Blackleg of Canola: Survey of virulence and race structure of the *Leptosphaeria maculans* pathogen population in Canada and evaluation of the genetic variation in the *L. maculans* global population

By

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ABSTRACT

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Phoma stem canker (aka Blackleg) caused by the fungal pathogen *Leptosphaeria maculans* is a major disease affecting Canola (*Brassica napus* L.). This study examined 674 *L. maculans* isolates collected in 2010 and 2011 from western Canada at ten avirulence gene loci. Overall, certain alleles were more prevalent with *AvrLm6* and *AvrLm7* present in >85% of isolates and *AvrLm3*, *AvrLm9*, and *AvrLepR2* present in <10% of isolates. This study also examined the genetic diversity of *Leptosphaeria maculans* populations around the world. Blackleg disease is found in most countries where Brassica spp. are cultivated and there are indications that *L. maculans* is an expanding species displacing the less aggressive *Leptosphaeria biglobosa*. Twenty two microsatellite primers were used to screen 96 isolates from 8 countries. A phylogenetic tree to assess the evolutionary relationship between regions was generated and the results indicated that genetic diversity was correlated with geographic location.

FOREWORD

This thesis is written in manuscript style. A general introduction and review of literature precedes manuscripts that comprise the main part of the thesis. Each manuscript consists of an abstract, introduction, materials and methods, results and discussion. A general discussion and conclusions, a list of references, and an appendix follow the manuscripts.

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CHAPTER 1

1.0 GENERAL INTRODUCTION

1.0 GENERAL INTRODUCTION

Blackleg (aka phoma stem canker) caused by the fungal pathogen *Leptosphaeria maculans* is a ubiquitous disease affecting oilseed rape and canola (*Brassica napus*). It is responsible for major yield losses around the world with the exception of China and is prevalent in North America, Western Europe and Australia. *L. maculans* is exceptional in its ability to maintain the genes necessary for both saprophytic and necrotrophic parastism lifestyles and transition from sexual to asexual stages providing the parasite with a high level of plasticity in survival. Various effector genes are involved in the interaction of the host and pathogen and a subset of these confer resistance to a pathogen avirulence gene if the plant has the corresponding resistance gene. Thus far at least 15 avirulence genes involved in the gene for gene interaction between Brassica species and *L. maculans* have been identified, 7 have been localized in the *L. maculans* genome, and 6 have been cloned: *AvrLm1*, *AvrLm6*, *AvrLm4*-7, *AvrLm11*, *AvrLmJ1/AvrLm5*, and *AvrLm2* (Gout *et al.*, 2006; Fudal *et al.*, 2007; Parlange *et al.*, 2009; Balesdent *et al.*, 2013; Van de Wouw *et al.*, 2013; Ghanbarnia *et al.*, 2014).

Leptosphaeria maculans has shown the evolutionary potential to breakdown novel resistance sources as anticipated by its combination of sexual recombination in the saprophytic ascospores and the large population sizes of the asexual pycnidiospores. This has been illustrated by its rapid adaptation to the *Rlm1* gene that was widely grown in France and broken down in three years (Rouxel *et al.*, 2003). Similarly the breakdown of sylvestris resistance in Australian cultivars lead to as much as 90% crop loss. (Li *et al.*,

2003). Despite its economic impact, research into the molecular basis of the effector induced immunity between *B. napus* and *L. maculans* is still in its infancy. However, the recent sequencing of the fungal genome (Rouxel *et al.,* 2011), cloning of several avirulence genes (Gout *et al.,* 2006; Fudal *et al.,* 2007; Parlange *et al.,* 2009), and a single resistance gene (Larkan *et al.,* 2012) has laid the groundwork for further studies.

Brassica napus (Canola) cultivars maintain a 'gene for gene' interaction with the Blackleg fungus, *Leptosphaeria maculans* in which a single major resistance (R) gene can detect and provide resistance against fungal isolates carrying a complementary avirulence (Avr) gene (Balesdent *et al.,* 2005). The 'gene for gene' model was described by Flor (1942) as a pair of matching genes in the host and pathogen that control for both resistance in the host and parasite ability in the pathogen. Host resistance genes are involved in recognizing pathogen avirulence genes and trigger a defense response. At least 14 major resistance genes (*Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm5*, *Rlm6*, *Rlm7*, *Rlm8*, *Rlm9, Rlm10, LepR1, LepR2, LepR3*, and *RlmS*) have been identified to date (Yu *et al.,* 2005, 2008; Delourme *et al.,* 2006; Larkan *et al.,* 2012). In addition to single major resistance genes, multiple minor genes have an additive effect and provide partial race-non-specific resistance in the adult plant (Hayward *et al.,* 2012). In spite of the availability and breeding of resistance genes into commercial cultivars, changes in the frequency of the corresponding avirulence genes in the pathogen population have led to the 'breakdown' of major resistance genes, as demonstrated by the examples above and the results of this study. The transition from sexual to asexual stages allows the pathogen to both incorporate new changes and rapidly shift population structure towards virulent

races. In addition recent sequencing of its genome has uncovered that the avirulence genes are subjected to increased mutational pressure due to their localization in gene poor isochores rich in transposable elements (Rouxel *et al.,* 2011). The increased rate of mutation has been suggested to contribute to the increased rate of deletions and mutations that allow pathogen avirulence genes to avoid host recognition (Van de Wouw *et al.,* 2010).

In Canada *L. maculans* remains among one of the most economically significant diseases of canola. Although resistance sources are not publically known, all cultivated *B. napus* varieties are considered to have some form of resistance (Rimmer, 2006). The disease has been managed thus far with several strategies including sowing resistant cultivars, rotations, stubble management, fungicides, etc. Due to the evolving nature of the fungal pathogen and its ability to avoid host recognition, it becomes necessary to generate data that will provide an overview of the relative risks imposed by the Canadian *Leptosphaeria maculans* population to the Canadian canola industry.

Fortunately Canadian canola cultivars have not experienced a Blackleg outbreak as severe as was reported in Australia and France (Rouxel *et al.,* 2003; Li *et al.,* 2003). This can be partially attributed to a shorter growing season and the reproductive nature of the pathogen (Ghanbarnia *et al.,* 2011; Ghanbarnia *et al.,* 2009). However, increasing disease pressure from larger acreages and tighter rotations have led to the emergence of new races in western Canada (Chen and Fernando, 2006; Kutcher *et al.,* 2007). Given that *L. maculans* can lose avirulence genes to become virulent, sustained use of any

single resistance gene cannot remain effective against dispensable alleles and a changing pathogen population. The rotation of cultivars with different resistance sources to mitigate pathogen adaptation is one emerging strategy that has thus far had some promising results in recent field trials (Marcroft *et al.*, 2012).

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Canola

2.1.1 Canola (Brassica napus)

Vegetable oil crops have been a common food source for thousands of years in the Mediterranean and Asia (Downey, 1983), but it is only in the latter half of the 20th century that Brassica plants have become a major oil for human consumption and more recently biofuel production. Records in India suggest oilseed Brassica species were cultivated as early as 4000BC, were reported in China and Japan 2000 years ago, and *B. napus* ssp. napobrassica was grown relatively recently in the Roman empire (Gupta and Pratrap , 2007; Snowdon *et al.*, 2007). Brassica is a genus of plants in the mustard family (Brassicaceae), and is comprised of approximately 3350 species in 340 genera that include some of the most important crops worldwide (Hayward *et al.*, 2012). Rapeseed (*Brassica napus* L; genome AACC, 2n=38) is the most widely cultivated and most economically important crop in the crucifer family (Brassicaceae).

Although commonly treated as a single commodity, rapeseed is a composite of two or three species. In Asia *Brassica rapa*, and *Brassica juncea* are widely grown whereas in Europe, Canada, and Australia, *Brassica napus* is most commonly cultivated species. The aforementioned species are closely related, and commonly represented in a triangle diagram (UN, 1935). *Brassica rapa* L. (n=10, AA), *Brassica nigra* L. (n=8, BB), and *Brassica oleracea* L. (n=9, CC), represent the three diploid species at the vertices, which intercross to produce three amphidiploid species, *Brassica carinata* L. (n=17, BBCC), *Brassica juncea* L. (n=18, AABB), and *Brassica napus* L. (n=19, AACC).

Spontaneous chromosome doubling following crosses of the amphidiploids Indian or brown mustard (*B. juncea*, AABB) and Abyssinian or Ethiopian mustard (*B. carinata*, BBCC) has been observed (Prakash & Hinata, 1980). This suggests that natural crosses between Black mustard (*B. nigra*, BB), *B. rapa*, and *B. oleracea* have occurred (McNaughton, 1995). However, no wild forms of *Brassica napus* are known, it is assumed that the species originated relatively recently in the Mediterranean where both its progenitor species can be found and was domesticated in southern Europe (Lamb, 1989). Although brassicas were used as vegetable crops for thousands of years, the use of its oil was first documented in Europe beginning around the 13th century for lighting lamps until it was largely replaced with petroleum in the 19th century, but it remained as a lubricant for industrial applications (Kimber and McGregor, 1995; Reiner *et al.*, 1995).

The high concentration of erucic acid, up to 50% in the oil, which can lead to cardiac damages and other related health issues (Conner, 1999), prevented *B. napus* from being utilized as a cooking oil except by the very poor. The first reduction of erucic acid occurred in the late 1960s resulting in cultivars with erucic acid levels under 1% (Stefansson and Hougen, 1964). Shortly thereafter, the first erucic acid free cultivar was derived by Dr. Stefansson in Canada from mutants of the German spring rapeseed Liho, and commercialized in the early 1970's. The value of the crop was however still reduced by the presence of glucosinolates, which made the meal toxic to livestock. The solution was found in the Polish spring rape variety Bronowski, which had low glucosinolate content. Successful backcrossing led to the first low erucic acid, low glucosinolate, high yielding cultivar named Tower in 1974 and was the introduction of Canadian low erucic

acid, ‘Canola’, varieties (Stefansson and Kondra, 1975). These varieties came to be known as ‘Canola’, a term registered by the Western Canadian Oilseed Crushers Association to represent varieties with less than 5% erucic acid and less than 3mg/g of glucosinolate content.

2.1.2 Growth and Development of Canola

In Europe and Asia, *B. napus* is cultivated primarily as a winter rapeseed, whereas in Canada, Australia, and parts of northern Europe only spring forms can be grown due to the colder climate. Winter canola requires vernalization, which triggers physiological changes that promote the onset of flowering. Winter oilseed rape is seeded in the fall and can survive winter by remaining dormant. Spring oilseed rape does not require vernalization but is also not winter-hardy and thus the crop must be sown in the spring.

Cotyledons emerge 4 to 7 days after seeding followed by true leaves and a rosette of broader leaves a few days later. Winter oilseed rape enters dormancy at this stage, until the spring when a vertical stem develops followed by flowering in the late spring. In spring oilseed rape, rapid elongation of the stem occurs a few weeks after seeding and is often greater than 1m (McGregor, 1981). Flower buds are produced as the stem elongates in a raceme inflorescence with bright yellow flowers. Indeterminate flowering begins at the lowest buds and moves upwards along the main raceme. *B. napus* is a facultative outcrossing species and will more frequently self-pollinate, however, cross pollination is more likely to occur when insect pollinators are abundant (Kasa & Kondra, 1986). When the flower is fertilized its petals drop and a cylindrical pod forms carrying 15-40 spherical

seeds (Chauhan, 1984). The duration of flowering and pod development will vary and can last between 6 to 8 weeks. (Smith & Scarisbrick, 1990).

2.1.3 Canola Trade & Nutrition

Rapeseed (mainly canola, *B. napus*) has become a major agricultural crop in the past 40 years. It is the world's third largest vegetable oil (after soybean and palm oil) and rapeseed oil production worldwide was in excess of 69 million metric tons in 2014 (USDA, 2014). This production occurs mainly in the European Union, followed by China, Canada, India, and Australia. It is used primarily as a cooking oil and is considered a 'healthy oil' due to its nutritional value, taste, and shelf life. Due to its low glucosinolates content, its meal is used to supplement the feed of cattle, poultry, swine and other animals. It has maintained continuous use in the oleo chemical industry, and is making gains as a competitive biofuel source. The yield per hectare of canola varies widely between countries, mainly due to the greater yields of winter canola vs spring canola, but also due to differences in climate, soil, and agricultural inputs.

Canola (*Brassica napus* L.) is an economically successful crop in the Canadian Prairies with nearly 19 million tonnes produced in 2014 (Canola Council, 2014). The canola industry contributes $19.3 billion dollars to Canada's economy and supports 52,000 farmers that actively grow canola. Approximately 85% of Canada's production is exported to markets around the world. Among the challenges to trade is the risk of the spread of the *Leptosphaeria maculans* pathogen into China (Fitt *et al.*, 2008). This issue

temporarily caused a trade barrier in 2009 and prompted greater research into the Blackleg pathogen in Canada.

Modern commercial canola cultivars yield in excess of 40% oil within the seed and have been extensively bred to remove any nutritionally undesirable traits such as long-chain fatty acids. Canola is highly regarded as a healthy oil due to its fatty acid profile and is often recommended as a means to reduce dietary saturated fat intake. Canola also contains the essential omega-6 and omega-3 fatty acids in a 2:1 ratio (Lands, 1997) and is a useful cooking oil due to its proportion of linoleic and α-linolenic acid. Canola is associated with a lower risk of coronary heart disease and canola oil based margarine is recommended as a major source of monounsaturated fatty acids (18:3n-3) in the prevention of coronary heart disease. (Trautwein, 1997, De Lorgeril *et al.* 2001).

2.1.4 Diseases of Canola

A recent review of oilseed rape diseases highlighted Sclerotinia stem rot and stem canker (aka Blackleg) as the major diseases of oilseed rape in Canada (Snowden *et al.*, 2007) although clubroot has recently become a concern in western Canada (Tewaria *et al.*, 2005). These two diseases are also commonly found in Europe, with the addition of Verticillium wilt in Sweden and Germany (Manitoba reported the first occurrence of the disease in one location in 2014), and leaf spot and clubroot in Scandinavian countries. China on the other hand mainly deals with Sclerotinia and a number of viral diseases, and only the less aggressive species of Blackleg, *Leptosphaeria biglobosa,* is present. Other less common diseases in Canada include white rust and staghead (*Albugo candida*),

Alternaria black spot (*Alternaria brassicae*), damping off and root rot (*Rhizoctonia solani, Fusarium* spp.), downy mildew (*Peronospora parasitica*), clubroot (*Plasmodiophora brassicae*) , and aster yellow disease (aster yellow phytoplasma) among many other less damaging bacterial, viral, and fungal diseases (Canola Council, 2014).

2.2 Phoma stem canker aka Blackleg

2.2.1 Significance of Blackleg

Leptosphaeria maculans is an economically important fungal pathogen of oilseed rape (*B. napus* L.). This filamentous ascomycete is the causal agent of phoma stem canker, commonly referred to as Blackleg. The disease is caused by a complex of Leptosphaeria species, the less aggressive *L. biglobosa*, and the more damaging *L. maculans*. Since the turn of the millennium, the worldwide acreage of canola has expanded significantly, from 26.9 million hectares in 1999 to more than 69 million hectares in 2013 (USDA, 2014). The expansion of canola production also led to the growth of pathogen population. Blackleg has been reported in Canada, USA, Mexico, Europe, Australia, Brazil, Argentina, and in Kenya (Fitt and al, 2006), among several other countries. However, the more aggressive *L. maculans* has yet to spread to China, the single largest producer of rapeseed worldwide.

Blackleg is not a new disease and despite breeding efforts, significant economic losses persist in major canola growing regions. Blackleg is estimated to cause a loss of $1000M per season globally (Fitt *et al.,* 2008). *L. maculans* has shown the evolutionary

ability to lose avirulence genes and gain virulence to resistance genes, allowing it to adapt to breeding efforts causing large economic losses as was seen with the `breakdown` of *Rlm1* cultivars in France and 'sylvestris' resistance in Australia (Rouxel *et al.,* 2003; Li *et al.,* 2003). Similarly, larger acreages and tighter rotations have led to the emergence of new races in western Canada (Chen and Fernando, 2006). Moreover, there is evidence that this pathogen is adaptable to different climates, since the disease is very damaging on both spring and winter (autumn-sown) types of oilseed rape in a wide geographic range (West *et al.,* 2001). These factors have led to increased research into Blackleg, specifically *L. maculans* that are providing new insights and solutions to disease management.

2.2.2 History and Taxonomy of *Leptosphaeria maculans*

The earliest record of symptoms similar to modern Blackleg was in 1791 and the pathogen was named *Sphaeria lingam* (Tode, 1791). This fungus was later identified as a pathogen of cabbage and renamed *Phoma lingam* by Desmazières (1849). The sexual stage of *P. lingam* was suggested to be *Leptosphaeria maculans* (Desm.) Ces. & De Not. by Tulasne (1863). There were various reports of Blackleg symptoms, Phoma stem canker, in the 19th century in the major cabbage growing areas of Europe, North America, and Australia. The first well documented epidemic of Blackleg, identified as *Phoma lingam*, was in Wisconsin, USA early in the 20th century (Henderson, 1918). Blackleg has since caused epidemics in France in the 1950's (Darpoux *et al.* 1957), Australia in the 1970's (Gugel & Petrie, 1992) and the USA in the 1990's (Lamey, 1995).

Early evidence of Phoma stem canker was reported in 1961 in western Canada (Vanterpool, 1961) and the first symptoms were described as superficial leaf and stem lesions on oilseed rape (McGee & Petrie, 1978). These were later known to be caused by the less aggressive *L. biglobosa*. The first report of *L. maculans*, the aggressive species responsible for most of the yield losses today, was in the prairies in Saskatchewan (McGee & Petrie, 1978). *L. maculans* was later also found in eastern Canada in Ontario (Peters &Hall, 1987).

The taxonomic history of *Leptosphaeria maculans* has been relatively unclear until recently. It was a challenge early on to correctly identify several distinct species of Leptosphaeria from different crucifer species such as *Thlaspi arvense, Sisymbrium spp, Descurainia spp Lepidium spp,* and *Erysimum spp* (Smith and Sutton, 1964). The Leptosphaeria genus is a putative monophyletic clade that includes *L. maculans, L. dryadis, L. weimeri, L. conferta, L. congesta*, and *L. doliolum* (Camara *et al.,* 2002). This clade encompasses a series of species that are pathogenic on dicotyledonous plants. *Phoma lingam* has been experimentally established as an anamorph of *L. maculans* based on findings from several independent groups utilizing different isolates from different hosts (Muller & Tomasevic, 1957; Smith and Sutton, 1964; Petrie, 1969).

The classification of the pathogen into subgroups was originally based on the phenotype on the host plant (Petrie, 1969), virulence on different brassica (Mithen, 1987; Koch *et al.,* 1989), and by chemical analysis of the secondary metabolites of *L. maculans*. The timing of new chemical analytical techniques allowed for the identification of

molecules involved in the host-pathogen interaction and the phytotoxin sirodesmin PL provided an early method to distinguish *L. maculans* strains (Balesdent *et al.,* 1992; Gugel and Petrie, 1992). The first common distinction was made between highly virulent or aggressive 'A' group strains that produced the phytotoxin sirodesmin PL and were also able to infect canola, and the weakly virulent or non-aggressive, 'B' group strains that did not produce sirodesmin PL nor could infect canola (Balesdent *et al.,* 1998). To clarify taxonomic issues and stimulate research into *L. maculans*, the International Blackleg of Crucifers network (IBCN) was established in 1994 with a collection of 90 IBCN isolates from around the world. Isolates were analyzed in terms of DNA, secondary metabolites, morphology, and pathotype groups to better categorize subgroups within the species.

Further research using the IBCN isolates, at the time referred to as *L. maculans*, unveiled that intercrossing between 'A' group and 'B' group isolates did not produce pseudothecia whereas intragroup crosses produced viable pseudothecia (Somda *et al.,* 1997). This distinction, along with morphological comparison of *in-vitro* pseudothecia and conidia led to the reclassification of the 'B' pathotype group as a separate species *Leptosphaeria biglobosa* (Shoemaker and Brun, 2001). This differentiation was further supported by RFLP studies between 'A' group and 'B' group isolates (Johnson & Lewis 1990; Balesdent *et al.,* 1992; Gall *et al.* 1995), AFLP studies (Purwantara *et al.,* 2000), isozyme and soluble protein studies (Gall *et al.,* 1995; Somda *et al.,* 1996), and DNA studies (Morales *et al.,* 1993; Balesdent *et al.,* 1998).

Leptosphaeria maculans, formerly 'A' group isolates, were first subdivided into pathogenicity groups (PG 2-4,T) based on their interaction with three different *B. napus* cultivars: Westar, Quinta, and Glacier (Koch *et al.*, 1991; Mengistu *et al.*1991). Later the addition of *Brassica napus* c.v. jet neuf classified *L. maculans* into six races, A1-A6 (Kuswinanti *et al.*, 1999). Further studies involving additional cultivars and the interaction of *B. napus, B. rapa*, or *B. juncea* identified several AvrLm genes involved in the host recognition of *L. maculans*. (Ansan *et al.*, 1995; Balesdent *et al.*, 2002). The numerous combinations of theoretical races possible from different AvrLm genes rendered the former three and six race classification groups obsolete with newer publications adopting any unique combination of avirulence genes as a separate race (Fitt *et al.*, 2006, Kutcher *et al.*, 2010)

2.2.3 Host Range

Leptosphaeria maculans infects a wide range of *Brassicae* species including cabbage and oilseed rape crops. The disease was first identified on Cabbage *(Brassica oleracea)* in Wisconsin (Henderson, 1918). Both *Leptosphaeria biglobosa* and *Leptosphaeria maculans* are known to cause infection on *Brassica oleracea* (Dilmaghani *et al.*, 2010). Most *B. napus* crops cultivated today have been bred to carry some level of resistance to *L. biglobosa* whereas *Leptosphaeria maculans* has adapted to *B. napus* and has caused several epidemics (West *et al.*, 2001). The emergence of the oilseed rape industry in the early 1970s has coincided with the increase of *L. maculans* displacing *L. biglobosa* as the dominant species in large ecological niches of North America and Europe (Fitt *et al.*, 2008).

While *L. biglobosa* is common on cabbage and *Brassica oleracea* (CC genome), it is less prevalent on *B. napus (AACC)* varieties today due to resistance derived from *B. rapa* (AA genome). Almost all known resistance genes to *L. maculans* in *B. napus* have been mapped to the A genome (*Rlm1, 2, 3, 4, 7, 9, LepR1, LepR2, LepR3, RlmS*) and no R genes to *L. maculans* has been found in the C genome (Hayward *et al.* 2011). *B. rapa* is known to carry increased resistance to *L. maculans* and blackleg is rare in India where *B. juncea* (BBAA) and *B. rapa* (AA) are cultivated (*West et al., 2001).* Resynthesized *B. napus* lines have successfully transferred new resistance from the *B. rapa* 'A' genome with commercial success (Yu *et al.,* 2008), termed sylvestris resistance. Most *B. juncea* varieties are very resistant to *Leptosphaeria maculans* due to resistance in the *B. genome* (Rlm 5, 6, 10) (Chèvre *et al.,* 1997). *B. nigra (BB)* is the most resistant species in the Brassica triangle and is a promising source for new blackleg resistance genes (Eber *et al.,* 2011)

Table 2.1: Location of mapped blackleg resistance genes on the Brassica napus genome. Source: Hayward *et al.*, 2011.

species	*Leptosphareria maculans* avirulence gene	resistance gene	location on *Brassica* chromosome
Brassica napus	–	*LmFr*$_1$	–
	–	*aRLMrb*	A7
	–	*cRLMj*	A7
	–	*aRLMj*	A7
	alm1	*LEM1=Rlm4 or 1?*	A7
	AvrLm1	*Rlm1*	A7
	AvrLm2	*Rlm2*	A10
	AvrLm3	*Rlm3*	A7
	AvrLm4	*Rlm4*	A7
	–	*cRLMm=Rlm4?*	A7
	AvrLm4	*LmR1=Rlm4?*	A7
	AvrLm4	*cRLMm=Rlm4?*	A7
	AvrLm4	*cRLMrb=Rlm4?*	A7
	AvrLm7	*Rlm7*	A7
	AvrLm9	*Rlm9*	A7
Brassica rapa	*AvrLm8*	*Rlm8*	–
Brassica rapa ssp. *sylvestris*	–	*LepR1*	A2
	–	*LepR2*	A10
	–	*LepR3*	A10
	–	*LepR4*	–
Brassica juncea	*AvrLm5*	*Rlm5*	–
	AvrLm6	*Rlm6=Jlm1*	–
	–	*rjLM2*	–
Brassica nigra	–	*Rlm10*	–

2.3 Blackleg of Canola

2.3.1 Morphology

L. maculans isolates typically have a small amount of short aerial mycelium and produce moderate amounts of pycnidia that are peppered on the plate, which in turn produce copious amounts of pink-purple pycnidiospores. Aside from the color of the pycnidiospores, the plate is relatively clear with no pigment production. *L. maculans* isolates cannot be distinguished in terms of pycnidia or pycnidiospores due to a high degree of variability in terms of size and shape (Petrie, 1969).

On the other hand, the less aggressive *L. biglobosa* isolates produce long fluffy or felty mycelium, and release yellow to brown-red pigments in water agar (Shoemaker & Brun, 2001). *L. biglobosa* produces fewer pycnidiospores, has faster germ tube elongation, and a faster growth rate than *L. maculans* both on agar and *in vitro*. It has also been shown that *L. biglobosa* isolates germinate interstitially compared to terminal germination of *L. maculans* (Huang *et al.,* 2001). There is no difference in hyphae septa in terms of number, size, or shape, between the two species. In terms of fruiting bodies, *L. biglobosa* isolates have a conspicuous beak on the ascomata that is greatly enlarged at the apex.

Pycnidia of both *L. maculans* and *L. biglobosa* are spherical, black, with a protruding concave beak, and approximately 150-200 x 100 µm (Shoemaker & Brun, 2001). Pycnidiospores are also similar for both species, elongated and cylindrical with a central bulge at approximately 4-5x 1.5-2 µm. Both species show bipolar heterothallism and require compatible partners to produce sexual spores. Ascomata are black,

cylindrical, 280-350 µm in diameter, and have a flat base. Asci are 100-140 x 12-16 µm in size, cylindrical with smaller ends, and carry 8 tetra seriate ascospores. Ascospores take longer to form in *L. biglobosa* than in *L. maculans* (Shoemaker & Brun, 2001).

2.3.2 *L. maculans* biochemistry

L. maculans produces several secondary metabolites, particularly phytotoxins that are helpful for resolving taxonomic conflicts, as well as shedding light on host pathogen interactions. The first identified secondary metabolite produced by the fungus was sirodesmin PL, a major dioxopiperazine phytotoxin containing an epipolysulfide bridge (Ferezou *et al.,* 1977). This phytotoxin was used to categorize *L. maculans* into Tox^{+} A and Tox^{0} B subgroups (William and Fitt, 1999), until *L. biglobosa* was later identified as a separate species (Shoemaker and Brun, 2001).

The phytotoxins produced by 'A' group or *L. maculans* isolates are largely comprised of the sulphur-containing epipolythiodioxopiperazine toxins of the Sirodesmin family. Sirodesmin PL is the main phytoxin, 50-70% of phytotoxic extracts, alongside sirodesmin J, sirodesmin K, and sirodesmin H, deacetylsirodesmin, phomalirazine phomapyrone A, phomalide, and l-valyl-l-tryptophan anhydride (Pedras *et al.,* 1990). *L. maculans* mutants lacking sirodesmin Pl production were shown to be less aggressive, with significantly reduced cotyledon and stem infection rates (Elliot *et al.*, 2007). *L. biglobosa* lacks sirodesmin, but produces other phytotoxins such as phomapyrons and cyclohexendions (Soledade *et al.,* 1997, Soledade *et al.,* 2000).

In addition to sirodesmin, *L. maculans* produces the host-selective toxin phomalide, which is structurally unrelated to sirodesmin, and causes lesions on *B. napus* leaves that closely resemble those caused by *L. biglobosa* (Pedras *et al.*, 1993). The host selectivity and correlation with disease resistance have made phomalide a promising target for Blackleg resistance breeding (Pedras and Biesenthal, 2000).

2.3.3 *L. maculans* genome

The Leptosphaeria Genome Consortium was established by INRA and Genoscope in 2004 (http://www.genoscope.cns.fr). Genoscope began whole genome shotgun sequencing of six cDNA libraries including infected samples in 2007 and the reads were aligned into 76 supercontigs and made available by INRA (http:/urgi.versailles.inra.fr/index.php/urgi/species/Leptosphaeria). An analysis of the genome structure and fungal effectors was recently published by Rouxel *et al.* (2011). They highlighted the distinct isochores structure of the genome with alternating AT rich and GC rich regions, speculated to be caused by the degenerative effects of transposable elements (TE's).

The *L. maculans* genome was found to be closely related to *Phaeosphaeria (Stagonospora) nodorum, Pyrenophora tritici-repentis,* and *Cochliobolus heterostrophus*, in terms of phylogeny (Rouxel *et al.*, 2011). Its genome is larger than the related Dothideomycetes at 45.12Mb scaffolded into 76 supercontigs with an inferred size of 17-18 chromosomes. The *Nuerospora crassa* genes necessary for repeat induced point mutations (RIPs), a fungal mechanism to inactivate repetitive DNA were previously identified in *L. maculans* and supported by experimental identification. Following the

sequencing of the genome, the presence of RIPs was statistically confirmed using RIPCAL software. The RIP mutations are a response to the presence of a few families of transposable elements (TEs) that duplicate and transpose themselves across the genome often leading to the inactivation of key genes and to general genetic degradation. Where strong selection pressure to maintain vital genes was present, multiple copies of genes resulted, or alternatively the TE regions were inactivated by RIP mutations. The resultant isochore structure of AT gene poor and GC gene rich blocks is similar to higher eukaryotes and the localization of effectors in the dynamic, gene poor AT rich regions is similar to oomycetes. (Haas *et al.,* 2009)

The *L. maculans* genome, like most phytopathogens contains numerous effectors that function as either avirulence genes, encode toxins, or suppress plant defenses. To date the only well characterized effectors are the phytotoxin sirodesmin PL and the cloned avirulence genes. The loss of either sirodesmin Pl or *AvrLm4-7* production has been associated with a fitness cost to the pathogen (Elliot *et al.,* 2007, Huang *et al.*2006).

Several putative small secreted proteins function as effectors and contribute to fungal growth during infection and suppression of plant defenses. In the genome of *L. maculans*, there are 57 genes identified in AT rich blocks encoding putative small secreted proteins with features indicating their role in pathogenicity, such as a lack of recognizable domains, absence of homologues, and high cysteine content. Of these, 41 are over expressed during infection and are candidates for pathogenicity effectors or avirulence genes (Rouxel *et al.,* 2011).

There are substantially more small secreted proteins in the GC rich regions (529) although a smaller number (19%) are overexpressed during infection. A higher percentage of the small secreted proteins in the GC regions (73% vs 60% in AT) carry an RxLR-like protein motif (Rouxel *et al.*, 2011) that mediates translocation into plant cells. This suggests the presence of pathogenicity factors in the GC rich regions although no avirulence genes have yet been identified in this region.

Pathogenicity effectors in AT rich regions promote infection but the loss of most effectors either has no effect or a fitness cost (Huang *et al.*2006), rather than inhibiting infection. There is necessarily some redundancy, other proteins either performing or compensating for the function of the lost avirulence proteins. This is supported by the presence or absence phenotype of small secreted proteins identified in AT rich blocks in field populations of *L. maculans* and other fungal species (Stukenbrock *et al.*, 2009). Likewise both virulent and avirulent isolates have been observed for all known avirulence genes (Hayward *et al.*, 2012). It's probable that the presence/absence phenotype occurs with many of the secreted protein effectors, not just avirulence genes. Thus the transposable elements and RIP machinery that is tolerated in *L. maculans* helps generate diversity for genes occurring in the AT-blocks of the genome, such as Avr genes, leading to increased genetic plasticity in those regions. This provides *L. maculans* with a distinct advantage in the evolutionary arms race to evolve effectors to avoid recognition faster than host resistance genes can evolve to recognize them.

2.4 Disease Epidemiology with *B. napus*

2.4.1 Disease Cycle & Symptoms

In western Canada, asexual conidiospores (aka pycnidiospores) are the primary inoculum source, and are released continuously during the growing season (Guo & Fernando, 2005) making Blackleg predominantly polycyclic. The sexual ascospores arise from canola stubble in late May to August after the long, cold winter, when they can infect leaves of the new spring oilseed rape crops (McGee & Petrie, 1979). Both ascospores and pycnidiospores adhere to leaf tissue and infect either through stomatal pores or tissue wounds and abrasions (Chen & Howlett, 1996). Leaf lesions may vary in appearance depending on the host resistance, isolate aggressiveness, stage of lesion development, and canopy climate. However, lesions develop similarly in Canada, Europe, and Australia, with the exception of seasonal timing which varies due to climate and differences between spring and winter canola. Lesions appear as pale spots which gradually enlarge in diameter and will either be bordered by a yellow chlorosis response or a dark border indicating some level of resistance. Lesions will contain tiny black pycnidia which produce asexual pycnidiospores that enable secondary infection of nearby plants by rain splash or wind dispersal. The absence of visible lesions does not indicate absence of infection, since the fungus has successfully been isolated from leaves without lesions (Hammond & Lewis, 1986). Thus there may be a poor correlation between incidence of visible symptoms early in the infection cycle, and the incidence of basal stem cankers at the end of the growing season. Infection that occurs early in seedling development, optimally the 2-4 leaf stage, allows the pathogen to travel the base of the

stem restricting nutrient and water uptake which impacts yield. In Australia infections up to the six leaf stage can cause a complete stem canker at the end of the growing season, but this is rare in Canada and in Europe (Barbetti & Khangura, 1999). Late infections generally do not have a significant impact on yield but stem lesions and phoma pod spots can cause premature ripening, and splitting (Petrie & Vanterppol, 1974).

The fungus undergoes a symptomless stage where it spreads down to the base of the plant, the crown, and even into the roots and severe Blackleg can lead to seedling blight as seen in Australia (Barbetti & Khangura, 1999). Visible black/brown lesions appear on the base of the stem and often at branching points which contrast well against the green hypocotyls or seedlings or older plants that are still green during flowering. These lesions are the reason the disease bears the common name of Blackleg. At the end of the growing season the fungus becomes necrotrophic and causes stem canker at the crown. This girdles the plant causing premature ripening and yield loss, or in severe cases lodging and death of the plant. Pod infections can occur and spread to the seed inside at <0.5% frequency (Fernando & Demoz, 2013). However, seed dockage that travels with shipments harbors significantly more *L. maculans,* up to 37% in some fields, and possess a greater risk of spreading the disease to new regions (Fernando & Demoz, 2013).

After harvest, the degrading stem tissue is rapidly colonized by Blackleg resulting in the formation of abundant pycnidia and followed by pseudothecia development if spores of both mating types are present. In Canada, pycnidiospores are more commonly found on remaining stubble, whereas in Australia ascospores are the primary cause of

new infections (Guo & Fernando, 2005). Ascospores travelled a distance of 25m and pycnidiospores were detected up to 45m from infected plants in Canada (Guo & Fernando 2005), while experiments in Australia found ascospores posed the greatest risk up to 500m from the source (Barbetti *et al.* 2000).

2.4.2 Environmental factors

Globally there is evidence that the two species causing phoma stem canker are adaptable, since the disease is very damaging on both spring and winter (autumn-sown) types of oilseed rape in a wide range of climates (West *et al.*, 2001). In Europe and North America, *L. maculans* often co-exists with *L. biglobosa* (West *et al.,* 2002), which may have evolved from a common ancestor (Gudel *et al.,* 2004). *Leptosphaeria biglobosa* is associated with upper stem lesions; whilst generally not damaging, they can cause serious losses in countries like Poland with high summer temperatures (Huang *et al.,* 2005).

The infection cycle varies in different growing regions. In autumn or fall, winter canola leaf spots appear in Eastern Canada and Western Europe, but are absent or rare Eastern Europe (Hall *et al.,* 1993; Jedryczka *et al.,* 1999). New spots will continue to develop in the winter in Western Europe but not in Eastern Europe due to a colder climate. In western Canada, leaf lesions occur on spring canola throughout the spring and summer. Leaf lesions occur throughout the growing season in Australia.

Local temperature and humidity conditions also influence Blackleg infection. Humidity is a necessary component of infection, and germination of *L. maculans*

ascospores prior to infection requires a minimum of 4 hours of wetness to produce leaf lesions (Hall, 1992; Biddulph *et al.* 1999). *L. maculans* is more tolerant to temperature fluctuations and ascospores were found to germinate within 4 hours at temperatures ranging from 4-28^{o}C (Hall, 1992). The optimal combination of temperature and humidity resulting in the greatest number of leaf lesions by *L. maculans* ascospores was found to be 48 hours at 12-20^{o}C. These humidity and temperature results may not necessarily correlate when infection is mediated by pycnidiospores, which would presumably be more sensitive to temperature and humidity fluctuations. Pycnidiospores are also able to infect wounded tissue under controlled environmental conditions which has allowed for phenotypic evaluation of blackleg resistance (Kutcher, 2007).

2.5 Disease management

2.5.1 Cultural practices

Farmers and growers of canola have relied on various practices to limit the risk of disease and yield loss in their fields. Rotations are generally favored since canola seeded into its own stubble yields 10-20% less than canola rotated with cereals or pulses (Canola Council, 2014), due to a variety of reasons including increased disease pressure. Several studies have found that the decay of canola stubble in longer rotations results in decreased inoculum production from colonized residue (West *et al.*, 2001). To reduce inoculum levels several recommendations for the destruction of stubble have been recommended such as burning and burying stubble which have been found to increase decomposition of stubble (Barbetti & Khangura, 1999; Kutcher & Malhi, 2010). Recently, soil conservations efforts have resulted in minimum or zero tillage farming

especially in drier climates. Nevertheless tillage is an effective method of managing Blackleg. Several studies have found that incorporation of canola stubble into the soil increased decomposition and resulted in decreased sporulation (Turkington *et al.*2000; Naseri *et al.*, 2008)

There are different recommendations of when to seed canola to avoid the peak of ascospores dispersal from coinciding with infection at the sensitive seedling stage. In France early sowing is recommended whereas in Australia late seeding is recommended (Lepage & Penaud, 1995; McGee, 1977). Adjusting the seeding date also impacts yield and is recommended when expected losses from disease outweigh any yield penalties incurred.

2.5.2 Chemical controls

Fungicides are a commonly used tool to control Blackleg symptoms and inhibit infection. Combinations of seed treatments, soil fungicides, and foliar fungicides are all tools growers use to minimize infection of their crops. Generally fungicides are recommended in crops with high yield potential and with low cultivar blackleg resistance. In Canada, pyraclostrobin (Headline ®), propiconazole (Tilt), and azoxystrobin (Quadris), are the main products used to control for fungicides including Blackleg. Kutcher *et al.*(2003) found that fungicide applications are effective in reducing Blackleg symptoms on susceptible canola varieties, but fungicide applications did not always show an economic benefit on blackleg resistant varieties in Canada. However, the greater yields

obtained in Western Europe can justify more expensive fungicide treatments which would be cost prohibitive or provide no gains in Canada and Australia.

The optimal time for foliar fungicides is at the two to four leaf stage in Canada (Liu, 2014). Poisson & Peres (1999) also showed that leaves 2 to 4 on a canola plant do not immediately show symptoms upon infection and the pathogen may have progressed to the stem if spray decisions are made after observing lesions. Hammond & Lewis (1986) have shown that fungicide applications past the initial seedling stage are not needed since late infections do not progress enough to produce severe crown canker. When comparing different classes, Qo inhibitors (QoI) were found to be more effective than demethylation-inhibitor (DMI) fungicides (Liu *et al.*, 2014). Fungicides effectively reduce disease incidence and severity but have not been shown to significantly improve yield on resistant or moderately resistant varieties (Kutcher *et al.*, 2005; Liu, 2014).

2.5.3 Biological controls

The use of biological controls is a popular consumer alternative to chemical controls and generally have a low impact on the environment. The birds nest fungi, *Cyanthus striatus* and *C. olla*, indirectly control *L. maculans* by competing for the stubble food base that *L. maculans* spores survive on thereby reducing inoculum production (Tewari *et al.*, 1997). Alternatively, the bacterium *Paenibacillus polymyxa* PKB1 directly inhibits *L. maculans* by producing two closely related antifungal peptides that significantly reduce germination and germ-tube length of pycnidiospores in culture

(Kharbanda *et al.*, 1999). In field experiments PKB1 significantly suppressed ascospores formation and decreased *L. maculans* survival on infected canola residue. More recently several soil bacteria were found to carry antifungal properties and significantly inhibit *L. maculans* growth in media assays and infection in cotyledon assays (Ramarathnam and Fernando, 2006)

2.5.4 Genetic Resistance

Genetic resistance offers the most cost effective management strategy for plant pathogens including *L. maculans*. Most breeding programs have focused on incorporating both adult plant resistance and seedling resistance towards developing *B. napus* lines that are resistant to Blackleg and durable in environments where conditions are favourable for disease. The pathogen population has increased in terms of disease incidence and severity steadily from 2011-2013 (Canola Council, 2013). This is in part due to the abundance of canola in the Canadian prairies, the long history of Blackleg, and recent decreases in crop rotations resulting from canola's increasing value, topping $1200/tonne in 2013 (Canola Council, 2014). The increasing levels of disease indicates that current resistance in commercial cultivars is degrading. Breeding programs are forced towards strategies that utilize both qualitative major gene resistance and quantitative adult plant resistance to maximize both the durability and effectiveness of their lines. Optimal management strategies should seek to reduce the effective population sizes while limiting selection towards more virulent races. Adult plant resistance reduces inoculum levels broadly among many races, while seeding resistance would limit spores of avirulent races. While selection pressure would remain, recent mathematical modelling

of cultivars with both seedling and adult plant resistance has shown that the combination of *Rlm6* and APR (cultivar 'Darmor-MX') increased the durability of resistance in comparison to *Rlm6* or APR alone and the first major symptoms of stem canker in Darmor-MX were recorded in the 5th year of the experiment (Brun *et al.*, 2010). In comparison the *Rlm6* cultivar lacking APR (cultivar 'EurolMX') displayed typical stem canker symptoms in the third year. The two year increase in durability was also supported by phenotypic data such as reduced number of pseudothecia formed and decreased leaf lesions throughout the 5 year experiment. This is consistent with other pathosystems such as the pepper-Potato virus Y interaction that demonstrated that the combined use of seedling and adult plant resistance delayed the emergence of virulent races (Palloix *et al.*, 2009).

Gene pyramiding is a potential method to develop strong cultivar resistance through the incorporation of multiple resistance genes that would provide resistance against multiple races of the pathogen population. However, this is expected to create significant selection pressure towards isolates virulent at multiple avirulence gene loci. If a virulent race exists for the combination of stacked R genes, it would eventually result in the breakdown of all the stacked R genes leaving the crop more susceptible. The competing strategy is to rotate cultivars containing one or a few R genes to shift selection pressure between different races avoiding selection of *L. maculans* races virulent towards multiple R genes. To utilize either strategy effectively, knowledge of both host R gene diversity, pathogen Avr diversity, and the interaction between specific Rlm-Avr genes is required. It is important to identify the most common avirulence genes in the pathogen

population to gauge the effectiveness of resistance genes. Pathogen surveys of avirulence gene frequencies would provide some of the information necessary to avoid stacking defeated R genes or stacking resistance genes that target the same avirulence gene. Similarly, knowledge of pathogen race structure could be used to rotate between resistance genes that target alternate pathogen races. Long term management of blackleg in canola requires both the use of the scientific data generated and the cooperation of many different industry groups with competing interests.

2.6 *L. maculans* - *B. napus* interaction

2.6.1 Gene-for-gene hypothesis

The *Leptosphaeria maculans-Brassica napus* pathosystem does not strictly follow the gene for gene interaction model. According to the classical interpretation of the gene for gene hypothesis each resistance gene detects and provides resistance against a pathogen isolate carrying the corresponding avirulence gene. However, two pairs of *B. napus* resistance genes have been found to detect a single avirulence gene. Both *Rlm4* and *Rlm7* from *B. napus* detect the *L. maculans AvrLm4-7* gene while both *Rlm1* and *LepR3* detect the *L. maculans AvrLm1* gene. This is problematic from a disease management point of view since the loss of a single avirulence gene can render two resistance genes ineffective. The reverse, where a single resistance gene detects multiple avirulence genes has not yet been shown.

In the case of the differential cultivar Surpass 400, there has been confusion in the literature about whether it contains *Rlm1* or *LepR3*. Surpass 400 showed a resistance reaction to isolates carrying *AvrLm1* and *AvrLmS* indicating two separate resistance genes (Van de Wouw *et al.,* 2009). The recent cloning of *LepR3* confirmed through host and pathogen transgenics that *LepR3* is indeed a separate gene from *Rlm1* and that both genes interact with *AvrLm1* (Larken *et al.,* 2012). Since *LepR3* detects *AvrLm1*, the hypothetical *AvrLepR3* gene does not exist. Surpass 400 is now known to carry both *LepR3* (detects *AvrLm1*) and *RlmS* (detects *AvrLmS*). These findings effect both the characterization of pathogen avirulence genes and host resistance genes. On the host side, a resistant reaction using a differential isolate carrying *AvrLm1* will be either due to *Rlm1* or *LepR3*. Thus, cultivars previously identified as having *Rlm1* may have *LepR3* instead or both. It becomes impossible to differentiate between *Rlm1* and *LepR3* in *B. napus* using differential isolates. However, since *LepR3* has been cloned a PCR approach will be able to detect whether *LepR3* is present in the cultivar. Additionally, a negative *LepR3* PCR result followed by a resistant reaction with an *AvrLm1* isolate will indicate the presence of *Rlm1* resistance in the tested cultivar. However, there is less value in discriminating between these two R since they act on the same avirulence genes. In addition to the overlap in avirulence gene targets by host resistance genes, there may also be overlap in the resistance genes mapped by different groups that fall into the same linkage group. Some examples of resistance genes likely to be the same include *LmR1=Rlm4, Jlm1=Rlm6, Blm1= LepR3, AvrLmJ1=AvrLm5* (Brun *et al.,* 2010; Hayward *et al.,* 2012; Larkan *et al.,* 2014). *BLMR1* and *BLMR2* identified by Long *et al.* (2011) in *B. napus* cultivar 'Surpass400' correspond *LepR3* and *RlmS* (Larkan *et al.,* 2013)

2.6.2 Host Resistance – Major Genes, APR, and QTL's

Until the mid-1980's *L. maculans* was considered as a necrotrophic pathogen that interacted with the host through a quantitative resistance mechanism and breeding relied primarily on field based assessments of disease resistance (Rouxel & Balesdent, 2005). Using this approach, major commercial cultivars were developed through field breeding that relied on single major R genes for resistance such as *Rlm4* cultivars in Australia and *Rlm1* cultivars in France; eventually both resistance sources become ineffective in those countries. With the advent of the 90 isolate International Blackleg of Crucifers network (IBCN) *L. maculans* collection in 1994, several differential plant pathogen gene-for-gene interactions were identified. Several Rlm genes and pathogen Avr genes contributing to qualitative resistance were identified. Mapping of related species that displayed 'complete resistance' such as *B. juncea* revealed that they also possessed single gene resistance (Balesdent *et al.* 2002). Similarly the *B. napus* resistance genes derived from the Australian *B. rapa* sylvestris cultivar was also found to be controlled by a single gene interaction. While some related crucifers are deemed to be fully resistant to *L. maculans*, the discovery that some Australian isolates are able to infect 'resistant' wild crucifers suggests that they may also be due to single gene resistance (Li *et al.*, 2005).

Research and breeding efforts have identified 14 major resistance genes for *B. napus* that are effective against *L. maculans* isolates with corresponding avirulence genes from the seedling stage to full maturity. They are *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm5*, *Rlm6*, *Rlm7*, *Rlm8*, *Rlm9*, *Rlm10*, *LepR1*, *LepR2*, *LepR3*, *RlmS* (Yu *et al.*, 2005, 2008; Delourme

et al., 2006; Larkan *et al.,* 2012). Mapping research has found that the majority of R-genes in *B. napus* clusters are inherited from the *B. rapa* genome (AA). The LepR1 resistance gene was mapped to A2 (Yu *et al.,* 2005). The A10 chromosome carries the Blackleg resistance genes *Rlm2, LepR2, & LepR3* (Rouxel *et al.,* 2003; Yu *et al.,* 2005; Rimmer, 2006). The resistance genes *Rlm1*, *Rlm3*, *Rlm4*, *Rlm7*, and *Rlm9* are found on A7 (Balesdent *et al.,* 2002). *Rlm3, Rlm4, Rlm7,* and *Rlm9* are tightly clustered together and may be either linked or allelic variants (Delourme *et al.,* 2004). There has been confusion in the literature in regards to the naming of resistance genes resulting in multiple competing names for the same resistance gene. Mapping by several different groups has resulted in multiple names for resistance genes that map to similar locations. This confusion is also due in part to the fact that two of the host Avr genes interact with more than a single resistance gene (*AvrLm1* with *Rlm1* and *LepR3*, and *AvrLm4-7* with *Rlm4* and *Rlm7*) resulting in a deviation from the classical gene for gene hypothesis (Parlange *et al.,* 2009, Larkan *et al.,* 2012). The mapping and cloning of pathogen *AvrLm1*, *AvrLm4-7,* and host *LepR3* has confirmed this phenomena.

In addition to these single major genes, *B. napus* also possesses adult plant resistance (APR) that is mapped to quantitative trait loci (QTL's) within the genome and have an additive effect that provides partial resistance to the adult plant (Hayward *et al.,* 2012). APR cultivars lacking qualitative seedling gene resistance (Rlm), will exhibit typical symptoms of *L. maculans* infection at the seedling stage, but disease symptoms and yield impact will be significantly reduced at crop maturity. APR operates at the symptomless growth stages of *L. maculans* as it travels in the leaf and stem tissue, and

can be assessed at crop maturity as reduced basal stem cankers. This partial adult plant resistance is also known as quantitative resistance in the literature in contrast to major gene seedling resistance which is also known as qualitative resistance since it manifests phenotypically as complete resistance when challenged by an isolate with the corresponding avirulence genes. In practice, unless all isolates in a field carry the corresponding avirulence gene, seedling resistance only provides complete resistance against a proportion of the pathogen inoculum, reducing effective disease pressure, and the combination of qualitative major gene resistance and minor gene quantitative resistance will determine the severity of cankering and yield loss.

APR is generally considered to be a more durable form of resistance due largely to the absence of examples of short term 3-4 year resistance breakdown seen in commercial crops relying on major seedling resistance genes. This results in resistance type (APR vs seedling Rlm) being assumed as a reliable predictor of the potential durability of resistance. Since R-gene mediated resistance is race specific, it exerts strong selection pressure against populations that are detected by the host R gene and thus these races cannot propagate resulting in rapid pathogen population shifts and the 'boom and bust' cycles commonly observed in agricultural pathosystems (Stuthman *et al.*, 2007). The reduced selection pressure of APR due to its partial resistance phenotype favors its long term durability against pathogen adaptation. However, this generalization cannot be used to compare the durability of varieties based solely on their resistance type. Experimental data has shown that fungal pathogens can adapt gradually to adult plant resistance (Andrivon *et al.*, 2007; Brun *et al.*, 2010) while conversely some single gene

resistance have had long term success (Poland *et al.*, 2008). The terminology of quantitative resistance and adult plant resistance has been used interchangeably in the literature suggesting a relationship between the observed disease phenotypes and their underlying genetic basis. That is to say, the assumption that disease phenotypes that display a continuous range of symptoms are due to QTLs, multiple loci segregating in a population. While this may be true, the genes responsible for adult plant resistance to Blackleg in *B. napus* have yet to be identified. There is disagreement in the literature whether adult plant resistance against Blackleg in *B. napus* is monogenic (Stringam *et al.*, 1994) or polygenic (Ferreria *et al.*, 1995; Rimmer, 2006). Fine mapping and cloning of the durable adult plant resistance gene in wheat *Lr34*, initially mapped as a QTL, showed that a single gene not multiple genes conferred a reduction in adult plants for three diseases; leaf rust (*Puccinia triticinia*), stripe rust (*Puccinia striiformis*), and powdery mildew (*Blumeria graminis*) (Krattinger *et al.*, 2009). Similarly, the other assumption that single genes are not durable or broad spectrum is not always true. Examples of monogenic durable resistance include the recessive gene *mlo* for powdery mildew resistance in barley, *Rpg1* gene for resistance to stem rust in barley, and the dominant *I* gene for resistance to bean common mosaic virus in common bean (Ayliffe *et al.*, 2008; Keller *et al.*, 2007; Kelly *et al.*, 2006). These single resistance genes, *Rpg1, I,* and *mlo* confer broad spectrum resistance to multiple isolates or races of their respective pathogens and have provided durable resistance over decades of use in crop breeding. In summary, it would be incorrect to assume the underlying genetic basis of resistance informs of the observed phenotypic range of resistance or conversely predicting the durability or specificity of resistance from the genetic basis of resistance.

2.6.3 Pathogen Avirulence genes (Avr genes), Pathotypes, and Races

Canola (*Brassica napus*) has two types of resistance against *L. maculans*. Quantitative resistance reduces disease severity and is effective against the general pathogen population while qualitative or major gene resistance is completely effective against certain races of the pathogen population. This second interaction is mediated by the combination of host resistance genes (Rlm) and pathogen avirulence genes (Avr). The host plant Rlm proteins are directly or indirectly involved in the recognition of pathogen avirulence genes signalling downstream processes resulting in host resistance, typically the hypersensitive response. The first genetic evidence for this type of gene for gene interaction model between Brassica species and *L. maculans* was identified between the *Rlm1* gene in *B. napus* cv. Quinta and the *AvrLm1* gene in *L. maculans* (Ansan-Melayah *et al.* 1998). The identification of *Rlm2* from *B. napus* cv. Glacier introduced the first categorization of Blackleg into pathogenicity groups (PG 2-4, and T) based on their interaction with three different *B. napus* cultivars, Westar, Quinta, and Glacier (Koch *et al.*, 1991). PG1 was avirulent on Westar, Quinta, and Glacier and was later distinguished as a separate species, *Leptosphaeria biglobosa*. PG2 isolates were classified as the more aggressive *Leptosphaeria maculans* and were virulent on 'Westar', but avirulent on both 'Quinta' and 'Glacier', they were common in the 1980's in the Canadian Prairies (Kutcher *et al.*, 1993). In 1998, PGT isolates that were virulent on 'Westar' and 'Quinta' but avirulent on 'Glacier' were identified (Keri *et al.*, 2001). PG3 isolates that were virulent on 'Westar' and 'Glacier' but avirulent on 'Quinta' were identified in southern Manitoba in 1999 followed by several PG4 isolates that were virulent on all three

cultivars in the 2000's (Chen & Fernando, 2006; Kutcher *et al.*, 2007). The PG system was limited to the resistance found in 'Quinta' and 'Glacier', which carry *Rlm1* and *Rlm2* respectively and both may carry *Rlm3* depending on the seed lot (Balesdent *et al.*, 2002). The addition of cv. Jet Neuf classified *L. maculans* into six races, A1-A6 (Kuswinanti *et al.*, 1999). However, the addition of any new differential cultivars would double the number of theoretically possible races, quickly leading the PG system to become impractical. The steady discovery of new resistance genes and avirulence genes lead to the replacement of the former three and six race classification systems in favor of designating any unique combination of avirulence genes as a separate race (Fitt *et al.*, 2006, Kutcher *et al.*, 2010)

To date, 14 avirulence genes have been hypothesized in *L. maculans* based on phenotypic interactions; these are *AvrLm 1, 2, 3, 4-7, 5 ,6, 8, 9, 10, 11, LepR1, LepR2, S, J1*. *AvrLmJ1* was found to confer avirulence towards *B. juncea* cultivars consistently, suggesting it may function in a species specific manner (Van de Wouw *et al.*, 2013). Thus far genetic studies have mapped *AvrLm1, 2, 3, 4-7, 6, 9, 11, LepR1, and AvrLmJ1*. Most of these are found in two gene clusters, the *AvrLm1*-2-6 cluster (Balesdent *et al.*, 2002) and the *AvrLm 3-4-7-9-AvrLepR1* cluster (Balesdent *et al.*, 2005, Ghanbarnia *et al.*, 2012). In addition to these, six avirulence genes have been cloned; they are *AvrLm1* (Gout *et al.* 2006), *AvrLm2* (Ghanbarnia *et al.*, 2014), *AvrLm6* (Fudal *et al.* 2007), *AvrLm4-7* (Parlange *et al.* 2009), *AvrLm11* (Balesdent *et al.* 2013), and *AvrLmJ1* (Van de Wouw *et al.* 2013). Most of the cloned Avr genes have been located through linkage mapping (Gout *et al.* 2006; Fudal *et al.* 2007; Parlange *et al.* 2009). Some other

techniques used RNA seq and comparative DNA seq (Van de Wouw *et al.* 2013; Ghanbarnia *et al.,* 2014).

In *L. maculans*, the avirulence genes are small proteins known as effectors that directly or indirectly are involved in initiating the host defense response in *B. napus* plants with the corresponding avirulence genes. Effectors in plant pathogenic fungi can have multiple functions such as altering host-cell structure, altering cell functions, facilitating infection (i.e. toxins), and may be detected by the host and trigger a defence response (i.e. avirulence genes). The cellular role of an avirulence gene or its role as a pathogenicity effector will determine if the loss of the avirulence gene will result in a fitness cost to the pathogen. A fitness penalty for the loss of an avirulence gene can be used as a reliable predictor of the durability of a resistance gene (Leach *et al.*, 2001). In *L. maculans* Huang *et al.*(2006) found a fitness cost associated with the *Avlm4-7* gene. In comparing near isogenic isolates of virulent *avrLm4-Avrlm7* and avirulent *avrLm4-AvrLm7* achieved through five generations of backcrossing, they found *AvrLm4-Avrlm7* isolates produced more lesions, larger lesions, and more pycnidia than *avrLm4-Avrlm7* isolates. They also found in field experiments that *AvrLm4* rebounded in the pathogen population from 5.4% to 20.5% in one year when cultivars lacking *Rlm4* were seeded.

L. maculans effectors share some common features. All avirulence genes that have been cloned are small secreted proteins (SSPs) that are cysteine-rich. These SSPs are polymorphic between isolates, and share no sequence similarity with genes from other species. They are localized in gene poor AT-isochores that are rich in degenerated copies

of transposable elements and they show increased expression *in planta* during the initial phase of infection (Gout *et al.,* 2006b; Fudal *et al.,* 2007; Parlange *et al.,* 2009). The *L. maculans* genome contains many other effector genes encoding SSPs that also show increased expression during infection, and are localized in gene poor AT-ischore regions (Rouxel *et al.,* 2011). These common features could be used as the basis of a rapid cloning strategy of selecting candidate avirulence genes following phenotyping, based on their genome location and induced expression *in planta.*

L. maculans avirulence genes can be 'lost' due to deletions or avoid detection through SNP mutations resulting in an increase in virulent races. These shifts have resulted in the 'boom and bust' cycles and the breakdown of major resistance sources. *L. maculans* maintains the genes necessary for both saprophytic and necrotrophic parasitism lifestyles and transitions from sexual to asexual stages providing the parasite with a high level of plasticity in survival. The combination of sexual recombination with ascospores and large population sizes of asexual pycnidiospores allows *L. maculans* to generate new virulent race combinations and rapidly shift populations towards virulent isolates.

The avirulence genes in *L. maculans* are localized in gene poor AT-rich regions of the genome alongside degenerated copies of transposable elements (TEs). Transpositions of transposons into coding regions, continual duplication, and chromosomal rearrangement all have a very significant impact on gene expression and evolution. Rouxel *et al.*(2011) hypothesized that the isochores genome structure of alternating AT and GC blocks in *L. maculans* is due to the massive invasion by and subsequent

degeneration of transposable elements (TEs). Ascomycete fungi employ repeat induced point mutations (RIP) mutations to inactivate TE's. RIP mutations alter multicopy DNA such as TEs through nucleotide changes of CpA to TpA and TpG to TpA during meiosis which often result in stop codons and deletions. Since all cloned *L. maculans* avirulence genes to date have been localized in proximity to TE's they are susceptible to RIP mutations. Fudal *et al.* (2009) found that virulence to *Rlm6* in 66% of isolates was due to deletions, possibly due to RIPs, while 24% was due to RIP mutations alone. Similarly, following the breakdown of *Rlm1* in Australia, Van de wouw *et al.* (2011) found RIP alleles present only in isolates collected after resistance conferred by *Rlm1* had broken down. Van de wouw *et al.* (2011) also found the degree of RIP mutations within single copy sequences in the *Rlm1* genomic region was proportional to their proximity to the degenerated transposable elements.

Surveys of the pathogen population have been used to geographically define the composition of avirulence genes in *L. maculans*. A survey in France at 9 avirulence loci, Avr1-9 found 11 races with the dominant race accounting for 65% of the population (Balesdent *et al.*, 2006). The French *L. maculans* population was entirely absent of *AvrLm2* and *AvrLm9* (all isolates virulent on corresponding Rlm genes) while *AvrLm6* and *AvrLm7* were greater than 99%. Recently, a collection of North and South American isolates was analyzed by Dilmaghani *et al.*(2009) who screened for the presence of *AvrLm*1, 2, 3, 4, 6, 7, 9, and *AvrLmX* which corresponded to unknown resistance in cv. 'Cooper'. They found that *AvrLm6* and *AvrLm7* prevalent in all locations although *AvrLm7* was lowest in Central Canada at around 50%. The Australian population carried

AvrLm1, *AvrLm6*, and *AvrLm7* at high frequencies while all other avirulence genes were rare or absent. The greatest diversity of races was in central Canada with the presence of some avirulence genes absent in Europe, and a greater frequency of *avrlm7* (virulent) isolates. Among all the isolates Dilmaghani *et al.*(2009) found that no isolate had fewer than 2 avirulence genes nor more than six avirulence genes. A similar European study by Statchowiak *et al.* (2006) found that *AvrLm*5, Av*rlm6*, and *AvrLm7* occurred very frequent at 86%, 100%, and 99% respectively. Statchowiak *et al.* (2006) found *AvrLm1* and *AvrLm4* at <10% and *AvrLm2*, *AvrLm3*, and *AvrLm9* absent in the European pathogen population.

A survey of 87 central Canadian isolates by Kutcher *et al.*(2010) examined 10 avirulence genes: *AvrLm1, 2, 3, 4, 5, 6, 7, 9, 10, S* (*AvrLmS* was listed as *AvrLepR3*). *LepR3* in the differential cultivar used, 'Surpass400' detects *AvrLm1* as demonstrated in a recent publication (Larken *et al.,* 2013), consequently there is no *AvrLepR3*. The other sylvestris derived resistance gene in 'Surpass400', *RlmS*, was responsible for the resistance attributed to *LepR3*. Kutcher *et al.*(2010) found that *AvrLm2* and *AvrLmS* were above 95% while *AvrLm6* and *AvrLm10* were fixed at 100%. The frequency of *AvrLm3*, *AvrLm4*, *AvrLm5*, and *AvrLm7* were all reported as under 30%. In total 16 races were found with 7 races accounting for nearly 90% of the pathogen population.

CHAPTER 3

RACE STRUCTURE OF THE CANADIAN *LEPTOSPHAERIA MACULANS* PATHOGEN POPULATION

3.0 Abstract

The fungus *Leptosphaeria maculans* (Desmaz.) Ces. & De Not is the causal agent of Phoma stem canker (aka blackleg,) and is a serious fungal disease of oilseed rape crops including canola (*Brassica napus* L.). This pathogen is prevalent across western Canada and is a consistent limiting factor in canola production. It is found in the major canola regions around the world and has been responsible for epidemics in Australia and Europe. Genetic resistance and extended crop rotation has provided an effective means of disease control in western Canada and a cultivar carrying a major resistance gene will be resistant to pathogen isolates carrying the corresponding avirulence gene. However, host genetic resistance can be overcome with population shifts and the emergence of new races. The gradual increase in both disease incidence and severity of Blackleg of canola in Canada is in part due to the emergence of new races virulent on widely used resistance genes. Maintaining genetic resistance to blackleg to minimize yield impacts is a priority for the canola industry. This study examines *L. maculans* isolates derived from canola stubble in commercial fields collected in 2010 and 2011 across western Canada for the presence and frequency of *Avr* genes. A total of 674 isolates were examined for the presence of avirulence (*Avr*) alleles *AvrLm1*, *AvrLm2*, *AvrLm3*, *AvrLm4*, *AvrLm6*, *AvrLm7*, *AvrLm9*, *AvrLepR1*, *AvrLepR2* and *AvrLmS* using a set of differential host genotypes carrying known resistance genes or PCR amplification of *AvrLm1, 6, 4 and 7*. Certain alleles were more prevalent in the pathogen population with *AvrLm6* and *AvrLm7* present in >85% of isolates while *AvrLm3*, *AvrLm9* and *AvrLepR2* were present in <10% of the isolates. A total of 55 races (different combinations of *Avr* alleles) were detected, the two most common being *AvrLm-2-4-6-7* and *AvrLm-2-4-6-7-S*. Races carrying as many as seven

and as few as one *Avr* allele against the 10 known resistance genes were detected, representing a wide virulence spectrum. Selection pressure from the race-specific resistance genes carried in canola varieties distributed in different regions is posited as a significant contributor to the variation in the *Avr* gene frequency observed. Recent increases in blackleg incidence and severity is posited to be due in part to the emergence of virulent races on widely used canola cultivars.

3.1 Introduction

Leptosphaeria maculans is an ascomycete fungus responsible for stem canker of crucifer crops. The fungus causes Phoma stem canker, commonly known as blackleg has become an economically important disease of canola (*Brassica napus* L.) in Canada and many other countries. Since the first report of *L. maculans* in Canada (McGee & Petrie, 1978), blackleg has spread rapidly and yield losses up to 50% were observed in individual fields (Gugel & Petrie, 1992). In the early 1990's, commercial varieties with genetic resistance to blackleg were released in Canada and with 3- to 4-year crop rotations, the disease was successfully controlled for many years. Changes in pathogen race structure were observed in the early 2000's (Chen & Fernando, 2006; Kutcher *et al.*, 2007), a circumstance likely attributable to the pathogen response to the resistance genes used in canola cultivars. In recent years producers and agronomists in western Canada have reported severe disease symptoms in varieties that were registered as resistant, and the overall level of disease has been increasing steadily (Canola Council, 2014). The damage in Canada, however, has not been as severe or widespread as seen in the USA (Lamey, 1995), France (Rouxel *et al.*, 2003) and Australia (Li *et al.*, 2003) in last 20

years. Changes in virulence have been attributed to pathogen adaptation to the resistance genes in canola varieties. The economic returns of canola have led to larger acreages and tighter rotations which have in turn led to the emergence of new races in western Canada (Chen and Fernando, 2006; Kutcher *et al.*, 2007). The decreased disease severity in Canada can in part be attributed to a shorter growing season, colder climate, and potentially asexual pycnidia as the dominant inoculum source (Ghanbarnia *et al.*, 2009; Ghanbarnia *et al.*, 2011).

In Canada, the vast majority of canola germplasm screened carry the resistance gene *Rlm3* while the other resistance genes were rarely detected (Zhang *et al.*, 2013). The risk posed by blackleg disease lies in the evolutionary potential of the pathogen to rapidly breakdown novel resistance sources through a combination of sexual recombination that increases genetic diversity and the large population sizes of the asexual pycnidiospores that can rapidly select for more fit races. While the loss of some *Avr* genes has been shown to carry a fitness cost (Huang *et al.*, 2006) and will therefore naturally remerge in the absence of selection pressure, others can simply be lost as a result of selection pressure from the resistant host or through genetic drift. In addition, sequencing of the *L. maculans* genome has shown that Avr genes are subjected to increased mutational pressure due to their localization in gene poor AC isochores rich in transposable elements (Rouxel *et al.*, 2011). The first three cloned Avr genes have all been localized in gene poor AC isochores (Gout *et al.*, 2006; Fudal *et al.*, 2007; Parlange *et al.*, 2009). The dual sexual and asexual lifecycles in combination with the underlying genetic plasticity of virulence factors provides the pathogen with an exceptional ability to rapidly overcome

new resistance sources, as demonstrated by the rapid `breakdown` of cultivars carrying *Rlm1* in France and *LepR3* in Australia (Rouxel *et al.,* 2003; Li *et al.,* 2003).

Resistance to blackleg often relies on recognition of the pathogen carrying avirulence (*Avr*) genes by corresponding *R* genes in the host, which can trigger cellular processes often resulting in localized cell death known as the hypersensitive response (del Pozo *et al.,* 1998). Pathogen *Avr* alleles, however, can evolve rapidly in response to *R* genes used, avoiding host detection and this phenomena have been observed in regions with high genome plasticity in the pathogen population (Guot *et al.,* 2006). Fungal *Avr* genes are studied to better understand the risks posed by different pathogen races and to assist in breeding efforts. The *Avr* genes from several ascomycete fungal pathogens have been cloned including *Magnaporthe grisea* (Orbach *et al.,* 2000; Farman *et al.,* 2002; Böhnert *et al.,* 2004; Yoshida *et al.,* 2009), *Cladosporium fulvum* (Rivas and Thomas, 2005), *Rhynchosporium secalis* (Rohe *et al.,* 1995), *Fusarium oxysporum* (Rep *et al.,* 2004; Houtermane*t al.,* 2008), and *Blumeria graminis* (Ridout *et al.,* 2006). To date, 14 *Avr* genes have been identified in *L. maculans*; i.e. *AvrLm1-AvrLm 11, LepR1-LepR3, AvrLmS and AvrLmJ1*. The *AvrLmJ1* conferred avirulence only towards *B. juncea* cultivars, suggesting it may function in a species-specific manner (Van de Wouw *et al.,* 2013). Genetic studies have mapped *AvrLm1, 2, 3, 4-7, 6, 9, 11, AvrLepR1-2 and AvrLmJ1*, and most of them are found in two clusters: The *AvrLm1*-2-6 (Balesdent *et al.,* 2002) and *AvrLm 3-4-7-9-AvrLepR1* (Balesdent *et al.,* 2005; Ghanbarnia *et al.,* 2012). Additionally, six *Avr* genes from *L. maculans* have been cloned; they are *AvrLm1* (Gout *et al.,* 2006), *AvrLm2* (Ghanbarnia *et al.,* 2014), *AvrLm6* (Fudal *et al.,* 2007), *AvrLm4-7*

(Parlange *et al.,* 2009), *AvrLm11* (Balesdent *et al.,* 2013) and *AvrLmJ1* (Van de Wouw *et al.,* 2013). Most of the cloned *Avr* genes have been located through linkage mapping (Gout *et al.,* 2006; Fudal *et al.,* 2007; Parlange *et al.,* 2009), although RNA sequencing (RNA-seq) and comparative DNA seq have also been used (Van de Wouw *et al.,* 2013; Ghanbarnia *et al.,* 2014).

Strategies for managing blackleg include crop rotation, stubble management, fungicides, and genetic resistance. Two types of resistance have been found in canola. Quantitative resistance sometimes referred to as adult-plant resistance (APR) reduces disease severity in the stem and is generally effective against different races in the pathogen population while qualitative or major-gene resistance (sometimes referred to as seedling resistance) is effective only against races carrying the corresponding *Avr* genes. Qualitative resistance is also effective at the adult plant stage against races carrying the corresponding *Avr* genes. At least 15 major *R* genes (*Rlm1-Rlm10*, *LepR1- LepR4* and *RlmS*) have been identified (Yu *et al.,* 2005, 2008, 2013; Delourme *et al.,* 2006; Larkan *et al.*, 2012). These *R* genes can be effective in the management of blackleg and some of the genes have been widely used in canola breeding programs. Due to their effectiveness, varieties carrying major *R* genes are often sown in large acreages, consequently resulting in strong selection pressure for virulent races in the pathogen population. The common sexual reproduction with *L. maculans* facilitates the high variation in the pathogen population through mutation, recombination and long range dispersal, while asexual reproduction (pycnidiospores) allows for rapid increases of virulent races (West *et al.,* 2001) locally. This process for the pathogen to adapt to *R* genes indicates the need for

continuous monitoring of the pathogen population in terms of the frequency of *Avr* genes, race structure, and regional variation. This information is informative in selecting resistance sources that are effective against the pathogen population in a region. The main objective of this study was to assess the presence and frequency of *Avr* genes in the *L. maculans* population in the Canadian Prairie provinces of Alberta, Saskatchewan, and Manitoba. The race structure and genetic variation at certain *Avr* alleles was also analyzed to characterize the pathogen population in western Canada. This information may be used by canola breeders and producers to select effective *R* genes against the current pathogen population in different regions.

3.2 Materials and Methods

3.2.1 Canola sample collection

Canola stubble samples with blackleg symptoms in the basal stem were collected primarily from hundreds of commercial farmers' fields as part of provincial disease surveys. These fields were located in the main canola production regions on the Canadian prairies and for most of the fields, the variety was unknown. A small number of samples were from field disease nurseries used for cooperative variety trials.

3.2.2 Fungal isolation

The diseased stubble pieces were surface sterilized with 70% ethanol for 5 sec, in 10% bleach for 2 minute and then rinsed in sterile water prior to incubation on V-8 juice agar [200 ml V8 juice (Campbell Soup Company Ltd. Toronto, ON), 800 ml distilled water, 15 g agar and 0.75 g calcium carbonate, 0.1 g streptomycin sulfate] amended with 10ml of streptomycin sulfate and placed on a light bench under cool white fluorescent light (100-150 E m^{-2} sec^{-1}) at 22-24℃ for 4-7 days. When masses of hyphae and conidia indicative of blackleg were observed, samples were selected from among other fungal growth and plated onto V8 juice agar media. After 5 days, the conidial ooze from a single pycnidia was picked using a fine wire under a dissecting microscope and plated onto a fresh V8 juice agar plate as a single spore isolate. Both *L. maculans* and *L. biglobosa* were isolated from diseased stubble tissues, and were distinguished by inoculating cotyledons of 'Westar' canola, which is resistant to *L. biglobosa* (Kutcher *et al.* 2010). Only one *L. maculans* culture was kept for each diseased stubble sample. The pathogen

inoculum was produced on V8-juice agar in Petri dishes on a light bench under cool white fluorescent light (100-150 E m^{-2} sec^{-1}) at 22-24℃ for 5-12 days.

3.2.3 Preparation of Fungal inoculum and DNA samples

Pycnidiospores were harvested by flooding *L. maculans* cultures with sterile distilled water, and scraping with a bent glass rod to dislodge spores. Spore suspensions were filtered through Miracloth into 50-ml sterile centrifuge tubes (Fisher Scientific, Pittsburgh, PA). The concentration was estimated using a hemocytometer (Hausser Scientific Company, Horsham, PA), and adjusted to 1×10^{-7} spores ml^{-1} and stored in sterile microcentrifuge tubes at -20^{o}C until use. The remaining mixture of hyphae, pycnidia, and spores on the agar plates were scraped off with a spatula and placed in sterile 1.5 ml microcentrifuge tubes for DNA extraction.

3.2.4 Differential cultivars

A set of *B. napus* and *B. juncea* lines or cultivars each carrying different single or multiple *R* genes were used as host differentials to identify *Avr* alleles in *L. maculans* isolates. The set included 'Westar' – no *R* genes (Delourme *et al.,* 2004), 'MT29' – *Rlm1,9* (Delourme *et al.,* 2008), 'Samouraï' – *Rlm2,9* (Rouxel *et al.,* 2003), 'Cooper' – *Rlm1,4* (Balesdent *et al.,* 2002), 'Glacier' – *Rlm2,3* (Balesdent *et al.,* 2002), 'Verona' – *Rlm2,4* (Balesdent *et al.,* 2002), 'Falcon' – *Rlm4* (Rouxel *et al.,* 2003), 'Cutlass' – *Rlm5,6* (AAFC), '01.23.2.1' – *Rlm7* (Delourme *et al.,* 2004), 'Darmor' – *Rlm9* (Delourme *et al.,* 2004), '1135' – *LepR1* (AAFC), '1065' – *LepR2* (AAFC), and 'Surpass 400' – *LepR3*, *RlmS* (Van de Wouw *et al.,* 2009; Yu *et al.,* 2008).

Table 3.1. Listing of *Brassica napus* differential cultivars with known blackleg resistance genes

Cultivar	Blackleg Resistance Genes
Westar	None
Quinta	*Rlm 1,3*
Cooper	*Rlm 1,4*
MT29	*Rlm 1,9*
Glacier	*Rlm 2,3*
Verona	*Rlm 2,4*
Samourai	*Rlm 2,9*
Quantum	*Rlm 3*
Falcon	*Rlm 4*
Cutlass (*B. juncea*)	*Rlm 5,6*
01.23.2.1	*Rlm 7*
Darmor	*Rlm 9*
1065	*LepR1*
1135	*LepR2*
Surpass 400	*LepR3, RlmS*

L. maculans isolates were inoculated onto this set of differential *Brassica napus* lines to observe the phenotypic reaction and deduce the corresponding avirulence genes carried in the isolates. The presence of multiple resistance genes in the host lines necessitates deduction from multiple phenotypic reactions to determine which resistance gene was responsible for the resistance response. For example, 'Quinta' contains *Rlm1* and *Rlm3* so from the reaction it alone cannot determine *AvrLm1* if the isolate also contains *AvrLm3* or vice versa, since any one of the two *R* genes can induce resistance against an *L. maculans* isolate carrying *AvrLm1, AvrLm3* or both. There were a few limitations with this set. First, hosts carrying *Rlm8, Rlm10* and *LepR4* were not available. Second, the differential set was unable to determine the presence or absence of *AvrLm1* and *AvrLm2* if the isolate also carried *AvrLm3*, *Avrm4*, and *AvrLm9*, but fortunately, no isolate was found to carry

all three of these *Avr* genes. 'Cutlass' appears to carry *Rlm5* and *Rlm6* plus unidentified *R* genes based on differential isolate screening (data not shown), therefore using it to identify *AvrLm5* and *AvrLm6* may overestimate the presence of the two *Avr* genes. Additionally, if both *Avr* genes were present in an isolate, 'Cutlass' would not be able to separate them. *AvrLm6* can be determined with PCR amplification of the cloned fungal gene using markers (Table 4). Finally, 'Surpass400' contains both *LepR3* and *RlmS*. *AvrLmS* could be deduced only for isolates without *AvrLm1* since *LepR3* is also able to interact with *Avrlm1* (Larkan *et al.*, 2012).

3.2.5 Pathogenicity tests

Host differentials were seeded into 96-cell flats filled with Pro-Mix BX w/Mycorrhizae (Premier Tech, Rivière-du-Loup, Québec). Flats were watered daily and maintained in growth chambers (22^{o}C/18^{o}C, day/night with a 16 h daily photoperiod). After 6 to 7 days, 6 seedlings of each differential line were inoculated with a suspension of *L. maculans* pycnidiospores. Each lobe of the cotyledons was wounded using a pair of modified forceps. A 10 µL droplet of inoculum was pipetted onto each of the two wounds on a cotyledon (four inoculated lobes per plant). Flats were returned to growth chambers once the inoculum droplets had air dried. The day following inoculation, the plants were fertilized using 20:20:20 (N:P:K), and emerging true leaves were removed to delay the senescence of cotyledons. Cotyledons were evaluated for interaction phenotype (IP) 12-14 days after inoculation on a rating scale of 0 to 9 based on lesion size, chlorosis or necrosis, and presence of pycnidia as described by Kutcher *et al.* (2007). The mean score from the 24 inoculated lobes was used to determine if a *L. maculans* isolate was avirulent

(IP 0 to 4.9) or virulent (IP 5 to 9). The results were analyzed and interactions of each isolate-host genotype combination considered to deduce the *Avr* genes carried by each isolate.

3.2.6 Diversity and evenness of the *L. maculans* population

These parameters were measured using Simpson's index of diversity (IOD) and index of evenness (IOE), respectively (Simpson, 1949). IOD is calculated by weighing the number of races relative to the total number of *L. maculans* isolates tested; an index of 1 is considered a completely diverse or random population, whereas an index of 0 would represent a single race. IOE measures the relative abundance of different races in the population, with 1 indicating a perfectly even representation of all races.

3.2.7 DNA extraction, PCR and sequencing

Extraction of DNA from purified *L. maculans* isolates used a mixture of pycnidia, conidia and hyphae harvested from 8-12 day old single-spore cultures. DNA was extracted using a modification of the procedure developed by Lee & Taylor (1990). The samples were mixed with a lysis buffer (Tris, EDTA, SDS and NaCl), lysed with mechanical beads at 5000 rpm for 30 seconds, incubated at 65°C for 30 minutes, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and precipitated with 5 M NaCl. Following the final centrifugation, the DNA pellet was dissolved in 100 µL of autoclaved distilled water.

DNA samples from *L. maculans* isolates were screened for the presence/absence of *AvrLm1*, *AvrLm6* and *AvrLm4-7* using the primers listed in Table 3.2. The PCR product for *AvrLm4*-7 was digested with the *HaeIII* enzyme (GG^CC) to detect the SNP mutation of C^{358} to G^{358} that leads to virulence against *Rlm4* (Parlange *et al.* 2009). Virulent isolates produced an additional band after digestion. PCR was performed on the Bio Rad T100^{TM} thermal cycler with the following conditions: 3 min @ 95ºC, 30 cycles of 45sec @ 95ºC, 30 sec at 61ºC, 1 min @ 72ºC, and finally 5 min @ 72ºC.

Table 3.2: Primers of *L. maculans* avirulence genes

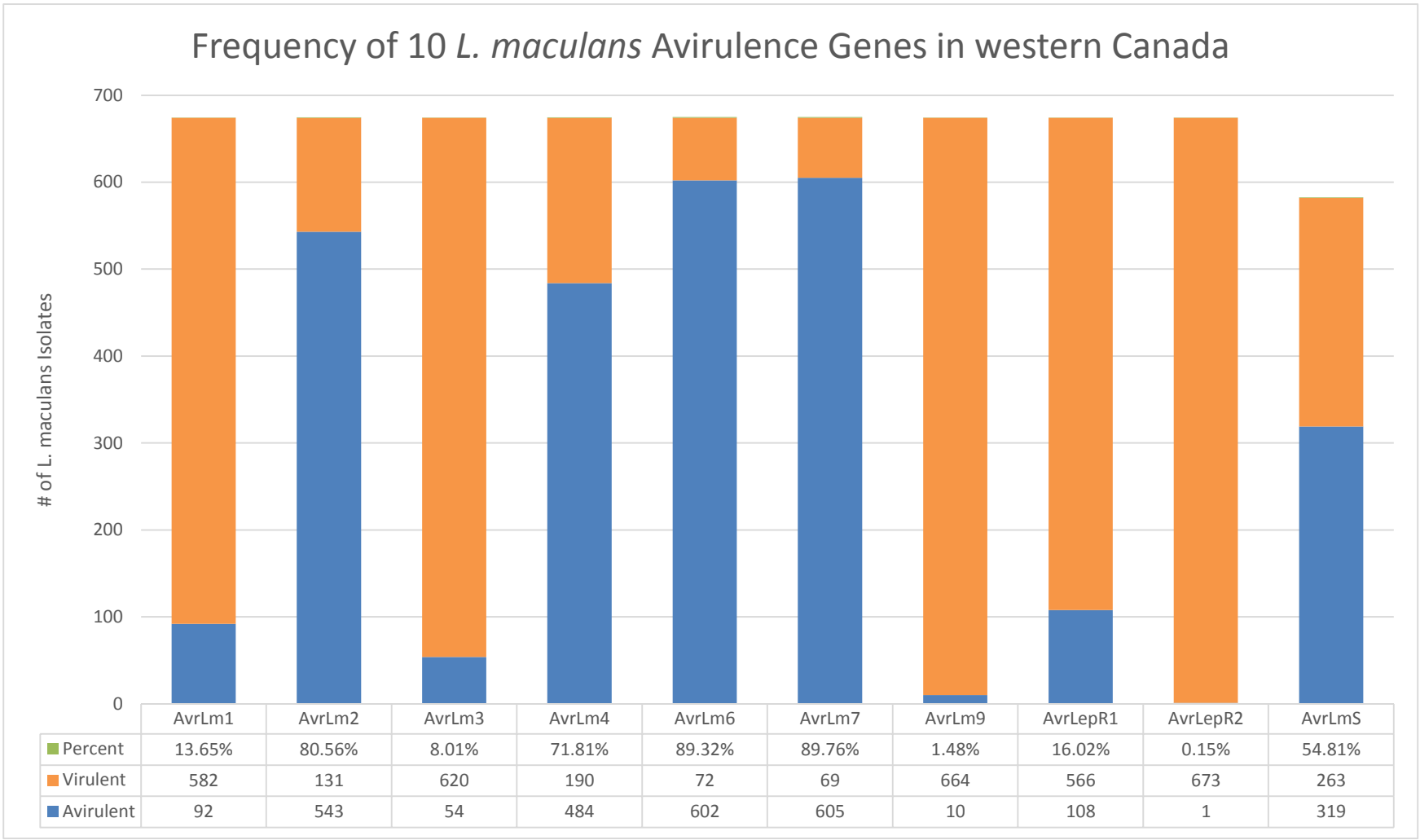
Avirulence genes	Primers (5'-3')	PCR Product Size (bp)
AvrLm1	13D2AvrLm1U: CTATTTAGGCTAAGCGTATTCATAAG	1123
	13D2AvrLm1L: GCGCTGTAGGCTTCATTGTAC	
AvrLm6	AvrLm6ext-U: TCAATTTGTCTGTTCAAGTTATGGA	774
	AvrLm6ext-L: CCAGTTTTGAACCGTAGTGGTAGCA	
AvrLm4-7	AvrLm4-7ext-Up: TATCGCATACCAAACATTAGGC	1433
	AvrLm4-7ext-Lo: GATGGATCAACCGCTAACAA	

The PCR products of the three cloned *Avr* genes were sequenced at Macrogen Inc. (Seoul, Korea), with 28 to 33 isolates subjected to single-pass sequencing for *AvrLm1*, *AvrLm6*, and *AvrLm4-7*. The sequences were aligned with the ClustalW (Higgins *et al.*, 1994) algorithm conducted within MEGA version 6 (Tamura *et al.* 2013). Pairwise alignment was set with a gap penalty of 15 and gap extension penalty of 6.66. The data was exported to BioEdit software where conserved regions with a minimum 15 residue length and a gap limit of 2 per segment were identified. BioEdit was also used to provide nucleotide positional summary and identify SNPs.

3.3 Results

3.3.1 Frequency of L. maculans Avirulence Genes in western Canada

A total of 674 *L. maculans* isolates were characterized for the presence of *AvrLm1*, *Avlm2*, *AvrLm3*, *AvrLm4*, *AvrLm6*, *AvrLm7*, *AvrLm9*, *AvrLepR1*, *AvrLepR2*, and *RlmS*. The presence or absence of *AvrLmS* could only be confirmed in 582 isolates due to the presence of *AvrLm1* in 92 isolates. The other nine *Avr* genes were analyzed for all 674 isolates. None of the ten examined avirulence genes were absent or fixed within the population although there was large variation observed between these genes. The frequency of avirulence genes ranged from as low as 0.2% in *AvrLepR2* to 89.8% in *AvrLm7*. Four other avirulence genes were detected at low frequencies; *AvrLm1* – 13.7%, *AvrLm3* – 8.0%, *AvrLm9* – 1.5%, and *AvrLepR1* – 16.0%. Along with *AvrLm7*, four other avirulence genes were detected in more than half the population: *AvrLm2* – 80.6%, *AvrLm*4 – 71.8%, *AvrLm6* – 89.3%, and *AvrLmS* – 54.8%. Figure 3.1 contains the frequency of avirulence genes of the 674 isolates examined in western Canada.



	AvrLm1	AvrLm2	AvrLm3	AvrLm4	AvrLm6	AvrLm7	AvrLm9	AvrLepR1	AvrLepR2	AvrLmS
Percent	13.65%	80.56%	8.01%	71.81%	89.32%	89.76%	1.48%	16.02%	0.15%	54.81%
Virulent	582	131	620	190	72	69	664	566	673	263
Avirulent	92	543	54	484	602	605	10	108	1	319

Figure 3.1: Frequency of *Leptosphaeria maculans* isolates containing each of the ten avirulence genes characterized in this study. A total of 674 isolates were examined for 10 avirulence genes with the exception of *AvrLmS* for which 582 isolates were characterized.

3.3.2 *L. maculans* Race Structure in western Canada

With 674 examined isolates at ten avirulence gene loci, there is a potential of 2^{10} combinations. Each combination of avirulence genes is a unique race of *L. maculans* following the nomenclature of Balesdent *et al.*(2005). In this study a total of 55 races were found ranging from as high as 22.7% to as low as 0.2% in a single isolate. A listing of western Canadian *L. maculans* races, # of isolates per race, and PG group designation is provided in Table 3.3. There were two main races at 22.70% and 22.55% which together account for almost half the population. These two races are followed by a drastic decline to 5.93% for the third most common race. Figure 3.2 illustrates the relative frequency of blackleg races in western Canada. The two main races differ only in the presence or absence of *AvrLmS* and both contain *AvrLm 2, 4, 6,* and *7.* The other 55 races are more varied and decline in frequency from 5.93% (40 isolates) to 15 races represented by a single isolate. If we group the races into pathogenicity groups we find that PGT is the dominant group at 70.92% of the sample population followed by PG2 at 10.1%, PG3 9.6%, and PG4 at 9.4%.

Table 3.3: Listing of *Leptosphaeria maculans* races among 675 characterized western Canadian isolates sampled in 2010 and 2011 based on differential lines and PCR amplification of avirulence genes. *AvrLmS* could not be determined in 92 isolates and a bracket signifies that the presence or absence of the avirulence gene is unknown.

# of Races	# of '*AvrLm*'	Race	# of isolates	# of isolates	Frequency	PG group
1	1	Avr-7	1	1	0.15%	PG4
2	2	Avr-2-6	4	24	0.59%	PGT
3		Avr-2-7	6		0.89%	PGT
4		Avr-4-7	1		0.15%	PG4
5		Avr-6-7	11		1.63%	PG4
6		Avr-7-S	1		0.15%	PG4

7		Avr-6-LepR1	1		0.15%	PG4
8	3	Avr-2-4-7	20	102	2.97%	PGT
9		Avr-2-6-7	23		3.41%	PGT
10		Avr-2-6-S	9		1.34%	PGT
11		Avr-2-7-S	7		1.04%	PGT
12		Avr-4-7-S	4		0.59%	PG4
13		Avr-4-6-7	8		1.19%	PG4
14		Avr-2-6-LepR1	3		0.45%	PGT
15		Avr-6-7-S	5		0.74%	PG4
16		Avr-1-6-7-(S)	4		0.59%	PG3
17		Avr-1-6-LepR1-(S)	1		0.15%	PG3
18		Avr-2-3-S	2		0.30%	PG2
19		Avr-1-3-6-(S)	3		0.45%	PG2
20		Avr-1-4-7-(S)	5		0.74%	PG3
21		Avr-6-7-LepR1	8		1.19%	PG4
22	4	Avr-2-4-6-7	153	299	22.70%	PGT
23		Avr-1-4-6-7-(S)	40		5.93%	PG3
24		Avr-2-6-7-LepR1	4		0.59%	PGT
25		Avr-2-6-7-S	38		5.64%	PGT
26		Avr-2-4-7-S	20		2.97%	PGT
27		Avr-4-6-7-S	15		2.23%	PG4
28		Avr-4-7-LepR1-S	2		0.30%	PG4
29		Avr-4-6-7-LepR1	1		0.15%	PG4
30		Avr-1-4-7-LepR1-(S)	1		0.15%	PG3
31		Avr-6-7-LepR1-S	1		0.15%	PG4
32		Avr-1-6-7-LepR1-(S)	1		0.15%	PG3
33		Avr-2-6-LepR1-S	3		0.45%	PGT
34		Avr-2-3-6-9	1		0.15%	PG2
35		Avr-2-3-6-S	19		2.82%	PG2
36	5	Avr-1-2-3-6-LepR1-(S)	4	222	0.59%	PG2
37		Avr-1-2-3-6-9-(S)	2		0.30%	PG2
38		Avr-2-3-6-7-S	2		0.30%	PG2
39		Avr-4-6-7-LepR1-S	3		0.45%	PG4
40		Avr-1-4-6-7-LepR1-(S)	14		2.08%	PG3
41		Avr-1-2-4-6-7-(S)	8		1.19%	PG2
42		Avr-2-3-6-LepR1-S	11		1.63%	PG2
43		Avr-2-4-6-7-S	152		22.55%	PGT

44		Avr-2-4-6-7-LepR1	18		2.67%	PGT
45		Avr-2-6-7-LepR1-S	6		0.89%	PGT
46		Avr-2-4-7-LepR1-S	2		0.30%	PGT
47	6	Avr-1-2-3-6-9-LepR1-(S)	3	25	0.45%	PG2
48		Avr-2-3-6-7-LepR1-S	1		0.15%	PG2
49		Avr-2-3-6-9-LepR1-S	2		0.30%	PG2
50		Avr-2-3-4-6-7-S	1		0.15%	PG2
51		Avr-2-3-6-LepR1-LepR2-S	1		0.15%	PG2
52		Avr-2-3-6-7-9-S	1		0.15%	PG2
53		Avr-2-4-6-7-LepR1-S	10		1.48%	PGT
54		Avr-1-2-4-6-7-LepR1-(S)	6		0.89%	PG2
55	7	Avr-2-3-6-7-9-LepR1-S	1	1	0.15%	PG2
Total			**674**	**674**	**100.00%**	

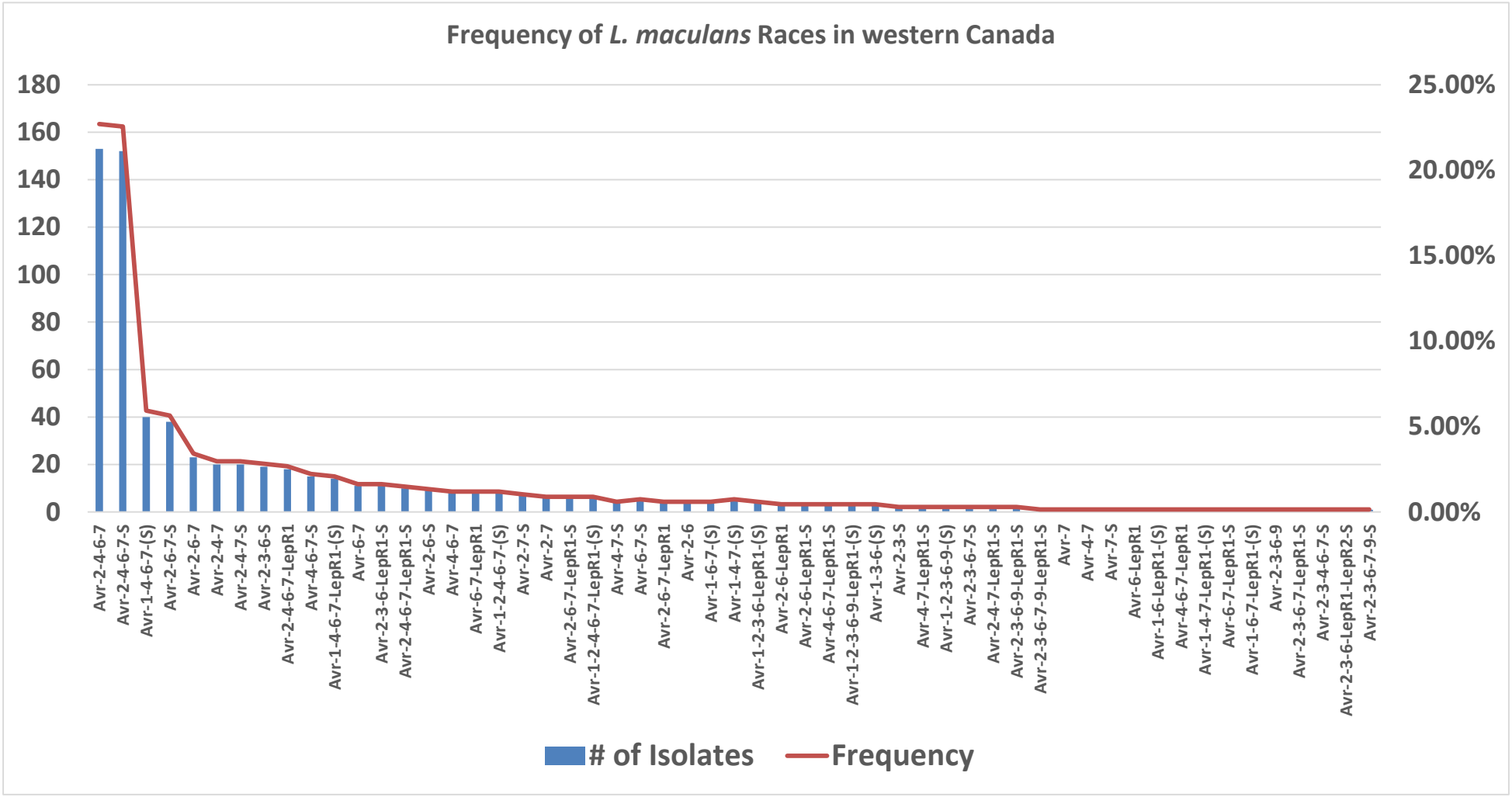


Figure 3.2: Frequency of 55 *Leptosphaeria maculans* races based on the ten avirulence genes characterized in this study. A total of 674 isolates were examined for all the avirulence genes with the exception of *AvrLmS* for which 582 isolates were characterized.

When we evaluate the race structure of the pathogen population in terms of complexity (# of avirulence genes carried per isolate) we observe that most isolates carry 4 or more avirulence genes with an average of 4.18 (Figure 3.3). Two races, *Avr2-4-6-7* at 22.7% and *Avr2-4-6-7-S* at 22.5% accounted for almost half the population, with the third most common race being detected in 5.9% of the sample population. More than 75% of the isolates carried 4-5 *Avr* genes, <4% carried 6 or more *Avr* genes and <20% carried 3 or fewer *Avr* genes (Figure 3.3). Only one isolate carried 7 *Avr* genes (*Avr2-3-6-7-9-S-LepR1*) and one isolate carried a single *Avr* gene, *AvrLm7*. The distribution is not normally distributed and is skewed to the right (Figure 3.3). The average complexity dropped slightly in 2011 when compared to 2010 (Figure 3.4)

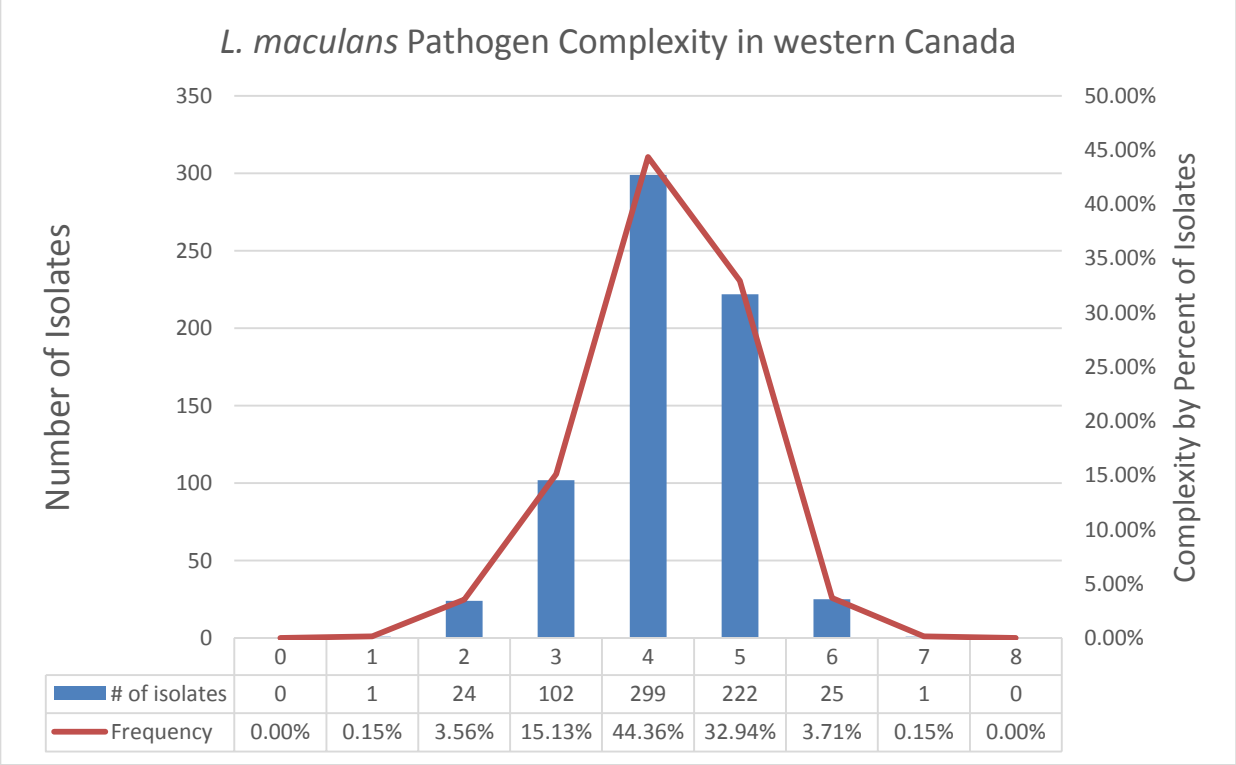
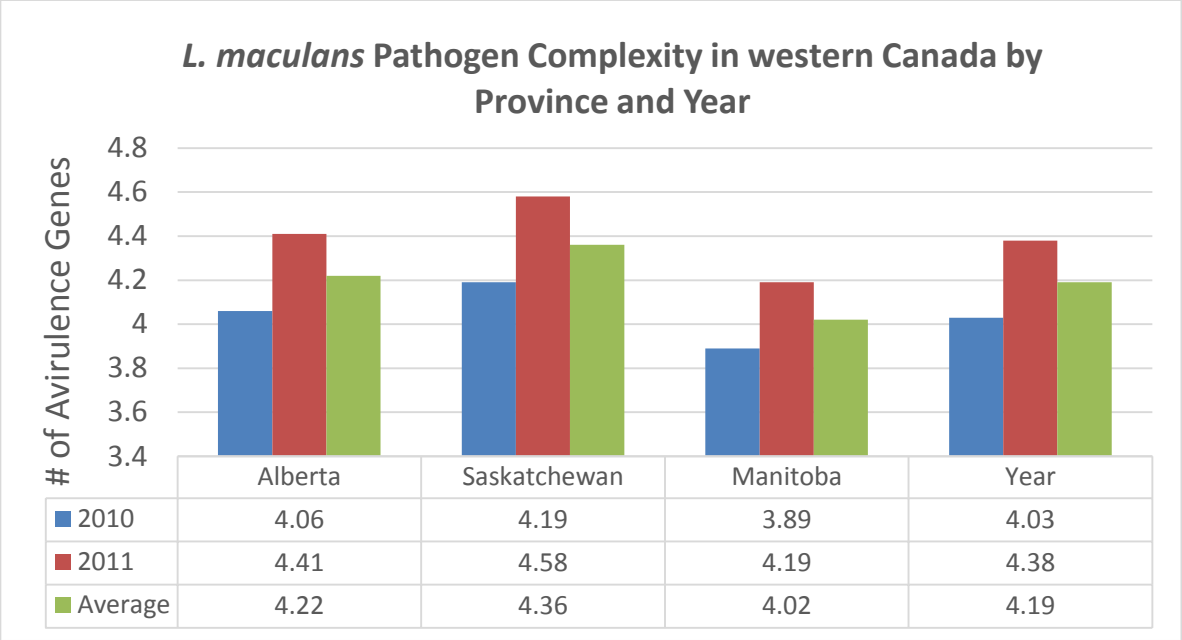


Figure 3.3: Average pathogen complexity; number of avirulence genes carried by *Leptosphaeria maculans* isolates. Race complexity based on a total of 674 isolates from western Canada in 2010-2011 assessed at 10 avirulence genes.



	Alberta	Saskatchewan	Manitoba	Year
2010	4.06	4.19	3.89	4.03
2011	4.41	4.58	4.19	4.38
Average	4.22	4.36	4.02	4.19

Figure 3.4: Pathogen Complexity of *L. maculans* in Canada by Province and Year. Race complexity based on a total of 674 isolates from western Canada in 2010-2011 assessed at 10 avirulence genes.

The *L. maculans* population appears genetically diverse as measured using Simpson's index of diversity and evenness (Simpson, 1949). The Simpson index of diversity (IOD) was calculated at 0.89 and weighs the number of races relative to the total number of samples. An index of 1 is a perfectly diverse or random population whereas an index of 0 would represent a single race. The Simpson index of evenness (IOE) measures the relative abundance of different races and was calculated at 0.71, indicating a somewhat even population. The two dominant races likely reduced the evenness score significantly. Simpson's IOD and IOE were variable among provinces: Manitoba had the most diverse and even *L. maculans* population, with IOD and IOE at 0.91 and 0.68, respectively. The respective scores of the other provinces were 0.87 and 0.61 for Alberta and 0.77 and 0.34 for Saskatchewan. However, IOD did not change significantly between the years, whereas IOE dropped slightly in 2011 (Table 3.4).

Table 3.4: : Simpson's Index of Diversity and Evenness for 674 *Leptosphaeria maculans* isolates collected from commercial canola fields in western Canada in 2010 and 2011. The assessment of evenness and diversity of *L. maculans* races was based on the presence/absence of 10 avirulence genes in the pathogen population

(A) Simpson's Diversity Index (IOD):

	MB	AB	SK	Years
2010	0.922	0.899	0.781	**0.868**
2011	0.891	0.832	0.755	**0.826**
Provinces	**0.906**	**0.865**	**0.768**	**0.885**

$$S = 1 - \sum (n_i^2 - n_i)/(N^2 - N)$$

(B) Evenness Index (IOE):

	MB	AB	SK	Years
2010	0.700	0.697	0.460	**0.619**
2011	0.667	0.519	0.317	**0.501**
Provinces	**0.684**	**0.608**	**0.389**	**0.712**

$$EH = H/\ln R, \quad \text{with } H = -\sum P_i x \ln P_i$$

The frequency of *Avr* genes showed some changes in certain *Avr* gene loci between 2010 and 2011, with both increases and decreases observed (Figure 3.5). *AvrLepR1* increased 12.4%, while *AvrLm2* declined by 6.7%. The *Avr* genes *AvrLm3*, *AvrLm7*, *AvrLm9*, and *AvrLepR2* shifted less than 5% between 2010 and 2011, while *Avr* genes *AvrLm6* and *AvrLepR1* shifted greater than 10% within a one year span. *AvrLm2*, *AvrLm4*, and *AvrLmS* changed by a 6.7% decline, 7.4% increase, and a 7.2% increase respectively. On average the frequency of avirulence genes increased by 3.4% from 2010 to 2011. The

increase in pathogen complexity and average avirulence gene frequency from 2010 to 2011 are indicators of less virulence in 2011, largely attributable to more favourable weather conditions in 2010. This observation is consistent with field survey data showing decreased incidence of blackleg stem canker in western Canada in 2011 when compared to 2010 (Figures 3.6-3.7).

The Avr-gene frequency varied somewhat in the pathogen population among provinces. On the provincial scale the differences range to as great as 31.8% more isolates carrying *AvrLm2* in Saskatchewan relative to Manitoba (Figure 3.8). The frequency of *AvrLm1, AvrLm2, AvrLepR1* and *AvrLmS* varied by greater than 20% among provinces, while *AvrLm3, Avlm7, AvrLm9*, and *AvrLepR2* varied less than 10%. Despite the variation, *AvrLm2, AvrLm4, AvrLm6* and *AvrLm7* were at high frequencies while *AvrLm1, AvrLm3, AvrLm9* and *AvrLepR2* were low in the pathogen population in all provinces. On average, avirulence gene frequency varied by 16.1% between provinces.

There were even greater variations in the frequency of *Avr* genes among different field sites based on the comparison of *L. maculans* isolates from 5 locations (Figure 3.9); *AvrLm9* and *AvrLepR2* were absent from all sites and the virulent alleles were predominant in the population. *AvrLm2* was the most variable *Avr* gene locus displaying large differences between sampling sites such as a difference of 78% between Melfort, Saskatchewan and Roland, Manitoba. Similarly for *AvrLm*1, the frequency of *AvrLm1* was 63% in Roland, but absent in Melfort. *AvrLm7* was the least variant locus and was

consistently above 90% at all five sites listed. On average, the 10 avirulence genes varied by 35% between the site with the maximum frequency and the site with the minimum frequency. A total of 36, 27, 29, 19, and 51 *L. maculans* isolates were characterized for the sites Snowflake (MB), Roland (MB), Plum Coulee (MB), Melfort (SK), and Vegreville (AB), respectively.

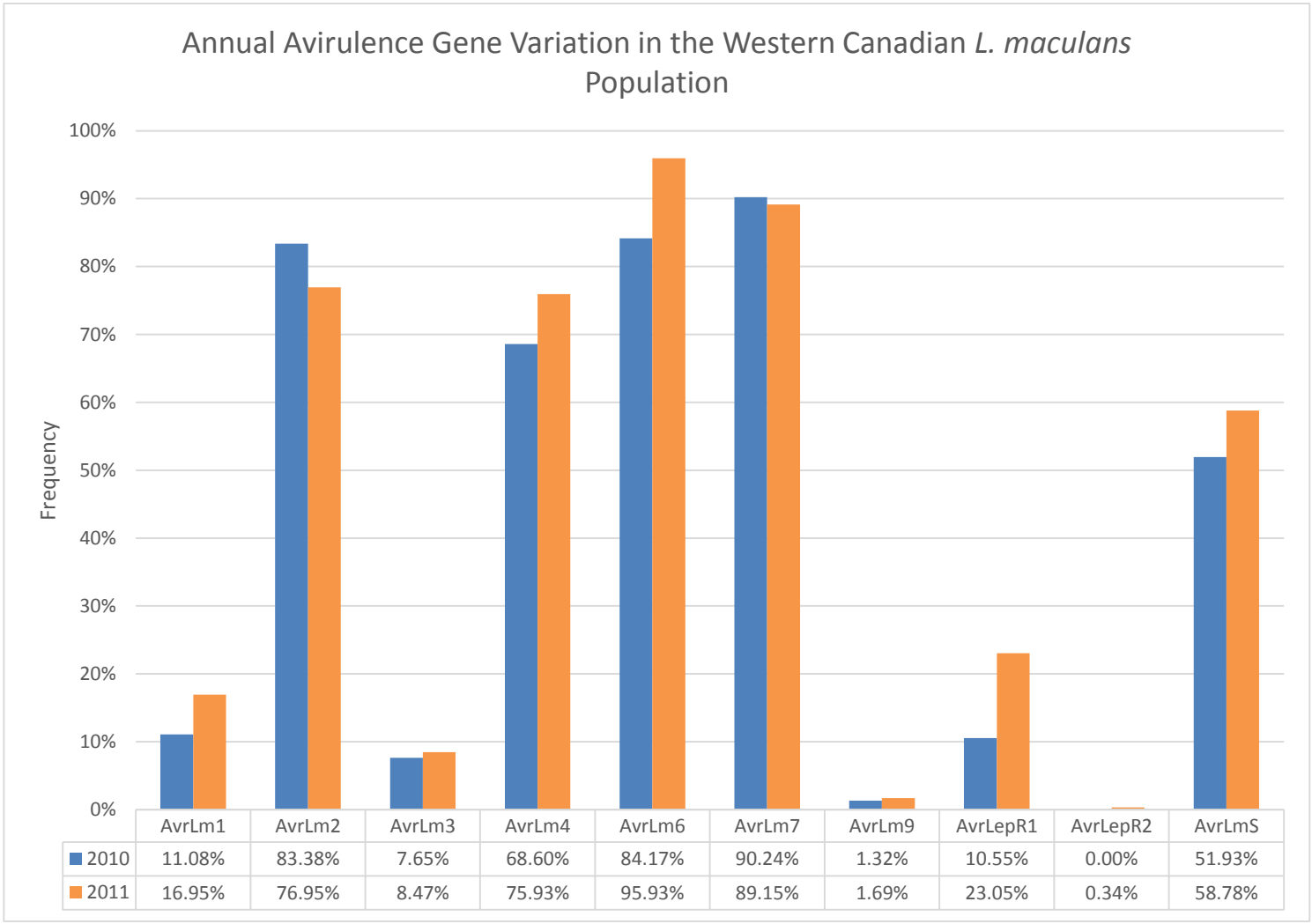


Figure 3.5: Comparison of *Leptosphaeria maculans* avirulence gene frequency in 2010 and 2011 in the Western Canadian pathogen population. A total of 674 isolates were examined for 10 avirulence genes with the exception of *AvrLmS* for which 582 isolates were characterized.

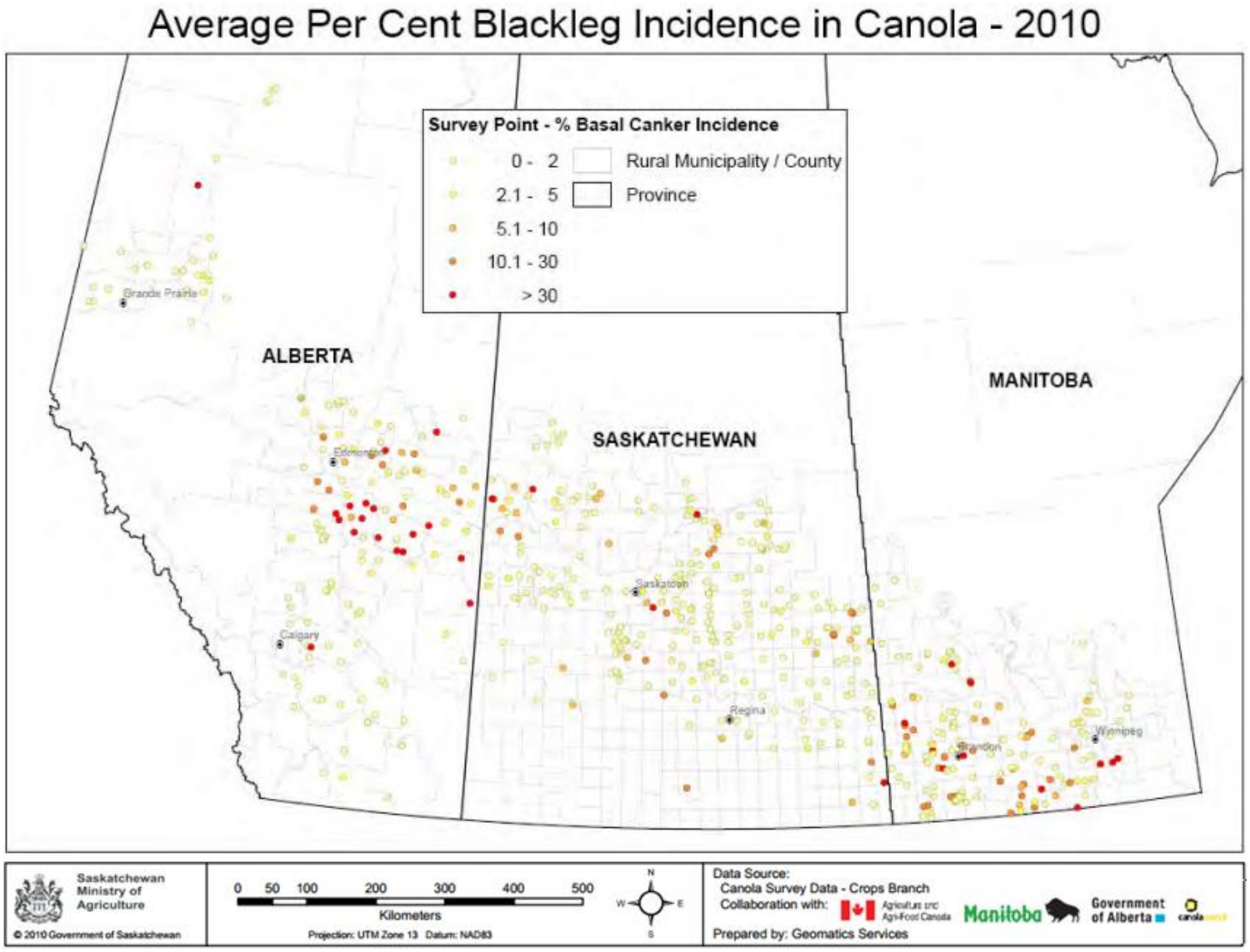


Figure 3.6: 2010 Blackleg disease incidence survey. Percent Basal stem canker infection rates in the Canadian Provinces of Alberta, Saskatchewan, and Alberta. Saskatchewan Ministry of Agriculture.

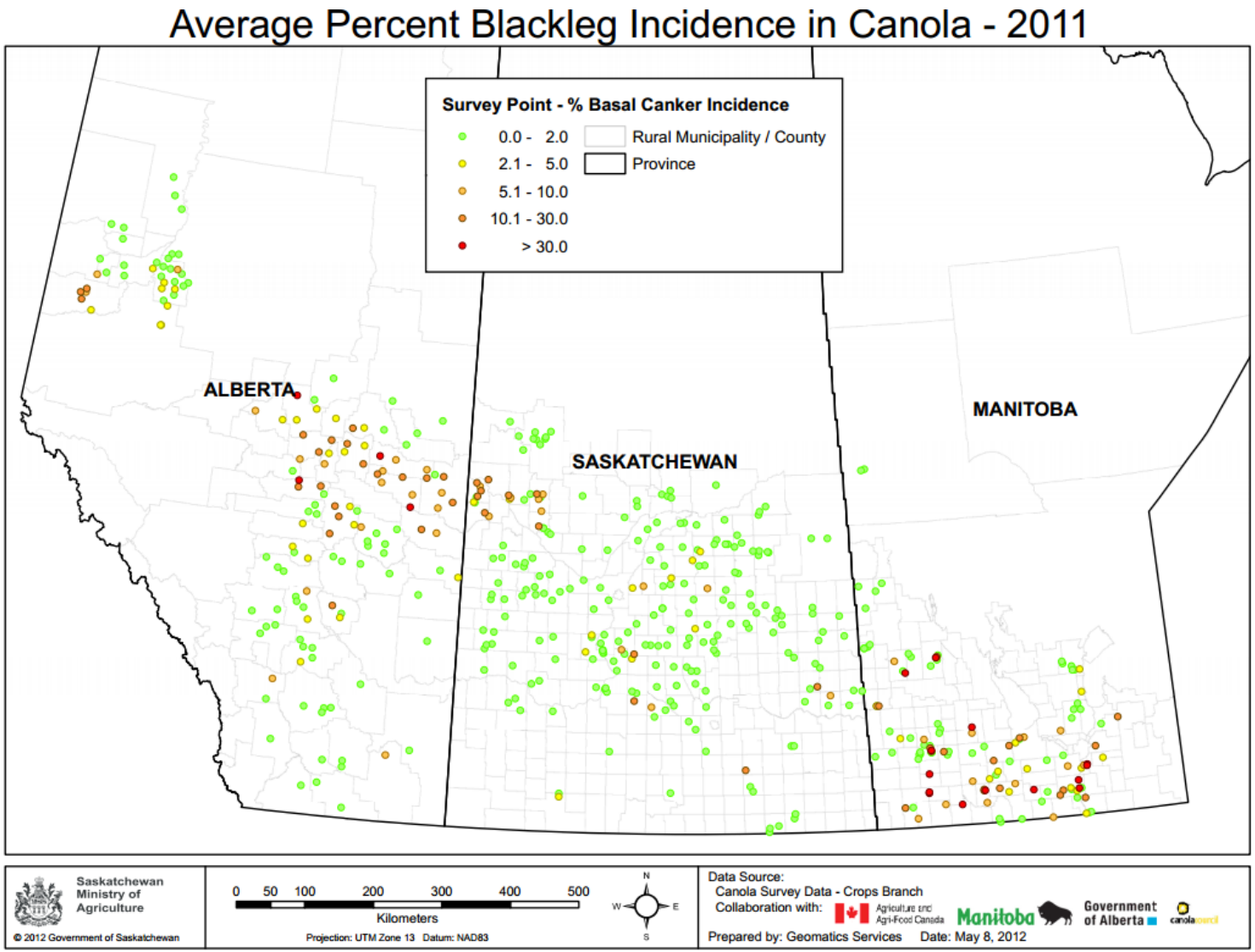
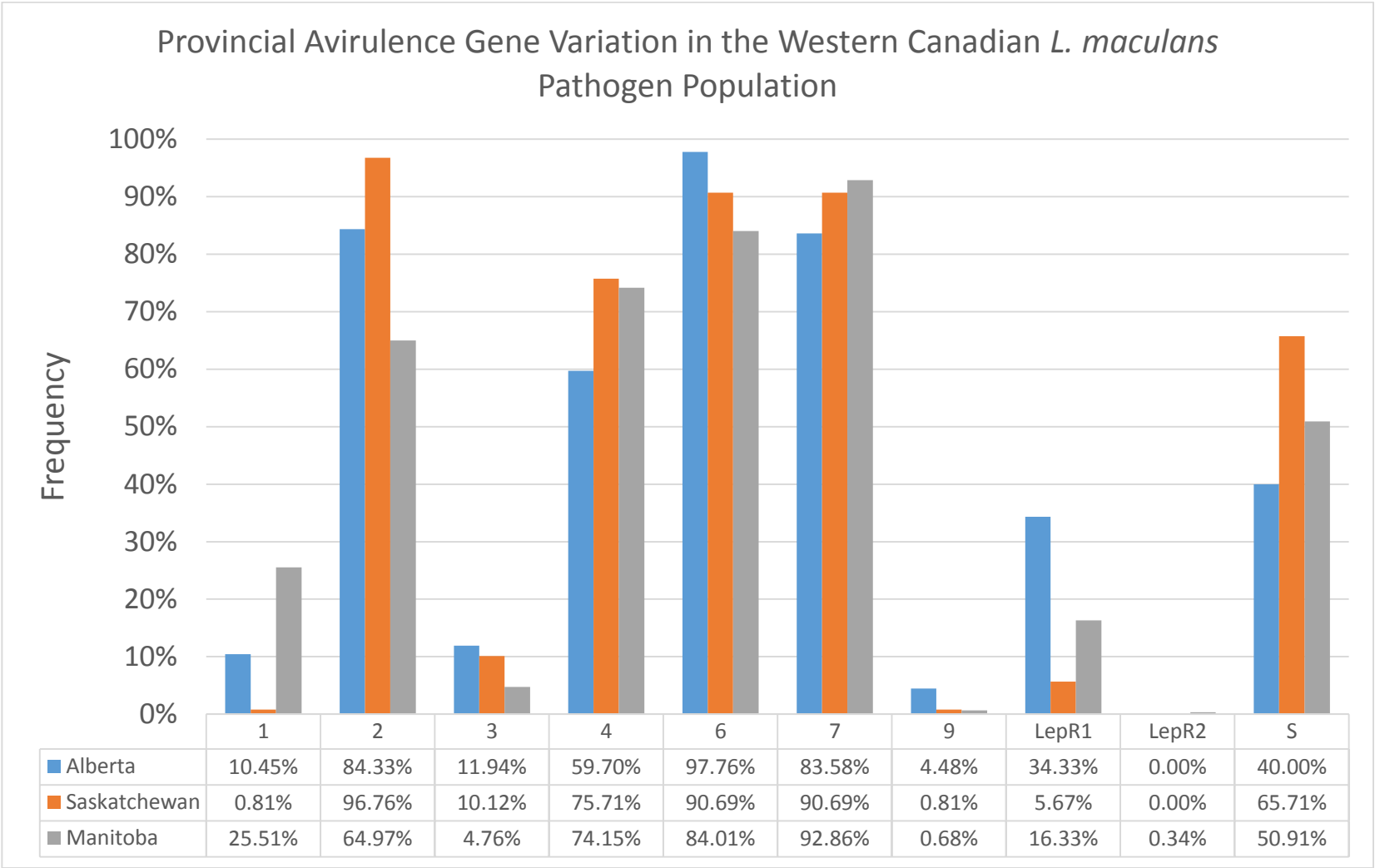
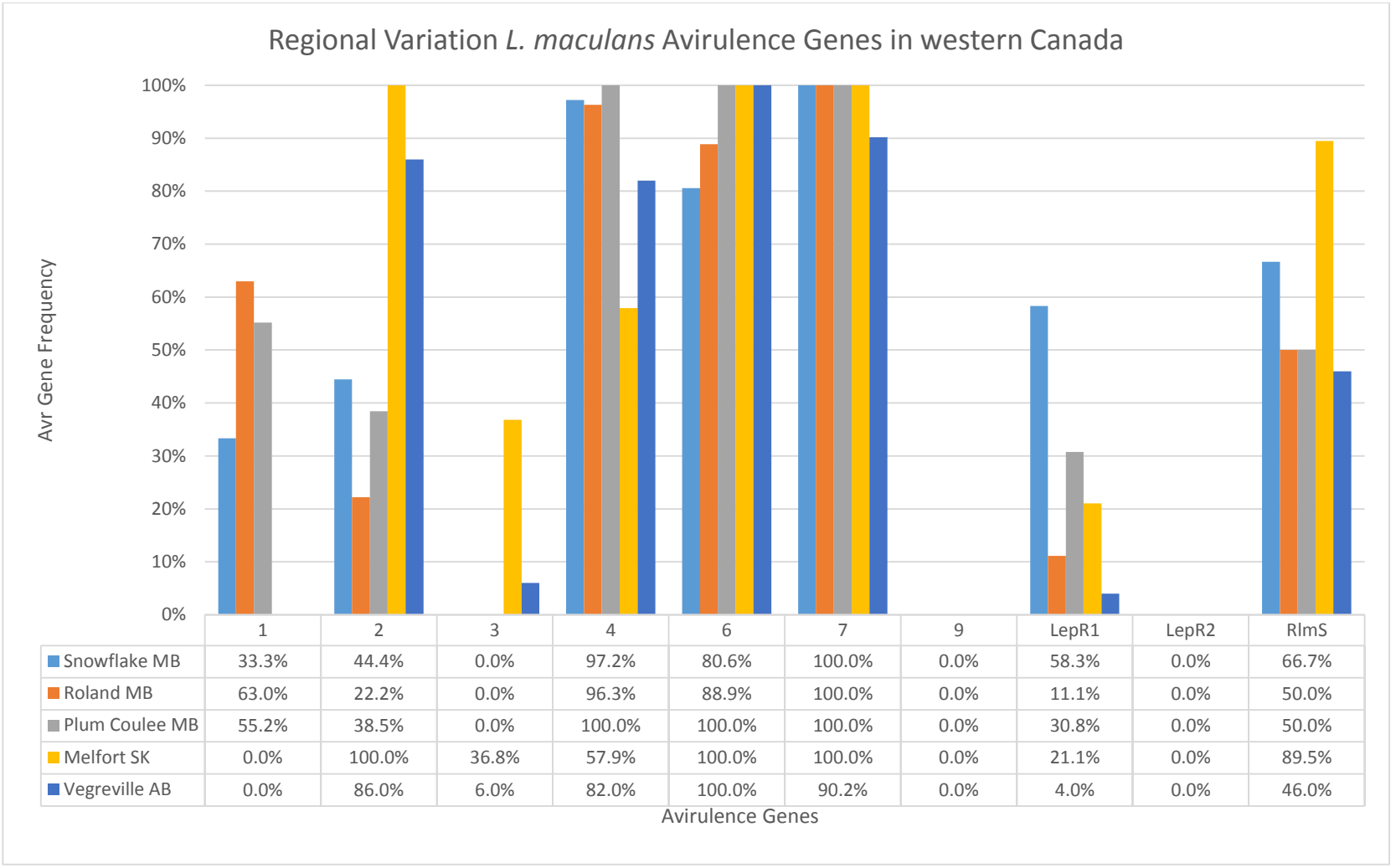


Figure 3.7: 2011 Blackleg disease incidence survey. Percent Basal stem canker infection rates in the Canadian Provinces of Alberta, Saskatchewan, and Alberta. Saskatchewan Ministry of Agriculture.



	1	2	3	4	6	7	9	LepR1	LepR2	S
Alberta	10.45%	84.33%	11.94%	59.70%	97.76%	83.58%	4.48%	34.33%	0.00%	40.00%
Saskatchewan	0.81%	96.76%	10.12%	75.71%	90.69%	90.69%	0.81%	5.67%	0.00%	65.71%
Manitoba	25.51%	64.97%	4.76%	74.15%	84.01%	92.86%	0.68%	16.33%	0.34%	50.91%

Figure 3.8: Comparison of *Leptosphaeria maculans* avirulence gene frequency between provinces in the Western Canadian pathogen population. A total of 674 isolates were examined for 10 avirulence genes with the exception of *AvrLmS* for which 582 isolates were characterized. A total of 124, 247, and 294 *L. maculans* isolates were characterized for each of Alberta, Saskatchewan, and Manitoba.



	1	2	3	4	6	7	9	LepR1	LepR2	RlmS
Snowflake MB	33.3%	44.4%	0.0%	97.2%	80.6%	100.0%	0.0%	58.3%	0.0%	66.7%
Roland MB	63.0%	22.2%	0.0%	96.3%	88.9%	100.0%	0.0%	11.1%	0.0%	50.0%
Plum Coulee MB	55.2%	38.5%	0.0%	100.0%	100.0%	100.0%	0.0%	30.8%	0.0%	50.0%
Melfort SK	0.0%	100.0%	36.8%	57.9%	100.0%	100.0%	0.0%	21.1%	0.0%	89.5%
Vegreville AB	0.0%	86.0%	6.0%	82.0%	100.0%	90.2%	0.0%	4.0%	0.0%	46.0%

Figure 3.9: Regional avirulence gene variation represented by 5 selected sites in the Western Canadian *Leptosphaeria maculans* pathogen population.

3.3.3 *L. maculans AvrLm1, AvrLm6,* and *AvrLm4-7* genetic variation

A total of 96 samples were amplified and sequenced at one of three avirulence gene loci: *AvrLm1*, *AvrLm6*, and *AvrLm4-7*. Table 3.5 contains a listing of the selected isolates.

Table 3.5: List of nucleotide samples from three avirulent gene loci (*AvrLm1*, *AvrLm6*, and *AvrLm4-7*) amplified from 96 selected blackleg isolates from the Western Canadian *Leptosphaeria maculans* population.

#	Isolate	Province	Year	PCR Product
1	DM2	MB	2010	*AvrLm1*
2	DM22A	MB	2010	*AvrLm1*
3	DM27	MB	2010	*AvrLm1*
4	DM32	MB	2010	*AvrLm1*
5	DM37	MB	2010	*AvrLm1*
6	DM47	MB	2010	*AvrLm1*
7	DM78	MB	2010	*AvrLm1*
8	DM91	MB	2010	*AvrLm1*
9	MB11 3-1	MB	2011	*AvrLm1*
10	MB11 8-1	MB	2011	*AvrLm1*
11	RL33	MB	2011	*AvrLm1*
12	MB11 39-1	MB	2011	*AvrLm1*
13	MB11 40-1	MB	2011	*AvrLm1*
14	MB11 45-2	MB	2011	*AvrLm1*
15	MB11 101-2	MB	2011	*AvrLm1*
16	RL2	MB	2011	*AvrLm1*
17	RL4	MB	2011	*AvrLm1*
18	RL6	MB	2011	*AvrLm1*
19	RL7	MB	2011	*AvrLm1*
20	RL8	MB	2011	*AvrLm1*
21	RL10	MB	2011	*AvrLm1*
22	RL20	MB	2011	*AvrLm1*
23	RL32	MB	2011	*AvrLm1*
24	SC6	MB	2011	*AvrLm1*
25	SC8	MB	2011	*AvrLm1*
26	SC10	MB	2011	*AvrLm1*
27	SC19	MB	2011	*AvrLm1*

28	A10 530A	AB	2010	*AvrLm1*
29	A10 537A	AB	2010	*AvrLm1*
30	MB11 106-1	MB	2011	*AvrLm1*
31	MB11 117-1	MB	2011	*AvrLm1*
32	MB11 128-5	MB	2011	*AvrLm1*
33	MB11 1-1	MB	2011	*AvrLm6*
34	MB11 3-1	MB	2011	*AvrLm6*
35	MB11 5-1	MB	2011	*AvrLm6*
36	MB11 8-1	MB	2011	*AvrLm6*
37	MB11 17-1	MB	2011	*AvrLm6*
38	MB11 21-2	MB	2011	*AvrLm6*
39	MB11 25-1	MB	2011	*AvrLm6*
40	MB11 30-3	MB	2011	*AvrLm6*
41	MB11 41-2	MB	2011	*AvrLm6*
42	SK11 25	SK	2011	*AvrLm6*
43	SK11 28	SK	2011	*AvrLm6*
44	SK11 29	SK	2011	*AvrLm6*
45	SK11 30	SK	2011	*AvrLm6*
46	SK11 31	SK	2011	*AvrLm6*
47	SK11 32	SK	2011	*AvrLm6*
48	SK11 33	SK	2011	*AvrLm6*
49	SK11 38	SK	2011	*AvrLm6*
50	SK11 39	SK	2011	*AvrLm6*
51	SK11 41	SK	2011	*AvrLm6*
52	SK11 46	SK	2011	*AvrLm6*
53	SK11 55	SK	2011	*AvrLm6*
54	SK11 64	SK	2011	*AvrLm6*
55	SK11 73	SK	2011	*AvrLm6*
56	A10 573A	AB	2010	*AvrLm6*
57	A10 597	AB	2010	*AvrLm6*
58	A10 610	AB	2010	*AvrLm6*
59	A10 611	AB	2010	*AvrLm6*
60	A10 642	AB	2010	*AvrLm6*
61	A10 643	AB	2010	*AvrLm6*
62	A11-695	AB	2010	*AvrLm6*
63	A11 700A	AB	2010	*AvrLm6*
64	A11 738	AB	2010	*AvrLm6*
65	DM8	MB	2010	*AvrLm4-7*
66	DM14	MB	2010	*AvrLm4-7*
67	DM20	MB	2010	*AvrLm4-7*
68	DM24	MB	2010	*AvrLm4-7*
69	RL34	MB	2011	*AvrLm1*

70	A11-745	AB	2011	*AvrLm6*
71	A11-750	AB	2011	*AvrLm6*
72	A11-779A	AB	2011	*AvrLm6*
73	DS4	SK	2010	*AvrLm4-7*
74	DS7	SK	2010	*AvrLm4-7*
75	DS14	SK	2010	*AvrLm4-7*
76	DS18	SK	2010	*AvrLm4-7*
77	DS27	SK	2010	*AvrLm4-7*
78	A10 680B	AB	2010	*AvrLm4-7*
79	DS43	SK	2010	*AvrLm4-7*
80	DS47	SK	2010	*AvrLm4-7*
81	DS62	SK	2010	*AvrLm4-7*
82	A10 509B	AB	2010	*AvrLm4-7*
83	A10 680C	AB	2010	*AvrLm4-7*
84	A10 527A	AB	2010	*AvrLm4-7*
85	A10 528A	AB	2010	*AvrLm4-7*
86	A10 528B	AB	2010	*AvrLm4-7*
87	A10 530A	AB	2010	*AvrLm4-7*
88	A10 530B	AB	2010	*AvrLm4-7*
89	A10 546A	AB	2010	*AvrLm4-7*
90	A10 546B	AB	2010	*AvrLm4-7*
91	A10 547A	AB	2010	*AvrLm4-7*
92	A10 559A	AB	2010	*AvrLm4-7*
93	A10 568	AB	2010	*AvrLm4-7*
94	A10 594	AB	2010	*AvrLm4-7*
95	A10 617	AB	2010	*AvrLm4-7*
96	A11 732	AB	2011	*AvrLm4-7*

The alignment of the three avirulence genes resulted in significant differences in terms of the number of SNPs and alleles while the size of the conserved region relative to the PCR product was relatively consistent between the three avirulence genes (Table 3.6)

Table 3.6: Summary of sequence variation among three avirulence gene loci sequenced in 96 isolates from the western Canadian *Leptosphaeria maculans* pathogen population.

	AvrLm1	***AvrLm6***	***AvrLm4-7***
# of Isolates	33	35	28
PCR Product Size (bp)	1123	774	1433
Conserved Regions	1	1	9
Conserved Regions Size (bp)	438	239	562
# of SNPs	58	11	118
% SNPs	13.24%	4.60%	21.00%
% of Canadian isolates with the virulent allele	86.52%	10.81%	28.15%
% of Canadian isolates with the avirulent allele	13.48%	89.19%	71.85%

The *AvrLm1* gene of 33 western Canadian *L. maculans* isolates was aligned to the reference French *L. maculans* isolate JN3 and resulted in a single conserved region of 438bp. A total of 58 SNPs were identified among the 33 isolates comprising 13.2% of the conserved region. The 58 SNPs arose from 3 alleles, with 31 isolates sharing a common allele and 2 isolates from Manitoba 2010 samples displaying unique haplotypes with many nucleotide substitutions (Table 3.7).

Alignment of the *AvrLm6* gene among 35 isolates with the reference genome identified a single conserved region of 239 bp with a total of 11 SNPs in 4 alleles. Thirty-two isolates shared a common allele and 2 isolates revealed SNPs in unique alleles. One isolate shared a SNP with the reference isolate JN3. The fewest SNPs (4.6%) were identified for *AvrLm6*, relative to the size of the conserved region.

Similar alignment of 28 isolates carrying the *AvrLm4-7* gene with the reference genome revealed 9 conserved regions separated by deletions involving a combined size

of 562 bp. This alignment detected SNPs at the highest frequency (21%) relative to the size of conserved regions, with 110 SNPs identified in 9 alleles. Fifteen isolates shared the most common allele, followed by 5 and 2 isolates sharing the next two most common alleles, respectively. The remaining 6 alleles were represented by 6 different isolates.

Table 3.7: Allele Frequency of *AvrLm1*, *AvrLm6*, and *AvrLm4-7* among 33, 36, and 28 western Canadian *Leptosphaeria maculans* isolates respectively.

		Number of isolates (frequency %)	
Gene	Allele	2010	2011
AvrLm1	0	9 (27.3)	22 (66.7)
	1	1 (3.03)	0 (0)
	2	1 (3.03)	0 (0)
AvrLm6	0	23 (65.7)	9 (25.7)
	1	0 (0)	1 (2.9)
	2	0 (0)	1 (2.9)
	3	0 (0)	1 (2.9)
AvrLm4-7	0	15 (53.6)	0 (0)
	1	5 (17.9)	0 (0)
	2	2 (7.1)	0 (0)
	3	1 (3.6)	0 (0)
	4	1 (3.6)	0 (0)
	5	1 3.6)	0 (0)
	6	1 (3.6)	0 (0)
	7	1 (3.6)	0 (0)
	8	1 (3.6)	0 (0)

The number of *L. maculans* isolates carrying *AvrLm1*, *AvrLm6* and *AvrLm4-7* genes were 33, 36, and 28, respectively.

3.4 Discussion

The current study examined the frequency of ten *Avr* genes in 674 *L. maculans* isolates from western Canada with a set of host differentials and PCR-based markers. A total of 55 races were identified following the approach of Balesdent *et al.* (2005. Two races appeared clearly dominant accounting for almost half of the population. Aside from the two dominant races, the other 53 races occurred at less than 6% frequency based on our sample size of 674 *L. maculans* isolates. An earlier study that characterized up to 96 isolates found 16 races of *L. maculans* in western Canada (Kutcher *et al.*, 2010). This difference suggests that sample size plays a significant role in detecting the presence of low-frequency races. At the other end of the scale, 15 races were represented by only a single isolate each. While 10 *Avr* gene loci were examined in the study, there were several *Avr* genes for which neither differential cultivars nor markers were available at the time this research was initiated; these include *AvrLm5, AvrLm8, AvrLm10, AvrLm11,* and *AvrLmJ1*. It is also possible that some *Avr* genes have not been identified for *L. maculans*. Therefore, *L. maculans* races are likely more diverse and complicated in western Canada than captured by this study.

This study isolated blackleg from commercial stubble directly to allow for a larger survey to capture the blackleg population across western Canada. Commercial cultivars with blackleg resistance will shift the pathogen population in favour of virulent isolates lacking corresponding avirulence genes. The selection pressure from commercial stubble is always present and the same pathogen population is expected if Westar was seeded into

commercial stubble, allowed to be infected, and re-isolated since Westar carries no blackleg resistance gene and would thus would not alter the blackleg population compared to direct isolation. However, since Westar is infected by inoculum generated from the previous canola crop, it would represent the pathogen population from last season's commercial stubble while isolating from commercial stubble will represent the current blackleg population.

The dominance of the two races (*AvrLm-4-6-7-S* and *AvrLm-4-6-7-S*) among the *L. maculans* isolates examined in this study indicates an uneven pathogen population, while the total number of races highlights the underlying diversity. The pathogen's race diversity points to blackleg posing a risk to canola production in western Canada since each resistance gene that corresponds to the 10 known *Avr* genes examined in the study can be overcome by at least one of the races identified. The Simpsons evenness index, which measures relative frequency of races, was generally lower in Saskatchewan and higher in Manitoba. The higher diversity and evenness indices observed among Manitoba *L. maculans* isolates may point to a larger risk of resistance erosion in that province, followed by Alberta and Saskatchewan where the indices were lower and lowest, respectively. It is surprising to find that Saskatchewan had both the least number of races along with the least even distribution of races given that *L. maculans* was first reported in the province in 1975 (McGee & Petrie, 1978) and that canola acreage is currently the largest in western Canada. However, agricultural practices such as crop rotation (Kutcher & Brandt, 2009), tillage (Turkington *et al.*, 2000) and nitrogen fertilizer application rates (Kutcher *et al.*, 2005) all play a role on pathogen reproduction, which

may influence the pathogen race structure and disease pressure in Saskatchewan. This assessment of disease risk based on the race structure is supported by field survey data that showed higher incidence of stem canker in Manitoba and Alberta in 2010 and 2011 (Figures 3.6-3.7) compared to Saskatchewan. Manitoba has long been considered in the industry as a blackleg 'hot spot' and the first evidence of new pathogen races (PG3 and PG4) was from southern Manitoba (Fernando & Chen, 2003; Chen & Fernando, 2006).

If we group the 55 races detected in this study into PG groups based on the presence of *AvrLm1*, *AvrLm2*, and *AvrLm3* the results can be compared to the findings of older studies. The PG rating system relies on the differential interaction of three different *B. napus* cultivars, Westar, Quinta, and Glacier (Koch *et al.*, 1991). PG1 is avirulent on Westar, Quinta, and Glacier and is distinguished as a separate species, *Leptosphaeria biglobosa*. PG2, 3, 4, and PGT are designated as the more aggressive species *Leptosphaeria maculans*. PG2 are virulent on 'Westar', but avirulent on both 'Quinta' and 'Glacier' and PGT isolates are virulent on 'Westar' and 'Quinta' but avirulent on 'Glacier'. PG3 isolates are virulent on 'Westar' and 'Glacier' but avirulent on 'Quinta' while PG4 isolates are virulent on all three differential cultivars. The PG system was limited to the resistance found in 'Quinta' and 'Glacier', which carry *Rlm1* and *Rlm2* respectively and both may carry *Rlm3* depending on the seed lot (Balesdent *et al.*, 2002). Since the PG system is limited to a few avirulence genes depending on the differential set adopted, the race classification system was adopted whereby any unique combination of avirulence genes is designated as a separate race (Balesdent *et al*; 2005). In this study, PGT isolates were the most common at 70.92% of the 2010-2011 sample population

followed by PG2, PG3, and PG4 at frequencies of 10.09%, 9.64%, and 9.35% respectively. Comparatively, Kutcher *et al.*(2007) found PG2 isolates to predominate, comprising 81.7% of the 1998-2000 Western Canadian *L. maculans* isolates surveyed followed by PGT at 18% and a single PG3 isolate. Interestingly, they observed that PGT isolates had increased from 9.7% in 1997 to 22.5% in 2000 which predicts to the current results with PGT comprising 70.9% of the population. Similarly, Chen & Fernando (2006) found that PG2 isolates comprised the major western Canadian pathogenicity group from 1984-2004 followed by PGT. Kutcher *et al.*(2010) examining 1997-2005 western Canadian *L. maculans* isolates were the first to report PGT isolates as the dominant pathogenicity group; they reported PGT and PG2 isolates at comprising 53% and 44% of their population respectively. More recently, a survey conducted by Dilmaghani *et al.*(2009) reported PGT isolates as the dominant pathogenicity group in 2005-2006 Western Canadian *L. maculans* isolates and characterized a single PG3 isolate and found no PG4 isolates in contrast to both Chen and Fernando (2006) and Kutcher *et al.*(2010) which detected a few PG3 and PG4 isolates. The increasing trend of PGT isolates first observed by Kutcher *et al.*(2007) and dominant since Dilmaghani *et al.*(2009) has been maintained by this studies finding where PGT isolates increased further to rise to 70.92% of the pathogen population. PGT isolates are avirulent on Glacier (Rlm2, Rlm3) but virulent on Quinta (Rlm1, Rlm3) and thus contain *AvrLm2* but not *AvrLm1* nor *AvrLm3*. This is consistent with a high frequency of *AvrLm2* in western Canada at 80.56% overall. Some 'Quinta' seed lots have been reported as carrying *Rlm4* (Balesdent *et al.,* 2001), which would result in isolates avirulent on Quinta being misclassified as PG2. However, the Canadian Quinta differential line shared between

Kutcher *et al.*(2007 & 2010) and this current study contains *Rlm1* and *Rlm3* but not *Rlm4*. Dilmaghani *et al.*(2009) did not use Quinta as a differential line. The two dominant races in this study that together comprise 45% of the population both lack *AvrLm1* and *AvrLm3* but maintain *AvrLm2*, in general this combination of avrlm1-*AvrLm*2-avrlm3 is most commonly found in L. maculans and explains the high frequency of PGT isolates.

The frequency of *AvrLm6* was determined primarily based on the presence/absence of the PCR gene product. Although the differential cultivar 'Cutlass' was also used, the presence of both *Rlm5* and *Rlm6* in our seed lot and lack of another host with either *Rlm5* or *Rlm6* alone meant that a resistant phenotype may be due to the presence of either *AvrLm5*, *AvrLm6*, or both in the isolate. *Rlm5* was not characterized in this study but Kutcher *et al.*(2007) reported the frequency of *AvrLm5* to be relatively low at 10.4% in western Canadian isolates collected from 1997-2005. While only deletions have been observed to lead to virulence at the *AvrLm1* locus, SNP mutations have been observed to cause virulence at the *AvrLm6* locus in several Australian isolates at a frequency of 2.5% (Van de wouw *et al.*, 2010). Thus, the frequency of "true" *AvrLm6* in western Canada may be slightly lower than the reported value if SNP mutations are taken into consideration. *AvrLmS* was determined through phenotypic interaction with 'Surpass400' which contains *RlmS* that interacts with *AvrLmS* and *LepR3* which provides resistance against isolates carrying *AvrLep3* or *AvrLm1* (Larkan *et al.*, 2012). Due to the lack of another differential carrying only *RlmS*, a total of 92 *L. maculans* isolates with *AvrLm1* could not be characterized for *AvrLmS* with the current differential set.

Most *L. maculans Avr* genes reside in two major gene clusters, the first of which is the *AvrLm1*-2-6 cluster (Balesdent *et al*, 2002). This study found that the frequency of *AvrLm1*, *AvrLm2* and *AvrLm6* to be 13.7%, 80.6% and 89.3%, respectively. The decrease in *AvrLm1* has been significant in western Canada, falling from 46% in 1997-2005 samples (Kutcher *et al*, 2010) to 12.6% in 2005-2006 samples (Dilmaghani *et al*, 2009), and to 13.7% in the 2010-2011 isolates of this study. *AvrLm2* declined moderately from 96.6% in 1997-2005 samples (Kutcher *et al*, 2010), to 87.02% in 2005-2006 samples (Dilmaghani *et al*, 2009), and to 80.56% in the 2010-2011 isolates of this study. *AvrLm6* declined only slightly from 100% in 1997-2005 samples (Kutcher *et al*, 2010), to 94.8% in 2005-2006 samples (Dilmaghani *et al*, 2009), and to 89.3% in the 2010-2011 isolates of this study. The host resistance genes *Rlm1* and *Rlm2* are present in 10% and 1% of Canadian commercial canola cultivars/breeding lines respectively (Zhang *et al*, 2013), but the actual acreage of cultivars carrying these *R* genes is unknown. It is possible that selection pressure along with genetic drift has led to the significant decline in *AvrLm1* and to a lesser extent *AvrLm2*, but this cannot be determined at this point. On the other hand *Rlm6* resistance is derived from *Brassica juncea* and has only been introgressed into experimental lines (Chèvre *et al.*, 1997). *AvrLm6* was at consistently high frequency in western Canada several years ago (Kutcher *et al.* 2010), and is one of the most frequently encountered *Avr* genes in the current study. Stachowiak *et al.* (2006) found *AvrLm6* to be fixed in the *L. maculans* population in Europe. This no longer appears to be the case because increased virulence at *AvrLm6* was observed after repeated uses of *Rlm6* cultivars in the same research plots in France (Brun *et al.* 2010). Dilmaghani *et al.* (2009) found *AvrLm6* to be at 99% and 96% in Chile and western Australia, respectively.

No Canadian cultivars with *Rlm6* were detected during germplasm screening (Zhang *et al,* 2013). In *L. maculans*, a 'hitch-hiking' effect was demonstrated when selection pressure on *AvrLm1* lead to increased virulence at *AvrLm6* in Australia despite the absence of any cultivars carrying *Rlm6* (Van de Wouw *et al.*, 2010). This is postulated to be due to the localization of *AvrLm1* and *AvrLm6* in gene poor regions with transposable elements (Rouxel *et al.*, 2011) where they are subjected to an increased frequency of repeat induced point (RIP) mutations.

The decline in *AvrLm1* in western Canada is most significant in the *AvrLm1-2-6* cluster and the proximity of *AvrLm2* and *AvrLm6* to repetitive DNA may have led to moderate to slight increases of virulence at those loci. In this study, the alignment of 33 *L. maculans* isolates at the *AvrLm1* locus identified 58 SNPs in the conserved region compared with 11 SNPs in a similar alignment of 28 isolates at the *AvrLm6* locus (Table 3.7). This indicates that *AvrLm1* is under increased pressure from RIP mutations which target repetitive DNA as a defense in ascomycota fungi against transposable elements (Rouxel *et al.*, 2011). Since no closely related paralogs exist for *AvrLm1* and *AvrLm6* (Gout *et al.* 2006; Fudal *et al.* 2009) within the genome, RIP mutations are not expected to target these single copy regions directly. RIP mutations target repetitive sequences over 400 bp in size, and consist of transitions from C:G to T:A (Watters *et al.* 1999) that often generate stop codons (Van de Wouw *et al.* 2010). RIP mutations have been reported in ascomycota fungi such as *Neurospora crassa* at distances of at least 930 bp from neighbouring repetitive sequences (Irelan *et al.*, 1994). Thus, the increased frequency of RIP mutations of non-repetitive single genes in *L. maculans*, such as *Avr*

alleles, is likely also due to their localization in gene poor isochores and their proximity to repetitive sequences. The RIP machinery and the location of *Avr* genes within repetitive regions has been suggested to enhance selection pressure at avirulence genes in other Ascomycota Fungi (Watters *et al.,* 1999; Farman *et al.,* 2007). In *L. maculans*, Van de wouw *et al.*(2010) observed that RIP mutations in avirulence genes are proportional to their proximity to flanking repetitive regions. These observations may explain the increased virulence at *AvrLm6* in Canadian *L. maculans* isolates despite the lack of selection pressure directly from cultivars carrying *Rlm6.*

The linkage of *AvrLm1-2-6* presents an interesting trend in terms of race structure; 90% of *L. maculans* isolates in western Canada that carried *AvrLm1* lacked *AvrLm2* and vice versa. Only 3.4% of isolates carried both *Avr* genes, while 6.5% lacked both *AvrLm1* and *AvrLm2.* The low frequency of *AvrLm1* at 13.7% in western Canada may have contributed to the relatively high frequency of *AvrLm2* (87.0%). This low rate of recombination may be explained by the observation of Ghanbarnia *et al.* (2009) that asexual pycnidiospores are the primary source of inoculum in western Canada. An inverse relationship between *AvrLm1* and *AvrLm2* is also found in Western Australia where Dilmaghani *et al.*(2009) reported the opposite, with *AvrLm1* at 91% and *AvrLm2* at 1%. *AvrLm6* did not significantly correlate with the presence or absence of either *AvrLm1* or *AvrLm2* in the current study. However, an experimental trial in France with recurrent sowing of *Rlm6* cultivars resulted in a decrease of *AvrLm6* frequency that correlated with an increase in *AvrLm1,* despite no direct selection pressure at *Avrlm1* (Brun *et al.,* 2010). The linkage of these genes likely plays a role in the inverse relationship. While the

fitness cost may be the first assumption, Dilmaghani *et al.*(2009) also found that almost all Chilean isolates lacked both *AvrLm1* and *AvrLm2;* both *Avr* genes were at <1% in 128 *L. maculans* isolates. This indicates that the frequency of both *AvrLm1* and *Avlm2* can be suppressed simultaneously given the selection pressure at both loci. Both *AvrLm*1 and *AvrLm*2 have been cloned and are not two variant alleles of the same gene (Gout *et al.,* 2006; Ghanbarnia *et al.,* 2014).

AvrLm4-7 is a single *Avr* gene that interacts with both *Rlm4* and *Rlm7*. Virulence to *Rlm4* is gained through a SNP mutation while virulence to *Rlm7* is gained through a deletion of *AvrLm4-7* (Parlange *et al.,* 2009). Our study did not find any isolates that were avirulent on *Rlm4* but virulent on *Rlm7,* while the reverse was relatively common. This supports the position by Parlange *et al.* (2009) that a deletion of *AvrLm4-7* results in virulence at *Rlm7* but a SNP mutation can cause virulence at *Rlm4*, and this is consistent with this study. The alignment of 28 western Canadian *L. maculans* isolates at the *AvrLm4-7* locus led to 118 SNPs in the conserved region and 8 alleles among the isolates (Table 3.6 & 3.7). Similar alignments lead to 58 SNPs and 3 haplotypes for *AvrLm1* and 11 SNPs and 4 haplotypes for *AvrLm6*. This demonstrates that the *AvrLm4-7* gene is subject the greatest amount of genetic variation among the 3 *Avr* genes sequenced, and yet this cannot be attributed to selection pressure since no *Rlm4* or *Rlm7* cultivars have been identified in Canadian commercial cultivars (Zhang *et al.*, 2013). *Rlm4* is the only resistance gene overcome primarily through a SNP mutation and the increased frequency of SNPs in the *AvrLm4-7 Avr* gene, in comparison to *AvrLm1* and *AvrLm6,* may explain this observation. Aside from *AvrLm4-7*, *AvrLm1* is the only other known avirulence gene

that interacts with two resistance genes, *Rlm1* and *LepR3*. *AvrLm1* should more accurately be referred to *AvrLm1-LepR3* to maintain consistent nomenclature.

The second major linkage group of *L. maculans* avirulence genes is the *AvrLm 3-4-7-9-AvrLepR1* cluster (Balesdent *et al.*, 2005, Ghanbarnia *et al.*, 2012). In this study *AvrLm3*, *AvrLm4*, *AvrLm7*, *AvrLm9*, and *LepR1* were observed at frequencies of 8.01%, 71.81%, 89.67%, 1.48%, and 16.02% respectively. The Frequency of *AvrLm3* dropped substantially in western Canada from 52.8% in 2005-2006 samples (Dilmaghani *et al.*, 2009) to 13.7% in the 2010-2011 isolates of this study. Selection pressure is likely the most significant factor since the most common resistance gene in Canadian cultivars is *Rlm3;* it is present in the majority of canola varieties (Zhang *et al.*, 2013). On the other hand, the frequency of the *AvrLm4-7* allele rose from 25% in 1997-2005 samples (Kutcher *et al.*, 2010) to 47.23% in 2005-2006 samples (Dilmaghani *et al.*, 2009), and to 89.76% in the 2010-2011 isolates of this study. The *avrlm4-AvrLm7* allele also rose. The increase of the *AvrLm4-7 gene* is too rapid to be due to random drift and *AvrLm4-7* isolates may have presumably been under positive selection pressure. When examining the race structure of *AvrLm3* and *AvrLm4-7*, we noticed that the same exclusivity of genes seen with *AvrLm1* and *AvrLm2*, about 96% of isolates examined carried one of *AvrLm3* or *AvrLm4-7*, and only 0.1% carry both *AvrLm3* and *AvrLm4-7* while 3% carry neither genes. This observation along with the rapid increase of *AvrLm4-7* supports the notion that the decline in *AvrLm3* led to the increase of *AvrLm4-7* in the Western Canadian *L. maculans* population. The prevalence of *Rlm3* in the Canadian commercial cultivars selected against isolates with *AvrLm3*, almost all of which did not carry

AvrLm4-7 and selected positively for isolates lacking *AvrLm3*, almost all of which carried *AvrLm4-7*. We see another rapid decline in *AvrLm9*, from 60.4% in 1997-2005 samples (Kutcher *et al.*, 2010) to 56.4% in 2005-2006 samples (Dilmaghani *et al.*, 2009), and to 1.5% in the 2010-2011 isolates of this study. All isolates with *AvrLm9* in this study also carried *AvrLm3*. Thus the decline of *AvrLm9* may also be attributed to negative selection at *AvrLm3*, and potential linkage of *AvrLm3* and *AvrLm9* in *L. maculans*. *AvrLm9* was reported as rare outside of central Canada (Hayward *et al.*, 2012), and the consistently low frequency in different canola growing regions as opposed to variation in the other *Avr* genes loci suggests that there is no fitness cost associated with the loss of this *Avr* gene.

AvrLepR1 is the last *Avr* gene in the *AvrLm 3-4-7-9-AvrLepR1* gene cluster and was observed at a relatively low frequency of 16.0%. The corresponding resistance gene, along with the other '*LepR*' resistance genes were first identified in re-synthesized *B. napus* lines that arose from *B. rapa* ssp. *sylvestris* × *B. oleracea* var. alboglabra interspecies cross (Crouch *et al.*, 1994). *LepR2* was also characterized in this study but surprisingly only a single isolate from 2010-2011 was found to carry this *Avr* gene. Several 2012 isolates were found to carry *AvrLepR2* and all isolates with *AvrLepR2* have only been found in Manitoba. The resistance phenotype of AAFC introgression lines '1065' (*AvrLepR1)* and '1135' *(AvrLepR2)* is muted compared to newer LepR1 and LepR2 introgression lines developed by AAFC and thus the frequency of these two resistance genes is likely underreported (N. Larkan, personal communication, 2014). The

host LepR3 resistance gene was shown to recognize *AvrLm1* in *L. maculans* rather than a separate *AvrLepR3* gene (Larkan *et al.*, 2012).

AvrLmS is the last avirulence gene characterized in this study, and it was detected in 54.8% of the *L. maculans* isolates surveyed. *AvrLmS* was characterized by Kutcher *et al.*(2010) with 'Surpass400' although it was mislabeled as *AvrLepR3* due to the confusion over the function of resistance genes carried by 'Surpass400' up until *LepR3* was cloned (Larkan *et al.*, 2012). Kutcher *et al.*(2010) estimated *AvrLmS* at 97% frequency in 47 1997-2005 isolates that did not carry *AvrLm1* compared to just 54.8% in the 2010-2011 *L. maculans* isolates of this study. The decline in *AvrLmS* is significant. The small sample size of the earlier study, the lack of mapping data, and the lack knowledge regarding the presence of *RlmS* in Canadian commercial cultivars make it difficult to infer anything more on the decline of *AvrLmS* in western Canada.

Overall the results indicate that the race structure of *L. maculans* may play a role in the frequency of *Avr* genes observed; an *Avr* gene can experience indirect selection pressure if the race also carries additional *Avr* genes that interact with *R* genes in canola cultivars. The significant rise in *AvrLm7* is likely due to >95% of races carrying the avrlm3-*AvrLm4-7* combination and the wide use of *Rlm3* in Canadian canola cultivars. The prevalence of certain avirulence gene combinations may be due to avirulence gene clusters reducing recombination between proximal genes, fitness cost (Huang *et al.*, 2006), or the result of initial founder race combinations and lower rates of sexual pseudothecia in Canada (Ghanbarnia *et al.*, 2009). The complexity of *Avr* genes in

western Canada varied from a single to seven *Avr* genes. Although the extremes were less frequent, there were about 25 isolates that carried 1 or 6 *Avr* genes, while the majority of the races carried 4 or 5. The average complexity varies slightly provincially, declining from 4.36 in Saskatchewan, to 4.22 in Alberta to 4.02 in Manitoba. The results in summary indicate that on average Manitoba isolates are more virulent due to an increased number of races and decreased average number of avirulence genes per isolate. This is supported by higher disease levels in these provinces compared to Saskatchewan in 2010 and 2011 field surveys (Figures 3.6-3.7). In 2011 the pathogen complexity rose significantly from an average of 4.03 in 2010 to 4.38 in 2011, making the average isolate slightly less virulent. Field survey data also indicates decreased stem canker incidence in 2011 compared to 2010 (Figures 3.6 & 3.7). However, only Saskatchewan reported decreased prevalence of blackleg from 2010 to 2011 while blackleg spread slightly in Manitoba and Alberta. In terms of avirulence genes, only four avirulence genes changed significantly in one year. The frequency of *AvrLm1*, *AvrLm2*, *AvrLm6*, and *AvrLepR1* shifted from 5.87%, -6.43%, 11.76%, and 12.5% respectively from 2010 to 2011. Interestingly, *AvrLm2* decreased by approximately the same frequency that *AvrLm1* increased which is consistent with the previous observations that most *AvrLm2* isolates lack *AvrLm1* and vice versa. *AvrLm6* also rose significantly, which may be due to its proximity to the other two avirulence genes as part of the *AvrLm1-2-6* genetic cluster. Van de Wuow *et al.*, (2010) previously found that a decline in *AvrLm1* also led to a decline in the frequency of *AvrLm6*, and a similar but opposite effect may be occurring.

Differences in *Avr* gene frequency were observed between the Prairie Provinces. Except for *AvrLm3*, *AvrLm9* and *AvrLepR2*, which were consistently low in each province, most of the other *Avr* genes varied slightly in frequency. *AvrLm2* had the greatest variation in frequency among provinces, at 97% in Saskatchewan, 84% in Alberta and 65% in Manitoba (Figure 3.8). *AvrLepR1* was also varied among provinces from 34% in Alberta to 6% in Saskatchewan. These differences in *Avr* gene frequency were even more dramatic when the five selected field sites were examined (Figure 3.9). The frequency of *AvrLm1* and *AvrLm2* showed the greatest variation from 0 to 63 % and 22% to 100%, respectively. *AvrLm3* was very low or absent at most sites with the exception of Melfort, where its frequency was 37%. *AvrLm7*, *AvrLm9* and *AvrLepR2* varied little among the five sites. Generally, greater differences were observed between sites in different provinces, although there were exceptions. These differences may be caused by local selection pressure and it would be interesting to pair pathogen race structure with the resistance genes in canola cultivars grown in these fields in future studies. The regional and provincial variation demonstrates that a single specific resistance gene would not provide effective control of blackleg at all sites across the Prairie Provinces except perhaps *Rlm6* and *Rlm7*, which consistently occurred at high frequencies in this study.

The number of *L. maculans* races detected and the geographic variation in *Avr* gene frequency indicates a diverse *L. maculans* population in western Canada. Since only *Rlm3* was found commonly in Canadian canola cultivars (Zhang *et al.*, 2013), the presence of virulent genes in isolates against resistance genes (*Rlm6*, *Rlm9*) not widely

used in commercial cultivars points to naturally existing virulent races in the absence of selection pressure. The frequency of avirulence genes varies widely such that the frequency of the most common avirulence gene is greater than the frequency of the five least common avirulence genes. This means that *Rlm7* would provide resistance against a greater proportion of the pathogen population than 4 weaker resistance genes combined: *Rlm1, Rlm3, Rlm 9, and LepR2.* While a cultivar with six resistance genes would generally be considered to be more durable, in Canada the two most common races lack the five least frequent avirulence genes. When races virulent to multiple resistance genes are present, mutation events have already occurred, and the durability of resistance is dependent on the balance between any fitness costs associated with the absence of avirulence genes and selection pressure.

Given that *L. maculans* can lose *Avr* genes to gain virulence, often with no fitness cost, sustained use of any single resistance gene will result in the loss of its effectiveness. This has been observed in western Canada with the increased of blackleg disease reported from surveys on varieties previously rated as resistant. Resistance genes may be stacked into a single variety to provide protection against multiple races of the pathogen, and this strategy would be most effective if there is a fitness cost associated with the pathogen carrying multiple virulence genes or virulent races are absent at the time of variety deployment. The presence of low frequency races virulent on stacked resistance risks the loss of multiple resistance genes simultaneously when selection pressure degrades resistance. Based on this study, stacking *Rlm6* and *Rlm7* would be effective against all races examined in western Canada. Similarly, stacking *Rlm2-7-LepR1* would provide

resistance against all isolates surveyed. In this case of *Rlm6* and *Rlm7* it would be advisable to stack these two genes together. Races able to infect *Rlm6* and *Rlm7* separately are present but no race was detected that can infect a cultivar carrying both *Rlm6* and *Rlm7* within our sample population. Stacking similar combinations such as *Rlm2-7-LepR1* would also work well. However, if virulent races are present in field populations, selection pressure and fitness cost would be the primary factors that determine how long until the virulent races dominate. In this study the combination of *Rlm1* and *Rlm2* would be more effective in western Canada than any single resistance gene. There are however 6.5% of isolates that lack both *AvrLm1* and *AvrLm2,* and long term deployment of stacked *Rlm1* and *Rlm2* could lead to a scenario such as in Chile where both *AvrLm1* and *AvrLm2* were <2% within 128 *L. maculans* isolates surveyed by Dilmaghani (2009). In this example, the second competing strategy of rotating resistance genes similar to rotating fungicides or herbicides would more likely increase the longevity of *Rlm1* and *Rlm2.* In western Canada, most blackleg isolates either carry *AvrLm1* or *AvrLm2,* fewer than 7% of the isolates lack both *AvrLm1* and *AvrLm2.* This may be a case where rotation of the resistance gene *Rlm1* and *Rlm2* would be more effective than stacking them to avoid a scenario found in Chile where both *AvrLm1* and *AvrLm2* were reported at <2% (Dilmaghani *et al.,* 2009). The race composition results also support rotating *Rlm3* with *Rlm4* or *Rlm7*. Rotating crops with different blackleg resistance has been shown to be effective in field experiments (Marcroft *et al.,* 2012).

In practice, it becomes difficult to separate the activities of stacking blackleg resistance genes and rotating varieties such that they become competing strategies.

Commercial crops would be disadvantaged if they do not carry multiple resistance genes due to the risk of resistance breakdown. Conversely, stacking too many resistance genes when virulent races are present at a detectable frequency would only risk losing the effectiveness of multiple resistance genes simultaneously. The compromise seems to be in balancing the number of R-genes used in commercial varieties and in selecting the right combinations for stacking and rotation. In western Canada, rotating *Rlm1* with *Rlm2* or rotating *Rlm3* with *Rlm4* or *Rlm7* would be preferable since the deployment of one of them strengthens the effectiveness of the other. Stacking *Rlm1* with *Rlm2,* or *Rlm3* with *Rlm4* or *Rlm7* risks increasing the frequency of isolates virulent on both resistance genes. Alternatively, stacking *Rlm1* with *Rlm4* instead of *Rlm1* with *Rlm2* would provide approximately the same level or resistance while maintaining the ability to rotate between *Rlm1* and *Rlm2*. Since avirulence genes in the *L. maculans* genome are clustered, *AvrLm1-2-6* and *AvrLm3-4-7-9-LepR1*, stacking resistance genes genetically distant is more advisable than those in proximity to reduce the risk of mutations and to take advantage of interactions of avirulence genes within clusters that can be exploited for rotation. In this case, *Rlm1* with *Rlm3* or *Rlm2* with *Rlm6* would present favorable stacking combinations in western Canada. Most avirulence genes have no observable correlation in terms of race structure and either stacking or rotation may work equally well. In some cases, a single *L. maculans Avr* gene interacts with multiple resistance genes in *B. napus,* such as *AvrLm1* that can be detected by both *Rlm1* and *LepR3,* and *AvrLm4-7,* which is detected by both *Rlm4* and *Rlm7*. In these cases, stacking *Rlm1* and *LepR3* or *Rlm4* and *Rlm7* would not be advisable since a single gene deletion in the pathogen would render two resistance genes ineffective.

The results of this study indicate that the *L. maculans* population in western Canada is diverse and consists of dozens of races, although two are dominant. The race combination and changes observed over earlier studies suggest that selection pressure against some resistance genes may alter the frequency of other *Avr* genes and this may be exploited in planning the deployment of resistance genes. The potential exists for using field survey data of pathogen race structure to guide breeding efforts and mitigate the risk of widespread resistance erosion. The geographic variation in race structure and some virulence at all avirulence gene loci means that no single R gene can perform consistently well against blackleg in all regions on the Canadian prairies. The linkage of *Avr* genes in the pathogen and the interplay between races and different resistance genes under selection pressure are two key factors to consider when breeding for blackleg resistance.

CHAPTER 4

GENETIC DISTANCE BETWEEN GLOBALLY ISOLATED SUBPOPULATIONS OF THE FUNGAL PATHOGEN *LEPTOSPHAERIA MACULANS*

4.0 Abstract

The fungal pathogen *Leptosphaeria maculans* is the causal agent of phoma stem canker (aka blackleg) and is an economically important disease affecting oilseed rape and canola (*Brassica napus* L.). This pathogen is a growing concern for the global canola industry and due to the emergence of new races it has adapted to several major resistant varieties. Reports of this fungus were first recorded in the early 1900's on cabbage in Wisconsin, USA and following the breeding of oilseed rape as a major crop for human consumption and bio-fuel production, it has since spread to major canola growing regions through natural dispersal and trade. It is now found in most countries where Brassica spp. are cultivated and in the last four decades *L. maculans* has caused significant yield losses in Canada, Europe, and Australia. Moreover there are indications that *L. maculans* is an expanding species displacing the less aggressive *Leptosphaeria biglobosa* which has historically colonized oilseed rape crops. The risk of spread to non-host countries (i.e. China) and growing yield losses raises question about the origin of the species and the genetic diversity between different geographic regions. Twenty two microsatellite primers designed from the reference genome of *L. maculans* were used to screen 96 isolates collected from 8 different countries. Over 200 unique alleles were observed and used to generate a phylogenetic tree to assess the evolutionary relationship between isolates. Overall, genetic diversity was correlated with geographic location.

4.1 Introduction

Leptosphaeria maculans is an ascomycete fungus responsible for stem canker of crucifer crops. The fungus causes Phoma stem canker, commonly known as blackleg and has become an economically important disease of canola (*Brassica napus L*.). It was thought to be caused by one species comprising a more aggressive and less aggressive type distinguished by factors such as the production of the phytotoxin sirodesmin PL and the ability to infect canola (Balesdent *et al.* 1992; Gugel and Petrie, 1992). The disease was later distinguished into a complex of at least two species *Leptosphaeria maculans* and *Leptosphaeria biglobosa*. *L. biglobosa* is most commonly observed on upper stem lesions that are less damaging to the plant (Rouxel & Balesdent, 2005). Both species have a wide host range and *L. maculans* has been cultured from several cruciferous weeds such as *Sisymbrium* spp., *Descurainia* spp., and *Erysimum* spp. (Williams & Fitt, 1999). *L. maculans* "brassicae" (referred to as *L. maculans* henceforth) is the major cause of yield loss to the oilseed crop canola. Little is known about the genetic diversity of *L. maculans*. The first well documented epidemic of Blackleg, identified as *phoma lingam*, was in Wisconsin, USA early in the 20th century (Henderson, 1918). The first report of *L. maculans,* the aggressive species responsible for most of the yield losses today, was in the prairies in Saskatchewan (McGee & Petrie, 1978). *L. maculans* was later found in eastern Canada in Ontario (Peters &Hall, 1987). *L. maculans* also causes severe losses in Europe and Australia and is adaptable to a range of different climates and is able to infect both spring and winter oilseed rape (West *et al.* 2001).

While both *L. biglobosa* and *L. maculans* occupy similar ecological niches, *L. maculans* is a newer species that has moved into areas where only the less damaging *L. biglobosa* was previously present (Fitt *et al.* 2006). The spread of *L. maculans* across Canada (1975-1998), and Eastward across Europe into Poland (1994-2007) has led to calls for it to be designated as an invasive species (Fitt *et al.* 2008, Dilmaghani *et al.* 2012). The spread of *L. maculans* has been favored by climate change and recent trends in agriculture such as reduced crop diversity and larger acreages of genetically homogeneous crops. It is likely that *L. maculans* has undergone several introduction events into new geographic areas through a combination of trade and natural dispersal. *L. maculans* is present in the major oilseed rape centers of the world but has yet to be identified within China, the country with the largest acreage of cultivated brassica where only *L. biglobosa* has been reported. The movement of phytopathogenic fungi into new areas is the major cause of the emergence of novel fungal diseases (Anderson *et al.,* 2004). Despite this, the origin, introduction, and genetic diversity of many phytopathogenic fungi are unknown. Investigating the genetic diversity of fungal species can be used to infer the origin of populations and to assess the evolutionary potential and success of introduced species (Gladieux *et al.,* 2010; Prentis *et al.* 2008).

There have been a several efforts to investigate the genetic variation within *L. maculans* populations around the world. The first publications used techniques such as AFLP, RFLP, and RAPD (Goodwin and Annis 1991; Jedryczka *et al.* 1999; Pongam *et al.* 1999; Purwantara *et al.* 2000). The main concern with these early approaches was that they were either not PCR based or generated complex banding patterns that did not allow

clear distinction. Techniques using variable number tandem repeat loci (VNTR) such as minisatellite and microsatellites are the most common for genetic distance studies and a recent paper based on 11 minisatellite markers was published (Dilmaghani *et al.* 2012). This project builds on previous work and reports the genetic diversity of *L. maculans* from eight different countries using 22 polymorphic microsatellite markers.

4.2 Materials and Methods

4.2.1 Canola sample collection

Leptosphaeria maculans isolates were received from various collaborators as dried filter disks or agar plugs. The cultures were received from the USA, Argentina, Brazil, United Kingdom, France, Poland, and Australia. Canadian canola samples were selected from the *L. maculans* pathogen collection at the University of Manitoba, Canada.

4.2.2 Fungal isolation

Fungal samples were re-isolated from agar plugs and dried filter disks. Preserved samples were plated onto V-8 juice agar [200 ml V8 juice (Campbell Soup Company Ltd. Toronto, ON), 800 ml distilled water, 15 g agar and 0.75 g calcium carbonate, 0.1 g streptomycin sulfate]. The petri dishes were placed under a light bench with cool white fluorescent light (100-150 E m^{-2} sec^{-1}) at 22-24℃ for 4-7 days. Fungal cultures were established by transferring spores from the ooze of a single pycnidia to V8-juice agar in a Petri dish under a dissecting microscope.

4.2.3 Preparation of Fungal inoculum and DNA samples

Pycnidiospores were harvested by flooding *L. maculans* cultures with sterile distilled water, and scraping with a bent glass rod to dislodge spores. Spore suspensions were filtered through Miracloth into 50-ml sterile centrifuge tubes (Fisher Scientific, Pittsburgh, PA). The concentration was estimated using a hemocytometer (Hausser Scientific Company, Horsham, PA), and adjusted to 1×10^{-7} spores ml^{-1} and stored in sterile microcentrifuge tubes at -20^{o}C until use. The remaining mixture of hyphae, pycnidia, and spores on the agar plates were scraped off with a spatula and placed in sterile 1.5 ml microcentrifuge tubes for DNA extraction.

4.2.4 DNA Extraction and PCR

Extraction of DNA from purified *L. maculans* isolates used a mixture of pycnidia, conidia and hyphae harvested from 8-12 day old single-spore cultures. DNA was extracted using a modification of the procedure developed by Lee & Taylor (1990). The samples were mixed with a lysis buffer (Tris, EDTA, SDS and NaCl), lysed with mechanical beads at 5000 rpm for 30 seconds, incubated at 65°C for 30 minutes, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and precipitated with 5 M NaCl. Following the final centrifugation, the DNA pellet was dissolved in 100 µL of autoclaved distilled water.

PCRs were performed on DNA samples from *L. maculans* in 10uL reactions [buffer 0.55uL; dNTP 0.45uL; MgCL2 0.45uL; m13 label 0.1uL; forward primer 0.05uL; reverse primer 0.15 uL, Taq 0.15uL; DNA 1 uL (30–80 ng/uL; 7uL water). PCR was run

on the ABI 3130XL with the following profile: 1 cycle of 3 mins @ 95oC; 10 cycles of 45sec @ 95oC, 30 secs at 65ºC-1ºC/cycle, 1min @ 72oC; 35 cycles of 45sec @ 95oC, 30 secs at 65ºC, 1min @ 72oC; 1 rep of 5mins @ 72oC. M13 tail was amended to forward primer sequences to allow multiplexing with 4 dyes.

4.2.5 Microsatellite identification and distance tree construction

MISA (microsatellite identification tool) was used to identify microsatellite sequences from among the 76 supercontigs of the sequenced French isolate JN3 (Rouxel *et al.* 2011). Microsatellite search parameters required a minimum of 6 repeats for 2bp SSRs and 5 repeats for 3bp+ SSRs. BatchPrimer3 (You *et al.* 2008) was used to design primer sequences and 192 potential SSRs were screened with 8 *L. maculans* isolates each from a different country to detect polymorphism. PCR products were run on the ABI 3130XL to determine PCR product size and computed on the ABI Gene mapper software. Values were exported to Genographer (Benham *et al.* 1999) and peaks were scored and tabulated. Twenty two Polymorphic markers were selected and run on 96 isolates from eight countries to determine genetic distance. SSR data points were formatted and imported into Poptree2 (Takezaki *et al.* 2010). Poptree2 calculated genetic distance based on the distance model by Goldstein *et al.* (1999) and neighbor joining clustering. The distance data was exported to MEGA 6 (Tamura *et al.* 2007) to generate an unrooted phylogenetic tree.

4.3 Results

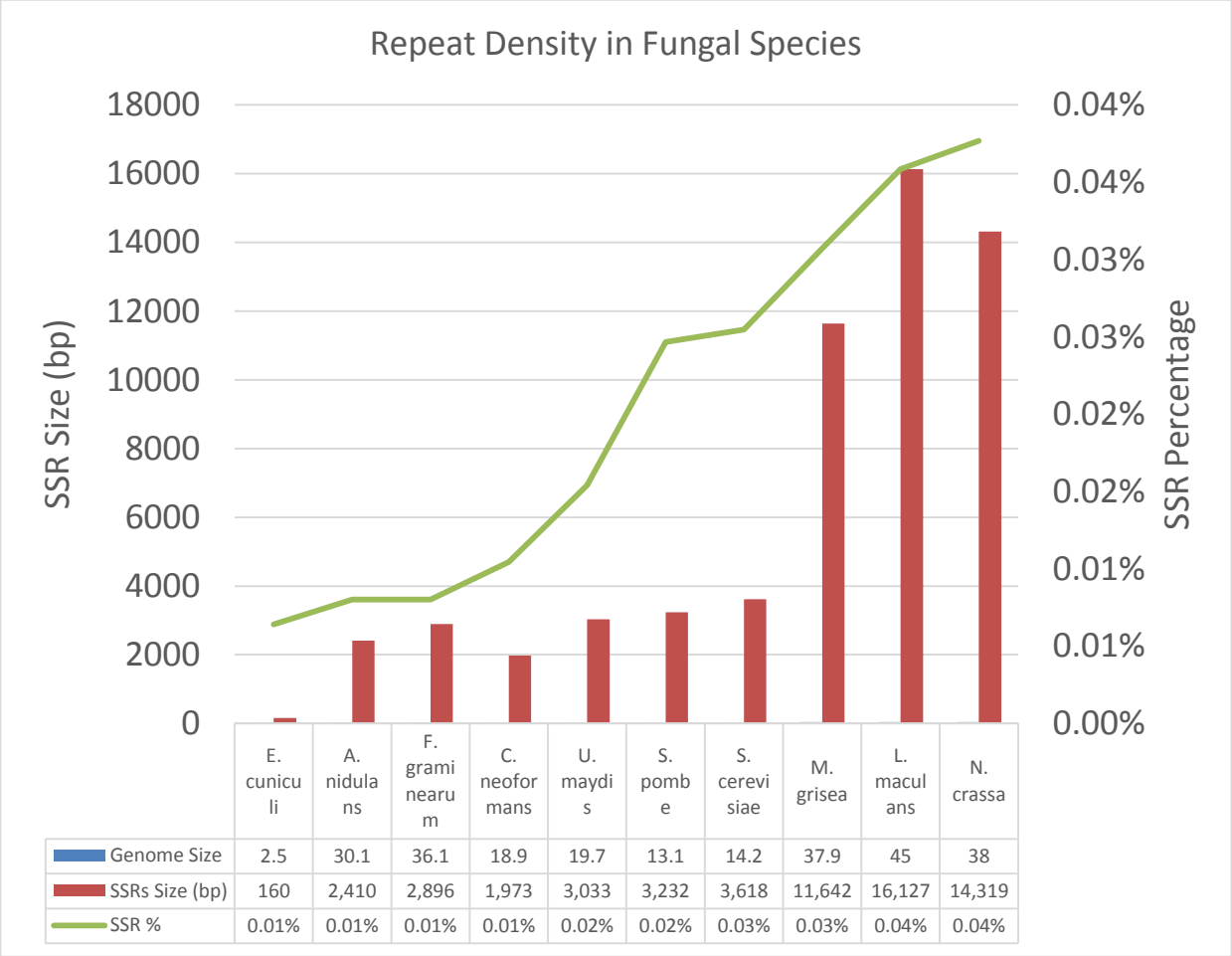
4.3.1 *L. maculans* SSR distribution and frequency

The ~45Mb genome of *L. maculans* contains over 5000 microsatellites comprising ~0.04% of the genome (Table 4.1). While the microsatellites are relatively abundant they are not evenly distributed and 10 supercontigs only carried a single SSR. Most of these SSRs were 2 or 3 bp repeats and relatively few 4-6bp SSRs found.

Table 4.1: Number and distribution of microsatellite sequences in the *Leptosphaeria maculans* genome

Microsatellite Parameters (unit size (bp) / minimum number of repeats): (2/6) (3/5) (4/5) (5/5) (6/5)		
Total number of sequences examined:	76	
Total size of examined sequences (bp):	45124619	
Total number of identified SSRs:	5650	
Number of SSR containing sequences:	43	
Number of sequences containing more than 1 SSR:	33	
Distribution to different repeat type classes		
Unit size	Number of SSRs	Size (bp)
2bp	2373	4746
3bp	2447	7341
4bp	363	1452
5bp	214	1070
6bp	253	1518
Total	**5650**	**16127**

The amount and proportion of SSRs in the genome of *L. maculans* was compared with 10 other fungal species: *Aspergillus nidulans, Cryptococcus neoformans, Encephalitozoon cuniculi, Fusarium graminearum, Magnaporthe grisea, Neurospora crassa, Saccharomyces cerevisiae, Schizosaccharomyces pombe,* and *Ustilago maydis*. The SSR results of *L. maculans* were amended to the data generated by Karaoglu *et al.* (2006) and *L. maculans* was observed to contain the greatest amount of SSRs and second largest as a percentage of its genome (Figure 4.1)



	E. cuniculi	A. nidulans	F. graminearum	C. neoformans	U. maydis	S. pombe	S. cerevisiae	M. grisea	L. maculans	N. crassa
Genome Size	2.5	30.1	36.1	18.9	19.7	13.1	14.2	37.9	45	38
SSRs Size (bp)	160	2,410	2,896	1,973	3,033	3,232	3,618	11,642	16,127	14,319
SSR %	0.01%	0.01%	0.01%	0.01%	0.02%	0.02%	0.03%	0.03%	0.04%	0.04%

Figure 4.1: Comparison of genomic repeat DNA size and proportion among 10 pathogenic fungal species. Amended from Karaloglu *et al.* (2006).

4.3.2 SSR Polymorphism in *L. maculans*

This study used a total of 22 *L. maculans* SSR markers from 196 potential SSRs that were screened for polymorphism. The 22 SSRs spanned 10 supercontigs. The sequence and primer details are listed in Table 4.2.

Table 4.2: Sequence and primer details of 22 selected polymorphic *Leptosphaeria maculans* microsatellite markers

#	CODE	SUPERCONTIG	MOTIF	SSR LENGTH	ORIENTATION	START	LEN	TM	GC%	PROD SIZE	PRIMER SEQUENCE (5'-3') (M13 TAIL FORWARD PRIMERS)
1	4RF	70	aca	18	FORWARD	1478	21	54.66	42.86	130-141	CACGACGTTGTAAAACGACGGTAGACACTGCTTGGTTTTA
	4RR	70	aca	18	REVERSE	1591	21	56.6	38.1		TAACGGTGTTAAAGGCTTTGT
2	2BF	18	ct	26	FORWARD	68352	20	55.49	50	181-192	CACGACGTTGTAAAACGACGGGACATGAGCTAACATGAC
	2BR	18	ct	26	REVERSE	68508	20	55.31	45		ATGATAGCCATGACAAGAGC
3	1BF	24	ag	20	FORWARD	159871	21	54.35	38.1	172-180	CACGACGTTGTAAAACGACAAAATTATACAGGCCTTCTCC
	1BR	24	ag	20	REVERSE	160032	21	55.47	42.86		TGTTAGTGAGGATGTTCTTGC
4	12BF	24	tc	24	FORWARD	164395	21	54.2	42.86	166-171	CACGACGTTGTAAAACGACCATACATGTAGACCATGTGGA
	12BR	24	tc	24	REVERSE	164542	21	54.79	42.86		GATGTGATGTGATGTATGCAG
5	6BF	25	tg 45	24	FORWARD	45175	20	55.01	50	204-215	CACGACGTTGTAAAACGACGAGTTGTGGGGATGAGAGTA

	6BR	25	tg 45	24	REVERSE	45355	21	54.93	42.86		ATACAGCAAAGGTGACAGGTA
6	4GF	31	ga	24	FORWARD	107716	21	55.15	38.1	209-223	CACGACGTTGTAAAACGACGATTGGTAGCCTTTTGAAGAT
	4GR	31	ga	24	REVERSE	107909	21	55.31	42.86		AATCTTCTACCGAGATTCGAG
7	3YF	13	ac	20	FORWARD	10773	21	55.16	42.86	238-255	CACGACGTTGTAAAACGACGTGGTTGCTATCATCTTGTGT
	3YR	13	ac	20	REVERSE	11001	21	54.85	33.33		TGTTGTCAAATGTTGTGTGTT
8	8BF	13	tgg	32	FORWARD	10129	21	55.2	47.62	198-208	CACGACGTTGTAAAACGACGGGAGTAAGTGTGAGTGTGAA
	8BR	13	tgg	32	REVERSE	10304	21	54.64	38.1		CTTTAACTGTTGTGGAAGGAA
9	4YF	13	ca	28	FORWARD	76599	21	55.66	47.62	239-250	CACGACGTTGTAAAACGACAGTACATCTTGGAGTGGGAAC
	4YR	13	ca	28	REVERSE	76831	21	54.9	42.86		GATCTATCTGATCGACCCTTT
10	7YF	26	ct	22	FORWARD	149583	21	54.93	42.86	285-293	CACGACGTTGTAAAACGACGCAATGCATATACACACACAC
	7YR	26	ct	22	REVERSE	149852	20	54.63	40		GCTAATCAATGTCTGGCTTT
11	12YF	0	ag	26	FORWARD	160690	21	56.07	47.62	246-256	CACGACGTTGTAAAACGACGCTAAGCAACTCAGCACTGTA
	12YR	0	ag	26	REVERSE	160925	21	54.95	52.38		TCCACTCTTACCTACCCTACC

12	5YF	25	tg	22	FORWARD	36248	21	55.07	52.38	285-296	CACGACGTTGTAAAACGACTACTCTGCTCTCTCCTCTCCT
	5YR	25	tg	22	REVERSE	36517	21	55.46	52.38		CACCAACCCACTACTACACAC
13	4-1-F	25	ca	28	FORWARD	287130	20	54.79	35	162-165	CACGACGTTGTAAAACGACAATCCAATTGACAAACATCC
	4-1-R	25	ca	28	REVERSE	287274	21	55.33	47.62		TGTGTGTGTGGATCTGTAG
14	4-2-F	24	ag	28	FORWARD	148527	21	55.02	42.86	176-183	CACGACGTTGTAAAACGACTGAAACTCTCTTAGCATCGAG
	4-2-R	24	ag	28	REVERSE	148691	21	55.57	57.14		GTACTCCCACTCCACTCTAGC
15	6-2-F	17	ag	20	FORWARD	157258	21	54.92	38.1	212-226	CACGACGTTGTAAAACGACAGTTGGAGACAACTTGTTGAA
	6-2-R	17	ag	20	REVERSE	157460	21	55.01	42.86		GGAGGAGGAAAGGTATTTGTA
16	1-3-F	17	ct	28	FORWARD	179510	21	55.2	42.86	192-203	CACGACGTTGTAAAACGACAGGAAATAAGCTGTGGGATAG
	1-3-R	17	ct	28	REVERSE	179686	21	55.22	38.1		AAGATTCATGTCACGATTGAG
17	3-3-F	31	gt	30	FORWARD	108249	21	54.95	52.38	226-236	CACGACGTTGTAAAACGACCAGGTACTGTCTGTGTGTGTG
	3-3-R	31	gt	30	REVERSE	108453	21	55.42	52.38		CTCCCACTTGTAACTCTACCC
18	6-3-F	5	ga	30	FORWARD	55149	21	55.26	42.86	267-284	CACGACGTTGTAAAACGACGTGCTATTCTCGGGTAAGTTT

	6-3-R	5	ga	30	REVERSE	55381	21	55.07	42.86		GATGCAAGTGTCCATGTCTAT
19	4-3-F	18	ac	18	FORWARD	92138	21	55.77	42.86	353-358	CACGACGTTGTAAAACGACACACATCACAAATCACACTCC
	4-3-R	18	ac	18	REVERSE	92330	21	55.02	47.62		ACCATATACACACCACTCTGC
20	2GF	29	tag	18	FORWARD	127543	20	55.96	50	231-238	CACGACGTTGTAAAACGACAGGAGCTCTAGGTGCCTTTA
	2GR	29	tag	18	REVERSE	127754	21	55.13	47.62		CCCTAAGCTTGCCTAATACTC
21	2-4-F	5	ga	30	FORWARD	55149	21	55.26	42.86	318-326	CACGACGTTGTAAAACGACGTGCTATTCTCGGGTAAGTTT
	2-4-R	5	ga	30	REVERSE	55381	21	55.07	42.86		GATGCAAGTGTCCATGTCTAT
22	3-4-F	5	ct	24	FORWARD	63157	20	54.3	40	260-263	CACGACGTTGTAAAACGACACCAAATACAATCAGGCTTC
	3-4-R	5	ct	24	REVERSE	63396	21	55.05	38.1		AGTTTTGTAATCTGGACAGCA

96 selected isolates from 8 countries were screened and analyzed with the 22 polymorphic microsatellite markers. The isolates were further grouped into 16 geographic regions to observe intraregional variations (Table 4.3). The number of polymorphic SSRs ranged slightly from 19 to 22 SSRs per region. In total 202 alleles were observed among the 22 SSRs. Some SSRs were more informative than others and the most informative marker carried 17 alleles while the least informative marker only had 3 alleles among the sample population. Similarly, different regions varied in the amount of polymorphic alleles, ranging from as few as 31 multilocus genotypes in Ontario (Canada) to 100 in Argentina.

The 22 SSR markers varied in terms of their polymorphism (Table 4.4). Not all markers consistently amplified in the 16 regions and the level of polymorphism, or alleles ranged from as many as 7 alleles per region per marker to a single common allele. Similarly the polymorphism between different population groups varied in the number of alleles generated with Argentina being the most polymorphic population with an average of 4.5 alleles per marker and Ontario being the least polymorphic population with an average of 1.4 alleles per marker.

Table 4.3: Allele distribution within 22 polymorphic microsatellites among 16 *Leptosphaeria maculans* pathogen population groups

	COUNTRY	REGION	NUMBER OF ISOLATES	NUMBER OF POLYMORPHIC SSRS	NUMBER OF POLYMORPHIC ALLELES
1	Canada	Alberta	5	22	46
2	Canada	Saskatchewan	3	19	41
3	Canada	Manitoba	4	22	38
4	Canada	Ontario	4	19	31
5	USA	North Dakota	11	22	96
6	USA	Georgia	4	20	51
7	Poland	North West	9	22	86
8	Poland	South East	3	20	38
9	United Kingdom	Oxfordshire	6	22	80
10	United Kingdom	Leicestershire	6	22	68
11	Argentina	n/a	14	22	100
12	Australia	South Australia	6	22	67
13	Australia	North South West	5	22	46
14	Australia	Victoria	5	21	35
15	Brazil	n/a	7	22	58
16	France	n/a	4	22	57
TOTAL			**96**	**/22**	**/202**

Table 4.4: Allele distribution within 22 polymorphic microsatellites among 16 *Leptosphaeria maculans* pathogen population groups

LOCUS	CAN_AB	CAN_SK	CAN_MB	CAN_ON	US_ND	US_WA	POL_NW	POL_SE	UK_OX	UK_LC	ARG	AUS_SA	AUS_NSW	AUS_VIC	BRZ	FRN	AVG
1	2	0	3	1	3	0	4	2	3	4	4	3	2	2	3	2	2.4
2	1	1	2	2	5	2	3	3	4	5	6	2	2	2	4	4	3.0
3	2	2	1	0	6	2	3	1	4	3	5	3	2	2	1	3	2.5
4	1	2	1	1	3	2	3	2	1	3	4	2	2	1	1	1	1.9
5	2	2	2	3	5	3	3	2	4	4	6	4	3	2	5	2	3.3
6	3	4	2	2	6	3	6	1	6	4	5	3	2	2	3	2	3.4
7	5	4	2	2	7	3	7	4	7	5	8	5	5	3	4	3	4.6
8	2	1	1	0	4	0	5	1	3	3	3	5	2	2	4	3	2.4
9	4	2	2	1	6	3	5	3	5	5	7	3	1	2	3	3	3.4
10	2	2	2	2	3	2	6	2	4	5	6	3	3	2	2	3	3.1
11	2	3	2	2	7	4	3	3	3	5	4	4	2	3	3	3	3.3
12	1	2	1	1	4	2	2	3	4	2	3	2	2	1	3	2	2.2
13	1	1	1	1	4	1	3	1	2	1	2	2	2	2	1	3	1.8
14	2	1	1	1	4	2	3	1	5	2	3	3	2	1	2	3	2.3
15	1	4	2	2	3	3	2	2	1	2	4	1	1	0	1	1	1.9
16	3	0	2	1	4	3	6	0	4	1	4	4	2	1	1	3	2.4
17	1	0	1	0	1	3	2	0	3	2	5	4	1	1	1	2	1.7
18	2	3	2	2	7	2	5	1	4	3	6	5	3	1	4	3	3.3
19	1	1	2	1	3	2	3	1	4	2	3	2	2	1	3	3	2.1
20	3	3	1	2	4	3	5	1	3	2	5	2	2	1	3	3	2.7
21	2	2	3	2	3	4	4	3	4	3	4	3	2	2	5	3	3.1
22	3	1	2	2	4	2	3	1	2	2	3	2	1	1	1	2	2.0
AVERAGE	2.1	1.9	1.7	1.4	4.4	2.3	3.9	1.7	3.6	3.1	4.5	3	2.1	1.6	2.6	2.6	
SE.	0.2	0.3	0.1	0.2	0.3	0.2	0.3	0.2	0.3	0.3	0.3	0.2	0.2	0.2	0.3	0.2	

The genetic distance between the 16 designated populations is represented in the distance matrix of Figure 4.2. The two most distal populations are the Ontario (Canada) and Georgia (USA) populations separated by a branch distance of 8.4. On the other hand, the populations of Oxfordshire (UK) was separated from South East Poland by 0.4 and from Leicestershire (UK) by 0.6. Similarly, Poland North West was separated from Poland SE by a branch length of 0.9 and the French population by 0.7. Population groups within the same geographic areas such as the European, South American, and Australian populations were separated by relatively small branch lengths whereas the Western Canadian population of Saskatchewan was genetically distant from the geographically proximal prairie populations of Manitoba and Alberta.

```
Number of loci compared 22
seed=2645 ninap=0

Distance matrix

          2     3     4     5     6     7     8     9    10    11    12    13    14    15    16
    1 1.997 1.721 2.056 1.744 3.689 0.619 1.534 1.416 1.500 1.321 0.736 0.992 1.252 2.240 1.325
    2       3.542 6.398 0.613 1.985 1.356 1.302 0.888 1.696 0.974 2.595 1.945 2.864 0.900 1.314
    3             2.407 3.513 6.038 1.860 3.396 3.166 3.194 2.803 1.844 1.923 2.664 3.920 2.942
    4                   5.579 8.367 1.873 4.566 4.091 4.821 4.423 1.460 2.266 3.147 5.841 3.972
    5                         0.944 1.225 0.900 0.444 1.181 0.568 2.053 1.846 2.038 0.691 1.333
    6                               2.796 1.767 1.489 2.598 1.769 4.344 4.456 4.585 1.971 2.942
    7                                     0.886 0.535 0.938 0.654 0.543 0.601 1.387 1.362 0.732
    8                                           0.431 0.881 0.713 2.085 1.720 2.134 1.207 1.023
    9                                                 0.595 0.527 1.278 1.110 1.702 0.735 0.910
   10                                                       1.182 1.549 1.264 1.683 1.234 1.101
   11                                                             1.574 1.351 1.687 0.680 0.736
   12                                                                   0.620 1.083 2.101 1.306
   13                                                                         1.070 1.601 1.188
   14                                                                               1.634 1.577
   15                                                                                     0.888
   16


(United_Kingdom_Oxfordshire:0.06566000,(((((((Canada_Alberta:0.07938700,
((Canada_Manitoba:0.62025800,Canada_Ontario:1.78696900):0.66778600,Australia_SA:-
0.21909400):0.40698500):0.28459600,Australia_VIC:0.68232500):0.04349800,Australia_NSW:0.26732100):0.328021
00,Poland_NW:-
0.01420300):0.27709900,France:0.37695100):0.15097600,United_Kingdom_Leicestershire:0.48475600):0.10289300,
Poland_SE:0.35881800):0.01116100,(((Canada_Saskatchwean:0.46877400,(United_States_ND:-
0.35887400,United_States_GA:1.30242400):0.35826900):0.13510600,Brazil:0.33132400):0.11626300,Argentina:0.2
0153700):0.17546200);
```

Figure 4.2: Genetic Distance matrix between 16 *Leptosphaeria maculans* populations based on 22 SSR markers.

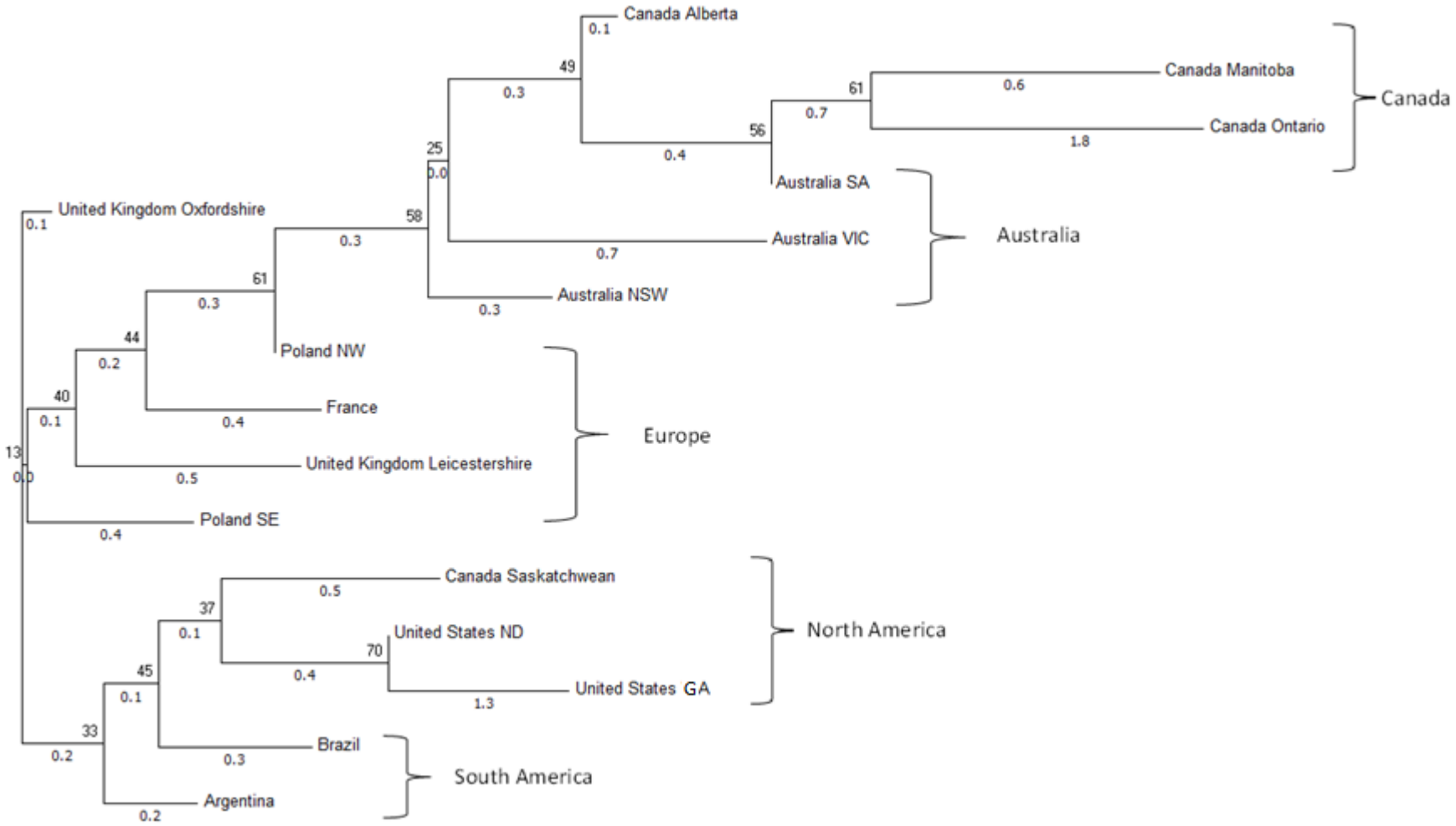
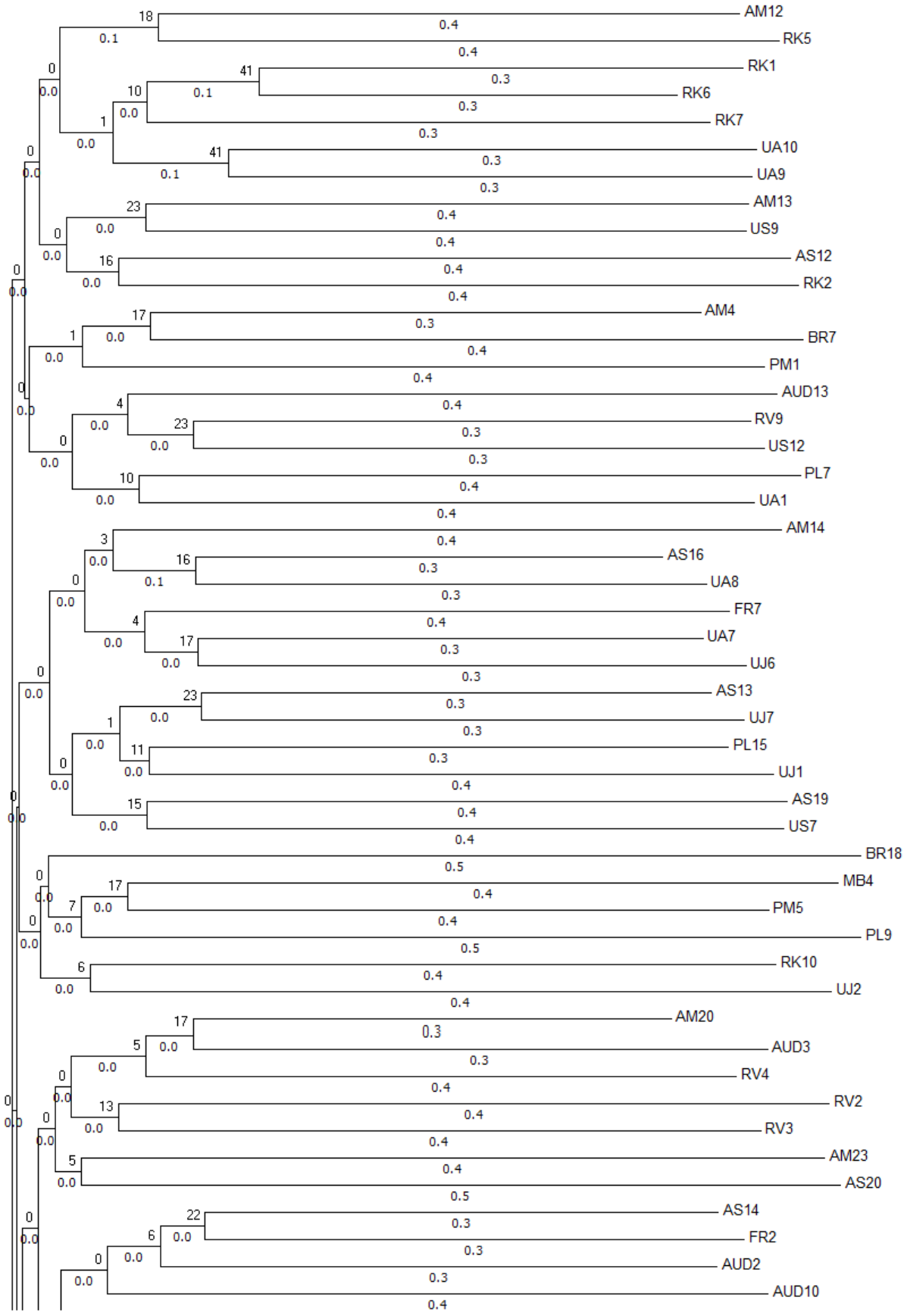


Figure 4.3: Genetic Tree of 16 *Leptosphaeria maculans* population groups constructed from 22 microsatellite markers.


AM12
RK5
RK1
RK6
RK7
UA10
UA9
AM13
US9
AS12
RK2
AM4
BR7
PM1
AUD13
RV9
US12
PL7
UA1
AM14
AS16
UA8
FR7
UA7
UJ6
AS13
UJ7
PL15
UJ1
AS19
US7
BR18
MB4
PM5
PL9
RK10
UJ2
AM20
AUD3
RV4
RV2
RV3
AM23
AS20
AS14
FR2
AUD2
AUD10

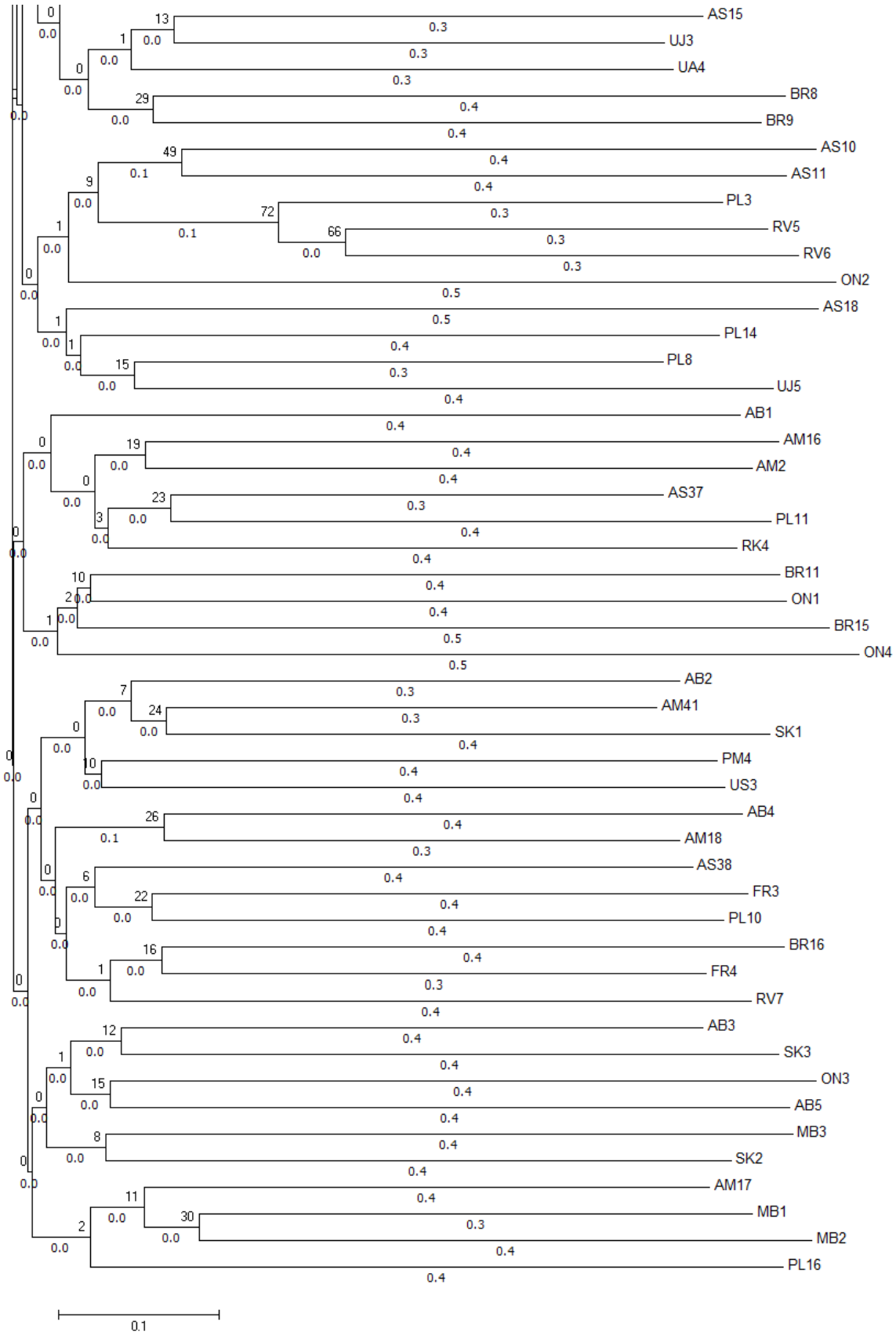


Figure 4.4: Genetic Tree of all 96 *Leptosphaeria maculans* isolates constructed from 22 SSR markers.

The genetic tree constructed using all 96 isolates individually results in low bootstrap values due to fewer polymorphic markers used to separate individual isolates (Figure 4.4). When the 96 isolates are pooled into 16 groups based on geographic origin, the average number of polymorphic SSR data points increases by 6x resulting in better separation and improved bootstrap results (Figure 4.3). When we examine the constructed genetic tree based on these 16 regions, we observe that most populations group by geographic origin (Figure 4.3). The European populations of Poland, UK, and France occupy the central regions of the tree while the South American populations of Argentina and Brazil are towards the lower half and the Australian population groups are located on the upper branches. However, the North American population appears in opposing ends of the genetic tree. The US populations and the western Canadian population of Saskatchewan group on the lower end of the tree with the South American populations. The Western Canadian provinces of Manitoba and Alberta, and the Eastern Canadian Ontario population groups are on the opposing upper end of genetic tree along with the Australian isolates. The USA Georgia and Canadian Ontario populations have the two largest branch lengths of 1.3 and 1.8 from the nearest node.

Table 4.5: Listing of the 96 *Leptosphaeria maculans* isolates used in this study.

#	Original ID	New ID	Country	Province / State	Cultivar	Year
1	A11-1	UA1	UK	Oxfordshire	Drakker	2011
2	A11-6	UA4	UK	Oxfordshire	Drakker	2011
3	A11-13	UA7	UK	Oxfordshire	Drakker	2011
4	A11-14	UA8	UK	Oxfordshire	Drakker	2011
5	A11-15	UA9	UK	Oxfordshire	Drakker	2011
6	A11-16	UA10	UK	Oxfordshire	Drakker	2011
7	J11-1	UJ1	UK	Leicestershire	Drakker	2011
8	J11-2	UJ2	UK	Leicestershire	Drakker	2011
9	J11-3	UJ3	UK	Leicestershire	Drakker	2011
10	J11-5	UJ5	UK	Leicestershire	Drakker	2011
11	J11-6	UJ6	UK	Leicestershire	Drakker	2011
12	J11-7	UJ7	UK	Leicestershire	Drakker	2011
13	AD2	AD2	AUS	n/a	n/a	2011
14	AD3	AD3	AUS	n/a	n/a	2011
15	AD10	AD10	AUS	n/a	n/a	2011
16	AD13	AD13	AUS	n/a	n/a	2012
17	11SMS001	AS10	AUS	SA	Monola76TT	2011
18	11SMS013	AS11	AUS	SA	44Y84CL	2011
19	11SMW009	AS12	AUS	NSW	Winifred	2011
20	11SMW015	AS13	AUS	SA	Hyola50	2011
21	11SMP009	AS14	AUS	SA	TawrifficTT	2011
22	11SMP017	AS15	AUS	SA	TawrifficTT	2011
23	11SMP026	AS16	AUS	SA	JardeeHT	2011
24	11SMP040	AS18	AUS	Vic	TawrifficTT	2011
25	11SMJ006	AS19	AUS	Vic	OasisCL	2011
26	11SMJ018	AS20	AUS	Vic	OasisCL	2011
27	10SMJ064	AS37	AUS	NSW	Oasis	2010
28	10SMJ068	AS38	AUS	NSW	Oasis	2010
29	RK1	RK1	Argentina	Tres Arroyos	Kisbye	2012
30	RK2	RK2	Argentina	Tres Arroyos	Kisbye	2012
31	RK4	RK4	Argentina	Tres Arroyos	Kisbye	2012
32	RK5	RK5	Argentina	Tres Arroyos	Kisbye	2012
33	RK6	RK6	Argentina	Tres Arroyos	Kisbye	2012
34	RK7	RK7	Argentina	Tres Arroyos	Kisbye	2012
35	RK10	RK10	Argentina	Tres Arroyos	Kisbye	2012
36	RV2	RV2	Argentina	Tres Arroyos	Verkuyl	2012

37	RV3	RV3	Argentina	Tres Arroyos	Verkuyl	2012
38	RV4	RV4	Argentina	Tres Arroyos	Verkuyl	2012
39	RV5	RV5	Argentina	Tres Arroyos	Verkuyl	2012
40	RV6	RV6	Argentina	Tres Arroyos	Verkuyl	2012
41	RV7	RV7	Argentina	Tres Arroyos	Verkuyl	2012
42	RV9	RV9	Argentina	Tres Arroyos	Verkuyl	2012
43	PL56	Pm1	Poland	Lower Silesia	n/a	1995
44	OJ43-3-1	Pm4	Poland	Opole	n/a	2003
45	MB158	Pm5	Poland	Great Poland	n/a	2009
46	POL2	Pl3	Poland	Great Poland	n/a	2003
47	POL109	Pl7	Poland	Mazovia	n/a	2004
48	POL46	Pl8	Poland	Great Poland	n/a	2003
49	MB150	Pl9	Poland	Great Poland	n/a	2009
50	MB133	Pl10	Poland	Great Poland	n/a	2009
51	POL156	Pl11	Poland	Great Poland	n/a	2004
52	POL17N	Pl14	Poland	Great Poland	n/a	2004
53	POL12	Pl15	Poland	Great Poland	n/a	2003
54	J-1-3-3	Pl16	Poland	Great Poland	n/a	2007
55	2027	AM2	United States	North Dakota, Rollette County	n/a	2010
56	2030	AM4	United States	North Dakota, Rollette County	n/a	2010
57	2108	AM12	United States	North Dakota, Ramsey County	n/a	2010
58	2109	AM13	United States	North Dakota, Ramsey County	n/a	2010
59	2111	AM14	United States	North Dakota, Ramsey County	n/a	2010
60	2131	AM16	United States	North Dakota, Ramsey County	n/a	2010
61	2136	AM17	United States	North Dakota, Kidder County	n/a	2011
62	2137	AM18	United States	North Dakota, Kidder County	n/a	2011
63	2139	AM20	United States	North Dakota, Kidder County	n/a	2011
64	2158	AM23	United States	North Dakota, Ward County	n/a	2012
65	2197	AM41	United States	North Dakota, Cavalier County	n/a	2012
66	us2012-3	US3	United States	Washington	n/a	2011
67	us2012-7	US7	United States	Washington	n/a	2011
68	us2012-9	US9	United States	Washington	n/a	2011
69	us2012-12	US12	United States	Washington	n/a	2011
70	Guelph2357	CO1	Canada	Guelph, ON	n/a	2003

71	Guelph2358	CO2	Canada	Guelph, ON	n/a	2003
72	Guelph2382	CO3	Canada	Guelph, ON	n/a	2003
73	OK 03-44-02	CO4	Canada	Kincardine, ON	n/a	2003
74	ICBN14	FR2	France	n/a	n/a	n/a
75	PWH1223	FR3	France	n/a	n/a	n/a
76	R2	FR4	France	n/a	n/a	n/a
77	JN3	FR7	France	n/a	n/a	n/a
78	Br7	Br7	Brazil	n/a	n/a	n/a
79	Br8	Br8	Brazil	n/a	n/a	n/a
80	Br9	Br9	Brazil	n/a	n/a	n/a
81	Br11	Br11	Brazil	n/a	n/a	n/a
82	Br15	Br15	Brazil	n/a	n/a	n/a
83	Br16	Br16	Brazil	n/a	n/a	n/a
84	Br18	Br18	Brazil	n/a	n/a	n/a
85	FR 1	MB1	Canada	Manitoba	n/a	2011
86	RL7	MB2	Canada	Manitoba	n/a	2011
87	SF5	MB3	Canada	Manitoba	n/a	2011
88	NN9	MB4	Canada	Manitoba	n/a	2011
89	A10-615	AB1	Canada	Alberta	n/a	2010
90	A10-619	AB2	Canada	Alberta	n/a	2010
91	A10-680A	AB3	Canada	Alberta	n/a	2010
92	J50	AB4	Canada	Alberta	n/a	2010
93	L8	AB5	Canada	Alberta	n/a	2010
94	ML3	SK1	Canada	Saskatchewan	n/a	2011
95	NB2	SK2	Canada	Saskatchewan	n/a	2011
96	SB1	SK3	Canada	Saskatchewan	n/a	2011

4.4 Discussion

The screening of the *L. maculans* genome for microsatellites showed an increased frequency of SSRs dispersed among the genome in comparison to 10 other pathogenic fungi (Karaoglu *et al.* 2006). As shown in Figure 4.1, it has the highest number of SSRs among the 10 species and the second highest frequency of SSRs relative to genome size, second only to *Neurospora crassa.* Repeat induced point mutation (RIP), the fungal mechanism to inactivate repetitive DNA were first identified in *N. crassa* (Cambareri, 1991) and the genes necessary for repeat induced point mutations (RIPs) in *L. maculans* were later identified by Rouxel *et al.*(2011). The high frequency of repetitive elements in these two species has been posited to be due to transposable elements (TEs) that duplicate themselves and proximal genes, then transpose themselves across the genome often leading to the inactivation of key genes and general genetic degradation. Many fungal effectors including avirulence genes are subjected to increased mutational pressure due to their localization in gene poor isochores rich in transposable elements (Rouxel *et al.,* 2011)

The relatively high frequency of repetitive DNA in the *L. maculans* genome allows for studies based on microsatellites and minisatellites to potentially generate many informative markers (Table 4.1). 22 SSRs were selected from 194 screened SSRs and used to compute genetic distance values between 16 designated population groups (Table 4.3). Geographically proximal populations were genetically proximal on the unrooted phylogenetic tree (Figure 4.3). Poland North West and Poland South east were separated

by a branch length of 0.9 and the two United Kingdom populations were separated by a branch length of 0.6. The three Australian regions were separated at most by a branch length of 1.1. Countries that were closer geographically also appeared nearby on the genetic tree with Brazil and Argentina separated by 0.9 while France and UK were separated by 1.1. However, when we examine the western Canadian population, we observe two genetically distal populations within a geographically small area. The Canadian Province of Saskatchewan has a *L. maculans* population that is very similar to the North Dakota population with a branch length separation of 0.6 while it is separated from the nearby Alberta population by a length of 2.0 and the Manitoba population by a length of 3.5. The Manitoba and Alberta provinces are separated by a branch length of 1.3 and make up a different genetic group. Thus, there are two genetically distinct populations of blackleg within the western Canada / Mid Northern US regions. The existence of two distinct populations in western Canada was also reported by Dilmaghani *et al.* (2012). This genetic separation within a relatively cohesive geographic region was not observed between other population groups and the reduced rate of of sexual recombination between these populations is likely aided by the colder climate and the prevalence of asexual pycnidiospores (Guo & Fernando, 2005; Ghanbarnia et. al 2011). The reduced rate of sexual reproduction through ascospores may have prevented any significant rates of genetic exchange and recombination allowing populations to maintain genetic structures similar to founder populations. The lack of recombination is also perceived from the low number of SSR alleles in western Canada (Table 4.4) with an average of 2.1, 1.9, and 1.7 alleles per marker in Alberta, Saskatchewan, and Manitoba

respectively. In comparison, all European regions with the exception of Poland SE have an average greater than 3 alleles per marker.

The Canadian Saskatchewan *L. maculans* population is genetically distant from the population found in the neighbouring provinces of Alberta and Manitoba. This difference was also observed in pathogenicity with Saskatchewan isolates being significantly less diverse and even (IOE of 0.389) in terms of avirulence genes (Figure 3.4) compared to Alberta (IOE of 0.608) and Manitoba (IOE of 0.684). Saskatchewan isolates are also the most complex carrying more avirulence genes than Alberta and Manitoba, and disease severity is generally milder on surveys (Figure 3.6 &3.7). The Saskatchewan *L. maculans* population is most closely related to the United States North Dakota population which is likely the source of introduction of blackleg into the province. The Alberta and Manitoba populations are more closely related on Ontario isolates and likely have different founder populations when compared to Saskatchewan. The largest acreage of canola in Canada is grown in Saskatchewan however blackleg disease nurseries are less common in the province due to lower incidence of disease. The genetic distance between populations within western Canada and the pathogenicity variance between provinces suggests the need to account for distinct populations when planning blackleg surveys and disease nurseries.

The Alberta isolates in western Canada are most closely related to Australian isolates and are separated from the Australian regions of South Australia, North

Australia, and Victoria by branch lengths of 0.7, 1.0, and 1.4 respectively. The two Canadian provinces of Manitoba and Alberta, and three Australian states of Victoria, North South West, and South Australia and clustered together on one end of the phylogenetic tree. South Australia has historically been a hot spot for blackleg disease, particularly the Eyre valley where crop losses up to 90% were reported (Li *et al.*, 2003). Similarly, the province of Manitoba and to a lesser degree Alberta has had the most severe blackleg symptoms in individual fields in western Canada. The very close genetic relationship between Alberta and South Australia suggests movement of isolates between these two regions. The closest population to the Manitoba isolates is Alberta with a branch length separation of 1.7 followed closely by South Australia with a separation of 1.8. The Manitoba isolates are interesting in that they have diverged significantly from the closest population group on the genetic tree suggesting that they may have diverged and adapted to an ecological niche. Similarly, the Ontario isolates are separated by branch length of 1.5 from their closest neighbour on the genetic tree which is South Australia followed by Alberta.

The greatest amount of genetic diversity exists between the populations of Ontario Canada and Georgia, United States, they are the two most distally separated populations with a branch separation of 8.4. This high level of genetic diversity between populations in North America compared to other continents suggests and origin of the pathogen in either USA or Eastern Canada. The low genetic variation within the Ontario isolates based on average SSR alleles of 1.4 compared to US populations of North Dakota and

Georgia with averages of 4.4 and 2.6 alleles per marker support the USA as a more likely point of origin followed by subsequent introduction into Ontario. A USA origin would be consistent with the first documented epidemics of the disease on cabbage in Wisconsin (Henderson 1918). This is also the scenario found most likely by Dilmaghani *et al.* (2012). Dilmaghani hypothesizes a single introduction from Ontario into Western Canada; this conflicts with the results of this study showing Saskatchewan isolates as genetically distal from Ontario blackleg isolates. Thus, this study favors two separate introductions from the USA, one through North Dakota into Saskatchewan and one from USA through Eastern Canada into Alberta and Manitoba. This would explain the two separate western Canadian populations observed by Dilmaghani *et al.* (2012) and this study.

The European populations are in the center of the genetic tree (Figure 4.3) and have very high levels of SSR allelic variation, they are closely related to one another. However, they have low levels of interregional variation compared to North America or Australia. The Polish isolates (excluding other European populations) are most closely related to western Canada isolates while the UK isolates (excluding other European populations) are most closely related to USA isolates, and the French isolates (excluding other European populations) are most closely related to the South American isolates. Overall this suggests multiple recurrent introductions of *L. maculans* in Europe which would explain the high levels of allelic variation. The grouping of the European isolates on the genetic tree also points to sexual recombination and admixture of the founder

populations. Similarly, the Australian populations are linked both to western Canadian and Polish populations also suggesting multiple introductions. The genetic clustering of European blackleg isolates suggests admixture of founder populations through sexual recombination.

The adaptation of local *L. maculans* populations within their current geographic regions has led to populations that can be separated genetically based on their location of origin. Despite the relatively low allelic diversity of Australian blackleg isolates compared to American or European populations, Blackleg in Australia was responsible for on the largest economic losses in canola production (Li *et al.,* 2003). This suggests that low genetic diversity in founder populations does not inhibit *L. maculans* from adapting under selection pressure to new regions, particularly if the environment is conducive to sexual reproduction.

Overall, the results of this work highlight the high levels of genetic diversity of the USA and Eastern Canadian *L. maculans* populations. The high levels of SSR allelic diversity in American isolates supports a model where USA isolates spread into Eastern Canada rather than the reverse. The two populations in western Canada are genetically distal and best explained by two distinct founding events that were likely maintained by an environment unfavourable to sexual reproduction. While the European and Australian populations also point to multiple introductions, they are also proximal on the genetic tree suggesting sexual recombination. Sexual reproduction is a regular feature of the life cycle

of *L. maculans* in Europe, Australia, and Canada (West *et al.*, 2001), however the low allelic diversity and genetically dissimilar proximal populations suggest the predominance of asexual reproduction in western Canada. Both mating types are present in western Canada and sexual pseudothecia are present but at a lower frequency than ascospores (Ghanbarnia *et al.* 2009). The shift to favour asexual ascospores is most likely due to epidemiological adaptation to unfavourable climatic conditions, primarily the shorter growing season inhibiting the sexual life cycle.

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSIONS

5.1 General Discussion and Conclusions

Blackleg of canola canola (*Brassica napus* L.) caused by the fungal pathogen *Leptosphaeria maculans* is a major disease affecting oilseed rape production in Canada and around the world. In the early 1990's, commercial cultivars with genetic resistance to blackleg were released and in combination with 3 to 4 year crop rotations, the disease was successfully controlled for many years. Changes in pathogen race structure was observed in the early 2000's (Chen & Fernando, 2006; Kutcher *et al.,* 2007), likely attributable to the pathogen responding to the resistance (*R*) genes in canola cultivars. The dual sexual and asexual lifecycles of *L. maculans* in combination with the underlying genetic plasticity of virulence factors provides the pathogen with an exceptional ability to rapidly overcome new resistance sources, as demonstrated in rapid `breakdown` of cultivars carrying *Rlm1* in France and *LepR3* in Australia (Rouxel *et al.,* 2003; Li *et al.,* 2003). In recent years producers and agronomists in western Canada have reported moderate to severe disease symptoms in cultivars that were previously registered as resistant, and the incidence and severity of the disease has increased steadily in disease surveys (Figures 3.6 & 3.7).

The first part of this is study examined the blackleg pathogen population in western Canada to identify the different races present, distribution of races, and changes within avirulence gene frequencies. This information may be used as a tool for breeders, growers, and regulators to develop both genetic resistance and the management tools to

effectively reduce the risk posed by *Leptosphaeria maculans*. The results of the first chapter of this work identified 55 races among 674 individual isolates based on the presence or absence of 10 avirulence genes. The pathogen's race diversity highlights the potential for blackleg to impact canola production in western Canada. All individual resistance genes corresponding to the 10 surveyed avirulence genes (*Rlm1, Rlm2, Rlm3, Rlm4, Rlm6, Rlm7, Rlm9, LepR1, LepR2,* and *RlmS*) can be overcome by at least one of the races detected in this study. Many stacked combinations of resistance genes would also be overcome by at least one race. However, certain combination such as *Rlm6+Rlm7,* and *Rlm2+Rlm7+LepR1* would be resistant to all of the 674 isolates surveyed. The presence of low frequency races virulent on stacked host resistance genes risks the loss of multiple *R* genes simultaneously when selection pressure degrades resistance, this risk is more pronounced when races virulent on the stacked combinations exist at the time of deployment.

AvrLm2, Avrlm4-7, Avrlm6, AvrLepR1 and *AvrLmS* were identified as the most frequent avirulence gens in the pathogen population and thus the corresponding resistance genes would be effective to manage blackleg in western Canada. On the other hand *AvrLm3*, *Avrlm9,* and *AvrLepR2* occur at low frequencies and the corresponding R genes would be ineffective against the majority of the pathogen population. Most avirulence genes, with the exception of *Avrlm6* and *Avrlm7* varied in their frequency across different sites in western Canada and should not be expected to perform well at all sites. The average isolate carried 4 of the 10 surveyed avirulence genes indicating that any one of the 4 corresponding R genes would be sufficient to induce resistance in the host. In terms

of pathogen complexity (# of avirulence genes) and diversity (# of races), Manitoba blackleg isolates are the most virulent followed by Alberta and then Saskatchewan, this corresponds to survey data (Figures S1 & S2).

AvrLm3, corresponding to the most common resistance gene in Canadian cultivars *Rlm3* (Zhang *et al.*, 2013), was shown to have declined significantly from 52.8% in 2005-2006 (Dilmaghani *et al.*, 2009) to 13.7% in this study. This shift was attributed to selection pressure since the most common resistance gene since Rlm3 is carried by more than half of Canadian germplasm screened (Zhang *et al.*, 2013). On the other hand the frequency of the *AvrLm4-7* avirulence gene increased from 25% in 1997-2005 (Kutcher *et al.*, 2010) to 47.2% in 2005-2006 (Dilmaghani *et al.*, 2009), and to 89.7% in this study. These two avirulence frequency shifts were hypothesized to be related, due to the observation that 96% of isolates carried either *AvrLm3* or *AvrLm4-7*, only 0.1% carried both, and 3% carried neither. A similar observation was seen with *AvrLm1* and *AvrLm2* where >90% of isolates carried either one or the other. These findings suggest that in western Canada, rotating *Rlm1* with *Rlm2* or rotating *Rlm3* with *Rlm4* or *Rlm7* would be preferable since the deployment of one of them strengthens the effectiveness of the other.

The second chapter of this study identified the genetic distance and relationship between 96 blackleg isolates from 8 countries. In general the blackleg populations grouped according to their geographic origin. Dilmaghani *et al.* (2012) reported two genetically distinct populations in western Canada and this study identified Saskatchewan isolates as clustering more closely with US North Dakota isolates than with Alberta or

Manitoba isolates. This genetic separation within a relatively cohesive geographic region was not observed between other population groups and may be influenced by the reduced rate of sexual recombination between these populations (Guo & Fernando, 2005; Ghanbarnia *et al.,* 2011). The genetic distance between populations within western Canada and the pathogenicity variance between provinces suggests the need to account for distinct populations in survey and management strategies.

The two Canadian provinces of Manitoba and Alberta and the three Australian states of Victoria, North South West, and South Australia clustered together on one end of the phylogenetic tree suggesting movement of isolates between Canada and Australia. The high level of SSR allelic variation and low level of interregional variation in European populations suggests sexual recombination and admixture of the founder populations. The genetic clustering of *L. maculans* populations generally within their geographic regions indicates adaptation of the pathogen to the local environment. *L. maculans* is adaptable to different climates and encompasses a wide geographic range due to its ability to infect both spring and winter oilseed rape (West *et al.,* 2001). The versatility of *L. maculans* coupled with selection pressure and favourable environmental conditions has allowed it to displace *L. biglobosa* as the dominant species in large ecological niches of North America and Europe (Fitt *et al.,* 2008). The genetic relationships in this study between isolates from different locations (Australia –western Canada & Ontario-Europe) suggest that the pathogen has moved between countries while the clustering of isolates within their geographic regions indicates the development of unique subpopulations.

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APPENDICES

Appendix 1: Blackleg (*Leptosphaeria maculans*) leaf lesion canola (*Brassica napus*). Bleaching of leaf tissue and *L. maculans* fruiting budies (pycnidia) visible within lesion. Faint outline indicates lack of resistance. Early infection will lead to migration of L. maculans into the stem through the leaf petiole. Colonization withing the stem restricts nutrient and water uptake and can lead to poor podfilling, lodging, or death of the plant.

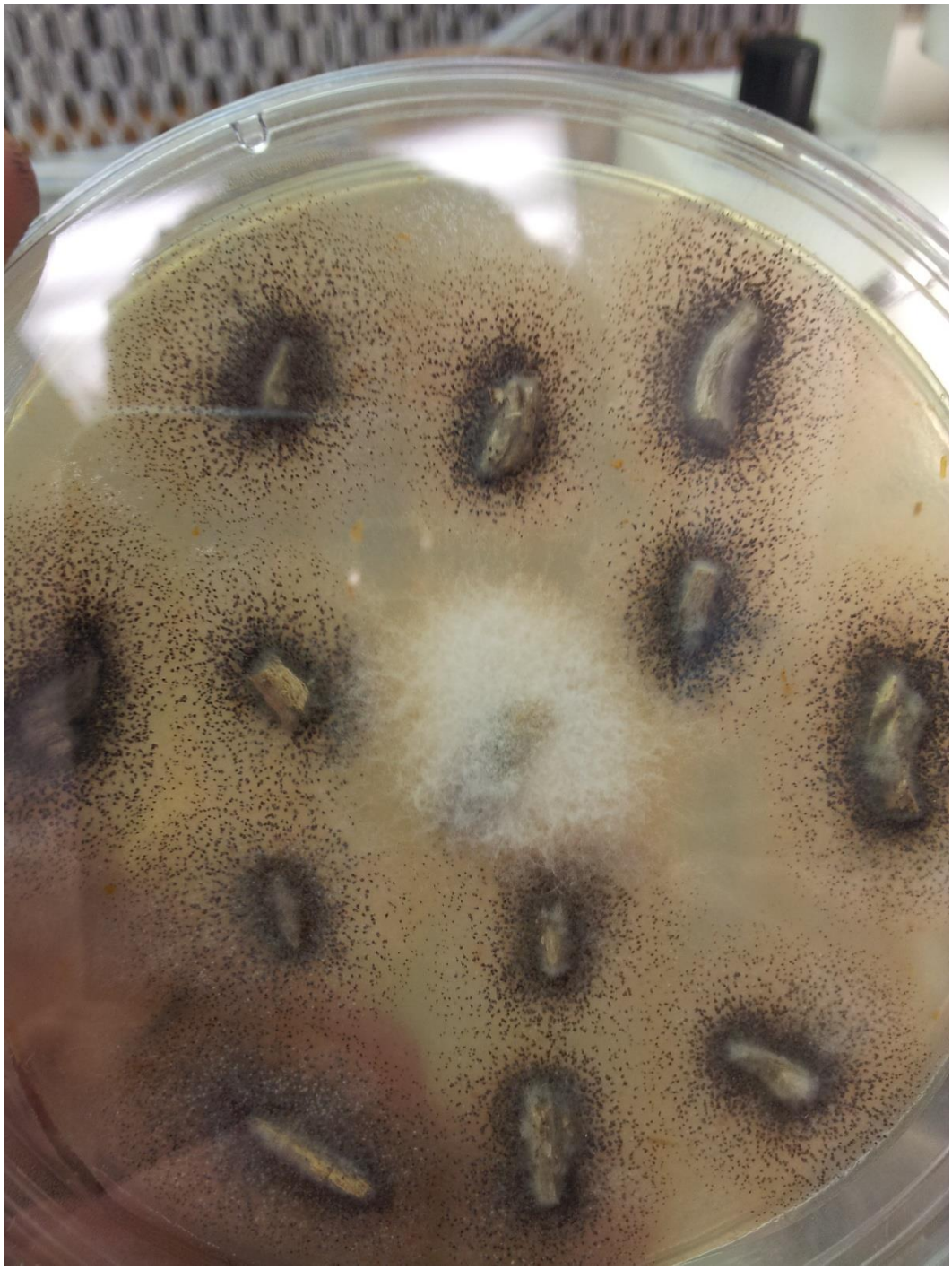
Appendix 2: Blackleg (*Leptosphaeria maculans*) upper stem canker on canola (*Brassica napus*). Bleaching of stem tissue and *L. maculans* fruiting budies (pycnidia) visible within lesion. Lesion is sourrounded by the outline of necrotic tissue.

Appendix 3: Blackleg (*Leptosphaeria maculans*) lower stem canker on canola (*Brassica napus*). Bleaching of stem tissue and *L. maculans* fruiting budies (pycnidia) visible within lesion. Lesion is sourrounded by the outline of necrotic tissue.

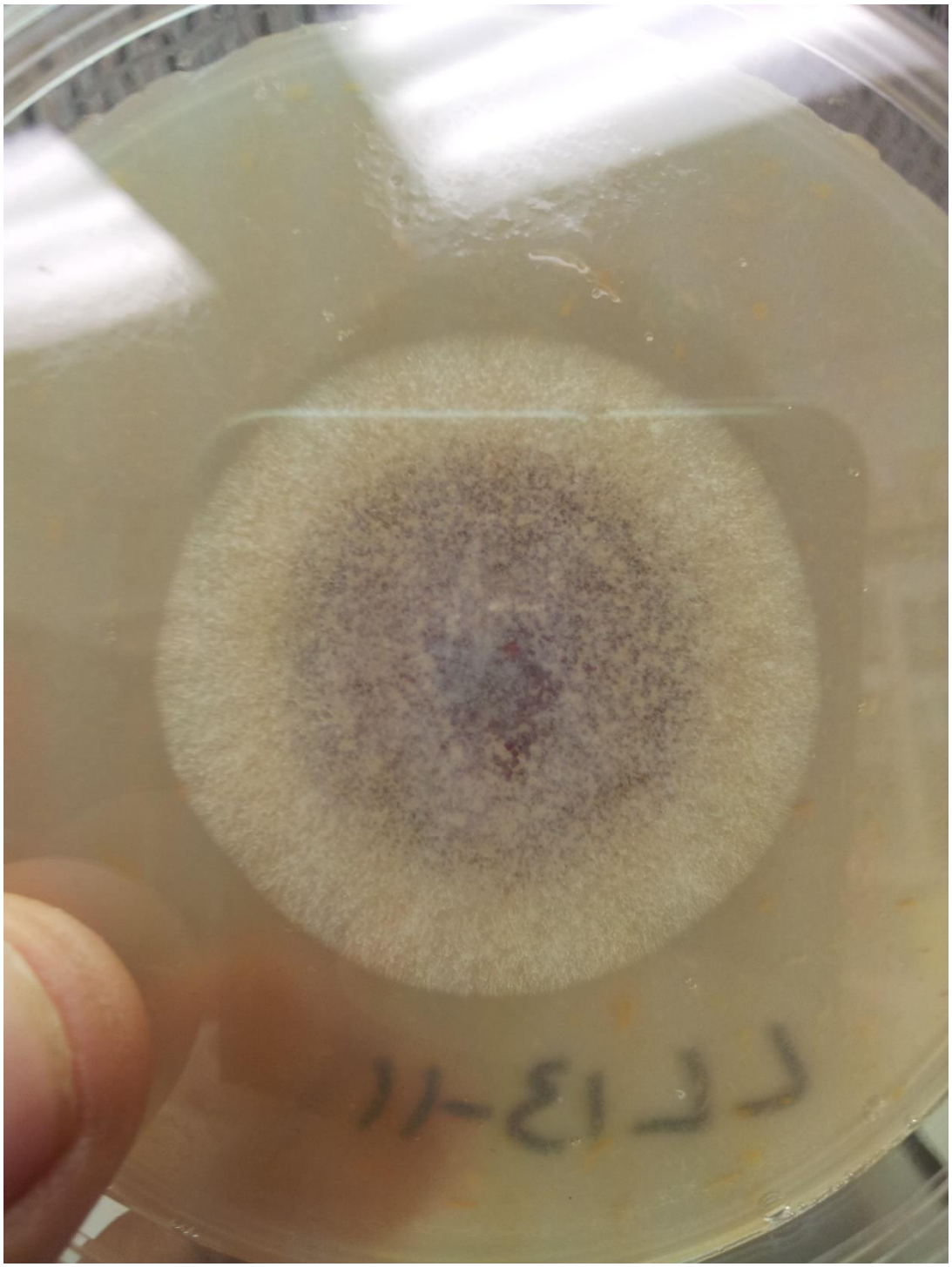
Appendix 4: Lodging of canola (*Brassica napus*) plants caused by Blackleg (*Leptosphaeria maculans*).

Appendix 5: Colonized stem canker on canola (*Brassica napus*) on lodged plant caused by Blackleg (*Leptosphaeria maculans*).

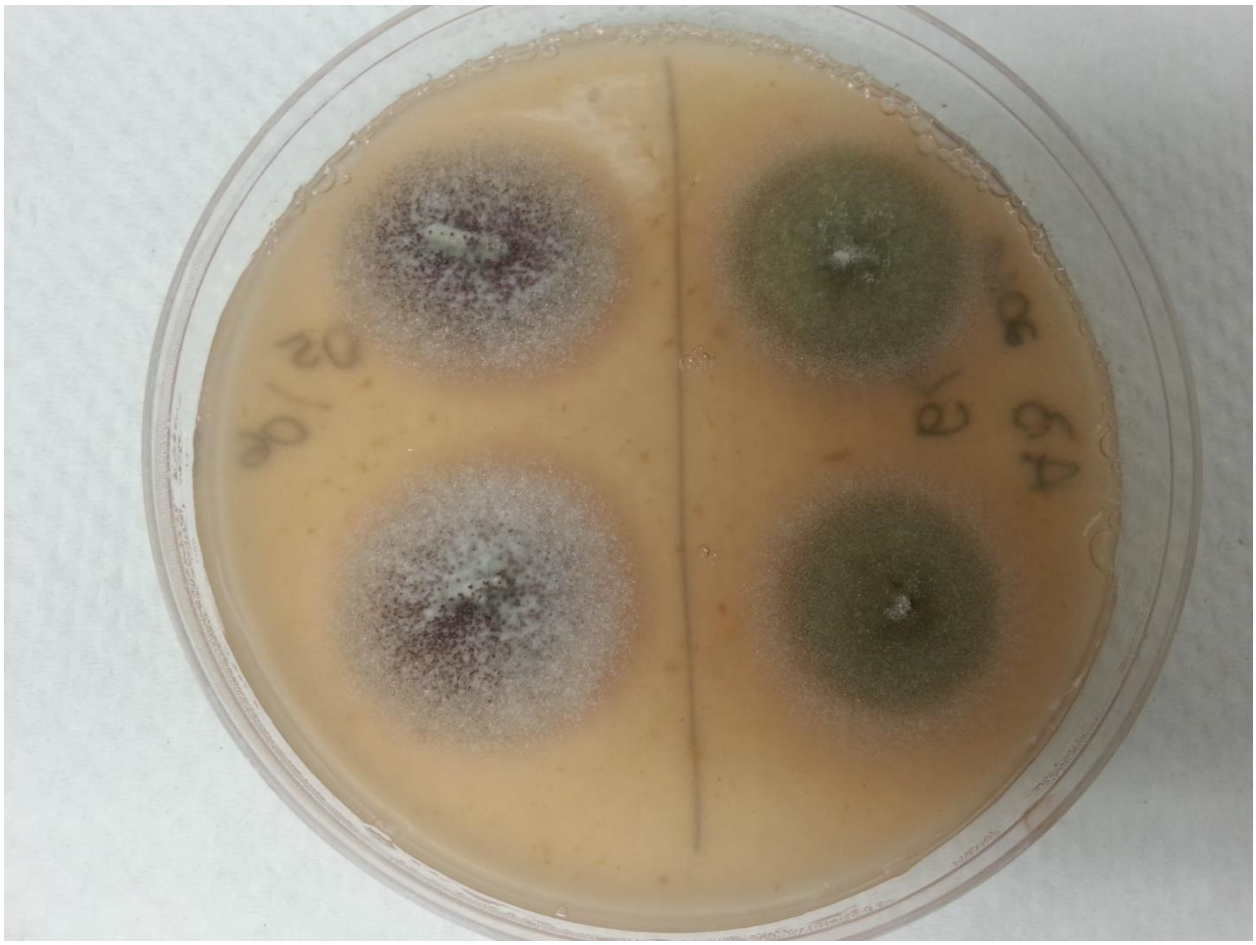
Appendix 6: Blackleg (*Leptosphaeria maculans* fruiting budies (pycnidia) visible on 1 year old canola (*Brassica napus*) stubble. Pycnidia will produce pycnidiospores after a rain event acting as an inoculum source to infect young canola plants.



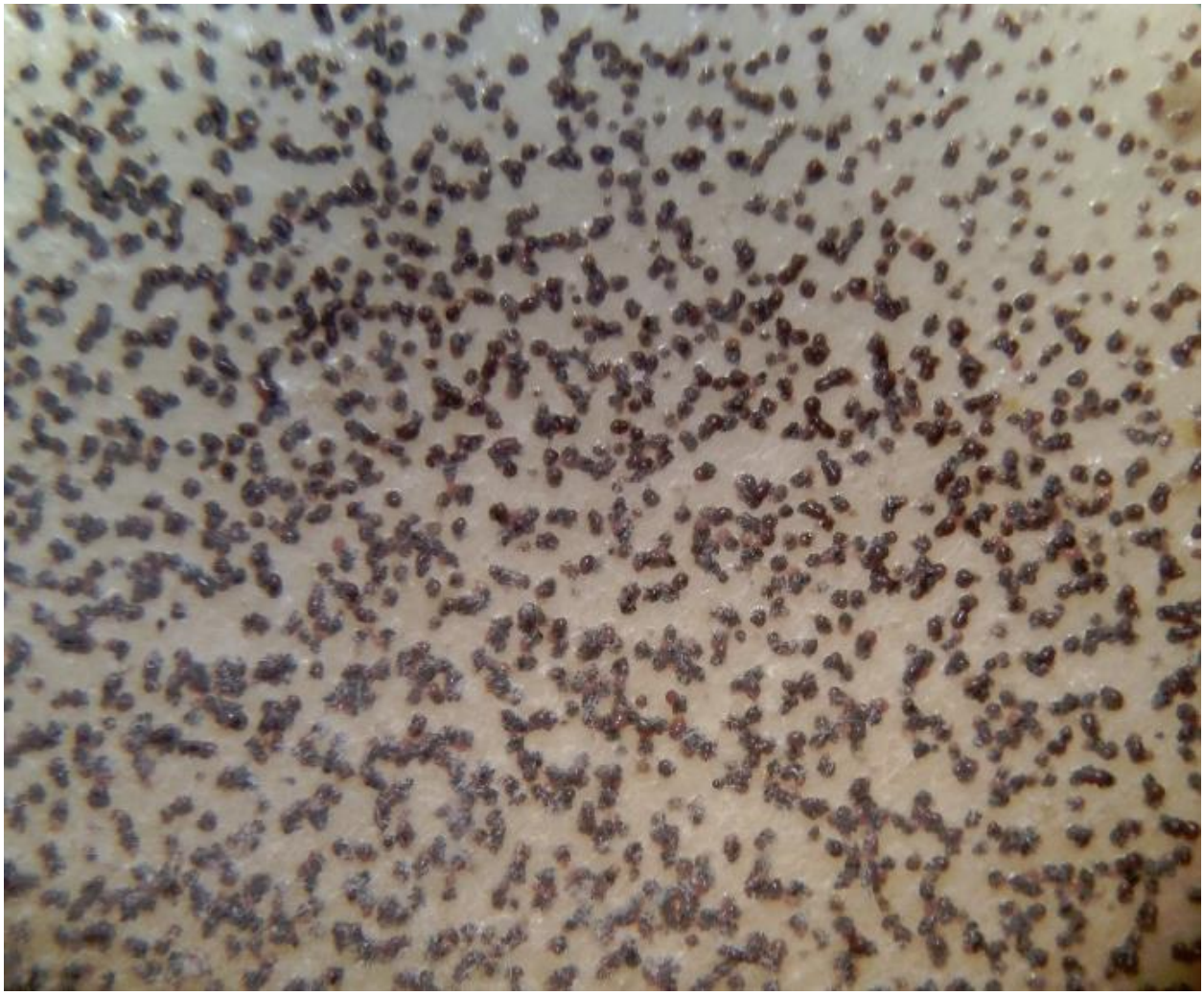
Appendix 7: 1 year old canola (*Brassica napus*) stubble surface sterilized with bleach and plated onto V8 agar media. Blackleg (*Leptosphaeria maculans* fruiting budies (pycnidia) are visible on the plate.



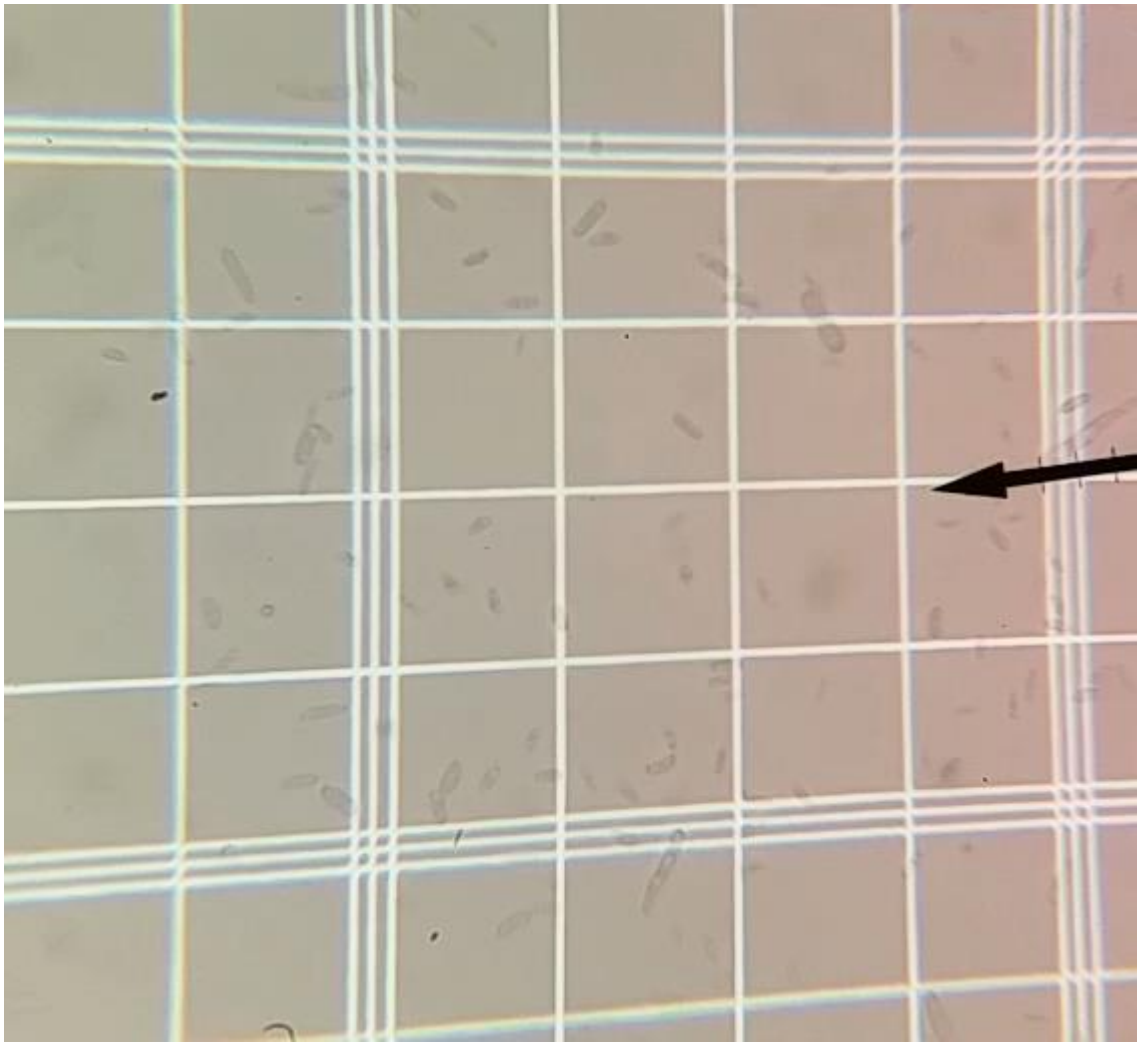
Appendix 8: Single spore *Leptosphaeria maculans* culture produced by selecting pycnidiospores froma a single pycnidia under a microscope.



Appendix 9: Plate showing *Leptosphaeria maculans* cultures on the left and *Leptopshaeria biglobosa* cultures on the right.



Appendix 10: Microscope image of *Leptosphaeria maculans* cultures on V8 agar media showing pycnidia producing pycnidiospores.



Appendix 11: Microscope image of *Leptosphaeria maculans* pycnidiospores. Pycnidospores are at varying maturity.

Appendix 12: 7 day old canola (*Brassica napus*) cotyledons inoculated with 10ul droplets of Blackleg (*Leptosphaeria maculans* pycnidiospores.

Appendix 13: Canola (*Brassica napus*) cotyledon inoculated with *Leptosphaeria maculans* pycnidiospores 14DAI. Closeup of resistant rating 1 on a 9 point scale.

Appendix 14: Canola (*Brassica napus*) cotyledon inoculated with *Leptosphaeria maculans* pycnidiospores 14DAI. Closeup of resistant rating 3 on a 9 point scale.

Appendix 15: Canola (*Brassica napus*) cotyledon inoculated with *Leptosphaeria maculans* pycnidiospores 14DAI. Closeup of intermediate rating 5 on a 1-9 point sclae.

Appendix 16: Canola (*Brassica napus*) cotyledon inoculated with *Leptosphaeria maculans* pycnidiospores 14DAI. Closeup of suscueptible rating 7 on a 1-9 point sclae.

Appendix 17: Canola (*Brassica napus*) cotyledon inoculated with *Leptosphaeria maculans* pycnidiospores 14DAI. Closeup of suscueptible rating 9 on a 1-9 point sclae.