (1995) found that *M. graminicola* isolates collected from California were more virulent on Californian cultivars than on Oregon cultivars, while Oregon isolates were more virulent on Oregon cultivars than on Californian cultivars.

The durum wheat cultivar Etit Hazera 38 was highly resistant to all isolates. Since these isolates were derived from infected leaves of a bread wheat cultivar, this reaction response agrees with other studies. Eyal et al. (1973) found that Etit Hazera 38 was

resistant to all isolates of *M. graminicola* obtained from hexaploid wheat, but it was susceptible to isolates which had been collected from durum wheat. van Ginkel and Scharen (1988) and Kema et al. (1996a, 1996b) reported that specialization on either bread wheat or durum wheat occurred in *M. graminicola.* They found that isolates of *M. graminicola* collected from durum wheat produced little disease on bread wheat and vice versa.

Cultivars Klein Titan and Colotana, which have the cultivar Frontana in their pedigree, showed a resistant reaction to most of the isolates. This agrees with published reports that South American and other wheat accessions with Frontana in their parentage expressed low pycnidial coverage to a wide range of *M. graminicola* isolates (Eyal et al. 1985; Eyal and Levy 1987; Eyal 1995).

Cultivar Veranopolis was susceptible to some isolates but resistant to others. Wilson (1979) reported that resistance in cultivar Veranopolis (Trintecinco/Frontana) to Australian isolates of *M. graminicola* was controlled by a single dominant gene. The cultivar Anza also showed a differential response to the isolates of *M. graminicola,* which agrees with the results of Kema et al. (1996a). The reaction of cultivars Veranopolis and Anza indicates that some isolates of *M. graminicola* carry virulence genes which were able to overcome their resistance genes. This finding was confirmed by significant isolate x cultivar interactions in the analyses of variance.

The occurrence of differential interactions between host and pathogen genotypes suggests the existence of a gene-for-gene relationship in the *M. graminicola*-wheat pathosytem. This hypothesis is supported by the results of other investigators (Eyal et al.1985; Kema et al. 1996a, 1996b; McCartney et al. 2002; Brading et al. 2002).

However, it is contrary to the results of van Ginkel and Scharen (1988) and Marshall (1985) who did not find significant interaction between isolates of the pathogen and host genotypes. Kema et al. (1996a) argued that the gene-for-gene relationship in the *M. graminicola*-wheat pathosystem is different from the model gene-for-gene system suggested by Person (1959). A model gene-for-gene relationship is typical of pathosystems, such as the wheat-stem rust or wheat-powdery mildew systems, that involve resistance and virulence genes with major effects and which result in distinct qualitative disease reactions. Parlevliet and Zadoks (1977) believed that where resistance and virulence are expressed quantitatively, a gene-for-gene interaction is inferred by the existence of a significant isolate x genotype interaction although the contribution of this interaction to the non-environmental variance was small.

Recent studies support the existence of race specificity in the wheat-*M. graminicola* pathosystem. Cowger et al. (2000) studied specific adaptation by *M. graminicola* to the three resistant wheat cultivars Gene, Madsen, and Stephens. They found that after three years, the highly resistant cultivar Gene became susceptible. They concluded that its resistance was race specific and that as a result of its commercial cultivation, strains of *M. graminicola* that could overcome its resistance were selected for.

Halperin et al. (1996) and Ezrati et al. (1999) reported that inoculation of specific genotypes with a mixture of avirulent and virulent isolates of *M. graminicola* resulted in a marked suppression of pycnidial production. They suggested that the presence of an avirulent isolate in the mixture triggered a defense mechanism in the host resulting in a significant reduction in the amount of pycnidial coverage. A recent study showed that

avirulence in *M. graminicola* is controlled by a single gene, a finding consistent with the gene-for-gene hypothesis (Kema et al. 2000).

This study has shown that, in addition to variability for the degree of pathogenicity (aggressiveness) reported in Chapter 3, there is also variability for virulence in *M. graminicola.* Variability for virulence was inferred from a significant isolate x cultivar interaction in the analysis of variance. However, because the relative magnitude of the variance of this interaction was small no attempt was made to classify the isolates into different races. Similar types of pathogenic variability have been reported for other plant pathogens. Rufty et al. (1981) showed that wheat cultivars differed in resistance to *Stagonospora nodorum* and pathogen isolates differed in pathogenicity and obtained a significant isolate x cultivar interaction. However, because the magnitude of the variance of interaction was low, no pathogen races were identified. Wroth (1998), in a study of variation in pathogenicity among and within populations of *Mycosphaerella pinodes,* the cause of ascochyta blight of pea in Australia, obtained similar results.

This experiment indicated that both variability for virulence and variability for degree of pathogenicity (aggressiveness) exist in *M. graminicola.* The presence of these types of variability suggests that the pathogen has the potential to adapt rapidly to race-specific resistance sources. Therefore, the best strategy for controlling this disease would be to use race non- specific resistance. In breeding for race non-specific resistance, it would be desirable to screen germplasm in locations where pathogen populations have the greatest genetic diversity. Another approach would be to expose germplasm to natural infection in different geographical regions in which different virulent isolates might occur. In addition to race non-specific resistance, pyramiding of

different resistance genes in one cultivar or using multilines or cultivar mixtures may provide adequate levels of resistance to *M. graminicola*.

CHAPTER 7

RELATIONSHIP BETWEEN MOLECULAR AND PATHOGENIC VARIABILITY IN A POPULATION OF *MYCOSPHAERELLA GRAMINICOLA*

7.1 Introduction

Molecular studies have shown that there is considerable genetic variability within a population of *Mycosphaerella graminicola* isolated at different sampling levels, even within a lesion (McDonald and Martinez 1990b, 1991). However, little is known as to whether the same degree of variability exists for pathogenicity.

Studies using RAPD (Chapter 4) and SSR markers (Chapter 5) showed that there was a high level of genetic variability at the molecular level among 90 isolates of the pathogen. When these isolates were tested for pathogenicity, as measured by incubation period, latent period and disease severity, significant differences were found among isolates for all these components of pathogenicity (Chapter 3). Testing a sub-sample of isolates on a set of differential cultivars also showed that there was variability for virulence among isolates, even among those collected from the same leaf (Chapter 6).

The objective of this experiment was to test whether variability at the molecular level is related to variability for pathogenicity in *M. graminicola*.

7.2 Materials and Methods

To study the association between pathogenic and molecular variability in *M. graminicola*, data (131 RAPD fragments) obtained from the RAPD analysis (Chapter 4)

and the means of the components of pathogenicity for 90 isolates (Appendix A) were used. In addition, the relationship between variability at the molecular level and virulence was investigated on a subset of 40 isolates.

To make a comparison, a similarity matrix for the RAPD data was constructed using the SIMQUAL (Similarity of Qualitative Data) program of NTSYS-pc and Jaccard's coefficient as described earlier (Section 4.2.6). To construct a similarity matrix from quantitative pathogenicity data, the data was first standardized to remove the effect of different rating scales (Sharma 1996) (Appendix F). The matrix of similarity was then calculated for all possible pairs of isolates using the general similarity coefficient of Gower (1971), which is defined as

$$S_G = \sum_{i=1}^{n} W_{ijk} S_{ijk} / \sum_{i=1}^{n} W_{ijk}$$

where, W_{ijk} is a weight for character i and is set to 1 when a comparison between two individuals is valid and to 0 when the value of character i is unknown for one or both of the individuals. For quantitative characters, S_{ijk} can be calculated as

$$S_{ijk} = (1-|X_{ij}-X_{ik}| / R_i)$$

where, X_{ij} and X_{ik} are the values of character i for individuals j and k, and R_i is the range of character i (Sneath and Sokal 1973).

Gower's coefficient of similarity is applicable to both qualitative and quantitative data. For two-state characters such as RAPD data (presence (1) or absence (0) of a band), S_{ijk} is equal to 1 for matches (1, 1) and to 0 for mismatches ((1,0) or (0,1)). When two individuals match for the negative state of a two-state character (0,0), W_{ijk} is set to zero. In a data matrix consisting of two-state characters, Gower's coefficient gives similar results to the coefficient of Jaccard (Sneath and Sokal 1973). Therefore, making

a comparison between a similarity matrix based on qualitative data and one based on quantitative data is considered valid. To find the relationship between molecular variability and components of pathogenicity, the two similarity matrices were compared using the MXCOMP program of NTSYS-pc.

Cluster analysis was performed for the components of pathogenicity and for the molecular variability data, and dendrograms were derived from similarity matrices using the UPGMA (unweighted pair group method of arithmetic averages) clustering method of the SAHN program of NTSYS-pc. To measure how a dendrogram was fit to its similarity matrix, a cophenetic value matrix was calculated by the COPH program of NTSYS-pc for each dendrogram. Then, using the MXCOMP program of NTSYS-pc, the correlation between the cophenetic matrix of each dendrogram and its corresponding similarity matrix was determined.

To study the relationship between molecular variability and variability for virulence on a subset of 40 isolates, a similarity matrix of molecular data (111 RAPD fragments) was constructed based on Jaccard's coefficient and compared to a similarity matrix of virulence data based on Gower's coefficient. The correlation between these two matrices was calculated using the MXCOMP program of NTSYS-pc.

7.3 Results

A poor correlation ($r = 0.018$) existed between the molecular and pathogenicity similarity matrices, indicating no relationship between the molecular data and the components of pathogenicity data, i.e., these two types of variability were independent.

studied pathogenic variation in *Rhynchosporium secalis* populations from commercial barley fields in Idaho and Oregon. They found that isolates from different populations, which had the same electrophoretic genotypes, were usually different pathotypes. Similarly, isolates of identical pathotypes from different populations had different electrophoretic genotypes. They concluded that there was no association between isozyme electrophoretic type and pathotype.

Peever and Milgroom (1993) did not find a consistent relationship between molecular genetic variability as revealed by RAPD markers, and variability for resistance to sterol-biosynthesis-inhibiting fungicides (SBIS) in *Pyrenophora teres*. They suggested that molecular markers cannot be used to predict variation in ecologically important traits such as fungicide resistance. Similar results have been reported in studies of *Drechslera teres* (Peltonen et al. 1996) and *Puccinia striiformis* (Chen et al. 1993) where no relationship was found between molecular variability and other morphological traits such as virulence and aggressiveness.

McDonald et al. (1995) suggested that it would be unlikely to find an association in a randomly mating population between neutral DNA markers and virulence or fungicide resistance genes. They believed that the best chance to find association between neutral DNA markers and other loci which are subjected to selection pressure (such as virulence or fungicide resistance) is in a population which reproduces exclusively by asexual means.

In this study molecular markers, RAPD (Chapter 4) and SSRs (Chapter 5), showed that there was a high level of genetic variability in the population and that the majority of the genetic variability was distributed at the lowest isolate sampling level. Since the

Correlation between the cophenetic value matrix and similarity matrix of molecular and the pathogenicity data was good ($r = 0.82$ and $r = 0.71$, respectively), suggesting that using the coefficient of Jaccard for cluster analysis of the qualitative molecular data and that of Gower for the quantitative pathogenicity data was appropriate. Figure 7.1 shows the dendrogram generated using RAPD data and is based on the similarity coefficient of Jaccard. Figure 7.2 depicts the dendrogram generated from the pathogenicity data and is based on the general similarity coefficient of Gower. The topology of the two dendrograms was different. In the dendrogram based on the pathogenicity data (Fig 7.2), three groups, groups 1, 2 and 3 composed predominantly of isolates with high, intermediate and low pathogenicity, respectively, were detected at the 72% similarity level (the lower level of the 95% confidence interval of the average of similarity was considered to be the cut-off point). However, no such grouping was detected at that similarity level in the dendrogram based on RAPD data (Fig 7.1). This suggests that the magnitude of molecular variability was higher than the magnitude of pathogenic variability.

When the isolates were compared based on their molecular and pathogenic similarity values, no relationship was found. The dendrogram based on molecular data (Fig. 7.1) showed that isolates St 7 and St 32 had the highest similarity value (80%). However, they occurred in different groups (group 1 and group 2, respectively) in the dendrogram based on pathogenicity data (Fig. 7.2). Similarly, isolates St 1 and St 3, sampled from the same leaf had a high degree of molecular similarity (76%), however, they also occurred in different pathogenicity groups, group 1 and group 2, respectively. Isolates

Figure 7.1 Dendrogram of similarity of 90 isolates of *Mycosphaerella graminicola* based on random amplified polymorphic DNA (RAPD) data generated by 15 random decamer primers. The UPGMA algorithm of the SAHN program of NTSYS-pc and similarity coefficient of Jaccard was used for cluster analysis.

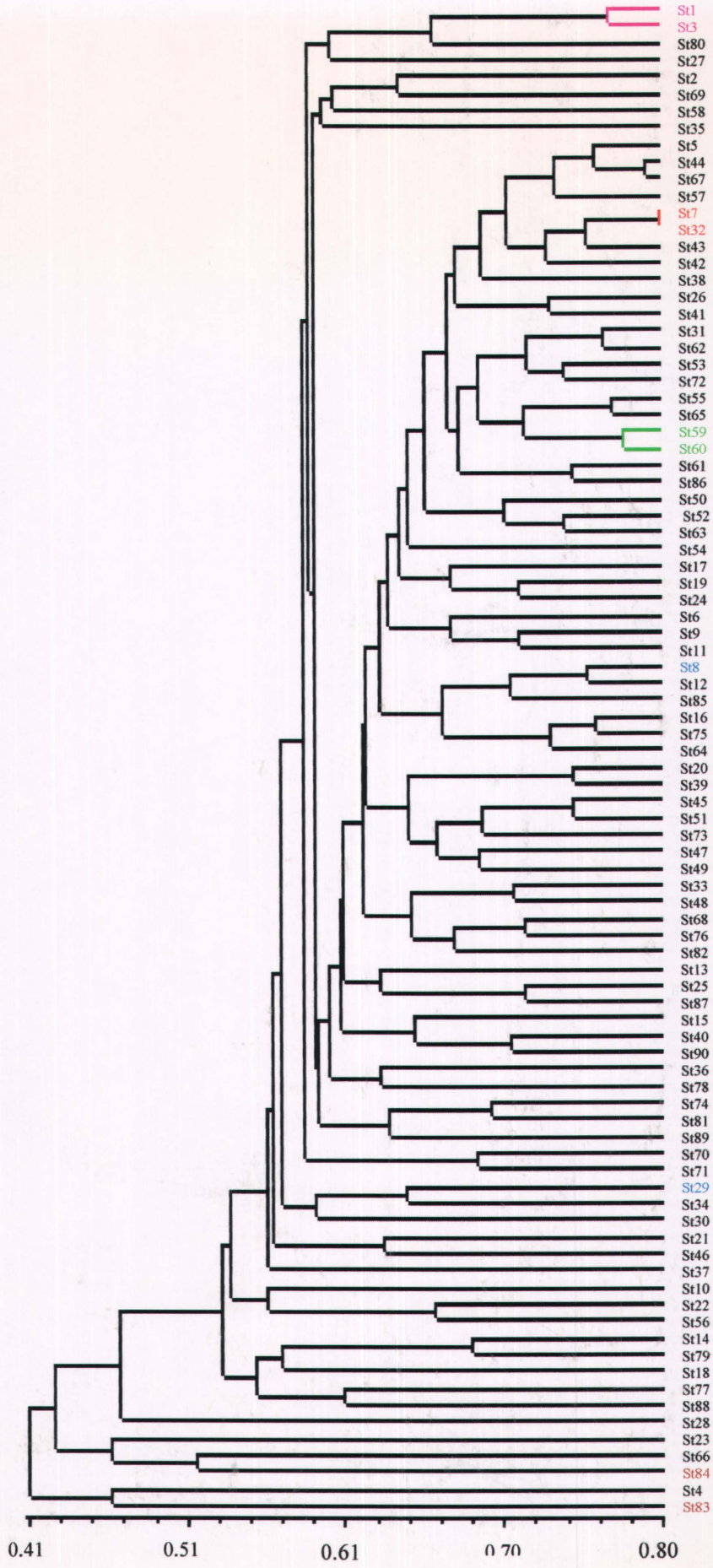
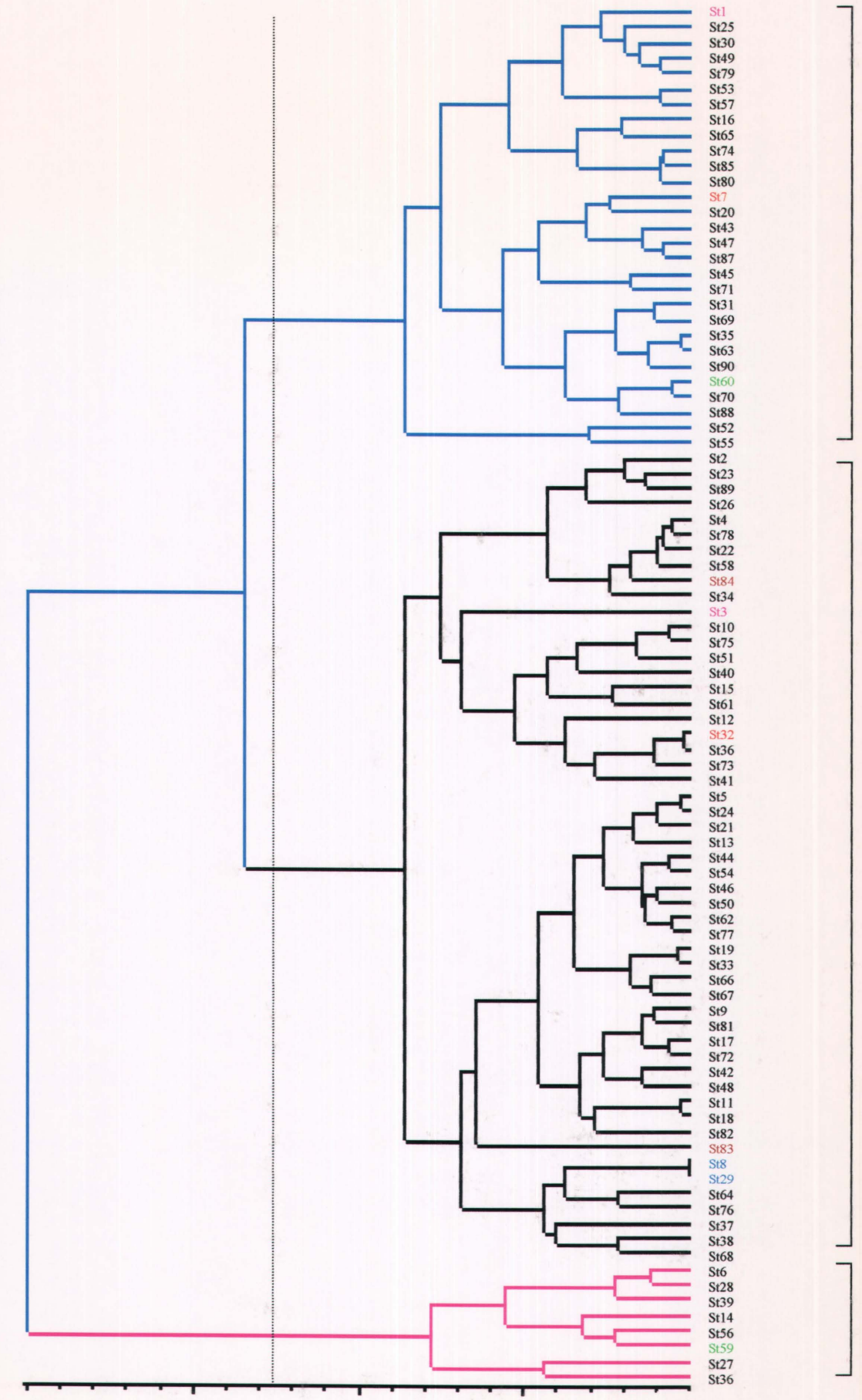

St1
St3
St80
St27
St2
St69
St58
St35
St5
St44
St67
St57
St7
St32
St43
St42
St38
St26
St41
St31
St62
St53
St72
St55
St65
St59
St60
St61
St86
St50
St52
St63
St54
St17
St19
St24
St6
St9
St11
St8
St12
St85
St16
St75
St64
St20
St39
St45
St51
St73
St47
St49
St33
St48
St68
St76
St82
St13
St25
St87
St15
St40
St90
St36
St78
St74
St81
St89
St70
St71
St29
St34
St30
St21
St46
St37
St10
St22
St56
St14
St79
St18
St77
St88
St28
St23
St66
St84
St4
St83
0.41
0.51
0.61
0.70
0.80
Jaccard's coefficient

Figure 7.2 Dendrogram of similarity of 90 isolates of *Mycosphaerella graminicola* based on the standardized data of components of pathogenicity (incubation period, latent period and disease severity) determined on the susceptible cultivar Conway. UPGMA algorithm of the SAHN program of NTSYS-pc and the general similarity coefficient of Gower (1971), was used for cluster analysis. The numbers 1, 2, and 3 represent three groups (groups 1, 2, and 3 composed predominantly of isolates with high and intermediate and low pathogenicity, respectively) detected at the 72% similarity level. The vertical dotted line represents the lower level of the 95% confidence interval of the average of similarity, which was considered to be the cut-off point).


St1
St25
St30
St49
St79
St53
St57
St16
St65
St74
St85
St80
St7
St20
St43
St47
St87
St45
St71
St31
St69
St35
St63
St90
St60
St70
St88
St52
St55
St2
St23
St89
St26
St4
St78
St22
St58
St84
St34
St3
St10
St75
St51
St40
St15
St61
St12
St32
St36
St73
St41
St5
St24
St21
St13
St44
St54
St46
St50
St62
St77
St19
St33
St66
St67
St9
St81
St17
St72
St42
St48
St11
St18
St82
St83
St8
St29
St64
St76
St37
St38
St68
St6
St28
St39
St14
St56
St59
St27
St36
1
2
3
0.56
0.67
0.78
0.88
0.99
Gower's coefficient

St 59 and St 60 with 78% molecular similarity (Fig. 7.1) each occurred in different pathogenicity groups, group 1 and group 3, respectively (Fig. 7.2).

Some isolates, which were very similar in their degree of pathogenicity, had low molecular similarity. For example, isolates St 8 and St 29 showed 99% similarity for pathogenicity, but their similarity based on molecular data was only 57%. There were also isolates which were low in both molecular and pathogenic similarity. Isolates St 83 and St 84, sampled from the same leaf, had the lowest molecular similarity (41%) in the population and they were also in very different pathogenicity subgroups.

7.4. Discussion

Comparison of similarity matrices of the molecular and pathogenicity data showed that there was no correlation ($r = 0.018$) between the two types of similarity matrices. Similar results were obtained when the molecular and virulence similarity matrices were compared ($r = -0.03$). In addition, visual comparison of the dendrograms based on pathogenicity and molecular data (Figs. 7.1, 7.2) and of the molecular and virulence data (Appendices G 1, G 2) indicated that their topology was different. Some isolates, which had the highest pathogenic similarity also had the highest genetic distance. Since genes involved in pathogenicity are only a small proportion of the genome, this type of relationship would be expected.

These types of relationship between the dendrograms and the low level of association between the similarity matrices suggested that there was no relationship between molecular and pathogenic variability in *M. graminicola* and that these two types of variability are independent. Similar results were reported by Goodwin et al. (1992) who

high degree of genetic variability and the type of distribution of the variability in the population suggested that air-borne ascospores are the primary source of inoculum, it was inferred that sexual reproduction of *M. graminicola* occurs in Saskatchewan (Section 3.4). This hypothesis was strongly supported by the recent report of the occurrence of the sexual state of *M. graminicola* in Manitoba (Hoorne et al. 2002). Therefore, due to gene re-assortment during meiosis, an association between neutral DNA markers and pathogenicity loci would not be expected unless such loci were closely linked.

Lack of association between molecular and pathogenic variability suggests that DNA fingerprinting has little value for monitoring the development of new virulent genotypes of the pathogen. Therefore, to detect different pathotypes or to monitor their frequencies in a population, use of neutral DNA markers will not be sufficient and isolates should be tested separately for their pathogenic variability on a set of appropriate differential cultivars.

CHAPTER 8

GENERAL DISCUSSION

Information about the genetic structure of pathogen populations and biology of the pathogen is necessary to understand how pathogen populations change and what factors are involved in their evolution. This knowledge is a prerequisite for implementing effective control strategies. Study of the genetic structure of a pathogen requires determination of how much genetic diversity exists in the pathogen population and how this genetic diversity is distributed within and among populations (McDonald et al. 1995; McDonald 1997). Molecular studies using RFLP markers showed that there was a high level of genetic variability within a population of *M. graminicola* (McDonald and Martinez 1990b, 1991), however, no attempt was made to relate this molecular variability to pathogenicity. The objectives of this study were to investigate a particular population of *M. graminicola* for both variability at the molecular level and variability for pathogenicity, and to investigate a possible relationship between these two types of variability.

There have been conflicting reports regarding pathogenic variation of *M. graminicola.* Most researchers believe that there are true virulence differences among isolates of the pathogen (Eyal et al. 1985; Eyal and Levy 1987; Kema et al. 1996a, 1996b; McCartney et al. 2002; Brading et al. 2002), however, others have shown that the only difference among isolates is in their degree of pathogenicity (aggressiveness) (Marshall 1985; van Ginkel and Scharen 1988; van Ginkel and Rajaram 1995). Since

the strategy for resistance breeding would be different, depending on which type of variability exists in the pathogen population, tests for both types of variability were conducted.

Significant differences were found for the degree of pathogenicity (aggressiveness) among isolates as measured by incubation period, latent period and disease severity on a single susceptible wheat cultivar Conway. Although there were significant differences among isolates for these components of pathogenicity, the magnitude of the variation was low. In addition, the distribution of means of these components appeared to be normal, suggesting that these components of pathogenicity were quantitative traits. Other investigators have reported similar results (Marshall 1985; van Ginkel and Scharen 1988; van Ginkel and Rajaram 1995). Quantitative variation for pathogenicity has also been reported in *Stagonospora nodorum* (Krupinsky 1997a, 1997b).

Variability for components of pathogenicity suggests that there are some isolates in the population that are more pathogenic than others. It is not known what causes these differences in pathogenicity. Recently, a number of enzymes such as proteases, glucanases, cutinases and xylanases, which are involved in the degradation of plant cell walls and in the acquisition of nutrients from the plant have been identified in *M. graminicola* (Palmer and Skinner 2002). One possibility is that the level of expression of these pathogenicity genes might be different in isolates with low and high pathogenicity. The other possibility is that the number of genes controlling pathogenicity might be different in highly and less aggressive isolates.

Partitioning the total variability of components of pathogenicity into variance components revealed that only the variation among lesions was significant and that

variations among leaves and locations were not significant. This type of distribution of variability suggests that there is no hierarchical structure for pathogenicity within the population of *M. graminicola.* If there was such a structure, significant differences for components of pathogenicity (incubation period, latent period and disease severity) among leaves and among locations would be expected. A hierarchical structure of pathogenic variation has been reported by Goodwin et al. (1992) in *Rhynchosporium secalis.*

The virulence study showed a significant isolate x cultivar interaction. This interaction indicated that race specificity exists in the wheat-*M. graminicola* pathosystem, a finding reported by others (Eyal et al. 1985; Kema et al. 1996a, 1996b; McCartney et al. 2002; Brading et al. 2002). However, the relative magnitude of this interaction was very low, suggesting the absence of physiological races. Similar results were reported by Krupinsky (1997a, 1997b) for *Stagonospora nodorum.* He speculated that a low level of cultivar specificity is due to a vigorous saprophytic phase in the life cycle of *S. nodorum* where there is no pressure on the population for the selection of highly specific types as occurs with obligate parasites. Perhaps a similar mechanism exists for *M. graminicola* which has the same type of life cycle.

The presence of an isolate x cultivar interaction suggests that a gene-for-gene relationship exists in the wheat-*M. graminicola* pathosystem. However, studies of the host-pathogen genetics of this pathosystem are required to prove this hypothesis. Recently, Brading et al. (2002) conducted this type of study and showed the existence of a gene-for-gene relationship in the wheat-*M. graminicola* pathosystem.

In this study, a significant isolate x cultivar interaction was obtained in tests conducted at the seedling stage under controlled environmental conditions. It is not known whether similar results would be obtained if similar testing was conducted in the field. van Ginkel and Rajaram (1995) believed that isolate x cultivar interactions are detected only under controlled environmental conditions, since in the field tests, cultivars are exposed to mixtures of isolates and differences in the virulence can not be detected. However, Kema and van Silfhout (1997) obtained significant isolate x cultivar interactions in tests conducted at the adult plant stage under field conditions.

The presence of isolate x cultivar interactions does not always indicate race specificity among isolates of a pathogen. Robinson (1987) reported that under certain circumstances, such as inaccurate measurement of disease severity and the type of host genotypes used as differentials, the analysis of variance can be affected resulting in false isolate x cultivar interactions. To avoid these problems it would be desirable to assemble a standard differential set with known resistance genes preferably in a common susceptible genetic background and develop a standard rating scale for scoring disease severity.

Parlevliet and Zadoks (1977) reported that a small isolate x cultivar interaction is common in host-pathogen systems where pathogenicity and host resistance are expressed quantitatively. A small isolate x cultivar interaction may be due also to incomplete expression of host resistance genes and the diversity of natural *M. graminicola* populations, which will mask the expression of race-specific resistance

genes. However, under controlled conditions, where tests for disease resistance are conducted with a single isolate, isolate x cultivar interactions become more evident (Kema and van Silfhout 1997).

This study has shown that variability for the degree of pathogenicity (aggressiveness) and for virulence can exist simultaneously in *M. graminicola*. Kema et al. (1996b) has also suggested that in the *M. graminicola*–wheat pathosystem expression of resistance and virulence appears to be both quantitative and qualitative. They found that there were some isolates whose average pycnidial production on a set of differential wheat genotypes was low but which exhibited a high variability for pycnidial production. However, there were some isolates, which had both low average pycnidial production and low variability for pycnidial production. They speculated that in the former group the relative proportion of cultivar-specific resistance genes was higher than the latter one.

Use of RAPD markers showed that there was a high level of molecular genetic variability within the population. Of the 131 RAPD fragments identified, 126 (95%) were polymorphic. The average gene diversity of the population was estimated to be approximately 0.18, while using SSR markers it was estimated to be 0.44. Similar results were reported by McDonald and Martinez (1990b) using RFLP markers in a Californian population of *M. graminicola* where the average gene diversity was 0.45. These results suggested that multi-allelic markers such as SSR and RFLP are more powerful than RAPD markers in detecting genetic diversity in plant pathogens. Since the use of RFLP markers is more laborious, time consuming and expensive, it would be better to use PCR-based multi-allelic markers such as SSR or SCAR in such studies.

Partitioning the total gene diversity into within- and among-location components revealed that only a small proportion of the variability (G_{ST} = 0.14) occurred among locations. Similarly, Boeger et al. (1993) found a very low level of genetic differentiation (G_{ST} = 0.04) between two geographically separated populations of *M. graminicola*. They showed that lack of differentiation between the populations was the result of gene flow. In this study, the low differentiation among locations was consistent with the proposal of Goodwin et al. (1993) that this would occur if the subpopulations were derived from a common source population.

Multilocus analysis detected 40 RAPD molecular phenotypes (haplotypes) in the population. These haplotypes were randomly distributed among the sampling locations, a type of distribution expected if little clonal structure existed within the population. Multilocus analysis with microsatellite markers detected 77 haplotypes among the 90 isolates, only 14% of which were identical. The cluster analysis based on RAPD data supported the finding that almost every isolate was a unique genotype. High genetic dissimilarity among some isolates sampled from different lesions of the same leaf suggested that every lesion was the result of infection by a genetically different ascospore. Chen and McDonald (1996) also have suggested that the primary source of inoculum was air-borne ascospores. Further evidence to support this finding was provided by the gametic disequilibrium analysis, where in allele-by-allele comparisons only 6% of the loci were in gametic disequilibrium and in locus-by-locus comparison none of the loci were associated implying that the population is randomly mating. Another approach to prove this hypothesis would be to estimate the ratio of different mating types in the pathogen population. Since *M. graminicola* is a heterothallic fungus

and produces two mating types (Kema et al. 1996c), detection of a 1:1 ratio of mating types would support the hypothesis of random mating. Waalwijk et al (2002) developed specific primers linked to each mating type of *M. graminicola*. In such studies, use of these primers would provide the potential for screening large numbers of isolates in a short period of time. The conventional method of detecting mating types by crossing different isolates would be very time consuming.

These results strongly suggest that sexual reproduction of *M. graminicola* occurs in Saskatchewan, a hypothesis supported by the recent detection of the sexual state of the pathogen in Manitoba (Hoorne et al. 2002). The presence of sexual reproduction implies that in each generation different genotypes with new combinations of virulence genes will be produced in the population. This allows the pathogen to adapt rapidly to race-specific resistance sources. Therefore, in breeding for resistance to *M. graminicola,* emphasis should be given to race non-specific resistance.

A poor correlation was found between variability for the degree of pathogenicity and molecular variability data, suggesting that there is no relationship between these two types of variability. A similar result was obtained when the relationship between virulence and molecular variability was examined. Visual comparisons of the dendrograms revealed that the clustering of isolates based on molecular data was different from the clustering based on pathogenicity and on virulence data, a further support for the independence of molecular and pathogenic variability in *M. graminicola.* This lack of association can be explained by (a) molecular RAPD data is based on amplification of parts of the genome which may not be linked or correlated with pathogenicity genes, and (b) since the pathogen most likely undergoes sexual

reproduction, and thus gene recombination, no association between any two loci would be expected unless they were closely linked. Therefore, the probability of finding isolates with similar molecular phenotype and the same level of pathogenicity should be very low. Therefore, association between pathogenic and molecular variability is not expected.

A similar lack of association between pathogenic and molecular variability has been reported in *Rhynchosporium secalis* (Goodwin et al. 1992), *Pyrenophora teres* (Peever and Milgroom 1993) and *Puccinia striiformis* (Chen et al. 1993). Lack of association between molecular and pathogenic variability suggests that randomly chosen molecular markers do not have the potential to detect variability for important traits such as fungicide resistance or virulence in *M. graminicola*. However, development of DNA markers closely linked to such traits would be useful for monitoring their variation and change in the population.

References

Ackermann, M. D., Stewart, S., and Ibanez, W. 1995. Pathogenic variability of *Septoria tritici* isolates from South America. Pages 41-51 in: The *Septoria tritici*: Proceedings of the Workshop. S. L. Gilchrist, M. van Ginkel, A. McNab, and G. H. J. Kema, eds. CIMMYT, Mexico, DF.

Adhikari, T. B., Mew, T. W., and Leach, J. E. 1999. Genotypic and pathotypic diversity in *Xanthomonas oryzae pv. oryzae* in Nepal. Phytopathology 89:687-694.

Ahmed, H. U., Mundt, C. C., and Coakley, S. M. 1995. Host-pathogen relationship of geographically diverse isolates of *Septoria tritici* and wheat cultivars. Plant Phathol. 44:838-847.

Arraiano, L. S., Worland, A. J., Ellerbrook, C., and Brown, J. K. M. 2001a. Chromosomal location of a gene for resistance to Septoria tritici blotch (*Mycosphaerella graminicola*) in the hexaploid wheat 'Synthetic 6X. Theor. Appl. Genet. 103:758-764.

Arraiano, L. S., Brading, P. A., and Brown, J. K. M. 2001b. A detached seedling leaf technique to study resistance to *Mycosphaerella graminicola* (anamorph *Septoria tritici*) in wheat. Plant Pathol. 50:339-346.

Bai, J., Choi, S. H., Ponciano, G., Leung, H., and Leach, J. E. 2000. *Xanthomonas oryzae* pv. *oryzae* avirulence genes contribute differently and specifically to pathogen aggressiveness. Mol. Plant Microbe Interact. 13:1322-1329.

Ballantyne, B. J., and Thompson, F. 1995. Pathogenic variation in Australian isolates of *Mycosphaerella graminicola*. Aust. J. Agri. Res. 46:921-934.

Ballantyne, B. J. 1989. Pathogenic variation in Australian cultures of *Mycosphaerella graminicola*. Page 54 in: Septoria diseases of cereals: Proceeding of the 3rd International Workshop. P. M. Fried ed. Zurich, Switzerland.

Bannon, F. J., and Cook, B. M. 1998. Studies on dispersal of *Septoria tritici* pycnidiospores in wheat-clover intercrops. Plant Pathol. 47:49-56.

Beck, J. J., and Ligon, J. M. 1995. Polymerase chain reaction assays for the detection of *Stagonospora nodorum* and *Septoria tritici* in wheat. Phytopathology 85:319-324.

Boeger, J. M., Chen, R. S., and McDonald, B. A. 1993. Gene flow between geographic populations of *Mycosphaerella graminicola* (anamorph *Septoria tritici*) detected with RFLP markers. Phytopathology 83:1148-1154.

Borer, S. O., Miller, L. M., and Kapuscinski, A. R. 1999. Microsatellites in walleye *Stizostedion vitreum*. Mol. Ecol. 8:335-346.

Brading, P. A., Verstappen, E. C. P., Kema, G. H. J., and Brown, J. K. M. 2002. A gene-for-gene relationship between wheat and *Mycosphaerella graminicola*, the Septoria tritici blotch pathogen. Phytopathology 92:439-445.

Brokenshire, T. 1976. The reaction of wheat genotypes to *Septoria tritici*. Ann. Appl. Biol. 82:415-423.

Brown, A. G. P., and Rosielle, A. A. 1980. Prospects for control of Septoria. W. Aust. J. Agric. 21:8-11.

Brown, J. S., Kellock, A. W., and Paddick, R. G. 1978. Distribution and dissemination of *Mycosphaerella graminicola* (Fuckel) Schröeter in relation to the epidemiology of speckled leaf blotch of wheat. Aust. J. Agri. Res. 29:1139-1145.

Burdon, J. J., and Roelfs, A. P. 1985. The effect of sexual and asexual reproduction on the isozyme structure of populations of *Puccinia graminis*. Phytopathology 75:1068-1073.

Cenis, J. L. 1992. Rapid extraction of fungal DNA for PCR amplification. Nucleic Acids Res. 20:2380.

Chen, R., and McDonald, B. 1996. Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola*. Genetics 142:1119-1127.

Chen, X. M., Line, R. F., and Leung, H. 1993. Relationship between virulence variation and DNA polymorphism in *Puccinia striiformis*. Phytopathology 83:1489-1497.

Chungu, C., Gilbert, J., and Townley-Smith, F. 2001. Septoria tritici blotch development as affected by temperature, duration of leaf wetness, inoculum concentration, and host. Plant Dis. 85:430-435.

Coakley, S. M., McDaniel, L. R., and Shaner, G. 1985. Model for predicting severity of *Septoria tritici* blotch on winter wheat. Phytopathology 75:1245-1251.

Cochran, W. G., and Cox, G. M. 1992. Experimental Designs. Johan Wiley and sons, Inc, New York.

Cohen, L., and Eyal, Z. 1993. The histology of processes associated with infection of resistant and susceptible wheat cultivars with *Septoria tritici*. Plant Pathol. 42:737-743.

Cook, R. J. 1999. Management by Chemicals. Pages 286-298 in: Septoria of Cereals: A Study of Pathosystems. J. A. Lucas, P. Bowyer, and H. M. Anderson, eds. CAB International, Wallingford, UK.

Cowger, C., Hoffer, M. E., and Mundt, C. C. 2000. Specific adaptation by *Mycosphaerella graminicola* to a resistant wheat cultivar. Plant Pathol. 49:445-451.

Cunfer, B. M., and Ueng, P. P. 1999. Taxonomy and identification of *Septoria* and *Stagonospora* species on small-grain cereals. Annu. Rev. Phytopathol. 37:267-284.

Czembor, P. C., and Arseniuk, E. 1996. Use of random amplified polymorphic DNA (RAPD) assay for differentiation among isolates of *Stagonospora* spp. and *Septoria tritici*. J. Appl. Genet. 37:239-251.

Danon, T., and Eyal, Z. 1990. Inheritance of resistance to two *Septoria tritici* isolates in spring and winter bread wheat cultivars. Euphytica 47:203-214.

Dubin, H. J., and Rajaram, S. 1996. Breeding disease-resistance wheats for tropical highlands and lowlands. Annu. Rev. Phytopathol. 34:503-526.

Duncan, K. E., and Howard, R. J. 2000. Cytological analysis of wheat infection by the leaf blotch pathogen *Mycosphaerella graminicola*. Mycol. Res. 104:1074-1082.

Edwards, A., Civitello, H., Hammond, H. A., and Caskey, C. T. 1991. DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. Am. J. Hum. Genet. 49:746-756.

Eyal, Z. 1999. Breeding for resistance to *Septoria* and *Stagonospora* diseases of wheat. Pages 332-344 in: Septoria of Cereals: A Study of Pathosystems. J. A. Lucas, P. Bowyer, and H. M. Anderson, eds. CAB International, Wallingford, UK.

Eyal, Z. 1995. Virulence in *Septoria tritici,* causal agent of Septoria tritici blotch of wheat. Pages 27-33 : in: The *Septoria tritici*: Proceedings of the Workshop. S. L. Gilchrist, M. van Ginkel, A. McNab, and G. H. J. Kema, eds. CIMMYT, Mexico, DF.

Eyal, Z., and Levy, E. 1987. Variation in pathogenicity patterns of *Mycosphaerella graminicola* within *Triticum* ssp. in Israel. Euphytica 36: 237-250.

Eyal , Z., Scharen, A. L., Prescott, J. M. and van Ginkel, M. 1987. The Septoria Diseases of Wheat: Concepts and methods of disease management. CIMMYT, Mexico, DF.

Eyal, Z., Scharen, A. L., Huffman, M. D., and Prescott, J. M. 1985. Global insights into virulence frequencies of *Mycosphaerella graminicola*. Phytopathology 75:1456-1462.

Eyal, Z. 1981. Integrated control of Septoria diseases of wheat. Plant Dis. 65:763-768.

Eyal, Z., and Brown, M. B. 1976. A quantitative method for estimating density of *Septoria tritici* pycnidia on wheat leaves. Phytopathology 66:11-14.

Eyal, Z., Amiri, Z., and Wahl, I. 1973. Physiologic specialization of *Septoria tritici*. Phytopathology 63:1087-1091.

Eyal, Z. 1971. The kinetics of pycnidiospore liberation in *Septoria tritici*. Can. J. Bot. 49:1095-1099.

Ezrati, S., Schuster, S., Eshel, A., and Eyal, Z. 1999. Interrelations among *Septoria tritici* isolates of varying virulence. Page 39 in: Septoria and Stagonospora Diseases of Cereals: A Compilation of Global Research. M. van Ginkel, A. McNab, and J. Krupinsky, eds. CIMMYT, Mexico, DF.

Fahima, T., Röder, M. S., Grama, A., and Nevo, E. 1998. Microsatellite DNA polymorphism divergence in *Triticum dicoccoides* accessions highly resistance to yellow rust. Theor. Appl. Genet. 96:187-195.

Fernandez, M. R., Zentner, R. P. McConkey, B. G., and Campbell, C. A. 1998. Effects of crop rotations and fertilizer management on leaf spotting diseases of spring wheat in southwestern Saskatchewan. Can. J. Plant Sci. 78:489-496.

Ganeshan, S. 1997. In vitro selection for resistance to net blotch of barley and molecular characterization of the pathogen. Ph.D. thesis. Department of Crop Science and Plant Ecology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Garcia, C., and Marshall, D. 1992. Observation on the ascogenous stage of *Septoria tritici* in Texas. Mycol. Res. 96:65-70.

Gilchrist, L., and Velazquez, C. 1994. Interaction to *Septoria tritici* isolate-wheat as adult plant under field conditions. Pages 111-114 in: Septoria of Cereals: Proceedings of the 4th International Workshop. E. Arseniuk, T. Goral, P. Czembor, eds. Radzikow, Poland.

Goodwin, S. B., and Kema, G. H. J. 1998. Population genetics of RAPD variation and the development of SCARs in *Mycosphaerella graminicola*. 7th International Congress of Plant Pathology, Edinburgh, Scotland, UK.

Goodwin, S. B., Saghai Maroof, M. A., Allard, R. W., and Webster, R. K. 1993. Isozyme variation within and among populations of *Rhynchosporium secalis* in Europe, Australia, and the United States. Mycol. Res. 97:49-58.

Goodwin, S. B., Allard, R. W., Hardy, S. A., and Webster, R. K. 1992. Hierarchical structure of pathogenic variation among *Rhynchosporium secalis* populations in Idaho and Oregon. Can. J. Bot. 70:810-817.

Gower, J. C. 1971. A general coefficient of similarity and some of its properties. Biometrics 27:857-74.

Grieger, A. P. 2001. Host-pathogen interactions in the wheat-*Mycosphaerella graminicola* pathosystem. M.S. thesis. University of Manitoba, Winnipeg, Manitoba, Canada.

Halama, P. 1996. The sexual state of *Septoria tritici*. The first occurrence in France. Phytoma 486:21-22.

Halperin, T., Schuster, S., Pnini-Cohen, S., Zilberstein, A., and Eyal, Z. 1996. The suppression of pycnidial production on wheat seedlings following sequential inoculation by isolates of *Septoria tritici*. Phytopathology 86:728-732.

Hamada, H. J., Petrino, M. G., and Kakanuga, T. 1982. A novel repeated element with Z-DNA-forming potential is widely found in evolutionarily diverse eukaryotic genomes. Proc. Natl. Acad. Sci. 79:6465-6469.

Hardwick, N. V., Jones, D. R., and Slough, J. E. 2001. Factors affecting diseases in winter wheat in England and Wales, 1989-98. Plant Pathol. 50:453-462.

Hess, D. E., and Shaner, G. 1987. Effect of moisture and temperature on development of *Septoria tritici* blotch in wheat. Phytopathology 77:215-219.

Hilu, H. M., and Bever, W. M. 1957. Inoculation, oversummering, and suscept-pathogen relationship of *Septoria tritici* on Triticum species. Phytopathology 47: 474-480.

Hoorne, C., Lamari, J., Gilbert, J., and Balance, G. M. 2002. Evidence of the sexual state of *Mycosphaerella graminicola* in Manitoba. Page 24 in: Proceeding of Canadian Phytopathological Society Annual meeting, June 16-19, 2002, Waterton Lakes Lodge, AB, Canada.

Huang, Z. Y., Smalley, E. B., and Guries. R. P. 1995. Differentiation of *Mycosphaerella dearnessii* by cultural characters and RAPD analysis. Phytopathology 85:522-527.

Hughes, G. R., and Hucl, P. 1993. CDC Teal hard red spring wheat. Can. J. Plant Sci. 73:193-197.

Hughes, G. R., and Hucl, P. 1992. Conway hard red spring wheat. Can. J. Plant Sci. 72:221-223.

Hunter, T., Coker, R. R., and Royle, D. J. 1999. Studies on the sexual phase of leaf blotch in UK winter wheat. Pages 108-114 in: Septoria of Cereals: A Study of Pathosystems. J. A. Lucas, P. Bowyer, and H. M. Anderson, eds. CAB International, Wallingford, UK.

Jacob, H. J., Lindpaintner, K., Lincoln, S. E., Kusumi, K., Bunker, R. K., Mao, Y. P., Ganten, D., Dzau, V. J., and Lander, E. S. 1991. Genetic mapping of a gene causing hypertension in the stroke-prone spontaneously hypersensitive rat. Cell 67:213- 224.

James, W. C. 1971. An illustrated series of assessment keys for plant diseases, their preparation, and usage. Can. Plant Dis. Surv. 51:39-65.

Jarne, P., and Lagoda, P. J. L. 1996. Microsatellites, from molecules to populations and back. Trends. Ecol. Evol. 11:424-429.

Jenns, A. E., and Leonard, K. J. 1985. Reliability of statistical analyses for estimating relative specificity in quantitative resistance in a model host-pathogen system. Theor. Appl. Genet. 69:503-513.

Jlibene, M., Mazouz, H., Farih, A., and Saadaoui, E. M. 1995. Host-pathogen interaction of wheat (*Triticum aestivum*) and *Septoria tritici* in Morocco. Pages 34-40 in: The *Septoria tritici*: Proceedings of the Workshop. S. L. Gilchrist, M. van Ginkel, A. McNab, and G. H. J. Kema, eds. CIMMYT, Mexico, DF.

Jlibene, M., Gustafson, J. P., and Rajaram, S. 1994. Inheritance of resistance to *Mycosphaerella graminicola* in hexaploid wheat. Plant Breeding 112:301-310.

Jonsson, R., Säll, T., and Bryngelsson, T. 2000. Genetic diversity for random amplified polymorphic DNA (RAPD) markers in two Swedish populations of *Pyrenophora teres*. Can. J. Plant Pathol. 22:258-264.

Kema, G. H. J., Goodwin, S. B., Hamza, S., Verstappen, E. C. P., Cavaletto, J. R., Van der Lee, T. A. J., de Weerdt, M., Bonants, P. J. M., and Waalwijk, C. 2002. A combined amplified fragment length polymorphism and randomly amplified polymorphic DNA genetic linkage map of *Mycosphaerella graminicola,* the septoria tritici leaf blotch pathogen of wheat. Genetics 161:1497-1505.

Kema, G. H. J., Verstappen, E. C. P., and Waalwijk, C. 2000. Avirulence in the wheat Septoria tritici leaf blotch fungus *Mycosphaerella graminicola* is controlled by a

single locus. Mol. Plant-Microbe Interact. 13:1375-1379.

Kema G. H. J., and van Silfhout, C. H. 1997. Genetic variation for virulence and resistance in the wheat–*Mycosphaerella graminicola* pathosystem. III. Comparative seedling and adult plant experiments. Phytopathology 87:266-272.

Kema, G. H. J., Annone, J. G., Sayoud, R., van Silfhout, C. H., van Ginkel, M., and de Bree, J. 1996a. Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. I. Interactions between pathogen isolates and host cultivars. Phytopathology 86:200-212.

Kema, G. H. J., Sayoud, R., Annone, J. G., and van Silfhout, C. H. 1996b. Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. II. Analysis of interactions between pathogen isolates and host cultivars. Phytopathology 86:213-220.

Kema, G. H. J., Verstappen, E. C. P., Todorova, M., and Waalwijk, C.1996c. Successful crosses and molecular tetrad and progeny analyses demonstrate heterothallism in *Mycosphaerella graminicola* . Curr. Genet. 30:251-258.

Kema, G. H. J., Yu, D., Rijkenberg, F. H. J., Shaw, M. W., and Baayen, R. P. 1996d. Histology of the pathogenesis of *Mycosphaerella graminicola* in wheat. Phytopathology 86:777-786.

Kema, G. H. J., van Silfhout, C. H., Annone, J. G., Sayoud, R., van Ginkel, M., and de Bree, J. 1995. Genetic variation for virulence in *Septoria tritici.* Pages 52-54 in: The *Septoria tritici*: Proceedings of the Workshop. S. L. Gilchrist, M. van Ginkel, A. McNab, and G. H. J. Kema, eds. CIMMYT, Mexico, DF.

King, J. E., Cook, R. J., and Melville, S. C. 1983. A review of Septoria diseases of wheat and barley. Ann Appl. Biol. 103:345-373.

Kolmer, J. A., Liu, J. Q., and Sies, M. 1995. Virulence and molecular polymorphism in *Puccinia recondita* f. sp. *tritici* in Canada. Phytopathology 85:276-285.

Krupinsky, J. M. 1999. Influence of cultural practices on Septoria/Stagonospora diseases. Pages 105-110 in: Septoria and Stagonospora Diseases of Cereals: A Compilation of Global Research. M. van Ginkel, A. McNab, and J. Krupinsky, eds. CIMMYT, Mexico, DF.

Krupinsky, J. M. 1997a. Aggressiveness of *Stagonospora nodorum* isolates obtained from wheat in the northern Great Plains. Plant Dis. 81:1027-1031.

Krupinsky, J. M. 1997b. Aggressiveness of *Stagonospora nodorum* isolates from perennial grasses on wheat. Plant Dis. 81:1032-1036.

Lalaoui, F., Halama, P., Dumortier, V., and Paul, B. 2000. Cell wall-degrading enzymes produced in vitro by isolates of *Phaeosphaeria nodorum* differing in aggressiveness. Plant Pathol. 49:727-733.

Le Cam, B., Massiot, P., and Rouxel, F. 1994. Cell wall polysaccharide-degrading enzymes produced by isolates of *Mycocentrospora acerina* differing in aggressiveness on carrot. Physiol. Mol. Plant Pathol. 44:187-198.

Leung, H., Nelson, R. J., and Leach, J. E. 1993. Population structure of plant pathogenic fungi and bacteria. Adv. Plant Pathol. 10:157-204.

Levy, M., Romao, J., Marchetti, M. A., and Hamer, J. E. 1991. DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in rice blast fungus. The Plant Cell 3:95-102.

Lewontin, R. C., and Kojima, K. 1960. The evolutionary dynamics of complex polymorphisms. Evolution 14:458-472.

Litt, M., and Luty, J. A. 1989. A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am. J. Hum. Genet. 44:397-401.

Loridon, K., Cournoyer, B., Goubely, C., Depeiges, A., and Picard, G. 1998. Length polymorphism and allele structure of trinucleotide microsatellites in natural accessions of *Arabidopsis thaliana.* Theor. Appl. Genet. 97:591-604.

Magboul, A. M., Geng, S., Gilchrist, D. G., and Jackson, L. F. 1992. Environmental influence on the infection of wheat by *Mycosphaerella graminicola.* Phytopathology 82:1407-1413.

Mann, C. E., Rajaram, S., and Villareal, R. L. 1985. Progress in breeding for *Septoria tritici* resistance in semidwarf spring wheat at CIMMYT. Pages 22-26 in: Septoria of cereals: Proceedings of the Workshop. A. L. Scharen, ed. Montana State University, Bozeman.

Marshall, D. 1985. Geographic distribution and aggressiveness of *Septoria tritici* on wheat in the United States. Phytopathology 75:1319.

McCartney, C. A., Brûlé-Babel, A. L., and Lamari, L. 2002. Inheritance of race-specific resistance to *Mycosphaerella graminicola* in wheat. Phytopathology 92:138-144.

McDermott, J. M., Brändle, U., Dutly, F., Haemmerli, U. A., Keller, S., Müller, K. E., and Wolfe, M. S. 1994. Genetic variation in powdery mildew of barley: Development of RAPD, SCAR, and VNTR markers. Phytopathology 84:1316-1321.

McDermott, J. M., and McDonald, B. A. 1993. Gene flow in plant pathosystems. Annu. Rev. Phytopathol. 31:353-373.

McDonald, B. A., and Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. Annu. Rev. Phytopathol. 40:349- 379.

McDonald, B. A., Zhan, J., and Burdon, J. J. 1999. Genetic structure of *Rhynchosporium secalis* in Australia. Phytopathology 89:639-645.

McDonald, B. A. 1997. The population genetics of fungi: tools and techniques. Phytopathology 87:448-453.

McDonald, B. A., Pettway, R. E., Chen, R. S., Boeger, J. M., and Martinez, J. P. 1995. The population genetics of *Septoria tritici* (teleomorph *Mycosphaerella graminicola*). Can. J. Bot. 73 (Suppl. 1): S292-S301.

McDonald, B. A., Miles, J., Nelson, L. R., and Pettway, R. E. 1994. Genetic variability in nuclear DNA in field populations of *Stagonospora nodorum*. Phytopathology 84:250-255.

McDonald, B. A., and McDermott, J. M. 1993. Population genetics of plant pathogenic fungi. BioScience 43: 311-319.

McDonald, B. A., and Martinez, J. P. 1991. DNA fingerprinting of the plant pathogenic fungus *Mycosphaerella graminicola* (anamorph *Septoria tritici*). Exp. Mycol. 15: 146-158.

McDonald, B. A., and Martinez, J.P. 1990a. Restriction fragment length polymorphisms in *Septoria tritici* occur at a high frequency. Curr. Genet. 17:133-138.

McDonald, B. A., and Martinez, J. P. 1990b. DNA restriction fragment length polymorphisms among *Mycosphaerella graminicola* (anamorph *Septoria tritici* isolates collected from a single wheat field. Phytopathology 80:1368-1373.

McIntosh, R. A., Hart, G. E., Devos, K. M., Gale M. D., and Rogers, W. J. 1998. Catalogue of gene symbols of wheat. Page 129 in: Proceeding of the 9th International Wheat Genetics Symposium, Vol. 5. Univ. Ext. Press, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Michelmore, R. W., and Hulbert, S. H. 1987. Molecular markers for genetic analysis of phytopathogenic fungi. Annu. Rev. Phytopathol 25:383-404.

Morgante, M., and Olivieri, A. M. 1993. PCR-amplified microsatellites as markers in plant genetics. The Plant Journal 3:175-182.

Mundt, C. C., Cowger, C., and Hoffer, M. E. 1999. Disease management using varietal mixtures. Pages 111-116 in: Septoria and Stagonospora Diseases of Cereals: A Compilation of Global Research. M. van Ginkel, A. McNab, and J. Krupinsky, eds. CIMMYT, Mexico, DF.

Mundt, C. C., Brophy, L. S., and Schmitt, M. S. 1995. Choosing crop cultivars and cultivar mixtures under low versus high disease pressure: a case study with wheat. Crop Prot. 14:509-515.

Nei, M. 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. 70: 3321-3323.

Nicholson, R. L., Turpin, C. A., and Warren, H. L. 1976. Role of pectolytic enzymes in susceptibility of living maize pith to *Colletotrichum graminicola.* Phytopathol. Z. 87:324-326.

Owen, P. G., Pei, M., Karp, A., Royle, D. J., and Edwards, K. J. 1998. Isolation and characterization of microsatellite loci in the wheat pathogen *Mycosphaerella graminicola.* Mol. Ecol. 7:1611-1612.

Palmer, C. L., and Skinner, W. 2002. *Mycosphaerella graminicola*: latent infection, crop devastation and genomics. Mol. Plant Pathol. 3:63-70.

Paran, I., and Mitchelmore, R. W. 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor. Appl. Genet. 85: 985-993.

Parlevliet, J. E., and Zadoks, J. C. 1977. The integrated concept of disease resistance: a new view including horizontal and vertical resistance in plants. Euphytica 26:5-21.

Pedersen, E. A., and Hughes, G. R. 1992. The effect of crop rotation on development of the septoria disease complex on spring wheat in Saskatchewan. Can. J. Plant Pathol. 14:152-158.

Pedersen, E. A. 1989. Epidemiology of the septoria diseases of wheat. M.Sc. thesis. Department of Crop Science and Plant Ecology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Peever, T. L., and Milgroom, M, G. 1994. Genetic structure of *Pyrenophora teres* populations determined with random amplified polymorphic DNA markers. Can. J. Bot. 72:915-923.

Peever, T. L., and Milgroom, M, G. 1993. Genetic correlations in resistance to sterol biosynthesis-inhibiting fungicides in *Pyrenophora teres*. Phytopathology 83: 1076-1082.

Peltonen, B. S., Jalli, M., Kammiovirta, K., and Karjalainen, R. 1996. Genetic variation in *Drechslera teres* popualtion as indicated by RAPD markers. Ann. Appl. Biol. 128:465-477.

Péros, J. P., Berger, G., and Lahogue, F. 1997. Variation in pathogenicity and genetic structure in the *Eutypa lata* population of a single vineyard. Phytopathology 87:799-806.

Person, C. 1959. Gene-for-gene relationships in host-parasite systems. Can. J. Bot. 37:1101-1130.

Powell, W., Machray, G. C., and Provan, J. 1996a. Polymorphism revealed by simple sequence repeats. Trends Plant Sci. 1:215-222.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., and Rafalski, A. 1996b. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol. Breed. 2:225-238.

Raeder, U., and Broda, P. 1985. Rapid preparation of DNA from filamentous fungi. Appl. Microbiol. 90:901-908.

Rillo, A. O., and Caldwell, R. M. 1966. Inheritance of resistance to *Septoria tritici* in *Triticum aestivum* subsp. *Vulgare*, Bulgaria 88: Phytopathology 56:597.

Robinson, R. A. 1987. Host management in crop pathosystems. Macmillan Publishing Company, New York.

Rohlf, F. J. 1998. NTSYS-pc: Numerical taxonomy and multivariate analysis system, Ver. 2.0. Applied Biostatistics, Inc. New York.

Rosewich, U. T., and McDonald, B. A. 1994. DNA fingerprinting in fungi. Methods Mol. Cell Biol. 5:41-48.

Rosielle, A. A. 1972. Sources of resistance in wheat to speckled leaf blotch caused by *Septoria tritici*. Euphytica 21: 152-161.

Royle, D. J., Parker, S. R., Lovell, D. J., and Hunter, T. 1995. Interpreting trends and risks for better control of *Septoria* in winter wheat. Pages 105-115 in: a vital role of fungicides in cereal production. G. H. Hewitt, D. Tyson, D. W. Hollomon, J. M. Smith, W. P. Davies, and K. R. Dixon, eds. BIOS Scientific Publisher, Oxford, UK.

Royle, D. J. 1994. Understanding and predicting epidemics: a commentary based on selected pathosystems. Plant Pathol. 43:777-78.

Rufty, R. C., Hebert, T. T., and Murphy, C. F. 1981. Variation in virulence in isolates of *Septoria nodorum*. Phytopathology 71:593-596.

Saadaoui, E. M. 1987. Physiologic specialization of *Septoria tritici* in Morocco. Plant Dis. 71:153-155.

Saari, E. E., and Prescott, J. M. 1975. A scale for appraising the foliar intensity of wheat diseases. Plant Dis. Rep. 59:377-380.

Salamati, S., Zhan, J., Burdon, J. J., and McDonald, B. A. 2000. The genetic structure of field populations of *Rhynchosporium secalis* from three continents suggests moderate gene flow and regular recombination. Phytopathology 90:901-908.

Sanderson, F. R., and Gaunt, R. E. 1980. Commercial control of speckled leaf blotch (*Mycosphaerella graminicola,* imperfect state *Septoria tritici*) on wheat using fungicides. Pages 554-557 in: Proceeding of 3rd International Wheat Conference. Madrid, Spain.

Sanderson, F. R., and Hampton, J. G. 1978. Role of the perfect states in the epidemiology of the common septoria diseases of wheat. N. Z. J. Agric. Res. 21:277-281.

Sanderson, F. R. 1972. A *Mycosphaerella* species as the ascogenous state of *Septoria tritici* Rob. And Desm. N. Z. J. Agric. Res. 14:359-360.

Santra, D. K., Singh, G., Kaiser, W. J., Gupta, V. S., Ranjekar, P. K., and Muehlbauer, F. J. 2001. Molecular analysis of *Ascochyta rabiei* (Pass.) Labr., the pathogen of ascochyta blight in chickpea. Theor. Appl. Genet. 102:676-682.

Schaffer, H. E., and Sederoff, R. R. 1981. Improved estimation of DNA fragment lengths from agarose gels. Anal. Biochem. 115:113-122.

Scharen, A. L., and Sanderson, F. R. 1985. Identification, distribution and nomenclature of the Septoria species that attack cereals. Pages 37-41 in: Septoria of Cereals: Proceedings of the Workshop. A. l. Scharen, ed. Montana State University, Bozeman.

Schilling, A. G., Moller, E. M., and Geiger, H. H. 1996. Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum, F. graminearum,* and *F. avenaceum*. Phytopathology 86:515-522.

Schnieder, F., Koch, G., Jung, C., and Verreet, J. A. 1998. The application of molecular markers for genetic characterization of *Septoria tritici* populations. Zeitschrift fur Pflanzenschutz 105:452-461.

Scott, P. R., Sanderson, F. R., and Benedikz, P. W. 1988. Occurrence of *Mycosphaerella graminicola*, teleomorph of *Septoria tritici* on wheat in the UK. Plant Pathol. 37:985-990.

Shaner, G., and Buechley, G. 1995. Epidemiology of leaf blotch of soft red winter wheat caused by *Septoria tritici* and *Stagonospora nodorum*. Plant Dis. 79:928-938.

Shaner, G., and Finney, R. E. 1976. Weather and epidemics of Septoria leaf blotch of wheat. Phytopathology 66:781-785.

Sharma, S. 1996. Applied multivariate techniques. John Wiley & Sons, Inc. New York.

Shaw, M. W., and Royle, D. J. 1989. Airborne inoculum as a major source of *Septoria tritici* (*Mycosphaerella graminicola*) infection in winter wheat crops in the UK. Plant Pathol. 38:35-43.

Shaw, M. W., and Royle, D. J. 1993. Factors determining the severity of epidemics of *Mycosphaerella graminicola* (*Septoria tritici*) on winter wheat in the UK. Plant Pathol. 42:882-899.

Shearer, B. L., and Wilcoxson, R. D. 1978. Variation in the size of macropycnidiospores and pycnidia of *Septoria tritici* on wheat. Can. J. Bot. 56:742-746.

Shipton, W. A., Boyd, W. R. J., Rosielle, A. A., and Shearer, B. I. 1971. The common Septoria diseases of wheat. Bot. Rev. 37:237-262.

Slatkin, M., and Excoffier, L. 1996. Testing for linkage disequilibrium in genotypic data using the Expectation-Maximization algorithm. Heredity 76:377-383.

Slatkin, M. 1994. Linkage disequilibrium in growing and stable populations. Genetics 137:331-336.

Sneath, P. H. A., and Sokal, R. R. 1973. Numerical taxonomy: the principles and practice of numerical classification. W. H. Freeman and Company, San Francisco.

Southern, E. M. 1979. Measurement of DNA length by gel electrophoresis. Anal. Biochem. 100:319-323.

Sprague, R. 1950. Disease of cereals and grasses in North America. Ronald Press, New York.

Sprague, R. 1944. Septoria disease of *Gramineae* in Western United States. Or. State Monogr., Stud. Bot. No. 6. Or. State Coll., Corvallis.

Sun, G. L., Salomon, B., and Bothmer, R. V. 1998. Characterization and analysis of microsatellite loci in *Elymus caninus* (*Triticeae: Poaceae*). Theor. Appl. Genet. 96:676-682.

Sun, G. L., Díaz, O., Salomon, B., and Bothmer, R. V. 1999. Genetic diversity in *Elymus caninus* as revealed by isozyme, RAPD, and microsatellite markers. Genome 42:420-431.

Tautz, D. 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acids Res. 17:6463-6471.

Tautz, D., Trick, M., and Dover, G. A. 1986. Cryptic simplicity in DNA is a major source of genetic variation. Nature 322:652-656.

Tautz, D., and Renz, M. 1984. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. Nucleic Acids Res. 12:4127-4138.

Teulat, B., Aldam, C., Trehin, R., Lebrun, P., Barker, J. H. A., Arnold, G. M., Karp, A., Baudouin, L., and Rognon, F. 2000. An analysis of genetic diversity in coconut (*Cocos nucifera*) populations from across the geographic range using sequence-tagged microsatellites (SSRs) and AFLPs. Theor. Appl. Genet. 100:764-771.

Tingey, S. V., and del Tufo, J. P. 1993. Genetic analysis with random amplified polymorphic DNA markers. Plant Physiol. 101: 349-352.

Tompkins, D. K., Fowler, D. B., and Wright, A. T. 1993. Influence of agronomic practices on canopy microclimate and septoria development in no-till winter wheat produced in the parkland region of Saskatchewan. Can. J. Plant Sci. 73: 331-344.

Thompson, J. R. 1989. The program GEL ver. 2/17/89. 7105 Hana RD., Edison, NJ.

Udupa, S. M., Weigand, F., Saxena, M. C., and Kahl. G. 1998. Genotyping with RAPD and microsatellite markers resolves pathotype diversity in the ascochyta blight pathogen of chickpea. Theor. Appl. Genet. 97:299-307

van Ginkel, M., and Rajaram, S. 1995. Breeding for resistance to *Septoria tritici* at CIMMYT. Pages 55-59 in: The *Septoria tritici*: Proceedings of the Workshop. S. L. Gilchrist, M. van Ginkel, A. McNab, and G. H. J. Kema, eds. CIMMYT, Mexico, DF.

van Ginkel, M., and Rajaram, S. 1999.Breeding for resistance to the Septoria/ Stagonospora blights of wheat. Pages 117-126 in: Septoria and Stagonospora Diseases of Cereals: A Compilation of Global Research. M. van Ginkel, A. McNab, and J. Krupinsky, eds. CIMMYT, Mexico, DF.

van Ginkel, M., and Scharen, A. L.1988. Host-pathogen relationships of wheat and *Septoria tritici*. Phytopathology 78:762-766.

van Silfhout, C. H., Arama, P. F., and Kema, G. H. J. 1989. International survey of factors of virulence of *Septoria tritici*. Pages 36-38 in: Septoria diseases of cereals: Proceeding of the 3rd International Workshop. P. M. Fried ed. Zurich, Switzerland.

Waalwijk, C., Mendes, O., Verstappen, E. C. P., de Waard, M. A., and Kema, G. H. J. 2002. Isolation and characterisation of the mating-type idiomorphs from the wheat septoria leaf blotch fungus *Mycosphaerella graminicola*. Fung. Genet. Biol. 35:277-286.

Weber, J. L. 1990. Informativeness of human (dC-dA)n.(dG-dT)n polymorphisms. Genomics 7:524-530.

Weber, J., L., and May, P. E. 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am. J. Hum. Genet. 44:388-396.

Weir, B. S. 1990. Genetic data analysis: methods for discrete population genetic data. Sinauer Assoc., Inc., Sunderland, Ma.

Wiese, M. V. 1987. Compendium of wheat diseases. APS Press, St. Paul, MN.

Wilson, R. E. 1979. Resistance to *Septoria tritici* in two wheat cultivars, determined by an independent, single dominant gene. Aust. Plant Pathol. 8:16-18.

Wroth, J. M. 1998. Variation in pathogenicity among and within *Mycosphaerella pinodes* populations collected from field pea in Australia. Can. J. Bot. 76:1955-1966.

Zelikovitch, N., Levy, E., and Eyal, Z. 1986. The effects of mixtures of *Mycosphaerella graminicola* isolates on the expression of symptoms on wheat seedling leaves. Phytopathology 76:1061 (Abstr.).

Appendix A. Means of incubation period, latent period and disease severity of 90 isolates of *Mycosphaerella graminicola* tested on the susceptible wheat cultivar Conway

Isolate #	Incubation period	Latent period	Disease severity
St 1	13.39	16.03	3.71
St 2	14.07	17.39	3.00
St 3	14.25	17.67	3.49
St 4	13.87	17.17	2.90
St 5	13.81	16.92	3.35
St 6	14.48	17.37	2.76
St 7	13.74	16.31	3.71
St 8	13.98	16.76	3.51
St 9	13.78	16.74	3.20
St 10	14.07	17.30	3.30
St 11	13.61	16.75	3.27
St 12	14.41	17.40	3.14
St 13	13.67	16.92	3.34
St 14	14.40	17.97	2.75
St 15	13.95	17.52	3.23
St 16	13.54	16.45	3.99
St 17	13.86	16.73	3.19
St 18	13.64	16.77	3.25
St 19	13.86	17.17	3.40
St 20	13.80	16.53	3.77
St 21	13.81	17.07	3.33
St 22	13.91	17.18	2.98
St 23	14.12	17.31	2.92
St 24	13.80	16.90	3.31
St 25	13.42	16.28	3.79
St 26	14.07	16.94	2.90
St 27	14.26	17.92	3.09
St 28	14.45	17.33	2.84
St 29	13.96	16.75	3.50
St 30	13.56	16.33	3.70
St 31	13.57	16.48	3.53
St 32	14.20	17.14	3.07
St 33	13.85	17.11	3.42
St 34	13.86	16.89	2.87
St 35	13.61	16.36	3.44
St 36	14.52	18.17	3.08
St 37	13.99	16.95	3.86
St 38	14.03	16.93	3.63
St 39	14.37	17.51	2.80
St 40	14.29	17.35	3.35

Appendix A. (cont.)

Isolate #	Incubation period	Latent period	Disease severity
St 41	14.30	17.09	3.21
St 42	13.92	16.75	3.08
St 43	13.76	16.51	3.60
St 44	13.96	16.82	3.37
St 45	13.82	16.33	3.89
St 46	13.90	16.97	3.30
St 47	13.82	16.68	3.55
St 48	13.98	16.70	3.15
St 49	13.50	16.24	3.74
St 50	13.92	16.83	3.28
St 51	14.08	17.34	3.18
St 52	13.31	16.06	3.49
St 53	13.54	16.59	3.61
St 54	13.91	16.85	3.39
St 55	13.41	15.88	3.40
St 56	14.65	18.06	2.78
St 57	13.54	16.59	3.72
St 58	13.87	17.04	2.97
St 59	14.60	17.84	2.73
St 60	13.77	16.43	3.39
St 61	14.00	17.74	3.17
St 62	14.01	16.91	3.33
St 63	13.60	16.43	3.43
St 64	13.75	16.85	3.70
St 65	13.67	16.31	3.99
St 66	13.81	17.30	3.42
St 67	13.81	17.27	3.30
St 68	14.07	17.22	3.62
St 69	13.67	16.45	3.52
St 70	13.76	16.41	3.33
St 71	13.87	16.48	3.94
St 72	13.88	16.72	3.25
St 73	14.25	17.29	3.09
St 74	13.46	16.18	4.00
St 75	14.04	17.38	3.31
St 76	13.94	16.81	3.69
St 77	13.96	16.95	3.32
St 78	13.86	17.15	2.96
St 79	13.47	16.19	3.69
St 80	13.48	16.11	4.05

Appendix A. (cont.)

Isolate #	Incubation period	Latent period	Disease severity
St 81	13.79	16.80	3.28
St 82	13.71	16.64	3.13
St 83	13.58	17.34	3.27
St 84	13.77	17.06	2.93
St 85	13.41	16.19	4.05
St 86	14.20	17.23	3.07
St 87	13.80	16.57	3.57
St 88	13.88	16.54	3.32
St 89	14.04	17.29	2.86
St 90	13.61	16.24	3.39
Average	13.88 ± 0.29	16.89 ± 0.49	3.35 ± 0.33

Appendix B. Frequency of positive alleles of RAPD loci within the population of *Mycosphaerella graminicola*

Primer	Locus	Frequency of positive allele within locations										f_T [a]
		1	2	3	4	5	6	7	8	9	10	
OPF6	1	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.022
OPF6	2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.000
OPF6	3	0.00	0.00	0.11	0.00	0.11	0.11	0.11	0.00	0.11	0.11	0.066
OPF6	4	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.011
OPF6	5	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.11	0.022
OPF6	6	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.011
OPF6	7	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.11	0.00	0.00	0.022
OPF6	8	0.00	0.11	0.22	0.11	0.11	0.11	0.33	0.11	0.11	0.00	0.121
OPF6	9	0.00	0.00	0.11	0.44	0.00	0.22	0.11	0.00	0.00	0.00	0.088
OPF6	10	0.56	0.89	1.00	1.00	1.00	1.00	1.00	1.00	0.56	0.67	0.868
OPF6	11	0.00	0.22	0.00	0.00	0.11	0.11	0.00	0.00	0.00	0.00	0.044
OPF6	12	0.00	0.00	0.22	0.00	0.11	0.00	0.00	0.33	0.00	0.78	0.144
OPG12	1	0.22	0.22	0.11	0.33	0.22	0.33	0.00	0.00	0.00	0.00	0.143
OPG12	2	0.67	1.00	1.00	0.78	0.78	0.89	0.89	0.78	0.89	0.78	0.846
OPG12	3	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.022
OPG12	4	0.33	0.78	0.22	0.44	0.56	0.44	0.67	0.33	0.22	0.56	0.455
OPG12	5	0.22	0.00	0.11	0.22	0.00	0.11	0.00	0.11	0.00	0.00	0.077
OPG12	6	0.67	0.67	0.44	0.78	0.67	0.67	0.89	0.67	0.44	0.67	0.657
OPG12	7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.011
OPG12	8	1.00	0.78	0.89	0.78	1.00	0.78	0.78	1.00	0.89	1.00	0.890
OPG12	9	0.11	0.11	0.11	0.22	0.56	0.22	0.11	0.11	0.22	0.33	0.210
OPG12	10	0.11	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.22	0.00	0.044
OPG12	11	0.00	0.00	0.22	0.22	0.11	0.22	0.11	0.22	0.00	0.00	0.110
OPG12	12	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.011
OPG13	1	0.33	0.44	0.44	0.33	0.33	0.22	0.00	0.44	0.44	0.44	0.341
OPG13	2	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.011
OPG13	3	0.67	0.56	0.67	0.67	0.67	0.67	1.00	0.56	0.56	0.44	0.647
OPG13	4	0.11	0.11	0.00	0.00	0.11	0.22	0.00	0.00	0.11	0.00	0.066
OPG13	5	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.011
OPG13	6	0.78	0.56	0.67	0.44	0.89	0.67	0.56	0.78	0.56	0.33	0.624
OPG13	7	0.56	0.44	0.78	0.67	0.89	0.67	0.78	0.67	0.22	0.44	0.612
OPH8	1	1.00	0.33	0.78	0.67	0.33	0.67	0.67	0.22	0.78	0.44	0.589
OPH8	2	0.11	0.11	0.00	0.00	0.00	0.00	0.11	0.11	0.11	0.00	0.055
OPH8	3	0.00	0.11	0.00	0.00	0.22	0.22	0.00	0.00	0.00	0.00	0.055
OPH8	4	0.33	0.44	0.44	0.78	0.44	0.33	0.44	0.33	0.33	0.11	0.397
OPH13	1	1.00	0.78	1.00	1.00	1.00	0.89	1.00	0.56	0.44	0.56	0.823
OPH13	2	0.11	0.33	0.11	0.22	0.22	0.11	0.00	0.00	0.11	0.00	0.121
OPH13	3	0.00	0.00	0.22	0.00	0.00	0.00	0.00	0.22	0.11	0.00	0.055
OPH13	4	0.89	1.00	0.89	1.00	1.00	1.00	1.00	1.00	0.89	0.78	0.945
OPH13	5	0.78	0.55	0.67	0.78	0.56	0.11	0.22	0.33	0.33	0.33	0.466
OPH13	6	1.00	1.00	1.00	0.78	0.89	1.00	1.00	0.78	0.78	0.67	0.890
OPH13	7	1.00	0.89	0.78	0.78	1.00	0.67	1.00	0.78	0.89	0.78	0.857

Appendix B. (cont.)

Primer	Locus	Frequency of positive allele within locations										f_T
		1	2	3	4	5	6	7	8	9	10	
OPI10	1	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.011
OPI10	2	0.00	0.11	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.022
OPI10	3	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.11	0.11	0.00	0.033
OPI10	4	0.22	0.11	0.00	0.00	0.11	0.00	0.11	0.00	0.11	0.11	0.077
OPI10	5	0.33	0.11	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.055
OPI10	6	0.89	0.78	0.56	0.89	0.67	0.89	1.00	0.89	0.89	0.67	0.813
OPI10	7	1.00	1.00	0.78	0.89	0.78	1.00	1.00	0.78	0.89	0.78	0.890
OPI10	8	0.22	0.11	0.00	0.00	0.11	0.00	0.11	0.11	0.11	0.00	0.077
OPI10	9	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.11	0.00	0.022
OPI10	10	0.67	0.56	0.56	0.56	0.56	0.44	0.89	0.78	0.22	0.56	0.580
OPI10	11	0.00	0.00	0.00	0.11	0.00	0.11	0.00	0.00	0.00	0.00	0.022
UBC648	1	0.22	0.22	0.11	0.11	0.11	0.00	0.11	0.00	0.11	0.22	0.121
UBC648	2	0.89	0.78	1.00	1.00	0.89	1.00	0.67	0.78	1.00	0.67	0.868
UBC648	3	0.89	0.67	0.78	0.78	0.78	0.89	1.00	0.89	0.78	0.56	0.802
UBC648	4	0.89	0.89	0.78	1.00	1.00	1.00	1.00	1.00	1.00	0.89	0.945
UBC726	1	0.22	0.33	0.22	0.44	0.22	0.33	0.33	0.33	0.22	0.11	0.275
UBC726	2	0.33	0.11	0.33	0.22	0.00	0.11	0.33	0.00	0.11	0.33	0.187
UBC726	3	0.67	0.78	0.56	0.78	0.89	0.67	0.78	0.78	0.89	0.56	0.736
UBC726	4	0.44	0.11	0.44	0.00	0.33	0.44	0.44	0.33	0.33	0.22	0.308
UBC726	5	1.00	1.00	0.89	0.78	1.00	0.88	0.89	1.00	1.00	1.00	0.944
UBC726	6	0.11	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.022
UBC726	7	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.000
UBC726	8	0.11	0.11	0.00	0.11	0.11	0.11	0.00	0.00	0.00	0.00	0.055
UBC726	9	0.78	0.89	0.78	0.56	0.67	0.44	0.78	0.78	0.78	1.00	0.746
UBC726	10	0.00	0.11	0.00	0.00	0.11	0.00	0.11	0.00	0.00	0.00	0.033
UBC736	1	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.22	0.055
UBC736	2	0.44	0.56	0.11	0.22	0.33	0.44	0.44	0.67	0.11	0.11	0.343
UBC736	3	0.78	0.78	0.78	1.00	0.78	1.00	0.89	1.00	0.78	0.67	0.846
UBC736	4	0.00	0.11	0.00	0.11	0.00	0.11	0.11	0.00	0.00	0.11	0.055
UBC736	5	0.78	0.67	0.67	0.56	0.78	1.00	0.44	0.67	1.00	0.56	0.713
UBC736	6	0.44	0.56	0.89	0.78	0.89	0.89	1.00	0.67	0.67	0.67	0.746
UBC736	7	0.78	0.78	0.89	0.89	0.56	0.89	1.00	1.00	1.00	0.67	0.846
UBC736	8	0.89	1.00	0.89	0.56	1.00	0.89	0.78	0.56	0.78	0.78	0.813
UBC737	1	0.56	0.33	0.67	0.89	1.00	0.89	0.89	0.89	0.44	0.33	0.689
UBC737	2	0.00	0.00	0.33	0.11	0.00	0.00	0.22	0.00	0.00	0.00	0.066
UBC737	3	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.011
UBC737	4	0.00	0.00	0.44	0.11	0.11	0.11	0.44	0.00	0.11	0.11	0.143
UBC737	5	0.00	0.00	0.11	0.11	0.00	0.11	0.22	0.00	0.11	0.11	0.077
UBC737	6	0.89	0.89	0.89	0.89	1.00	0.89	0.78	1.00	0.89	0.89	0.901
UBC737	7	0.00	0.00	0.11	0.11	0.11	0.00	0.00	0.00	0.00	0.00	0.033
UBC737	8	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.011
UBC737	9	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.011
UBC737	10	0.67	0.67	0.56	0.67	0.67	0.78	0.22	0.56	0.78	0.56	0.614
UBC737	11	0.00	0.11	0.33	0.00	0.00	0.00	0.11	0.00	0.00	0.22	0.077
UBC737	12	0.44	0.11	0.00	0.11	0.11	0.11	0.00	0.00	0.00	0.11	0.099

Appendix B. (cont.)

Primer	Locus	Frequency of positive allele within locations										f_T
		1	2	3	4	5	6	7	8	9	10	
UBC757	1	0.89	0.56	0.56	0.67	1.00	1.00	0.89	0.78	0.56	0.56	0.747
UBC757	2	0.00	0.33	0.22	0.33	0.00	0.00	0.00	0.11	0.00	0.00	0.099
UBC757	3	0.67	0.33	0.22	0.67	0.58	0.22	0.11	0.33	0.33	0.33	0.377
UBC757	4	1.00	1.00	0.89	1.00	1.00	1.00	1.00	0.89	0.89	0.89	0.956
UBC757	5	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.011
UBC757	6	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.33	0.00	0.00	0.044
UBC757	7	0.11	0.44	0.33	0.56	0.33	0.67	0.11	0.00	0.11	0.11	0.277
UBC757	8	0.33	0.44	0.22	0.33	0.22	0.22	0.44	0.44	0.56	0.22	0.342
UBC757	9	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.011
UBC757	10	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.011
UBC757	11	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.011
UBC757	12	0.00	0.00	0.22	0.11	0.22	0.11	0.11	0.22	0.00	0.00	0.099
UBC757	13	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.022
UBC758	1	0.00	0.22	0.78	0.11	0.22	0.44	0.11	0.22	0.00	0.11	0.221
UBC758	2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.000
UBC763	1	0.00	0.11	0.11	0.11	0.11	0.11	0.00	0.00	0.11	0.00	0.066
UBC763	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.011
UBC763	3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.011
UBC763	4	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.000
UBC763	5	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.011
UBC763	6	0.89	0.89	0.89	0.89	1.00	1.00	1.00	1.00	0.89	1.00	0.945
UBC763	7	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.011
UBC767	1	0.78	0.67	0.78	0.89	1.00	1.00	0.89	0.67	1.00	0.78	0.846
UBC767	2	0.00	0.00	0.00	0.11	0.00	0.00	0.11	0.00	0.11	0.00	0.033
UBC767	3	0.56	0.44	0.89	0.44	0.89	0.89	0.33	0.56	0.89	0.44	0.633
UBC767	4	0.56	0.22	0.33	0.78	0.67	0.78	0.67	0.56	0.56	0.22	0.535
UBC767	5	0.11	0.11	0.00	0.11	0.11	0.00	0.00	0.00	0.00	0.00	0.044
UBC767	6	1.00	1.00	0.78	0.56	1.00	0.56	0.78	0.78	0.89	0.89	0.824
UBC767	7	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.011
UBC767	8	0.00	0.00	0.00	0.22	0.22	0.00	0.11	0.00	0.00	0.11	0.066
UBC767	9	0.89	0.67	0.78	0.89	1.00	0.89	0.89	0.89	0.89	0.78	0.857
UBC767	10	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.011
UBC767	11	0.89	1.00	1.00	1.00	1.00	1.00	0.89	1.00	1.00	1.00	0.978
UBC772	1	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.011
UBC772	2	0.33	0.33	0.11	0.67	0.89	0.11	0.11	0.00	0.00	0.11	0.266
UBC772	3	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.022
UBC772	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.011
UBC772	5	1.00	1.00	0.89	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.989
UBC772	6	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.000
UBC772	7	0.00	0.11	0.11	0.00	0.22	0.00	0.00	0.00	0.00	0.00	0.044
UBC772	8	0.00	0.00	0.00	0.11	0.00	0.11	0.00	0.00	0.00	0.00	0.022
UBC772	9	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.011
UBC772	10	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.11	0.00	0.022
UBC772	11	0.00	0.00	0.00	0.11	0.11	0.00	0.00	0.00	0.00	0.00	0.022

[a] Average frequency of the positive allele for each RAPD locus within the population.

Appendix C. Total gene diversity value of each RAPD locus and partitioning into within and among location components

Primer	Locus	Total gene diversity	Gene diversity Within locations [a]	Gene diversity among locations
OPF6	1	0.043	0.039	0.004
OPF6	2	0.000	0.000	0.000
OPF6	3	0.123	0.117	0.006
OPF6	4	0.022	0.020	0.002
OPF6	5	0.043	0.039	0.004
OPF6	6	0.022	0.020	0.002
OPF6	7	0.043	0.039	0.004
OPF6	8	0.213	0.196	0.017
OPF6	9	0.161	0.123	0.038
OPF6	10	0.229	0.162	0.067
OPF6	11	0.084	0.073	0.011
OPF6	12	0.247	0.132	0.114
OPG12	1	0.245	0.211	0.034
OPG12	2	0.261	0.240	0.020
OPG12	3	0.043	0.039	0.004
OPG12	4	0.496	0.433	0.063
OPG12	5	0.142	0.127	0.015
OPG12	6	0.451	0.418	0.033
OPG12	7	0.022	0.020	0.002
OPG12	8	0.196	0.176	0.019
OPG12	9	0.332	0.294	0.037
OPG12	10	0.084	0.073	0.011
OPG12	11	0.196	0.176	0.019
OPG12	12	0.022	0.020	0.002
OPG13	1	0.449	0.413	0.036
OPG13	2	0.022	0.020	0.002
OPG13	3	0.457	0.418	0.039
OPG13	4	0.123	0.113	0.011
OPG13	5	0.022	0.020	0.002
OPG13	6	0.469	0.418	0.051
OPG13	7	0.475	0.403	0.072
OPH8	1	0.484	0.373	0.111
OPH8	2	0.104	0.098	0.006
OPH8	3	0.104	0.088	0.016
OPH8	4	0.479	0.428	0.051
OPH13	1	0.291	0.202	0.090
OPH13	2	0.213	0.191	0.022
OPH13	3	0.104	0.088	0.016
OPH13	4	0.104	0.093	0.011
OPH13	5	0.498	0.398	0.099
OPH13	6	0.196	0.167	0.029
OPH13	7	0.245	0.221	0.024

Appendix C. (cont.)

Primer	Locus	Total gene diversity	Gene diversity Within locations	Gene diversity among locations
OPI10	1	0.022	0.020	0.002
OPI10	2	0.043	0.039	0.004
OPI10	3	0.064	0.059	0.005
OPI10	4	0.142	0.132	0.010
OPI10	5	0.104	0.083	0.021
OPI10	6	0.304	0.270	0.034
OPI10	7	0.196	0.176	0.019
OPI10	8	0.142	0.132	0.010
OPI10	9	0.043	0.039	0.004
OPI10	10	0.487	0.428	0.059
OPI10	11	0.043	0.039	0.004
UBC648	1	0.213	0.201	0.012
UBC648	2	0.229	0.196	0.033
UBC648	3	0.318	0.290	0.028
UBC648	4	0.104	0.093	0.011
UBC726	1	0.399	0.383	0.016
UBC726	2	0.304	0.270	0.034
UBC726	3	0.389	0.363	0.025
UBC726	4	0.426	0.384	0.043
UBC726	5	0.106	0.095	0.011
UBC726	6	0.043	0.039	0.004
UBC726	7	0.000	0.000	0.000
UBC726	8	0.104	0.098	0.006
UBC726	9	0.379	0.334	0.045
UBC726	10	0.064	0.059	0.005
UBC736	1	0.104	0.088	0.016
UBC736	2	0.451	0.379	0.072
UBC736	3	0.261	0.235	0.025
UBC736	4	0.104	0.098	0.006
UBC736	5	0.409	0.349	0.060
UBC736	6	0.379	0.324	0.055
UBC736	7	0.261	0.221	0.040
UBC736	8	0.304	0.260	0.044
UBC737	1	0.429	0.310	0.119
UBC737	2	0.123	0.098	0.025
UBC737	3	0.022	0.020	0.002
UBC737	4	0.245	0.196	0.049
UBC737	5	0.142	0.132	0.010
UBC737	6	0.178	0.171	0.007
UBC737	7	0.064	0.059	0.005
UBC737	8	0.022	0.020	0.002
UBC737	9	0.022	0.020	0.002
UBC737	10	0.474	0.428	0.046
UBC737	11	0.142	0.118	0.024
UBC737	12	0.178	0.147	0.031

Appendix C. (cont.)

Primer	Locus	Total gene diversity	Gene diversity Within locations	Gene diversity among locations
UBC757	1	0.378	0.315	0.063
UBC757	2	0.178	0.142	0.036
UBC757	3	0.470	0.403	0.067
UBC757	4	0.084	0.078	0.006
UBC757	5	0.022	0.020	0.002
UBC757	6	0.084	0.064	0.020
UBC757	7	0.401	0.310	0.091
UBC757	8	0.450	0.423	0.027
UBC757	9	0.022	0.020	0.002
UBC757	10	0.022	0.020	0.002
UBC757	11	0.022	0.020	0.002
UBC757	12	0.178	0.162	0.017
UBC757	13	0.043	0.039	0.004
UBC758	1	0.344	0.245	0.099
UBC758	2	0.000	0.000	0.000
UBC763	1	0.123	0.117	0.006
UBC763	2	0.022	0.020	0.002
UBC763	3	0.022	0.020	0.002
UBC763	4	0.000	0.000	0.000
UBC763	5	0.022	0.020	0.002
UBC763	6	0.104	0.098	0.006
UBC763	7	0.022	0.020	0.002
UBC767	1	0.261	0.231	0.030
UBC767	2	0.064	0.059	0.005
UBC767	3	0.465	0.369	0.096
UBC767	4	0.498	0.418	0.080
UBC767	5	0.084	0.078	0.006
UBC767	6	0.290	0.241	0.049
UBC767	7	0.022	0.020	0.002
UBC767	8	0.123	0.108	0.015
UBC767	9	0.245	0.230	0.015
UBC767	10	0.022	0.020	0.002
UBC767	11	0.043	0.039	0.004
UBC772	1	0.022	0.020	0.002
UBC772	2	0.390	0.231	0.160
UBC772	3	0.043	0.034	0.009
UBC772	4	0.022	0.020	0.002
UBC772	5	0.022	0.020	0.002
UBC772	6	0.000	0.000	0.000
UBC772	7	0.084	0.073	0.011
UBC772	8	0.043	0.039	0.004
UBC772	9	0.022	0.020	0.002
UBC772	10	0.043	0.039	0.004
UBC772	11	0.043	0.039	0.004
Means [b]		0.179	0.154	0.025

[a] Average gene diversity for each RAPD locus within 10 locations of the field; [b] Average of gene diversity over 131 RAPD loci and the ratios of within and among location components.

Appendix D1. Allelic frequency of eight microsatellite loci of *Mycosphaerella graminicola* within 10 locations and in the total population of a single wheat field located in Saskatoon

SSR loci	Allele	Location										
		1	2	3	4	5	6	7	8	9	10	Mean
ST1A2	1	0.33	0.33	0.67	0.33	0.67	0.33	0.44	0.56	0.11	0.67	0.44
	2	0.44	0.44	0.00	0.56	0.11	0.33	0.33	0.33	0.56	0.00	0.31
	3	0.22	0.22	0.33	0.11	0.11	0.33	0.11	0.11	0.22	0.11	0.19
	4	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.11	0.22	0.04
	5	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.01
ST1A4	1	0.56	0.11	0.22	0.22	0.44	0.56	0.56	0.22	0.33	0.67	0.39
	2	0.44	0.67	0.67	0.56	0.56	0.33	0.44	0.78	0.56	0.33	0.53
	3	0.00	0.22	0.11	0.22	0.00	0.11	0.00	0.00	0.11	0.00	0.08
ST2E4	1	0.89	0.78	0.89	0.78	1.00	0.89	0.78	0.78	1.00	1.00	0.88
	2[n]	0.11	0.22	0.11	0.22	0.00	0.11	0.22	0.22	0.00	0.00	0.12
ST1E3	1	0.00	0.22	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03
	2	0.44	0.44	0.89	0.75	0.78	0.67	0.78	0.78	0.56	0.78	0.69
	3	0.56	0.33	0.00	0.25	0.22	0.33	0.22	0.22	0.44	0.22	0.28
ST2C10	1	0.33	0.00	0.22	0.22	0.33	0.22	0.56	0.33	0.33	0.22	0.28
	2	0.00	0.00	0.11	0.00	0.00	0.11	0.00	0.00	0.11	0.00	0.03
	3	0.33	0.56	0.56	0.67	0.56	0.44	0.33	0.67	0.44	0.67	0.52
	4[n]	0.33	0.44	0.11	0.11	0.11	0.22	0.11	0.00	0.11	0.11	0.17
ST1G7	1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.78	1.00	1.00	0.98
	2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.00	0.00	0.02
ST1E7	1	0.44	0.67	0.67	0.44	0.56	0.22	0.22	0.78	0.33	0.44	0.48
	2	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.02
	3	0.56	0.22	0.33	0.56	0.44	0.78	0.78	0.22	0.67	0.44	0.50
ST1D7	1	0.67	0.78	0.89	0.89	0.67	0.67	0.67	0.78	0.56	0.56	0.71
	2	0.11	0.22	0.00	0.11	0.11	0.22	0.33	0.11	0.44	0.33	0.20
	3	0.22	0.00	0.11	0.00	0.11	0.11	0.00	0.11	0.00	0.11	0.08
	4	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.01

n: null allele

Appendix D2. Allelic frequency of seven microsatellite loci within the population of *Mycosphaerella graminicola* used for gametic disequilibrium analysis

SSR Loci	Allele no.	Average frequency of each allele within the population
ST1A2	1	0.44
	2	0.31
	3	0.19
	4 [a]	0.06
ST1A4	1	0.39
	2	0.53
	3	0.08
ST2E4	1	0.88
	2 [n]	0.12
ST1E3	1	0.03
	2	0.69
	3	0.28
ST2C10	1	0.28
	2	0.03
	3	0.52
	4 [n]	0.17
ST1E7	1	0.48
	2	0.02
	3	0.50
ST1D7	1	0.71
	2	0.20
	3 [a]	0.09

[a] Alleles with frequencies of less than 0.1 were pooled into a single category.
[n] Null allele

Appendix E. Buffers and solutions used in RAPD and microsatellite studies

TAE (Tris-Acetate EDTA) 50X:	Tris base	242 g
	Glacial Acetic Acid	57.1 ml
	0.5 M EDTA (pH 8.0)	100 ml
TBE (Tris-Borate EDTA) 10X:	Tris base	108 g
	Boric Acid	55 g
	0.5 M EDTA (pH 8.0)	40 ml
TE (Tris- EDTA):	1M Tris (pH 8.0)	10 ml
	0.5M EDTA (pH 8.0)	2 ml
Gel Loading Buffer (6X):	Glycerol	50 ml
	Bromophenol blue	40 mg
	Water	50 ml
6% polyacrylamige gel:	Urea	44.1 g
	10 X TBE (buffer)	9.0 ml
	40% 19:1 Acrylamide / bis-Acrylamide	13.5 ml
	Water	36.0 ml

Heat the solution until the urea dissolved,
- filter through 40 micron syringe filter and de-gas it under strong vacuum.
- prepare a 25% solution of ammonium persulfate (0.0625g/250µl)
- add 90µl of the 25% ammonium persulfate solution and 90µl of TEMED just before casting the gel and mix gently.

Developing solution: - dissolve 60 g of sodium carbonate in 2 litters of double-distilled Water.
-chill the solution on an ice bath and add 3 ml of 37% formaldehyde and a 400 µl aliquot of sodium thiosulfate (10 mg / ml) to the solution before immediate use.

Appendix F. Standardized means of incubation period, latent period and disease severity of 90 isolates of *Mycosphaerella graminicola* tested on the susceptible wheat cultivar Conway

Isolate #	Incubation period	Latent period	Disease severity
St 1	-1.70[a]	-1.76	1.10
St 2	0.64	1.01	-1.06
St 3	1.26	1.58	0.43
St 4	-0.05	0.56	-1.37
St 5	-0.25	0.05	0.00
St 6	2.05	0.97	-1.79
St 7	-0.49	-1.19	1.10
St 8	0.33	-0.27	0.49
St 9	-0.36	-0.31	-0.46
St 10	0.64	0.83	-0.15
St 11	0.94	-0.29	-0.24
St 12	1.81	1.03	-0.64
St 13	-0.73	0.05	-0.03
St 14	1.77	2.19	-1.82
St 15	0.23	1.27	-0.36
St 16	-1.18	-0.90	1.95
St 17	-0.08	-0.33	-0.49
St 18	-0.84	-0.25	-0.30
St 19	-0.08	0.56	0.15
St 20	-0.29	-0.74	1.28
St 21	-0.25	0.36	-0.06
St 22	0.09	0.58	-1.12
St 23	0.81	0.85	-1.31
St 24	-0.29	0.01	-0.12
St 25	-1.59	-1.25	1.34
St 26	0.64	0.09	-1.37
St 27	1.29	2.09	-0.79
St 28	1.94	0.89	-1.55
St 29	0.26	-0.29	0.46
St 30	-1.11	-1.15	1.07
St 31	-1.08	-0.84	0.55
St 32	1.09	0.50	-0.85
St 33	-0.12	0.44	0.21
St 34	-0.08	-0.01	-1.46
St 35	-0.94	-1.08	0.27
St 36	2.18	2.59	-0.82
St 37	0.36	0.11	1.55
St 38	0.50	0.07	0.85
St 39	1.67	1.25	-1.67
St 40	1.39	0.93	0.00

Appendix F. (cont.)

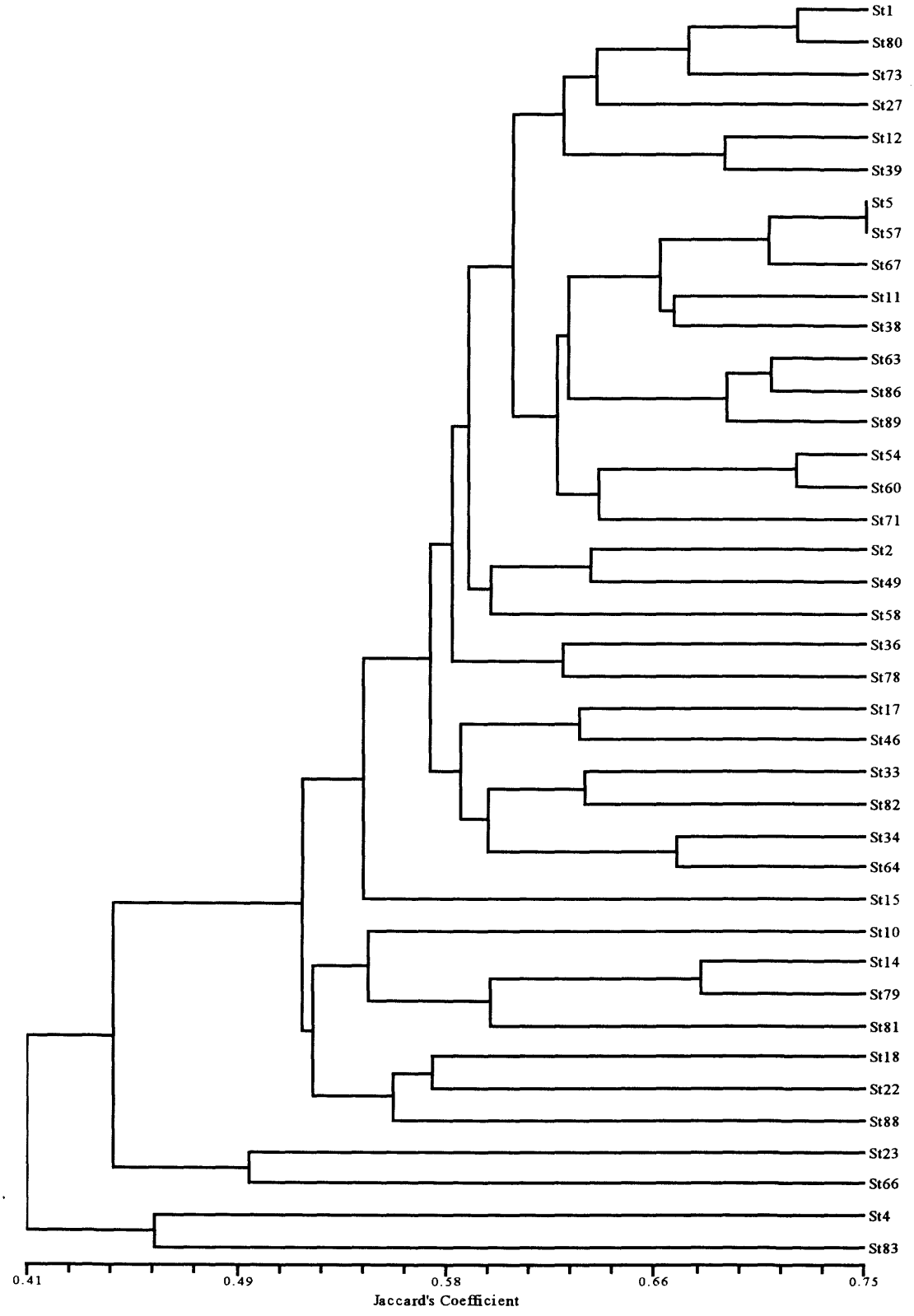
Isolate #	Incubation period	Latent period	Disease severity
St 41	1.43	0.40	-0.46
St 42	0.12	-0.29	-0.82
St 43	-0.42	-0.78	0.76
St 44	0.26	-0.15	0.06
St 45	-0.22	-1.15	1.64
St 46	0.06	0.16	-0.15
St 47	-0.22	-0.43	0.61
St 48	0.33	-0.39	-0.61
St 49	-1.32	-1.33	1.19
St 50	0.12	-0.13	-0.21
St 51	0.67	0.91	-0.52
St 52	-1.97	-1.69	0.43
St 53	-1.18	-0.62	0.79
St 54	0.09	-0.09	0.12
St 55	-1.63	-2.06	0.15
St 56	2.63	2.37	-1.73
St 57	-1.18	-0.62	1.13
St 58	-0.05	0.30	-1.16
St 59	2.46	1.92	-1.89
St 60	-0.39	-0.94	0.12
St 61	0.40	1.72	-0.55
St 62	0.43	0.03	-0.06
St 63	-0.97	-0.94	0.24
St 64	-0.46	-0.09	1.07
St 65	-0.73	-1.19	1.95
St 66	-0.25	0.83	0.21
St 67	-0.25	0.77	-0.15
St 68	0.64	0.66	0.82
St 69	-0.73	-0.90	0.52
St 70	-0.42	-0.98	-0.06
St 71	-0.05	-0.84	1.80
St 72	-0.01	-0.35	-0.30
St 73	1.26	0.81	-0.79
St 74	-1.45	-1.45	1.98
St 75	0.54	0.99	-0.12
St 76	0.19	-0.17	1.04
St 77	0.26	0.11	-0.09
St 78	-0.08	0.52	-1.19
St 79	-1.42	-1.43	1.04
St 80	-1.39	-1.59	2.13

Appendix F. (cont.)

Isolate #	Incubation period	Latent period	Disease severity
St 81	-0.32	-0.19	-0.21
St 82	-0.60	-0.52	-0.67
St 83	-1.04	0.91	-0.24
St 84	-0.39	0.34	-1.28
St 85	-1.63	-1.43	2.13
St 86	1.09	0.68	-0.85
St 87	-0.29	-0.66	0.67
St 88	-0.01	-0.72	-0.09
St 89	0.54	0.81	-1.49
St 90	-0.94	-1.33	0.12

[a] The value of incubation period, latent period and disease severity of each isolate was standardized to the mean and standard deviation of the mean in the population.

Appendix G 1. Similarity of 40 *Mycosphaerella graminicola* haplotypes based on RAPD data, using the coefficient of Jaccard



Appendix G 2. Similarity of 40 *Mycosphaerella graminicola* haplotypes based on virulence data, using the coefficient of Gower (1971)