Virulence and genetic structure of *Plasmodiophora brassicae* populations in Alberta, Canada

by

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Abstract

Clubroot, caused by *Plasmodiophora brassicae* Woronin, is one of the most important diseases of canola (*Brassica napus* L.) in western Canada. While the disease is managed most effectively by planting clubroot resistant (CR) canola cultivars, genetic resistance has been overcome in more than 200 fields in Alberta since 2013. Thirty-four single-spore isolates of *P. brassicae* were purified from 9 field isolates collected from CR canola crops across the province, and evaluated for pathotype classification on the Canadian Clubroot Differential (CCD) set, which includes the differentials of Williams and Somé et al. Using an index of disease of 50% (+/- 95% confidence interval) to distinguish between resistant and susceptible host reactions, 13 pathotypes could be distinguished based on the CCD system, seven on the differentials of Williams, and three on the hosts of Somé et al. Novel pathotypes, not reported in Canada previously, were identified among the isolates. The genetic structure of the isolate collection was evaluated by simple sequence repeat (SSR) marker analysis, which indicated a low level of genetic diversity. Polymorphisms were detected in 32 loci with the identification of 93 distinct alleles. Haploid linkage disequilibrium and number of migrants suggested that recombination and migration were rare or almost absent in the tested *P. brassicae* population. A relatively clear relationship was found between the genetic and virulence structure of some of the pathotypes, and isolates from northern and southern Alberta were genetically distinct from each other and from those from central Alberta. Further testing on a suite of seven CR canola cultivars indicated that the field isolates consisted of a mixture of virulent and avirulent pathogen genotypes. Significant genetic differentiation was detected among the pathotypes and between virulent and avirulent populations. Genetically homogeneous single-spore isolates provided a more complete and clearer picture of *P. brassicae* virulence and genetic structure. The deployment of genetically resistant canola should

be combined with other management tactics, such as longer rotation intervals out of susceptible hosts, for the sustainable management of clubroot in Alberta.

Preface

This dissertation is an original work by Homa Askarian in partial fulfilment of the requirements for the degree of Doctor of Philosophy. Mrs. Askarian conducted all of the experiments and prepared the first drafts of all chapters. The chapters were then examined by Mrs. Askarian's supervisors, Dr. Stephen Strelkov and Dr. Sheau-Fang Hwang, who provided suggestions, comments and editorial revisions for each chapter, which were then incorporated by Mrs. Askarian. Dr. Alireza Akhavan provided assistance with data analysis in the various chapters.

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Mrs. Askarian was responsible for the design of the study, method development, conducting the experiments, collecting and analysing the data, and manuscript composition. Drs. Strelkov and Hwang developed the initial research plan and assisted with manuscript editing and interpretation of the results. Dr. Akhavan provided expertise with the NTSys-pc analyses and contributed to manuscript editing. Dr. Manolii collected all samples during the clubroot survey in 2014 and assisted in conducting greenhouse experiments. Dr. Cao also assisted in conducting greenhouse experiments.

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Chapter 1. Introduction and literature review

1. 1 General introduction

Clubroot, caused by the obligate biotroph *Plasmodiophora brassicae* Wor., is a devastating soil-borne disease of vegetable and oilseed crops in the Brassicaceae family (Dixon 2009b; Karling 1968). Tewari et al. (2005) first identified clubroot on canola (*Brassica napus* L.) in Alberta, Canada, in 2003, in 12 fields in Sturgeon County. More than 3,000 fields with confirmed clubroot infestations had been reported in Alberta by 2018, highlighting the rapid spread of the pathogen (Strelkov et al. 2019b). This dissemination suggests that clubroot poses a significant threat to the economically important canola industry in Canada, since yield losses have been estimated to be as high as 30-100% in fields with severe infestations (Strelkov and Hwang 2014). Management of the disease is challenging, and most cultural or chemical control methods are either not practical or too expensive to implement. The deployment of clubroot resistant (CR) canola cultivars has been the most convenient and cost-effective way to manage the disease, but the recent identification of novel *P. brassicae* pathotypes capable of overcoming this resistance represents an additional challenge to clubroot management (Strelkov et al. 2016, 2018).

Field isolates of *P. brassicae* can be a mixture of pathotypes, making assessments of host resistance and the genetics of host-pathogen interactions difficult. Therefore, it seems necessary to obtain genetically homogeneous single-spore isolates, which may more clearly represent the pathogen population structure. This will not only allow improved understanding of host-pathogen relations, but will also enable comparison and characterization of the genetic and virulence structure of *P. brassicae* single-spore and field isolates able to overcome host resistance.

This chapter will focus on the biology of *P. brassicae* with respect to clubroot disease of canola, concentrating on past studies of the genetic and virulence spectrum of the pathogen and its ability to overcome host resistance.

1. 2 Clubroot disease

1. 2. 1 Distribution and economic importance

Clubroot has long been a constraint in the production of cruciferous crops. While the first report of clubroot is from 4th century Italy (Watson and Baker 1969), the disease is now established worldwide and is a major cause of yield and quality losses (Dixon 2009a). In Canada, clubroot has been an issue on *Brassica* vegetables in British Columbia, Ontario, Quebec and the Maritimes for over a century, and was likely introduced by European settlers (Howard et al. 2010). The first report of clubroot on canola occurred in Quebec in the late 1990s (Morasse et al. 1997), soon followed by the identification of the disease on canola in Alberta in 2003 (Tewari et al. 2005). More recently, clubroot has also been reported on canola in Manitoba, Saskatchewan and Ontario (Al-Daoud et al. 2018; Cao et al. 2009; Dokken-Bouchard et al. 2012). By 2018, clubroot infestations had been confirmed in more than 3000 fields throughout Alberta (Strelkov et al. 2019b), with additional cases reported in Saskatchewan and Manitoba. This disease causes crop losses of 10-15% worldwide (Dixon 2006). In replicated field trials in Quebec, Pageau et al. (2006) estimated yield losses of up to 91% yield in canola, with additional declines in seed weight and oil content. Yield losses of 30-100% have been reported in severely infested commercial canola crops in Alberta (Strelkov and Hwang 2014).

1. 2. 2 Causal agent

Plasmodiophora brassicae is a soil-borne obligate parasite and the casual agent of clubroot of crucifers (Woronin 1878). While traditionally regarded as a fungus, its taxonomic classification has been revised various times. Currently, *P. brassicae* is placed within the Plasmodiophorida in the phylum Cercozoa under the Rhizaria supergroup of the Protista (Bulman and Braselton 2014), based mainly on molecular phylogenetic analyses of the small subunit (SSU) ribosomal RNA (Bulman et al. 2001; Castlebury and Domier 1998), actin and polyubiquitin genes (Archibald and Keeling 2004), and mitochondrial sequences of the *COX1* gene (Daval et al. 2019). The Rhizaria supergroup includes several other economically important plant pathogens, including *Spongospora subterranea* (Wallr.) Lagerh., cause of powdery scab of potato, and *Polymyxa* spp., the vector of several plant viruses (Braselton 1995; Bulman et al. 2001). A comparison of the amino acid sequences of 12 protein-coding genes of 67 organisms demonstrated a close relationship between *P. brassicae* and *S. subterranea* (Stjelja et al. 2019). The economic impact of *P. brassicae*, combined with its ability to infect the model plant *Arabidopsis thaliana* (L.) Heynh., makes it the most studied member of the Plasmodiophorida (Siemens et al. 2009).

The clubroot pathogen is believed to infect all vegetable and oilseed crops in the Brassicaceae, including Brussels sprouts (*Brassica oleracea* L. var. *gemmifera)*, cabbage (*B. oleracea* L.var. *capitata)*, cauliflower (*B. oleracea* L. var. *botrytis)*, kale (*B. oleracea* L. var. *sabellica*), turnip (*B. rapa* subsp. *rapa* L.), turnip rape (*B. rapa* L. subsp. *oleifera* (DC.) Metzg), swede turnip (*B. napobrassica* (L.) Mill), canola/oilseed rape (*B. napus* L.), radish (*Raphanus sativus* (L.) Domin), and broccoli (*B. oleracea* L. var. *italica*) (Dixon 2009a). It is also a parasite of wild crucifers such as *Sinapis alba* L., *Matthiola* spp, *Capsella bursa-pastoris* (L.) Medik., *Cardamine* sp., *Thlaspi arvense* L., *Erysimum cheiri* (L.) Crantz., and *A. thaliana* (Buczacki and

Ockendon 1979; Mithen and Magrath 1992; Tanaka et al. 1993). *Plasmodiophora brassicae* also infects the root hairs of several non-cruciferous hosts in the Poaceae, Rosaceae, Papaveraceae, Polygonaceae, Resedaceae, and Fabaceae, although further disease development appears to be arrested (MacFarlane 1952).

Resting spores are the main survival structures of *P. brassicae*. They are extremely resilient to harsh environmental conditions and can survive for many years (Wallenhammar 1999). While the half-life of the resting spores has been estimated at 3-6 years, recent studies from Canada suggest large declines in soil inoculum levels in the first two years following a susceptible crop (Ernst et al. 2019; Peng et al. 2015). Resting spore longevity is influenced by soil moisture, temperature and the position of the spores in the soil profile (Dixon 2009b). Given its soilborne nature, *P. brassicae* is disseminated mainly via the movement of infested soil (Strelkov and Hwang 2014). In Australia, Donald and Porter (2009) reported transmission of *P. brassicae* on farm machinery, boots, the hooves of grazing animals, infected transplants, and in surface floodwater. In western Canadian cropping systems, the movement of *P. brassicae*-infested soil on farm machinery appears to be the main mechanism of pathogen dispersal (Cao et al. 2009). Nonetheless, resting spores were also found in windblown dust from infested fields, likely contributing to local dispersal of *P. brassicae* (Rennie et al. 2015). In addition, the pathogen was detected by quantitative PCR analysis on the surface of the seeds and tubers of various crops, suggesting the potential for seedborne transmission, although commercial seed cleaning reduced infestation to near negligible levels (Rennie et al. 2011).

As an obligate parasite, *P. brassicae* cannot be readily cultured on growth media; therefore, studies of the pathogen biology, genome and gene expression can be difficult to conduct (Sundelin et al. 2011). Nevertheless, *P. brassicae* has been reported to grow in cultures containing plant cells

as either callus or hairy root cultures, or as a cell culture suspension (Asano and Kageyama 2006; Graveland et al. 1992; Ikegami et al. 1978). Bulman et al. (2011) found that *P. brassicae* could be maintained for long periods on *B. rapa* cell callus cultures in the absence of exogenous plant growth regulators, although development of new infected cells was uncertain. Recently, dual tissue culture of *P. brassicae* and Shanghai pak choy (*B. rapa* L. *ssp. chinensis* Rupr.) was reported as an excellent source of pathogen DNA for genome sequencing (Sedaghatkish et al. 2019).

1. 2. 3 Symptoms

On infected plants, *P. brassicae* causes malformation of both the tap and lateral roots, and occasionally also the base of the stem, resulting in galls or clubs that are the most characteristic symptom of the disease (Fig. 1.1). This galling reflects cellular hyperplasia and hypertrophy in the infected roots (Kageyama and Asano 2009; Voorrips 1995). At temperatures of 20-25°C, full symptom development occurs within 5-6 weeks after host inoculation (Sharma et al. 2011). The malformation of infected root tissues leads to a reduced capacity for water and nutrient uptake, and affected plants may wilt even under minor moisture stress. Severe infections can also result in foliar chlorosis and necrosis, plant stunting, and ultimately major yield losses (Dixon 2009a; Voorrips 1995). Pathogen resting spores form within the infected tissues and are released into the surrounding soil as the clubs decompose (Kageyama and Asano 2009). In highly susceptible plants, as many as 1×10^{10} resting spores can be produced per g of galled root tissue (Hwang et al. 2013).

The clubroot pathogen relies on its host as a source of carbon. The host also supplies *P. brassicae* with organic nitrogen, vitamins and minerals (Ludwig-Müller and Schuller 2008). Rolfe et al. (2016) identified 11 thiamine transporters in the *P. brassicae* genome and concluded that the pathogen obtains thiamine from its host. *Plasmodiophora brassicae* also profoundly alters host

physiology (Ludwig-Müller and Schuller 2008), and there is an expectation for it to secrete a variety of effector proteins that promote growth and the enlargement and differentiation of infected host cells (Pérez-López et al. 2018). Development of galls in infected plants depends primarily on auxin and cytokinin (growth promoting plant hormones) (Devos et al. 2005; Siemens et al. 2006). It was demonstrated that the amount of cytokinin is higher in infected vs. healthy roots (Dekhuijzen 1980). Secondary plasmodia were reported to synthesize cytokinin (Dekhuijzen 1981; Müller and Hilgenberg 1986), and it has been postulated that a combination of the downregulation of genes involved in cytokinin degradation in the host and the production of cytokinin by *P. brassicae* results in greatly enhanced host cell division (Siemens et al. 2006). Cytokinin also is involved in inducing a strong metabolic sink for the attraction of nutrients (Ludwig-Müller and Schuller 2008). An increase in auxin activity and extensive synthesis of host auxin precursors was also reported in infected roots (Butcher et al. 1974, 1984). Cell division is the main process at the start of gall formation, while the enlargement of infected cells occurs at later stages (Kobelt et al. 2000; Siemens et al. 2006).

1. 2. 4 Management

The persistence of *P. brassicae* resting spores in the soil makes the management of clubroot difficult. Cultural strategies, including long rotations out of susceptible hosts and exclusion of the pathogen via sanitization of field equipment, can be effective but have not been widely adopted by growers, at least not in a western Canadian context (Howard et al. 2010; Hwang et al. 2014; Strelkov et al. 2011). Nonetheless, once clubroot becomes established in a field, the integration of multiple management methods is recommended (Donald and Porter 2009; Howard et al. 2010). Since the development of clubroot is favored by lower pH soils, the application of lime to increase soil alkalinity represents one of the oldest methods to control this disease (Donald and Porter 2009;

Karling 1942). Unfortunately, liming is not always sufficient to keep clubroot severity at low levels (Gossen et al 2013; Murakami et al. 2002), and the cost and time required to lime the soil may be prohibitive in the canola cropping systems of western Canada (Hwang et al. 2014). Nonetheless, the application of lime as a spot treatment or to supplement genetic resistance may have some potential as another tool in the integrated management of clubroot on canola (Fox 2019). Calcium and boron also have been reported to inhibit clubroot, but results from field studies have been mixed (Deora et al. 2011; Dixon 1996; Donald et al. 2006; Gossen et al. 2014; Page 2001; Ruaro et al. 2009). A number of fungicides, including cyazofamid, fluazinam, benomyl, pentachloronitrobenzene, trichlamide and flusulfamide, have also been evaluated for the management of clubroot, but while effective at reducing the disease in controlled environments, they have been less effective in field studies (Adhikari 2010; Naiki and Dixon 1987; Tanaka et al. 1999; Townley and Fox 2003). Furthermore, the use of soil-applied fungicides for the control of clubroot in broad acre crops such as canola is not economical nor eco-friendly due to cost and environmental concerns (Hwang et al. 2014).

The challenges associated with clubroot management have generated interest in the use of biological control strategies for this disease. Application of Serenade® (*Bacillus subtilis*) and Prestop® (*Clonostachys rosea* f. *catenulate*) reduced clubroot severity on canola in greenhouse studies, but did not significantly suppress resting spore populations under field conditions (Lahlali et al. 2011, 2013; Peng et al. 2011). At present, there are no biocontrol agents registered for clubroot management in Canada (Peng et al. 2014b). The planting of bait crops to decrease *P. brassicae* inoculum levels, by inducing resting spore germination in the soil, has also been evaluated. While bait crops have been found to reduce clubroot severity in some studies, their efficacy has not always been consistent (Ahmed et al. 2011; Friberg et al. 2006; Hwang et al. 2015;

Ikegami 1985; Murakami et al. 2000, 2001; Robak 1996; Rod and Robak 1994). Furthermore, the costs associated with seed purchase and cultivation of the bait crop, together with the length of time during which the land is not productive as the bait crop is cultivated, limit the potential utility of bait crops for clubroot control (Donald and Porter 2009).

The deployment of resistant host varieties represents the most effective and practical approach to clubroot management (Rahman et al. 2014; Peng et al. 2014a, b). In western Canada, the first CR canola cultivar was released in 2009, with multiple varieties becoming available over the next few years. While the genetic basis for the resistance in commercial cultivars is not in the public domain, there is strong evidence to suggest that in most cultivars, this resistance was derived from the European winter oilseed rape 'Mendel' (Fredua-Agyeman et al. 2018). Soon after their introduction, the planting of CR canola cultivars became the most widely used clubroot management strategy on the Prairies (Strelkov et al. 2016, 2018). Despite evidence that this resistance was not particularly durable (LeBoldus et al. 2012), CR canola was grown in short rotations in many fields, resulting in the emergence of new pathotypes of *P. brassicae* able to overcome resistance (Strelkov et al. 2016). Indeed, there has been a proliferation of resistance-breaking pathotypes since 2013 (Strelkov et al. 2016, 2018), which collectively represent one of the biggest threats to sustainable canola production in western Canada.

1. 3 Life cycle of *Plasmodiophora brassicae*

There are three main stages in the *P. brassicae* life cycle (Fig. 1.2); these include survival in the soil, root hair infection (primary infection) and cortical infection (secondary infection) (Ingram and Tommerup 1972; Kageyama and Asano 2009). The most active part of the life cycle occurs intracellularly, with the production of intracellular plasmodia, which in turn leads to the development of resting spores (Ludwig-Müller et al. 2015).

1. 3. 1 Resting spore germination

The first step in the infection process begins with germination of the resting spores and the release of spindle-shaped, pyriform, biflagellate primary zoospores that attack the root hairs (Kageyama and Asano 2009). The germination rate of the resting spores is greatly influenced by their maturity (Ayers 1944; Honig 1931). In addition, soil pH, texture, moisture and temperature are other key factors affect spore germination. A temperature range of 20-25°C is most favourable for resting spore germination (Ikegami et al. 1981). Bremer (1924) found enhanced germination in wet acidic soils versus alkaline soils (Gossen et al. 2014), although severe clubroot can also develop in alkaline soils if spore levels are very high (Colhoun 1953; Karling 1968; Strelkov et al. 2007). Ingram and Tommerup (1972) reported that resting spore germination commenced after 12-18 hours of incubation, while Kageyama and Asano (2009) reported that approximately 2 days were required. The disappearance of the refractile globules usually found in the resting spores is the first sign that germination has been initiated, and likely reflects the degradation of energy reserve materials via increased enzymatic activity (Dixon 2009b).

Host root exudates (as well as exudates from some non-host plants) stimulate resting spore germination (Bochow 1965; Chupp 1917; Feng et al. 2010; Hooker et al. 1945; MacFarlane 1970; Niwa et al. 2008), although spores can also germinate in the absence of any host (Honig 1931). It seems that carbohydrates in the root exudates prompt germination, and may serve as an external source of energy for the primary zoospores (Dixon 2009b). Feng et al. (2010) cloned a serine protease gene (*PRO1*) that may play a role in *P. brassicae* pathogenesis, by stimulating the germination of resting spores via its proteolytic activity. The involvement of G-protein-coupled receptors (GPCRs) in recognizing signals from the host and soil environment has also been suggested to influence spore germination (Bi et al. 2019).

1. 3. 2 Root hair infection (primary infection)

The primary zoospores attach to and encyst on the root hairs. A tubular structure called the Rohr develops to protect the stachel (sting), a sharp, pointed rod which penetrates the host cell wall. A bulbous adhesorium forms at the end of Rohr and strongly adheres to the cell wall. In the zoospore, the vacuole enlarges, creating pressure that pushes the stachel into the host cell wall, injecting the pathogen protoplasm into the root hair (Aist and Williams 1971). Once inside the host, a multinucleate primary plasmodium develops, and following several endomitotic divisions, cleaves into a zoosporangium that gives rise to 4-16 uninucleate secondary zoospores (Ingram and Tommerup 1972; Williams et al. 1971). The secondary zoospores are morphologically similar to the primary zoospores (Kageyama and Asano 2009), but have been suggested to differ on a molecular level, particularly with respect to gene expression (Feng et al. 2013).

Root hair infection also can be caused by secondary zoospores (Naiki et al. 1984). Dobson and Gabrielson (1983) stated that primary zoospores are not able to infect cortical tissues; however, Mithen and Magrath (1992) reported the cortical infection in *A. thaliana* by primary zoospores. Feng et al. (2013) also demonstrated that both primary and secondary zoospores are able to cause primary and secondary infections.

Clubroot symptoms are not obvious during the primary infection stage (Howard et al. 2010). The only visible symptoms are subtle, and include curling and deformation of the root hairs (Samuel and Garrett 1945). Root hair infection was also observed in non-host plants (Deora et al. 2012; Feng et al. 2012; Ludwig-Müller 1999; MacFarlane 1952) and resistant hosts (Deora et al. 2013; Diederichsen et al. 2009; Fei et al. 2016; Gludovacz et al. 2014; Peng et al. 2019). Therefore, root hair infection appears to be less host-specific than cortical infection. Feng et al. (2013) proposed that root hair infection plays a role in overcoming the basal resistance of the plant to

cortical infection. McDonald et al. (2014) showed that initiation of the host resistance reaction in a resistant canola cultivar occurred during primary infection.

1. 3. 3 Cortical infection (secondary infection)

The process of cortical infection is one of the least understood stages of the *P. brassicae* life cycle. It is unclear whether the secondary zoospores are released into the soil and infect the cortical tissues from the outside, and/or if they infect the neighboring cortical cells from inside the root (Kageyama and Asano 2009). Furthermore, there is uncertainty regarding potential fusion between pairs of secondary zoospores. Fusion of two zoospores to form a binucleate zoospore was reported by Ingram and Tommerup (1972) and Tommerup and Ingram (1971) prior to penetration of the host, while McDonald et al. (2014) did not observe any fusion or binucleate zoospores in a more recent study. Narisawa et al. (1996) and Diederichsen et al. (2016) also found that fusion of the secondary zoospores is not required for cortical infection. Regardless, following penetration, the zoospore develops into a multinucleate secondary plasmodium (Ingram and Tommerup 1972; Kageyama and Asano 2009; Tommerup and Ingram 1971). Active movement of a motile phase (myxoamoeba) inside the root was reported by several research groups (Dekhuijzen 1979; Kunkle 1918; Rochlin 1933; Mithen and Magrath 1992), while other researchers suggested that intracellular spread of the plasmodium occurs by cytoplasmic streaming during root cell division (Buczacki 1983; Kageyama and Asano 2009; Tommerup and Ingram 1971). Development of the visible root galls takes place during secondary infection as *P. brassicae* proliferates within the host.

The formation of the resting spores represents the final stage of the *P. brassicae* life cycle. Tommerup and Ingram (1971) stated that fusion of two haploid nuclei may occur inside the multinucleate secondary plasmodia, resulting in diploid nuclei that immediately undergo meiosis

to produce haploid resting spores. This suggestion, however, is not universally accepted (Kageyama and Asano 2009). Nonetheless, at the start of sporogenesis, the outer membrane of the secondary plasmodium disintegrates, leaving only the inner (plasmodial) membrane (Williams and McNabola 1967). At this stage, several vacuoles become visible, resulting from invaginations of the plasmodial membrane. The vacuoles line up and merge next to cleavage planes around each nucleus, which is surrounded by cytoplasm and vacuolar membrane and develops into a spiny resting spore (Williams and McNabola 1967). The *P. brassicae* resting spore wall consists of five layers, with protein, lipid and chitin as its major components (Buczacki and Moxham 1983; Moxham and Buczacki 1983).

1. 4 Evolution of *Plasmodiophora brassicae*

Physiological specialization has long been observed in *P. brassicae* (Honig 1931), and several race or pathotype-specific resistance genes have been identified in its hosts (Piao et al. 2009). This suggests that the clubroot pathosystem follows the gene-for-gene model (Crute et al. 1983; Feng et al. 2014), although several quantitative trait loci (QTLs) have also been reported to be involved in resistance to *P. brassicae* (Chen et al. 2013; Sakamoto et al. 2008; Werner et al. 2008). Horizontal gene transfer (HGT) has been suggested to contribute to the evolution of the clubroot pathogen and emergence of new pathotypes (Bryngelsson et al. 1988; Feng et al. 2014; Zhang et al. 2015). For example, Zhang et al. (2015) suggested that the presence of the *Cr811* gene exclusively in pathotype 5 isolates of *P. brassicae* may have resulted from horizontal gene transfer. In a recent study, however, no evidence for horizontal gene transfer events between *P. brassicae* and its host or other soil microorganisms was detected (Stjelja et al. 2019).

The changes in the frequency of the available strains of the pathogen without any changes of genome structure is the most immediate evolutionary response of the pathogen against host

resistance (Anderson et al. 2010). Such evolutionary changes are regularly reported in the clubroot pathosystem (Crute and Pink 1989; Jones et al. 1982; Seaman et al. 1963; Strelkov et al. 2016, 2018; Tanaka and Ito 2013). In experiments under controlled conditions, LeBoldus et al. (2012) showed the rapid adaptation of *P. brassicae* isolates to a variety of host genotypes, while Cao et al. (2020) reported that continuous planting of CR canola resulted in a fast increase in virulent pathotypes. Repeated cultivation of resistant cultivars leads to increases in the proportion of the pathogen population virulent on those cultivars, eventually resulting in a loss of resistance (Hwang et al. 2017; Tanaka and Ito 2013). The loss or erosion of clubroot resistance has been reported in cabbage (Seaman et al. 1963), Chinese cabbage (Hatakeyama et al. 2006; Osaki et al. 2004), the oilseed rape 'Mendel' (Diederichsen et al. 2014; Oxley 2007) and most recently in Canadian canola (Strelkov et al. 2016, 2018).

Relatively little is known regarding the molecular basis of variation among strains of *P. brassicae*; however, mutation, genetic drift, gene flow and migration may contribute to this diversity. Bi et al. (2019) found that genetic diversity in *P. brassicae*, assessed as single-nucleotide polymorphisms, was not influenced by transposable elements (TEs) in the genome. Therefore, alternative mechanisms may be involved in maintaining genetic diversity in the pathogen. Yang et al. (2018) reported a high level of polymorphism in a comparison of 'old' and 'new' virulent pathotypes of *P. brassicae* from Alberta, and suggested that the emergence of new virulent pathotypes may involve more than the loss of an avirulence gene or single-point mutations. Holtz et al. (2018) suggested that *P. brassicae* populations in Canada are clonal, and that a group of virulent isolates identified in 2013 (and able to overcome resistance in CR canola) may have been introduced recently or were present at low frequencies, increasing as a result of selection pressure from the cultivation of CR cultivars. Calculation of linkage disequilibrium, however, indicated a

high frequency of recombination in the Canadian *P. brassicae* population, at least based on the isolates analyzed in a recent study (Sedaghatkish et al. 2019). Balancing selection was suggested to occur in *P. brassicae* (Strelkov et al. 2018), which may help the pathogen adapt to its environment by preventing loss of low-frequency alleles or those that are less fit. Data also suggest that both old and new pathotypes of *P. brassicae* were already present on the Canadian Prairies before the introduction of CR canola, with populations of compatible pathotypes selectively increased by the selection pressure imposed by resistant varieties (Sedaghatkish et al. 2019; Strelkov et al. 2019a).

1. 5 Genome of *Plasmodiophora brassicae*

Five chromosomes were reported in *P. brassicae* using a karyotyping technique (Tommerup and Ingram 1971). Later, an electron microscopy study of the pathogen showed 20 synaptonemal complexes (SC), which are meiosis-specific nuclear structures between two pairs of sister chromatids (Braselton 1982). An electrophoretic karyotype of *P. brassicae* generated using spheroplasts indicated 13 chromosomal bands in the range of 750 -1900 kb (Ito et al. 1994), while only six bands in the range of 680-1700 kb were found using plasmodia (Bryan et al. 1996). Graf et al. (2001) reported 16 chromosomal bands in the range of 680 to 2200 kb and suggested the possibility of more than 16 chromosomes in *P. brassicae*. Most recently, 20 contigs representing the nuclear genome were identified in the clubroot pathogen, from which 13 chromosomes were characterized 'from telomere to telomere' (Stjelja et al. 2019).

Gene identification and knowledge of gene organization and expression are critical for understanding the interaction between plant pathogens and their hosts (Sundelin et al. 2011). In the case of *P. brassicae*, very few genes were functionally well-characterized prior to 2010 (Feng et al. 2010). The ribosomal small subunit (SSU) gene was the first *P. brassicae* DNA fragment to

be amplified and sequenced (Castlebury and Domier 1998). Around the same time, another 11 DNA fragments of *P. brassicae* were sequenced (Ito et al. 1998). Ando et al. (2006) reported the full sequence at the genomic and mRNA level of a serine/threonine kinase-like gene (*PbSTKL1*) from *P. brassicae*, and concluded that *PbSTKL1* plays a role in the proliferation of the pathogen in the host. Approximately 44 kb of the *P. brassicae* genome, consisting of 24 genes, was sequenced by Bulman et al. (2007), who found the genes to be rich in spliceosomal introns. Twenty-four *P. brassicae* genes were identified by suppression subtractive hybridization in infected Chinese cabbage (Sundelin et al. 2011), from which three genes (*PbsHSP1*, *PbGST1* and *PbSUR2*) were found in common with genes reported by Bulman et al. (2006) in infected *Arabidopsis* plants. This led the authors to suggest that these genes may play a role in pathogenicity. Sundelin et al. (2011) also showed that the *P. brassicae* genes they characterized possessed multiple short introns and relatively short exons. Niwa et al. (2011) reported the complete sequence of the small subunit (SSU), 5.8S and large subunit (LSU) rDNAs of *P. brassicae*, as well as the internal transcribed spacer and intergenic spacer regions. In a subsequent analysis, however, Schwelm et al. (2016) determined the sequence of *P. brassicae* rDNA and concluded that half of the Niwa et al. (2011) sequence did not belong to *P. brassicae*. A mitochondrial genome sequence of 102,962 bp was recently published for the clubroot pathogen (Bi et al. 2019). An independent study also found that the *P. brassicae* mitochondrial genome is large (114,663 bp), gene-dense and intron rich (Stjelja et al. 2019).

Based on the number and size of the chromosomes, Graf et al. (2001) estimated the *P. brassicae* genome to be 20.3 Mb. More recently, Schwelm et al. (2015) reported a 25.5 Mb draft genome (in 165 scaffolds) for the European single-spore isolate e3, in the first published comprehensive analysis of the entire *P. brassicae* genomic sequence. In a concurrent study of

Canadian isolates of *P. brassicae*, a highly compact genome 24.2 Mb in size was reported for pathotypes 3 (109 scaffolds) and 6 (356 scaffolds), with the compact nature of the genome attributed to a significant reduction of intergenic space, a low number of repetitive elements, and overlapping untranslated regions (Rolfe et al. 2016). Bi et al. (2016, 2019) reported a 24.1 Mb (667 scaffolds) genome for *P. brassicae* isolate ZJ-1 from China, and Daval et al. (2019) published a 24.6 Mb genome for a second European isolate (eH) of the pathogen. The GC-content was reported to be 58.7% for isolate e3 (Schwelm et al. 2015), with a similar value (59%) found for other genomes of *P. brassicae* (Bi et al. 2016; Daval et al. 2019; Rolfe et al. 2016). The highest (12,811) and lowest (9,730) number of predicted genes were found by Daval et al. (2019) and Schwelm et al. (2015), respectively, while 10,851 (Canadian pathotype 3), 10,070 (Canadian pathotype 6) and 10,951 (Chinese ZJ-1 isolate) predicted genes were reported by Rolfe et al. (2016) and Bi et al. (2016, 2019). On average, *P. brassicae* genes contain four introns, with only 5.4% repetitive and transposable elements (Schwelm et al. 2015). All of the recent genomic analyses have noted the small size and high gene density (with a low rate of repetitive elements) of the *P. brassicae* genome.

1. 6 Importance of *Plasmodiophora brassicae* single-spore isolation

Given its biotrophic nature, single-spore isolates of *P. brassicae* can be difficult to obtain. As such, most studies have been conducted with field isolates (syn. populations) of the pathogen, which represent all of the resting spores recovered from a single gall or root of an infected plant. Field isolates, while relatively easy to collect, can be heterogeneous mixtures of various pathotypes (Jones et al. 1982; Tinggal and Webster 1981; Xue et al. 2008). These pathotypes may proliferate selectively on different host genotypes, making assessments of host resistance and the genetics of host-pathogen interactions difficult. Furthermore, pathotypes found at lower frequencies may not

be detected on differential host genotypes, since host reactions could be masked by the predominant pathotypes (Jones et al. 1982). The heterogeneity of some field isolates may also lead to intermediate or fluctuating host reactions to inoculation (Jones et al. 1982; Scott 1985; Strelkov et al. 2006 and 2007; Tinggal and Webster 1981; Xue et al. 2008), resulting from competition between virulent and avirulent pathotypes for infection sites, and/or modulation of host resistance by the avirulent pathotypes (Jones et al. 1982; Voorrips 1996). In light of these issues, single-spore isolates may more clearly reflect the pathogen population structure, particularly rare or infrequent pathotypes, which can enable an improved understanding of host-pathogen relations and *P. brassicae* population dynamics.

To date, the isolation of single-spores of *P. brassicae* has been achieved using agar/agarose-based techniques (Jones et al. 1982; Manzanares-Dauleux et al. 1994; Scott 1985; Somé et al. 1996; Tinggal and Webster 1981) or with droplets of highly diluted spore suspensions (Buczacki 1977; Voorrips 1996; Xue et al. 2008). An alternative approach for single-spore isolation was described by Diederichsen et al. (2016), in which *Brassica* seedlings were inoculated with single root hairs infected by *P. brassicae*. The efficacy of single-spore isolation procedures can vary, depending on the condition of the spore suspension, particularly the percentage of mature and viable spores, as well as technical differences in the protocols (Buczacki 1977; Voorrips 1996). Since resting spores of *P. brassicae* are haploid (Tommerup and Ingrain 1971), the descendants of a single resting spore should be genetically homogeneous except for mutations (Voorrips 1995, 1996). *Plasmodiophora brassicae* single-spore isolates were shown to be genetically stable after three generations based on random amplified polymorphic DNA (RAPD)-PCR analysis (Heo et al. 2009).

1. 7 Virulence spectrum of *Plasmodiophora brassicae*

An updated knowledge of diversity in the virulence of local populations of *P. brassicae*, particularly in regions where resistant varieties are cultivated, is important for the judicious and effective deployment of the currently-known resistance genes (Manzanares-Dauleux et al. 2000). Diversity in the virulence of *P. brassicae* was first suggested by Honig (1931), although it had been suspected much earlier (see Buczacki et al. 1975). Since then, many studies have reported significant variation in collections of *P. brassicae* single-spore and field isolates (Buczacki 1977; Crute et al. 1980; Jones et al. 1982; Manzanares-Dauleux et al. 1994; Scott 1985; Somé et al. 1996; Tinggal and Webster 1981; Voorrips 1996; Xue et al. 2008; Strelkov et al. 2018).

Since isolates of *P. brassicae* are morphologically identical, they could be distinguished only by virulence tests on sets of differential hosts (Ayers 1957; Buczacki et al. 1975; Kuginuki et al. 1999; Somé et al. 1996; Williams 1966), at least prior to the advent of molecular tools. A differential genotype may effectively discriminate between populations of *P. brassicae* in one region, but may lack differentiating capacity at another location (Somé et al. 1996). Thus, the prudent selection of differential host genotypes is required for the successful study of population structure, both between and within pathogen field isolates, in a particular region (Somé et al. 1996; Strelkov et al. 2007).

Various sets of differential hosts have been proposed over the years to identify pathotypes of *P. brassicae*. Ayers (1957) identified six pathotypes (races) in a collection of isolates from the USA and Canada, which were distinguished based on their virulence on a differential set consisting of wild and cultivated crucifers. Williams (1966) developed a differential system based on the reaction of two cabbage (‘Jersey Queen’ and ‘Badger Shipper’) and two rutabaga (‘Laurentian’ and ‘Wilhelmsburger’) genotypes, which allowed for the identification of a theoretical maximum

of 16 pathotypes. An effort to develop an international system for pathotype classification resulted in the development of the European Clubroot Differential (ECD) set, which includes a total of 15 hosts (five *B. napus*, five *B. rapa* and five *B. oleracea* genotypes) (Buczacki et al. 1975). Somé et al. (1996) introduced another set of differentials, consisting of the *B. napus* cultivars 'Nevin', 'Wilhelmsburger' and 'Brutor', to identify pathotypes of *P. brassicae* from France.

While the systems of Williams, the ECD set and Somé et al. have been used quite extensively to characterize physiologic specialization in *P. brassicae*, they cannot distinguish between isolates that are virulent or avirulent on clubroot resistant (CR) brassicas (Strelkov et al. 2018; Yano et al. 1997). Therefore, Kuginuki et al. (1999) proposed a new differential set consisting of CR Chinese cabbage cultivars showing clear resistant or susceptible reactions to Japanese isolates of *P. brassicae*. Osaki et al. (2008) also used CR Chinese cabbage cultivars to classify Japanese isolates of the pathogen into four groups. In Canada, the need for a novel system to classify isolates of *P. brassicae* became clear following the identification of pathogen strains that were virulent on CR canola (Strelkov et al. 2016), resulting in the development of the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018). The CCD set includes the differentials of Williams (1966) and Somé et al. (1996), selected hosts of the ECD set, and several *B. napus* hosts including CR canola. This system has significant differentiating capacity, and enabled the identification of 17 pathotypes from a collection of *P. brassicae* isolates from Canada, versus two and five pathotypes, respectively, on the differentials of Somé et al. and Williams (Strelkov et al. 2018). In Alberta, pathotype 3, as defined on the differentials of Williams, is prevalent on canola (Strelkov and Hwang 2014; Strelkov et al. 2018). On the CCD Set, several distinct variants of this pathotype have been identified, including pathotype 3A, which can overcome the resistance in CR

canola cultivars, and pathotype 3H, which corresponds to the 'old' pathotype 3 and is avirulent on CR canola (Strelkov et al. 2018).

In Canada, evaluation of virulence diversity of *P. brassicae* field isolates began in the 1950s (Ayers 1957). There has been a strong attempt to determine virulence spectrum of *P. brassicae* in Canada since the first report of disease on canola in Alberta in 2003. A summary of *P. brassicae* pathotypes reported in Canada is included in Table 1.1.

1. 8 Population genetic structure

Information on the genetic structure of pathogen populations is helpful in developing durable plant resistance and assessing the risk of those populations overcoming host resistance (McDonald and Linde 2002). Genetic diversity of plant pathogens can be related to host availability and their ability to infect multiple hosts (Travadon et al. 2011) or to their survival as resting spores between two growing seasons (Strehlow et al. 2014). Moreover, mutations and large effective population sizes are among the major sources of gene diversity that can lead to new virulent strains (McDonald and Linde 2002). The longevity of the resting spores (Wallenhammar 1996), along with a high reproduction rate per each generation (Diederichsen et al. 2009), can result in a large effective population size and may help to establish highly diverse *P. brassicae* populations (Strehlow et al. 2014). High gene diversity can also indicate high mutation rates in the pathogen life cycle (Strehlow et al. 2014).

Diversity also can be influenced by the occurrence of sexual reproduction. There is, however, some uncertainty regarding sexual recombination in the life cycle of *P. brassicae*. Tommerup and Ingram (1971) noted the possibility of sexual reproduction in the clubroot pathogen, since they observed nuclear fusion in vegetative plasmodia. In addition, a high level of genetic diversity among the single-spores derived from one gall may highlight the importance of

sexual recombination in the life cycle of *P. brassicae* (Manzanares-Dauleux et al. 2001). Given the formation of synaptonemal complexes in vegetative plasmodia (Braselton 1982), and the occurrence of re-infection by dikaryotic plasmodia (Kobelt 2000), Fähling et al. (2004) also noted the possibility of sexual recombination in *P. brassicae*. However, their comparison of molecular fingerprints and virulence patterns of two single-spore isolates as parents with new sets of single-spore isolates derived from mixed-infected roots suggested an absence of sexual recombination in the life cycle of this pathogen (Fähling et al. 2004). In contrast, Stjelja et al. (2019) identified seven active gene candidates encoding proteins associated with the synaptonemal complex, which suggests potential genetic recombination events during meiotic division.

In one of the first studies evaluating the genetic diversity of *P. brassicae*, Möller and Harling (1996) compared RAPD profiles of three single-spore isolates with their virulence patterns on the ECD set (Buczacki et al. 1975), and found that only one of 40 primers tested gave a profile consistent with the pathotype classification of the isolate. Also using RAPD analysis, Yano et al. (1997) assessed the genetic diversity of *P. brassicae* field isolates collected from cruciferous crops in Japan, and identified two major clusters. One cluster consisted of isolates classified as pathotype 9, as defined on the system of Williams (1966), and another consisted of pathotypes 1 and 4, leading the authors to suggest that RAPD analysis was effective for the identification of pathotype 9. RAPD profiling could not, however, distinguish isolates that were virulent or avirulent on CR Chinese cabbage (Yano et al. 1997). Osaki et al. (2008) also used RAPD analysis to detect relatively high levels of genetic variation among 17 Japanese field isolates, but found no relationship between DNA polymorphisms, isolate virulence or geographic origin. Similarly, Manzanares-Dauleux et al. (2001) also reported a high level of genetic diversity, but no clear

relationship between the molecular analysis and pathotype classification, host or geographic origin, in a RAPD analysis of *P. brassicae* field and single-spore isolates from France.

Comparisons of large subunit (LSU) rDNA sequences from eight Japanese field isolates also revealed variation among the isolates, which was related to their geographic distribution (Niwa et al. 2011). Schwelm et al. (2016), however, found no variation in the LSU rDNA sequences from *P. brassicae* isolates representing a worldwide collection, and suggested that the polymorphisms reported by Niwa et al. (2011) may have been derived from other (contaminating) soil organisms. Hence, the LSU rDNA region is not likely to be a suitable marker for distinguishing pathotypes of *P. brassicae* (Schwelm et al. 2016). In an amplified fragment length polymorphism (AFLP) analysis, Strehlow et al. (2014) detected genetic variability among 59 *P. brassicae* field isolates from three regions of Germany having different rapeseed cropping histories, acreages, and clubroot incidences. The authors concluded that *P. brassicae* in Germany is a genetically diverse species, which may reflect high mutation rates, sexual recombination, and large effective population sizes. However, cropping history, acreage or clubroot incidence were not found to influence the genetic diversity of the isolates tested.

Simple sequence repeat (SSR) markers were used to study genetic variation among 24 Japanese isolates of *P. brassicae* (Kubo et al. 2017). Eleven of 23 SSR markers showed polymorphism. The polymorphic markers came from intergenic regions and none of the genic markers revealed any variation among the isolates. A relatively low level of diversity was detected, which suggested low divergence of SSR repeats among the *P. brassicae* isolates. Using whole genome single nucleotide polymorphism (SNP) profiles, Rolfe et al. (2016) reported a correlation between haplotype clustering, host range and geographical distribution among five pathotypes of *P. brassicae* in Canada. Pathotypes 2, 3, 5 and 8, which were virulent on *B. napus* and collected

from the Prairies, were highly similar and grouped together. In contrast, pathotype 6, which is prevalent in British Columbia and Ontario and avirulent on *B. napus*, was more divergent (Rolfe et al. 2016). Comparison of the whole genome sequences of pathotypes 3 and 6 with the reference genome of the European isolate e3 (Schwelm et al. 2015) also indicated that e3 and pathotype 6 clustered together, while pathotype 3 did not (Rolfe et al. 2016). In another study, restriction site-associated DNA sequencing (RADseq) grouped Canadian *P. brassicae* single-spore and field isolates into two major clusters, corresponding to isolates that were virulent or avirulent on CR canola (Holtz et al. 2018). There was, however, a low level of genetic variation among the isolates within each group (Holtz et al. 2018). A SNP analysis of field and single-spore isolates of *P. brassicae* from Canada, the United States, and China indicated no clear grouping of isolates based on geographical origin, host or virulence (Sedaghatkish et al. 2019). However, isolates from Alberta that were virulent on CR canola separated clearly from the others (Hotz et al. 2018; Sedaghatkish et al. 2019).

1. 9 Molecular identification of *Plasmodiophora brassicae*

1. 9. 1 Molecular identification at the species level

Multiple approaches have been used to test for the presence of *P. brassicae* in soil and plant samples, including light and fluorescence microscopy (Takahashi and Yamaguchi 1988), bioassays with susceptible hosts (Colhoun 1957), and serological methods (Orihara and Yamamoto 1998; Wakeham and White 1996). However, these techniques require considerable effort, time, and highly specific knowledge. An alternative approach involves molecular detection assays, which are more sensitive, rapid, and less expensive. In one of the first molecular assays for *P. brassicae*, a single tube nested PCR for detecting pathogen-infested soil was developed based on the specific amplification of putative *ipt* genes encoding isopentenyl transferase (Ito et al.

1999). This method was reported to be sensitive enough to detect the presence of the target DNA from one resting spore per gram of soil. However, the sensitivity of the assay was reduced by the presence of humic acids in the soil; therefore, a double PCR technique (a combination of two PCR amplifications in which the product of the first amplification is used as DNA template for the second amplification) was developed and shown to be an effective tool for identifying infested fields (Ito et al. 1999). Faggian et al. (1999) developed a nested PCR assay, with primers designed based on the sequence of the ribosomal internal transcribed spacer (ITS) region, for the specific detection of *P. brassicae* in soil, water and plant tissue. This method had a detection level of 1×10^3 spores per gram of soil. Wallenhammar and Arwidsson (2001) also published a nested PCR assay to detect *P. brassicae* DNA in soil samples collected from infested fields, which could successfully amplify pathogen DNA from a variety of soil types.

Cao et al. (2007) published a simple, one-step PCR protocol to detect *P. brassicae* in plant and soil samples. Pathogen-specific primers were designed based on a *P. brassicae* partial 18S ribosomal RNA (rRNA) gene and ITS regions of the rDNA repeat. The assay was specific for the clubroot pathogen, since no amplicons were generated from non-infected host plants, non-infested soil, or common soil fungi and bacteria. In addition, these primers could consistently detect the pathogen in host roots 3 days after inoculation and as few as 1×10^3 resting spores per gram of soil. The ability of this protocol to detect such low levels of *P. brassicae* DNA likely reflected the high copy number of the rDNA sequences (Russel 1984) from which these primers were designed.

1. 9. 2 Molecular identification at the pathotype level

Bioassays are a reliable way to study variation in the virulence of *P. brassicae* populations and classify isolates into pathotypes. However, bioassays are also labor intensive and time consuming, and require ample space to grow large numbers of plants (Siemens et al. 2009). As

noted earlier, molecular techniques are useful for studying genetic variation in pathogen populations, but may also be valuable for distinguishing *P. brassicae* pathotypes or virulence types. One of the first pathotype-specific markers was published by Manzanares-Dauleux et al. (2000), who developed a sequence characterized amplified region (SCAR) marker for the specific identification of *P. brassicae* pathotype P_1, as classified on the system of Somé et al. (1996). More recently, Zhang et al. (2015) identified a gene, designated *Cr811*, which appeared to occur only in isolates representing Williams' pathotype 5. Testing of additional isolates indicated that, in addition to pathotype 5, *Cr811* was found in multiple *P. brassicae* strains capable of overcoming resistance, regardless of their Williams' pathotype designation (Feng et al. 2016). While an evaluation of a larger collection of isolates is still needed, the results suggested that *Cr811* could serve as a marker to detect new strains of *P. brassicae* before an erosion or loss of resistance is observed (Feng et al. 2016). In another study, three sets of primers designed based on the 18S/ITS rDNA region could distinguish 'pathotype 5-like' strains of *P. brassicae* able to over the resistance in CR canola from other isolates avirulent on these hosts (Zhou et al. 2018). One of these primer sets was used in a quantitative PCR assay with a TaqMan probe to measure pathogen DNA *in planta* starting at 4 days after inoculation (Zhou et al. 2018).

The publication of the *P. brassicae* genome has facilitated pathotype-specific marker development in recent years. For example, a duplex RNase H-dependent PCR (rhPCR) protocol was developed (Yang et al. 2018) by assessing DNA polymorphisms among 85 genes of *P. brassicae* and their corresponding sequences in the two whole genomes of the isolates e3 (Schwelm et al. 2015) and ZJ-1 (Bi et al. 2016). This assay could distinguish between pathogen strains virulent or avirulent on CR canola, although only a limited number of isolates was assessed. Furthermore, since the tested field isolates likely consisted of both virulent and avirulent *P.*

brassicae genotypes, Yang et al. (2018) suggested that single-spore isolates or field isolates propagated on specific CR cultivars would be required for successful detection by rhPCR. A set of SCAR markers also was developed by comparing the whole genome sequences of *P. brassicae* isolates from Korea with the genome of the European isolate e3 (Jeong et al. 2018). These markers successfully differentiated Korean isolates with different virulence patterns on non-CR and CR cultivars of Chinese cabbage.

1. 9. 3 Molecular quantification of *Plasmodiophora brassicae*

Quantitative or 'real-time' PCR (qPCR) is an effective tool to quantify the amount of *P. brassicae* found in plant, soil or water samples for basic studies as well as for disease management or resistance screening programs. Sundelin et al. (2010) developed a SYBR Green I qPCR assay, employing primers designed based on the ITS region, which could detect *P. brassicae* in plants just 10 days after germination of seeds planted in infested soil. In another study, Rennie et al. (2011) used primers specific for a partial 18S rRNA gene sequence (Cao et al. 2007) to design a SYBR Green I qPCR assay for measuring the amount of *P. brassicae* resting spores present as external contaminants in seeds and tubers. Wallenhammar et al. (2012) designed a TaqMan fluorogenic probe and species-specific primers to amplify a region of *P. brassicae* ribosomal DNA for detection and quantification of the clubroot pathogen in naturally and artificially infested soil samples. This protocol had a detection threshold as low as 500-1000 resting spores per gram of soil, and could detect as few as three or four copies of the target DNA. Cao et al. (2014) also developed and used a qPCR-based protocol based on a TaqMan probe to quantify *P. brassicae* DNA in the roots of hosts with different levels of resistance at multiple time-points following inoculation. Since qPCR-based techniques cannot distinguish between viable and non-viable resting spores, Al-Daoud et al. (2017) published a protocol in which spores were pre-treated with

propidium monoazide (PMA) before PCR analysis, preventing amplification of DNA from nonviable spores. PMA-PCR could also be used to estimate resting spore viability, facilitating assessments of the efficacy of chemical or cultural methods to control the pathogen (Al-Daoud et al. 2017).

1. 10 Significance of the research

The research to be presented in this thesis has generated a collection of single-spore isolates and detailed information on the genetic and virulence structure of *P. brassicae* populations able to overcome clubroot resistance in canola, helping to identify emerging pathotypes and providing important information for the judicious and effective deployment of currently known resistance genes. This information will also help to estimate the evolutionary potential of *P. brassicae* and its ability to overcome host resistance, aiding in assessments of the longevity of resistance sources. The identification of strain-specific alleles may also prove useful in the development of pathotype-specific markers for *P. brassicae*. Finally, from a technical perspective, this research led to the development of an optimized method for obtaining single-spore isolates of *P. brassicae*.

1. 11 Hypotheses and objectives

This thesis had five main objectives:

1- To obtain single-spore isolates of *P. brassicae* from field isolates virulent on CR canola cultivars

2- To determine the pathotype structure of single-spore and field isolates of *P. brassicae* from Alberta

3- To evaluate the virulence spectrum of the *P. brassicae* single-spore and field isolates on resistant canola cultivars, and assess if all single-spore isolates derived from a single club cause similar reactions on CR canola cultivars

4- To determine the genetic structure of *P. brassicae* populations from Alberta, and to assess the genetic differentiation between and within field isolates virulent and avirulent on CR canola

5- To explore the relationship between DNA polymorphisms and virulence patterns, with the aim of developing pathotype-specific primers

I hypothesized that:

1- Analysis of genetically homogeneous single-spore isolates will generate a clearer profile of the *P. brassicae* pathotype structure, compared with those obtained using field isolates, and will uncover novel pathotypes

2- The virulence spectrum of *P. brassicae* field and single-spore isolates will differ, even between an individual field isolate and its corresponding single-spore isolates

3- The virulence of *P. brassicae* field and single-spore isolates collected from the same fields prior to and following cultivation of CR canola cultivars will have shifted

4- Pathotypes able to overcome resistance on CR canola cultivars were present in commercial fields prior to the introduction of the first registered CR canola cultivar in Alberta

5- Genetic structure of *P. brassicae* varies greatly among and between counties and also fields in Alberta

6- There is a strong relationship between genetic and virulence structure of *P. brassicae*

1. 12 Tables

Table 1.1. Pathotype composition of *Plasmodiophora brassicae* in Canada

Province	Pathotype[a]		Reference(s)
	Williams	CCD	
Alberta	2, 3, 5, 6, 8	2B, 2F, 3A, 3D, 3H, 3O, 5C, 5G, 5I, 5K, 5L, 5X, 6M, 8E, 8J, 8N, 8P	Cao et al. 2009; Strelkov et al. 2006, 2007, 2016, 2018; Xue et al. 2008
British Columbia	6	6M	Ayers 1972; Strelkov et al. 2006, 2018; Williams 1966; Xue et al. 2008
Manitoba	5	-	Cao et al. 2009
Maritimes	1, 2, 3	-	Ayers 1972
Nova Scotia	1, 2, 3	-	Hildebrand and Delbridge 1995
Ontario	2, 3, 5, 6, 8	5I, 8N	Al-Daoud et al. 2018; Ayers 1972; Cao et al. 2009; Reyes 1974; Strelkov et al. 2006, 2018; Xue et al. 2008
Prince Edward Island	4	-	Ayers 1972
Quebec	1, 2, 4, 5, 6	-	Ayers 1972; Williams 1966; Cao et al. 2009

[a] Pathotypes were designated based on their virulence on the differential hosts of Williams (1966) and the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018).

1. 13 Figures

Figure 1.1. The most characteristic clubroot symptom is the malformation of the tap and lateral roots, and sometimes even the base of the stem, leading to the formation of galls. Symptoms result from cellular hyperplasia and hypertrophy.

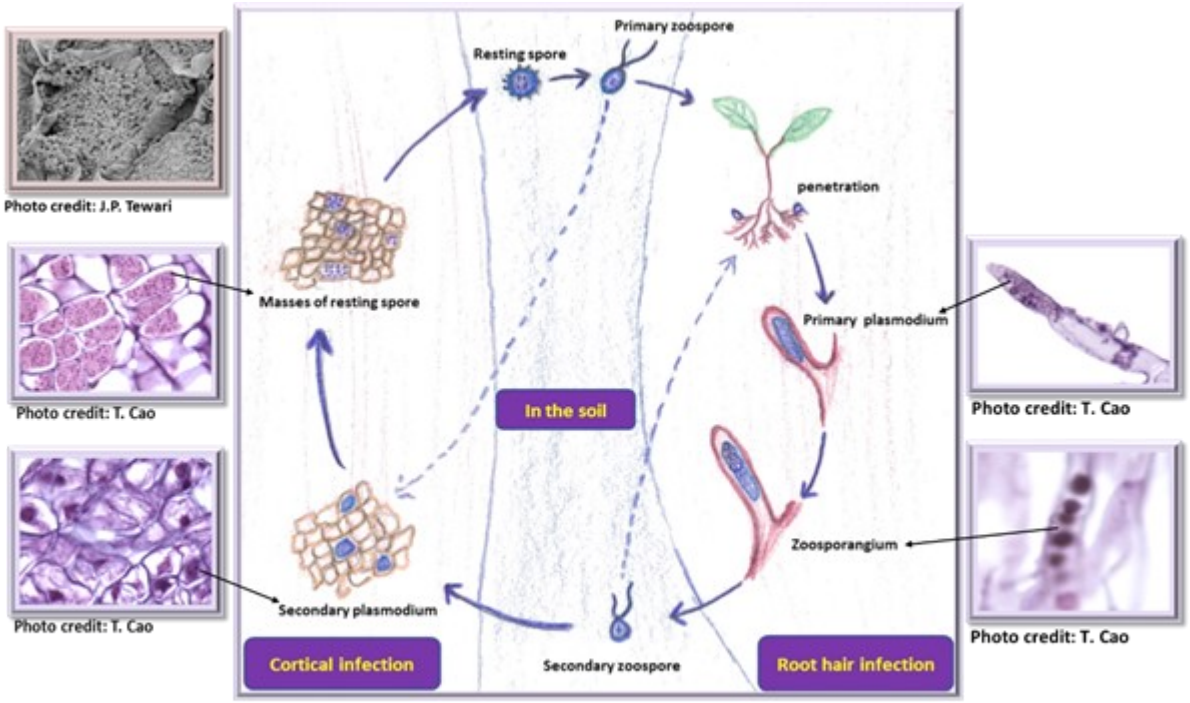

Figure 1.2. Three main stages in the *P. brassicae* life cycle: survival in the soil, root hair infection and cortical infection (Kageyama and Asano 2009). The infection process starts with resting spore germination and the formation of biflagellate primary zoospores that attack root hairs. Subsequently, primary plasmodia and multinucleate zoosporangia that contain 4-16 secondary zoospores are formed in root hairs. The secondary zoospores are similar to the primary zoospores in morphology and are released into the surrounding soil environment. These re-infect the host, with the pathogen invading root cortical cells and forming secondary plasmodia. Eventually the plasmodia develop into millions of long-lived resting spores.

1. 14 References

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Chapter 2. Virulence spectrum of single-spore and field isolates of *Plasmodiophora brassicae* able to overcome resistance in canola (*Brassica napus* L.)

2. 1 Introduction

Clubroot, caused by the obligate parasite *Plasmodiophora brassicae* Woronin, is one of the most significant soilborne diseases of the Brassicaceae (Dixon 2009a; Karling 1968). On infected plants, *P. brassicae* causes the malformation of the tap and lateral roots, resulting in the development of clubs or galls that are the most characteristic symptom of the disease (Kageyama and Asano 2009; Voorrips 1995). As root galling becomes more severe, the uptake of water and nutrients is reduced and affected plants may wilt, even under moderate moisture stress. Severe infections result in stunting, premature plant senescence, accelerated flowering, and ultimately major yield losses (Dixon 2009a; Voorrips 1995). In Canada, clubroot has been a constraint to the production of *Brassica* vegetables for over a century (Howard et al. 2010), but the disease was not identified on canola (*Brassica napus* L.) in the Prairies region until 2003, when it was found in 12 fields in central Alberta (Tewari et al. 2005). By 2018, clubroot infestations had been confirmed in > 3,000 fields throughout Alberta (Strelkov et al. 2019), with additional infestations reported in Saskatchewan, Manitoba and North Dakota. Clubroot can cause yield losses of 30-100% in susceptible canola (Strelkov and Hwang 2014) and poses a major threat to the production of this important crop (Rempel et al. 2014). The most effective clubroot management strategy is the deployment of genetically resistant cultivars (Diederichsen et al. 2009; Hirai 2006; Peng et al. 2014), which relies on an understanding of the virulence spectrum of *P. brassicae* populations. The occurrence of physiologic specialization is well documented in the clubroot pathogen, with

multiple races or pathotypes identified based on their virulence patterns on various sets of differential hosts (Ayers 1957; Buczacki et al. 1975; Kuginuki et al. 1999; Somé et al. 1996; Strelkov et al. 2018; Williams 1966).

The prudent selection of differential host genotypes is important for the accurate identification of virulence phenotypes in specific regions (Somé et al. 1996; Strelkov et al. 2007). In Canada, the differentials of Williams (1966) have been used most commonly to characterize *P. brassicae* populations, although other systems, including the European Clubroot Differential (ECD) set (Buczacki et al. 1975) and the hosts of Somé et al. (1996) also have been employed occasionally. While these differential sets have been fairly effective for detecting the predominant pathotypes of *P. brassicae* in Canada, each has its own limitations in the context of canola cropping systems (Strelkov and Hwang 2014). Most importantly, these systems do not distinguish between those strains of *P. brassicae* that can overcome the resistance in clubroot resistant (CR) canola cultivars and those that cannot. Given the recent emergence of *P. brassicae* strains able to overcome resistance (Strelkov et al. 2016), a new set of differential hosts, the Canadian Clubroot Differential (CCD) set, was proposed to characterize *P. brassicae* populations (Strelkov et al. 2018). This system includes the differentials of Williams (1966), Somé et al. (1996) and selected hosts of the ECD set, as well as several *B. napus* hosts with differential resistance profiles. The inclusion of hosts from other systems, especially those of Williams (1966) and Somé et al. (1996), allows rapid comparisons of pathotype designations among systems.

A challenge for the identification of *P. brassicae* pathotypes are the intermediate and fluctuating disease reactions often observed on many of the differential hosts (Jones et al. 1982a; Scott 1985; Strelkov et al. 2006, 2007; Tinggal and Webster 1981; Toxopeus et al. 1986; Xue et al. 2008). These reactions may reflect heterogeneity and potentially quantitative resistance in some

of the hosts, but may also indicate heterogeneity in *P. brassicae* field isolates (Scott 1985; Somé et al. 1996; Tinggal and Webster 1981; Xue et al. 2008). Most studies on the virulence of *P. brassicae* have examined field isolates (syn. field populations), which represent a collection of pathogen resting spores originally made from a single root gall or plant. While field isolates are straightforward to obtain and propagate, they often represent a heterogeneous mix of pathotypes that can proliferate selectively on resistant host genotypes. Therefore, pathotypes found at lower frequencies within a field isolate may be masked by the presence of other pathotypes found at higher frequencies (Jones et al. 1982b). In addition, competition between virulent and avirulent pathotypes for infection sites and/or the effect of avirulent pathotypes on host resistance may complicate the interpretation of results obtained with *P. brassicae* field isolates (Jones et al. 1982b; Voorrips 1996).

The use of *P. brassicae* single-spore isolates in studies of clubroot may improve the consistency of host reactions and reflect true virulence patterns present in pathogen populations. The objectives of this study were to obtain single-spore isolates of the clubroot pathogen from field isolates virulent on CR canola, and to investigate the virulence diversity within and between field isolates that overcome host resistance. Different thresholds of disease severity were also compared in order to distinguish between susceptible and resistant reactions in those hosts that still gave intermediate reactions.

2. 2 Materials and methods

2. 2. 1 Clubbed root materials

The galled roots originated from nine commercial fields (Fig. 2.1) sown to clubroot resistant (CR) canola cultivars (‘74-47 CR’, ‘45H29’, ‘74-54 RR’, ‘L135 C’ or ‘1960’) with a relatively high incidence of clubroot (Table 2.1). Seven of the fields were located in central

Alberta, at the centre of the clubroot outbreak (one field from each of the City of Edmonton and Red Deer and Leduc counties, and two fields from each of Sturgeon and Westlock counties), another was located in northern Alberta (Athabasca County), and the ninth field was in southern Alberta (Newell County). The samples were collected during a clubroot survey conducted in 2014 (Strelkov et al. 2015), air-dried at room temperature and stored at 4°C in a cold room.

2. 2. 2 Single-spore isolation

Single-spores were produced by a combination of the methods of Manzanares-Dauleux et al. (1994) and Xue et al. (2008) with several modifications. One dried gall was selected randomly from each field and placed in a 50 mL Falcon conical centrifuge tube (Fisher Scientific, Rochester, NY) containing 20 mL cold sterile distilled (SD) water. The gall was softened by incubating it in the SD water for 1 h at 4°C, washed several times as necessary to remove any surface soil or debris, and then homogenized in SD water with a sterile mortar and pestle. The homogenate was filtered through 10 layers of cheesecloth (American Fiber & Finishing Inc., Albemarle, NC). The resting spore concentration in the filtrate quantified with a hemocytometer (VWR, Mississauga, ON) and the final concentration was adjusted to 100 spores per mL by adding sterile 5% glycerol. This stock was used to isolate individual resting spores via inoculation of 50, 5-day-old Chinese cabbage (*Brassica rapa* L. var. *pekinensis* 'Granaat') seedlings, which had been germinated on moistened sterile filter paper in 9 cm-diam. Petri dishes. Briefly, the rootlets of each seedling were placed with tweezers on a coverslip inside a 6 cm-diam. Petri dish containing two pieces of sterile, moistened filter paper and put aside for subsequent use. Six square slices (approximately 1 mm^2 each) of a 1% agarose gel were excised with a scalpel and placed on a microscope slide (disinfected with 70% ethanol), with 0.5 µL of the spore suspension added to the center of each gel slice. After 1 min, the slices were examined under a light microscope at 400X magnification. This procedure

was repeated until 50 slices each containing one round (ca. 4 µm diam.), robust and intact resting spore, which was considered potentially mature and viable, had been obtained from each field isolate.

Each agarose slice was then taken from the microscope slide with an insect pin and carefully placed on the root hairs of an individual Chinese cabbage seedling, which had been previously placed in a 6 cm-diam. Petri dish as described above. One drop of SD water was placed near the gel slice. The Petri dishes were wrapped with clear plastic food wrap (Clorox, Canada) and incubated in darkness at 23°C for 72 h. Subsequently, each inoculated seedling was transferred to a small plastic pot (6.5 cm × 6.5 cm × 9 cm) containing moistened potting mix (Sunshine Mix LA7, Sunshine Growers) and incubated for 8 weeks in a greenhouse maintained at about 24°C/ 18°C day/night under natural light supplemented with artificial lighting (16 h photoperiod). The potting mix was kept saturated for 6 weeks by soaking the pots separately in water; over the remaining two weeks, the plants were watered as required and fertilized once a week. Great care was taken throughout all of the steps to avoid cross-contamination between isolates. The stock spore suspensions from which the single-spore isolations were made was retained and used as inoculum to propagate the original field isolates on Chinese cabbage 'Granaat' and on the same resistant cultivar from which each field isolate was initially recovered.

2. 2. 3 Propagation of single-spore isolates

The inoculated plants were removed carefully from the potting mix and inspected for the presence of root galls. If a gall was observed, it was sectioned in two; one-half was stored at -20°C, while the other half was homogenized in a mortar and pestle as above. The homogenate was filtered through four layers of cheesecloth and the resting spore concentration quantified with a hemocytometer and adjusted to 10^7 spores per mL in SD water. Seven-day-old Chinese cabbage

'Granaat' seedlings (140 per resting spore suspension) were inoculated by the root dip method (Strelkov et al. 2006), planted in potting mix, and grown in a greenhouse as above, except that the potting mix was kept saturated for 2 weeks and the roots were harvested after 6 weeks. The galls were washed in SD water and stored at -20°C until further processing.

2. 2. 4 Pathotype classification

The virulence patterns of the field and single-spore isolates were assessed on the 13 host genotypes (Table 2.2) of the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018), which include the differentials of Williams (1966) and Somé et al. (1996). Inoculations were conducted following Strelkov et al. (2006) with 20 g of galls representing each field isolate or putative single-spore isolate. The inoculated seedlings were grown for 6 weeks in the greenhouse under the conditions described above, with the potting mix kept saturated with water for the first 2 weeks after inoculation. Experiments were conducted with four replicates for each host genotype-pathogen isolate combination and 12 plants per replicate (experimental unit); treatments were arranged in a split-plot design with isolates as main plots and differential hosts as subplots. One single-spore isolate of each pathotype identified was selected and retested on the differentials an additional three times.

In the original description of the CCD set (Strelkov et al. 2018), isolates of *P. brassicae* with unique virulence patterns were assigned a letter (A, B, C, etc.) to denote different pathotypes. Each isolate could also be assigned a pathotype number based on the system of Williams (1966), and while the two designations were often used together (e.g., pathotype 5X) they were not formally linked. Given the identification of multiple novel pathotypes in this report (see below), and following consultation among Canadian clubroot workers, the nomenclature for new pathotypes of *P. brassicae* has been modified slightly. In this and subsequent papers, the official

pathotype designation will indicate the Williams' number first, followed by a letter denoting the CCD designation. While previously a letter was used only once and assigned independently of the Williams' designation, now the entire alphabet may be applied to distinguish multiple variants of a single Williams' pathotype (e.g., pathotype 3A, 3B, 3C). In order to avoid confusion, the designations for pathotypes that have already been published (Strelkov et al. 2018), such as 3A, 3H and 5X, will be retained, with new letters used to denote new variants.

2. 2. 5 Disease assessment

Seedlings were uprooted 6 weeks after inoculation, with the roots washed with water and evaluated for clubroot symptom development. Each root was rated on a 0-to-3 clubroot severity scale (Kuginuki et al. 1999), where: 0: no galling, 1: a few small galls, 2: moderate galling, and 3: severe galling. An index of disease (ID) severity was calculated for each replicate based on the formula of Horiuchi and Hori (1980) as modified by Strelkov et al. (2006):

$$\text{ID (\%)} = \{[\sum (n \times 0) + (n \times 1) + (n \times 2) + (n \times 3)]/ N \times 3\} \times 100\ \%$$

Where n is the number of plants in each class, N is the total number of plants, and 0, 1, 2 and 3 are the symptom severity classes.

2. 2. 6 Analysis

For each host genotype-pathogen combination, mean IDs and 95% confidence intervals (CI) were calculated with Microsoft Excel 2016. Three thresholds were compared for the classification of resistant and susceptible reactions: ID <50% (hosts classified as resistant if ID < 50% and the 95% CI does not overlap 50%) (LeBoldus et al. 2012); ID < 33% (host classified as resistant if ID < 33% and the 95% CI does not overlap 33%), and ID <25% (host reaction classified as resistant if ID < 25% and 95% CI does not overlap 25%). Each field and single-spore isolate

was then classified into pathotypes based on their virulence patterns on the differential hosts (CCD, Williams and Somé et al.).

Data were also converted into a 0-1 matrix with 0 as resistant and 1 as susceptible to generate a similarity matrix with NTSys-pc v 2.2 (Exeter Software, New York, NY). The similarity matrix was then subjected to cluster analysis by the unweighted pair-group method of arithmetic means (UPGMA) and simple similarity coefficient. The UPGMA analysis was conducted with the SAHN program of NTSys-pc and dendrograms were generated using the Tree Plot program of the same software package (Akhavan et al. 2016). The Mantel test was used to calculate the correlation between the virulence matrices generated with threshold IDs of 25%, 33% and 50% on the hosts of each of the 3 differential sets, using the MXCOMP program in NTSys-pc v. 2.2q with 999 permutations (Rohlf 2009).

2. 3 Results

2. 3. 1 Single-spore isolation

Four hundred fifty Chinese cabbage 'Granaat' seedlings were inoculated with single resting spores of *P. brassicae* representing nine 'mother' field isolates. Each infected plant was assumed to represent a single-spore isolate. The severity of root infection was scored as 1, 2 or 3, with a rating of 3 found to be the most common reaction type (65%). The infection rate varied from 2-22% with an average of 7.6% for inoculations with single-spores derived from different field isolates. The highest infection rate (22%) was obtained with resting spores derived from field isolate 187-14-P, followed by inoculations with resting spores from the field isolates 183-14-P (14%) and 1-14-P (10%). Three plants (6%) with clubroot symptoms were obtained following inoculation with spores from each of the field isolates 41-14-P and 331-14-P. Two plants (4%) developed symptoms following inoculation with resting spores derived from 4-14-P. One of 50

(2%) seedlings inoculated with spores from each of the field isolates 6-14-P, 175-14-P and 290-14-P developed galls. Thirty-four single-spore isolates, representing nine field isolates, were recovered from the 450 inoculated seedlings (Table 2.3).

2. 3. 2 Isolate virulence and host reactions

The mean ID and lower and upper limits of the associated 95% CI for each host genotype and pathogen combination are summarized in (Supplementary Table 2.1). All single-spore and field isolates were highly virulent (IDs of 92-100%) on ECD 05, the universal suscept, and on the spring oilseed rape 'Brutor' (IDs of 73- 100%). All but one isolate were highly virulent on the spring canola 'Westar' (IDs of 79-100%), with only the single-spore isolate 290-14-SS1 causing low levels of disease (ID = 8%). Similarly, all single-spore and field isolates with the exception of 290-14-SS1 were virulent on the differential ECD 08 (IDs of 75-100%). In contrast, the *B. rapa* genotype ECD 02 was highly resistant to all of the isolates tested (IDs of 0-9%), developing little to no symptoms of clubroot. The *B. napus* hosts ECD 06, ECD 09, ECD 10, 'Laurentian', 'Mendel' and '45H29', and *B. oleracea* hosts ECD 11 and ECD 13, varied in their responses to specific field and single-spore isolates, and were able to differentiate pathotypes of *P. brassicae*.

The UPGMA cluster analysis using the simple similarity coefficient and a threshold ID of 25% to distinguish resistant vs. susceptible reactions resulted in four, eight and 18 pathotype groups among the single-spore and field isolates tested on the differentials of Somé et al., Williams and the CCD set, respectively (Supplementary Fig. 2.1). In contrast, at a threshold ID of 33%, four, nine and 20 pathotype groups were obtained on each differential set, respectively (Supplementary Fig. 2.2). A threshold ID of 50% resulted in three, seven and 13 pathotype groups, respectively, on the differential sets of Somé et al. (Fig. 2.2), Williams (Fig. 2.3) and the CCD (Fig. 2.4).

The percentage of isolates scored as virulent on ECD 05 (100%), 'Brutor' (100%) and 'Westar' (98%) was similar with all three threshold IDs. In general, however, the number of isolates classified as 'virulent' on the other hosts increased as the threshold ID was lowered from 50% to 25%. Nevertheless, the number of isolates considered virulent on ECD 06, ECD 08, ECD 09, ECD 13 and 'Laurentian' did not change dramatically with the three different thresholds. ECD 02 was resistant to all isolates at all thresholds with the exception of isolate 183-14-SS5 when the threshold ID was set to 25%. Only one (2%) of the isolates (183-14-SS4) was regarded as virulent on ECD 10 at a threshold of 50%, but this increased to 11 (26%) and 22 (51%) of the isolates at 33% and 25%, respectively. Similarly, there was a rise in the number of isolates classified as virulent on ECD 11, 'Mendel', and '45H29' as the threshold ID was lowered from 50% to 33% and 25% (Fig. 2.5).

The Mantel test indicated significant and strong correlations between virulence matrices generated using threshold IDs of 25%, 33% and 50% on each of the three differential sets (Table 2.4). The highest correlation (90%) was found between matrices produced with thresholds of 25% and 33% on the CCD set. A comparison of associated matrices generated with thresholds of 25% and 33% vs. 50% on the CCD set also showed high correlations (81% and 87%, respectively). The lowest, but still significant, correlation (51%) was observed between matrices generated using threshold IDs of 25% and 50% on the hosts of Williams. On the differentials of Somé et al., a correlation of 57% was found in pairwise comparisons of the matrix generated with a threshold ID of 25% vs. each of the matrices produced with thresholds of 33% or 50%. A correlation of 74% was observed between matrices generated with threshold IDs of 33% and 50% on these hosts (Table 2.4).

2. 3. 3 Pathotype designations

At a threshold of 50%, three pathotypes could be distinguished on the differentials of Somé et al., namely P_1, P_2 and P_3, with P_2 representing more than half the isolates. On the system of Williams, seven pathotypes were identified, including pathotypes 2 (5 isolates (12%)), 3 (18 isolates (42%)), 4 (1 isolate (2%)), 5 (5 isolates (12%)), 6 (12 isolates (28%)), 7 (1 isolate (2%)), and 8 (1 isolate (2%)). The seven Williams' pathotypes could be subdivided further into 13 pathotypes with the CCD set. These included pathotypes 2A (2 isolates (5%)), 2F (3 isolates (7%)), 3A (2 isolates (5%)), 3D (7 isolates (16%)), 3H (9 isolates (21%)), 4A (1 isolate (2%)), 5G (1 isolate (2%)), 5X (4 isolates (9%)), 6A (1 isolate (2%)), 6B (10 isolates (23%)), 6C (1 isolate (2%)), 7A (1 isolate (2%)) and 8E (1 isolate (2%)).

In most cases, multiple pathotypes were identified from single-spores derived from one field isolate. Pathotypes P_2, P_3/2F, 3H, 6A were recovered from the five single-spore isolates obtained from field isolate 1-14-P, which was classified as pathotype P_2/3D on the systems of Somé et al. and Williams/CCD, respectively. These single-spore isolates shared similar virulence patterns with 1-14-P on the differential hosts ECD 02, ECD 05, ECD 08, ECD 10, ECD 13, 'Brutor', 'Mendel' and 'Westar'. However, while ECD 06, ECD 09 and 'Laurentian' were susceptible to most of the single-spore isolates and the original field isolate, these hosts were resistant to 1-14-SS2. The single-spore isolates 1-14-SS1 and 1-14-SS4 were virulent on ECD 11, which was resistant to the original field isolate. Only the single-spore isolate 1-14-SS2 was virulent on the CR canola '45H29' like 1-14-P.

One of two single-spore isolates (4-14-SS2) derived from field isolate 4-14-P shared the same virulence pattern on all 13 differential hosts, retaining the pathotype classifications P_3/6B on the systems of Somé et al. and Williams/CCD. The other single-spore isolate, 4-14-SS1, gave different reactions on ECD 06, ECD 09, 'Laurentian', 'Mendel' and '45H29', and was designated

as pathotype P_2/3H. Only one single-spore isolate was derived from field isolate 6-14-P; this single-spore possessed different virulence patterns on the hosts ECD 06, ECD 09, 'Laurentian' and 'Mendel' relative to its parent. Among the three single-spores derived from field isolate 41-14-P, which was virulent on '45H29' and classified as pathotype P_2/3D, the single-spores 41-14-SS1 and 41-14-SS3 shared the same virulence pattern on all hosts with the exception of '45H29,' resulting in a pathotype designation of P_2/3H. The single-spore isolate 41-14-SS2 generated distinct reactions on ECD 06, ECD 09, 'Laurentian' and 'Mendel' relative to the original field isolate and was classified as pathotype P_3/6B. While the differentials ECD 06, ECD 09, 'Laurentian' and '45H29' were susceptible to field isolate 175-14-P, they were resistant to 175-14-SS1, the sole single-spore derived from this field isolate.

None of the single-spore isolates derived from the field isolate 183-14-P shared an identical virulence pattern with the parent on all 13 differential hosts. While 183-14-P was classified as pathotype P_2/8E, multiple pathotype classifications were obtained among the single-spore isolates, including P_1, P_2, P_3/2A, 3D, 3H, 4A, 6B. Most notably, all single-spores were virulent on ECD 13, which was resistant to the field isolate. On the hosts ECD 02, ECD 05, ECD 08, 'Brutor' and 'Westar', the virulence patterns of the single-spores were the same as the original field isolate. The single-spore isolate 183-14-SS4 was virulent on all host genotypes except ECD 02.

The most single-spore isolates (11) were obtained from the field isolate 187-14-P, which was classified as P_2/3A. Only one of those single-spores (187-14-SS10), however, shared all three pathotype classifications with 187-14-P. The other single-spore isolates were classified as various pathotypes including P_2, P_3/3A, 3H, 5X, 6B. The eleven single-spores had the same virulence patterns as the parent field isolate on the hosts ECD 02, EDC 05, ECD 08, ECD 10, ECD 11, 'Brutor' and 'Westar'. However, two of the single-spores (187-14-SS7 and 187-14-SS9) were

avirulent on the CR canola '45H29'. 'Laurentian' was susceptible to the field isolate (187-14-P) and three of the single-spores (187-14-SS7, 187-14-SS9 and 187-14-SS10). These three single-spores were virulent on ECD 06 and ECD 09 as was the parent field isolate. The differential host ECD 13 was resistant to four single-spores (187-14-SS1, -SS2, -SS3, -SS4). Contrasting reactions were observed on 'Mendel' in response to inoculation with the isolates 187-14-SS7 and 187-14-SS9.

Only one single-spore isolate (290-14-SS1) was obtained from the field isolate 290-14-P (classified as P_3/6B). The single-spore shared the same virulence pattern as 290-14-P on ECD 02, ECD 05, ECD 09, ECD 10, ECD 13, 'Brutor' and 'Laurentian', but the reactions of ECD 08, ECD 11, 'Mendel', 'Westar' and '45H29' were different, resulting in a pathotype classification of P_2/7A for the single-spore isolate. The field isolate 331-14-P, classified as pathotype P_3/5G, yielded single-spore isolates belonging to a variety of pathotypes (P_2/2F, 3D, 3H). While 331-14-P was avirulent on ECD 06, ECD 09, ECD 13 and 'Laurentian', all single-spores derived from this parent were virulent on these hosts. The CR canola '45H29' was resistant to two of the single-spores (331-14-SS1 and 331-14-SS2) and susceptible to the original field isolate and the third single-spore (331-14-SS3). One single-spore isolate, 331-14-SS2, was virulent on ECD 11. The differentials ECD 02, ECD 05, ECD 08, ECD 10, 'Brutor', 'Mendel' and 'Westar' exhibited similar reactions to the field isolate and all three single-spores.

2. 4 Discussion

The emergence of *P. brassicae* strains capable of overcoming host resistance represents a significant challenge to the management of clubroot on canola (Strelkov et al. 2016, 2018). An accurate assessment of the pathotype composition of *P. brassicae* populations is a prerequisite for the identification of new and effective sources of genetic resistance. Field isolates can represent

heterogeneous mixtures of pathotypes; therefore, the characterization of single-spore isolates is important for such assessments. In the current study, 34 single-spore isolates were obtained from pathogen collections made from CR canola. The infection rate obtained by inoculation of single resting spores recovered from the various field isolates ranged from 2-22%. Resting spore maturity and viability can vary among field isolates of *P. brassicae*, which may help to explain the variability in these results. Kageyama and Asano (2009) indicated that resting spore populations may be at various stages of maturation and that their ability to germinate is linked to this maturity. In the present study, the average infection rate was 7.6%, which compares favorably with the infection rates that have been reported previously (Buczacki 1977; Diederichsen et al. 2016; Jones et al. 1982b; Manzanares-Dauleux et al. 1994; Scott 1985; Somé et al. 1996; Tinggal and Webster 1981; Voorrips 1996; Xue et al. 2008). The efficacy of any single-spore isolation procedure may depend on the condition of the spore suspension, particularly the percentage of the mature and viable spores, as well as technical differences between procedures (Buczacki 1977; Voorrips 1996).

Field isolates of *P. brassicae* are often maintained and propagated on the universally susceptible Chinese cabbage 'Granaat'. It has been suggested that all pathotypes represented in one gall will propagate similarly on this line without any obvious change in the pathotype composition of the gall (Buczacki et al. 1975). However, Jones et al. (1982a) and Fähling et al. (2003) stated that even on a susceptible line, environmental circumstances might encourage a selective multiplication of a subset of all existing pathotypes within a single field isolate. Furthermore, if there is a fitness cost to the pathogen for virulence on a particular source of resistance, it is possible that those virulent isolates will be selected against on a completely susceptible host (Crute et al. 1980; Diederichsen et al. 2009; Dixon 2009b; Montarry et al. 2010).

In the current study, to reduce the possibility of changes in pathotype composition or shifts in the virulence of the field isolates, single-spore isolation was carried out directly from the field-collected galls.

To date, the isolation of *P. brassicae* single-spores has been performed with agar/agarose-based techniques (Manzanares-Dauleux et al. 1994; Scott 1985; Somé et al. 1996; Tinggal and Webster 1981), or by using droplets of highly diluted spore suspensions (Buczacki 1977; Voorrips 1996; Xue et al. 2008). There are some difficulties associated with the employment of either of these methods. The need for special equipment and micro-manipulative devices to cut and lift the agar or agarose gel pieces containing single-spores is one of the disadvantages of agarose-based methods. The fact that standard compound microscopes are designed to capture light emitted from a 2-dimensional object, while a droplet is a 3-dimensional (spherical) object with multiple focal planes, is a limitation of the use of droplet suspensions. Evaporation of the droplet and surface tension are additional concerns. The current study combined these two approaches, with some modifications, to simplify the procedure. Small pieces of agarose gel were cut with a scalpel and placed on a microscope slide, and then a highly diluted spore suspension was laid on the center of each gel slice. The use of small pieces of agarose gel, rather than the spread of the spore suspension over the entire surface of an agarose plate, reduced the chances that multiple rather than single-spores were obtained, since additional spores could be concealed on the edge of the agarose during the cutting procedure. In addition, since *P. brassicae* resting spores tend to aggregate, the resting spore suspension included 5% (vol/vol) glycerol as recommended by Xue et al. (2008).

Several studies suggest that the presence of primary plasmodia and/or pathogen DNA can be detected beginning 3 days after inoculation (Devos et al. 2005; Ingram and Tommerup 1972; Kageyama and Asano 2009). Hence, seedlings were incubated for 72 h in darkness before they

were transferred to a potting mixture, rather than 48 h as has often been reported (Somé et al. 1996; Voorrips 1996; Xue et al. 2008). Since temperature significantly influences the germination of the resting spores, incubation of the seedlings was conducted at 23°C, following reports that the optimum temperature range for infection is 20-25°C (Ikegami et al. 1981; Wellman 1930).

The occurrence of intermediate and fluctuating host reactions can hinder the study of physiologic specialization in *P. brassicae* (Jones et al. 1982a; Scott 1985; Strelkov et al. 2006, 2007; Tinggal and Webster 1981; Toxopeus et al. 1986; Xue et al. 2008), and a major aim of single-spore isolation is to obtain genetically homogeneous pathogen inoculum. Nevertheless, in this and earlier studies, intermediate host reactions were still observed following inoculation with single-spore isolates. This may reflect heterogeneity in some of the differential hosts (Jones et al. 1982a; Kuginuki et al. 1999), and suggests that a refined clubroot differential set that includes homozygous inbred lines would be valuable, helping to facilitate understanding of the genetics of resistance in the clubroot pathosystem. In the case of the *B. oleracea* differentials, intermediate disease reactions could reflect the occurrence of polygenic resistance (Crute et al. 1980; Piao et al. 2009). The latter is consistent with the observation in the current study that intermediate IDs most frequently occurred on ECD 11 and ECD 13. It is also worth noting that while *P. brassicae* inoculum derived from single-spores is considered to be genetically homogeneous, this has not been demonstrated by genetic or molecular means. The possibility that heterogeneity could be introduced during propagation of *P. brassicae* (i.e., increasing from one to billions of spores over an infection cycle) cannot be ruled out. For instance, in a study of haploid and diploid yeast populations, Sharp et al. (2018) found that haploids were subject to more single-nucleotide mutations (SNMs), especially for DNA replicated later in the cell cycle.

Since intermediate host reactions are often observed in response to inoculation with the clubroot pathogen, several threshold IDs have been proposed to discriminate between a resistant and a susceptible response. For example, Somé et al. (1996) used a threshold ID of 25% when pathotyping field and single-spore isolates from France. In contrast, in a study from Canada, Strelkov et al. (2006) found that most hosts developed IDs from 0% to ca. 45% and from ca. 65% to 100%, with only a few genotypes exhibiting IDs of 45-65%. Hence, they took an ID of 50% as the threshold between a resistant and susceptible reaction (Strelkov et al. 2006). In the current study, three thresholds (25%, 33% and 50%) were compared. As expected, the threshold applied influenced the number, type and frequency of pathotypes identified among the single-spore and field isolates examined. Use of thresholds set at 25% and 33% resulted in the identification of larger numbers of putative pathotypes, but often based on small differences in ID. Similar results were reported in an analysis of *P. brassicae* field isolates from the Czech Republic and Poland, when thresholds of 25% and 50% were compared (Ricarová et al. 2016).

In the present analysis of field and single-spore isolates from Alberta, the mean IDs for most hosts ranged from 0 to ca. 45% and from 65% to 100%, although the reactions of ECD 11, ECD 13 and '45H29' occasionally fell between 45-65%. This was consistent with the earlier report of Strelkov et al. (2006), and therefore a threshold ID of 50% to discriminate between resistant and susceptible host reactions gave the most consistent results. Indeed, most recent studies from Canada have designated reactions as resistant if the ID is < 50% and the 95% CI does not overlap 50% (Al-Daoud et al. 2018; Cao et al. 2020; LeBoldus et al. 2012; Strelkov et al. 2016, 2018), and these were the criteria applied here to classify *P. brassicae* isolates into pathotypes. Nonetheless, similarity matrices were also generated for each differential set and threshold ID (25%, 33% and 50%). The highest correlation coefficients were observed among the matrices generated on the

CCD set, which suggests the choice of a specific threshold has the least impact on this set compared with the differentials of Williams or Somé et al. It may be valuable in the future to compare the genetic structure of isolates with these virulence datasets, to determine which threshold has a higher correlation with the genetic background information.

Thirteen pathotypes were identified with the CCD set, while seven and three pathotypes, respectively, were detected on the differentials of Williams and Somé et al. This confirms a greater differentiating capacity with the CCD relative to the two other systems. Many of the CCD pathotypes reported by Strelkov et al. (2018) from Alberta and other regions of Canada, including pathotypes 3A, 3D, 3H, 5G and 8E, also were found in this study. In addition, several novel pathotypes, designated 2A, 4A, 6A, 6B, 6C and 7A, were recovered among the single-spore isolates (Table 2.5). These pathotypes may have been newly introduced to these fields, or they may have been present at a low frequency but not detected previously. Indeed, the single-spores were derived from field isolates shown to be a mixture of pathotypes, and therefore their virulence patterns may have been masked by pathotypes occurring at higher frequencies (Jones et al. 1982b). Four of the novel pathotypes were virulent on one or both of the CR *B. napus* cultivars tested ('45H29' and 'Mendel'), and likely were selectively increased by the cultivation of CR hosts.

Pathotype 6B (virulent on 'Mendel' and '45H29') and pathotype 3H (corresponding to the 'original' pathotype 3 and avirulent on 'Mendel' and '45H29') were found with the almost same frequency and were predominant in the current study. In contrast, pathotype 3A, which can overcome resistance and was found with increasing frequency among field isolates from Alberta in 2014-2016 (Strelkov et al. 2018), was rare. This may reflect the fact that the field isolates used for single-spore isolation in the present study included only one classified as pathotype 3A. Pathotype 2F (corresponding to the 'original' pathotype 2; Strelkov et al. 2018) was also identified

among the single-spore isolates, as was pathotype 5X. The latter represents the first pathotype identified in Canada that could overcome resistance, although it was not found again in pathogen collections made from 2014 to 2016 (Strelkov et al. 2016, 2018). Interestingly, the four single-spore isolates classified as pathotype 5X were derived from 187-14-P, which was the field isolate classified as pathotype 3A.

On the hosts of Williams and Somé et al., pathotypes 3 and P_2 were predominant, respectively, a finding consistent with previous reports (Cao et al. 2009; Strelkov et al. 2006, 2007; Xue et al. 2008). In addition, several additional pathotypes, as defined on these systems and not previously reported from Canada, also were recovered. These included pathotype P_1 (*sensu* Somé et al. 1996) and pathotypes 4 and 7 (*sensu* Williams 1966).

As in earlier assessments, more isolates appeared to overcome the resistance in the CR canola '45H29' than in the CR winter oilseed rape 'Mendel' (Strelkov et al. 2018). At a threshold ID of 50%, 29 of the isolates in the present study were virulent on '45H29' vs. only 16 isolates that were virulent on 'Mendel'. Fredua-Agyeman et al. (2018) suggested that most CR canola cultivars from Canada derive their clubroot resistance from 'Mendel,' which is controlled by a major dominant gene (Diederichsen et al. 2006). Nevertheless, 'Mendel' may also possess additional unmapped resistance genes, which could have been lost during development of the commercial CR cultivars, resulting in increased clubroot susceptibility in some cases (Fredua-Agyeman et al. 2018; Strelkov et al. 2018). In addition to the many canola cultivars that appear to possess 'Mendel'-derived resistance, several cultivars have recently been released that are advertised as possessing "2nd generation resistance". The nature of 2nd generation resistance has not been disclosed, but appears to be distinct from 'Mendel'. It will be important to monitor these new varieties for their performance in *P. brassicae*-infested fields. Interestingly, the older open-

pollinated canola cultivar 'Westar', which was reported to be susceptible to all isolates of *P. brassicae* from Canada (Strelkov et al. 2016, 2018), was found to be resistant to one single-spore isolate (290-14-SS1) in the current study.

As previously reported (Cao et al. 2009; Strelkov et al. 2007, 2016, 2018; Xue et al. 2008), ECD 02 was highly resistant to all of the isolates tested and developed the lowest IDs among the hosts studied. In earlier studies, the differential ECD 10 was found to be resistant to all field and single-spore isolates of *P. brassicae* from Canada (Cao et al. 2009; Strelkov et al. 2007; Xue et al. 2008), including those recovered from fields with resistance issues (Strelkov et al. 2016, 2018). In the present experiment, however, this host, which has been reported to carry the *crr1* gene on chromosome A08 (Hasan and Rahman 2016), was found be moderately susceptible (ID = 59%) to the single-spore isolate 183-14-SS4. Hence, while ECD 10 may be an effective source of resistance to many pathotypes of the clubroot pathogen, it does not appear to be universally resistant to Canadian *P. brassicae* populations.

Most of the single-spore isolates examined in this study were obtained from collections made in central Alberta, with only one each from northern (Athabasca) and southern (Newell) Alberta. Therefore, it is difficult to make conclusions regarding the relationship between pathogen virulence patterns and geographic origins. Nevertheless, the single-spore isolates from northern and southern Alberta generated distinct virulence patterns (P_3/6C and P_2/7A) relative to those from the heart of the clubroot outbreak in the central part of the province. While the amount of data is limited, these findings are consistent with earlier studies, which suggested that field isolates or populations from southern Alberta (Cao et al. 2009) or from northern and southern Alberta (Strelkov et al. 2018) tended to have distinct virulence patterns relative to those from central Alberta.

The results of the current study indicate significant diversity in the virulence of *P. brassicae* single-spore and field isolates from Alberta, which was identified most clearly with the CCD set. The identification of multiple new pathotypes underscores the need for ongoing monitoring of this pathogen in western Canada. The collection of characterized single-spore isolates resulting from this work may serve as a reference and be useful in breeding for clubroot resistance. It could also be of interest in studies of *P. brassicae* biology and evolution. Genetic characterization of this collection using whole genome sequencing may also provide new insights into pathotype biology and relationships.

2. 5 Tables

Table 2.1. Origin of *Plasmodiophora brassicae* field isolates included in the current study. All of the field isolates were collected from clubroot resistant canola crops in Alberta in 2014.

Field ID[a]	Field isolate	Location	Original host [b]
F1-14	1-14-P	City of Edmonton	74-47 CR
CDCN#4	4-14-P	Westlock	L135 C
CDCN#6	6-14-P	Westlock	L135 C
F41-14	41-14-P	Red Deer	45H29
F175-14	175-14-P	Athabasca	74-54 RR
F183-14	183-14-P	Sturgeon	L135 C
F187-14	187-14-P	Sturgeon	45H29
CDCS	290-14-P	Newell	45H29, L135C, 74-47 CR, 1960
F331-14	331-14-P	Leduc	L135 C

[a] Field ID(s) as designated by Strelkov et al. (2018)

[b] Cultivars of canola (*Brassica napus*) which were regarded as resistant to all field and single-spore isolates of *Plasmodiophora brassicae* collected before 2013 (Strelkov et al. 2016).

Table 2.2. Host genotypes included in the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018).

Host genotype [a]	Differential
ECD 02 (*Brassica rapa* subsp. *rapifera* line AAbbCC)	CCD
ECD 05 (*Brassica rapa* var. *pekinensis* 'Granaat')	CCD
ECD 06 (*Brassica napus* var. *napus* 'Nevin')	CCD, Somé et al.
ECD 08 (*Brassica napus* var. *napus* 'Giant Rape' selection)	CCD
ECD 09 (*Brassica napus* var. *napus* New Zealand resistant rape)	CCD
ECD 10 (*Brassica napus* var. *napus* 'Wilmesburger')	CCD, Somé et al., Williams
ECD 11 (*Brassica oleracea* var. *capitata* 'Badger Shipper')	CCD, Williams
ECD 13 (*Brassica oleracea* var. *capitata* 'Jersey Queen')	CCD, Williams
'Laurentian' (*Brassica napus* var. *napobrassica*)	CCD, Williams
'Brutor' (*Brassica napus*)	CCD, Somé et al.
'Mendel' (*Brassica napus*)	CCD
'45H29' (*Brassica napus*)	CCD
'Westar' (*Brassica napus*)	CCD

[a] The Canadian Clubroot Differential (CCD) set includes the differentials of Williams (1966), Somé et al. (1996), an additional four genotypes from the European Clubroot Differential (ECD) set (Buczacki et al. 1975), the clubroot resistant (CR) winter oilseed rape 'Mendel', the open-pollinated spring canola 'Westar', and the CR hybrid canola '45H29'.

Table 2.3. Efficiency of single-spore isolation and summary of pathotype designation of *Plasmodiophora brassicae* field isolates and their single-spores.

Field isolate	Single-spore isolate	#Inoculated plants	#Infected plants	Infection rate	Pathotype[a]		
					Somé et al.	Williams	CCD set
1-14-P		50	5	10%	P_2	3	3D
	1-14-SS1				P_2	2	2F
	1-14-SS2				P_3	6	6A
	1-14-SS3				P_2	3	3H
	1-14-SS4				P_2	2	2F
	1-14-SS5				P_2	3	3H
4-14-P		50	2	4%	P_3	6	6B
	4-14-SS1				P_2	3	3H
	4-14-SS2				P_3	6	6B
6-14-P		50	1	2%	P_3	6	6B
	6-14-SS1				P_2	3	3D
41-14-P		50	3	6%	P_2	3	3D
	41-14-SS1				P_2	3	3H
	41-14-SS2				P_3	6	6B
	41-14-SS3				P_2	3	3H
175-14-P		50	1	2%	P_2	3	3D
	175-14-SS1				P_3	6	6C
183-14-P		50	7	14%	P_2	8	8E
	183-14-SS1				P_2	2	2A
	183-14-SS2				P_2	3	3D
	183-14-SS3				P_2	2	2A
	183-14-SS4				P_1	4	4A
	183-14-SS5				P_3	6	6B
	183-14-SS6				P_2	3	3D
	183-14-SS7				P_2	3	3H
187-14-P		50	11	22%	P_2	3	3A
	187-14-SS1				P_3	5	5X
	187-14-SS2				P_3	5	5X
	187-14-SS3				P_3	5	5X
	187-14-SS4				P_3	5	5X
	187-14-SS5				P_3	6	6B
	187-14-SS6				P_3	6	6B
	187-14-SS7				P_2	3	3H
	187-14-SS8				P_3	6	6B
	187-14-SS9				P_2	3	3H
	187-14-SS10				P_2	3	3A
	187-14-SS11				P_3	6	6B
290-14-P		50	1	2%	P_3	6	6B
	290-14-SS1				P_2	7	7A

Field isolate	Single-spore isolate	#Inoculated plants	#Infected plants	Infection rate	Pathotype[a]		
					Somé et al.	Williams	CCD set
331-14-P		50	3	6%	P_3	5	5G
	331-14-SS1				P_2	3	3H
	331-14-SS2				P_2	2	2F
	331-14-SS3				P_2	3	3D

[a] Each field and single-spore isolate was classified into pathotypes based on their virulence patterns on the differential hosts of the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018), Williams (1966) and Somé et al. (1996). A threshold ID of 50% was considered to distinguish resistant vs. susceptible reactions, hosts classified as resistant if ID < 50% and the 95% CI does not overlap 50% (LeBoldus et al. 2012).

Table 2.4. Correlation between virulence matrices derived from datasets generated using threshold indices of disease of 25%, 33% and 50% on the differential hosts of the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018), Williams (1966) and Somé et al. (1996).

Differential set	Thresholds	Correlation
CCD	25% and 33%	90%
	25% and 50%	81%
	33% and 50%	87%
Williams	25% and 33%	71%
	25% and 50%	51%
	33% and 50%	66%
Somé et al.	25% and 33%	57%
	25% and 50%	57%
	33% and 50%	74%

Table 2.5. The reaction of the novel pathotypes of *Plasmodiophora brassicae* found in the current study on the 13 host genotypes of the Canadian Clubroot Differential (CCD) (Strelkov et al. 2018).

	Pathotype designation[a]					
CCD	2A	4A	6A	6B	6C	7A
Williams	2	4	6	6	6	7
Somé et al.	P_2	P_1	P_3	P_3	P_3	P_2
Host genotypes	Reaction[b]					
ECD 02	-	-	-	-	-	-
ECD 05	+	+	+	+	+	+
ECD 06	+	+	-	-	-	+
ECD 08	+	+	+	+	+	-
ECD 09	+	+	-	-	-	-
ECD 10	-	+	-	-	-	-
ECD 11	+	+	-	-	-	+
ECD 13	+	+	+	+	+	+
'Brutor'	+	+	+	+	+	+
'Laurentian'	+	+	-	-	-	-
'Mendel'	-	+	-	+	-	-
'Westar'	+	+	+	+	+	-
'45H29'	+	+	+	+	-	-

[a] Pathotype designations are based on the systems of the Canadian Clubroot Differential (CCD) Set, Williams (1966) and Somé et al. (1996).

[b] A plus (+) sign denotes a susceptible host reaction, while a minus (-) sign denotes a resistant reaction. Hosts classified as resistant if ID < 50% and the 95% CI does not overlap 50% (LeBoldus et al. 2012).

2. 6 Figures

Figure 2.1. Map of Alberta, Canada, showing the distribution of fields from which field and single-spore isolates of *Plasmodiophora brassicae* were collected for the current study.

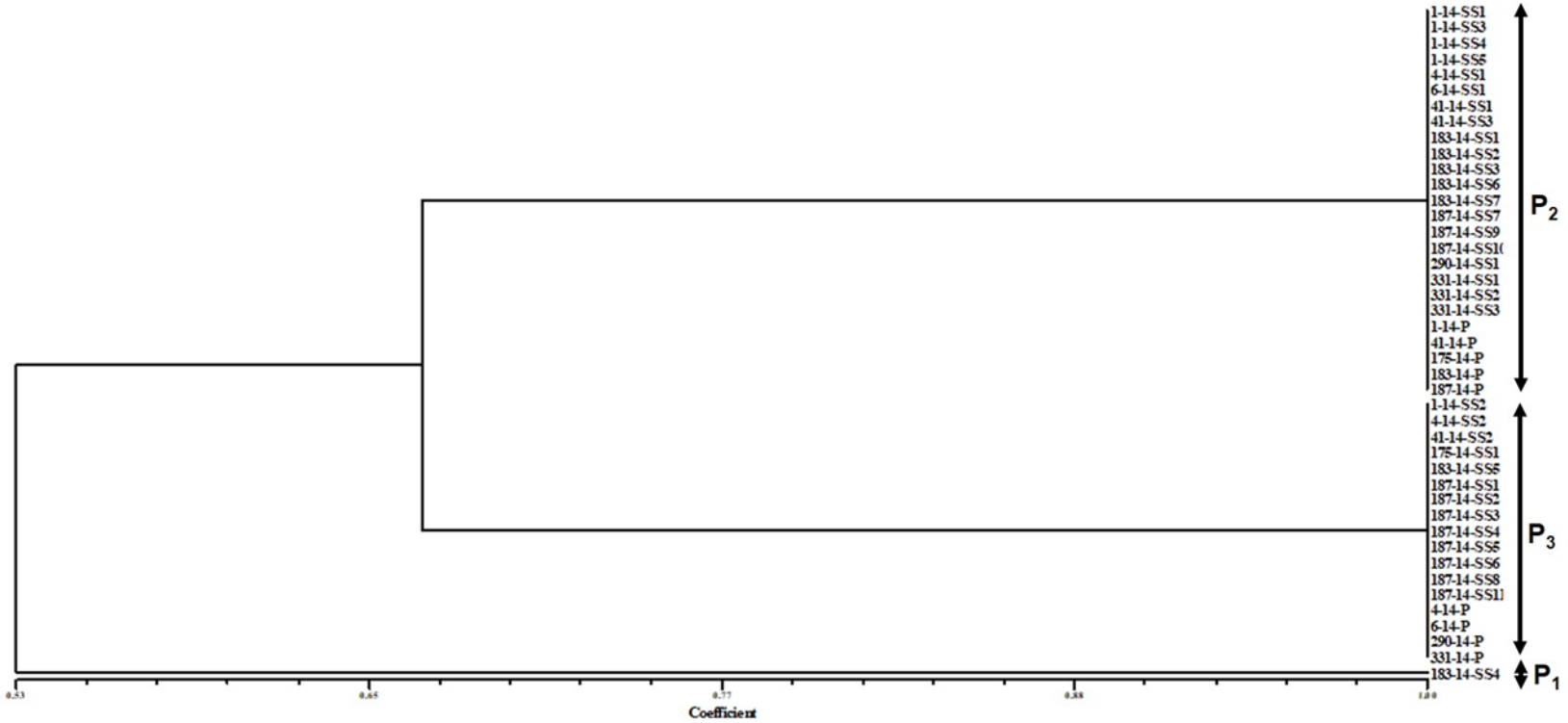

Figure 2.2. Pathogenic similarity of *Plasmodiophora brassicae* isolates on the differential hosts of Somé et al. (1996) based on a threshold index of disease (ID) = 50%. The dendrogram was constructed employing the unweighted pair group method with arithmetic mean procedure and simple similarity coefficient with NTSys-pc ver. 2.2. Three pathotypes P_1, P_2 and P_3 were identified among the isolates tested, with P_2 found to be predominant.

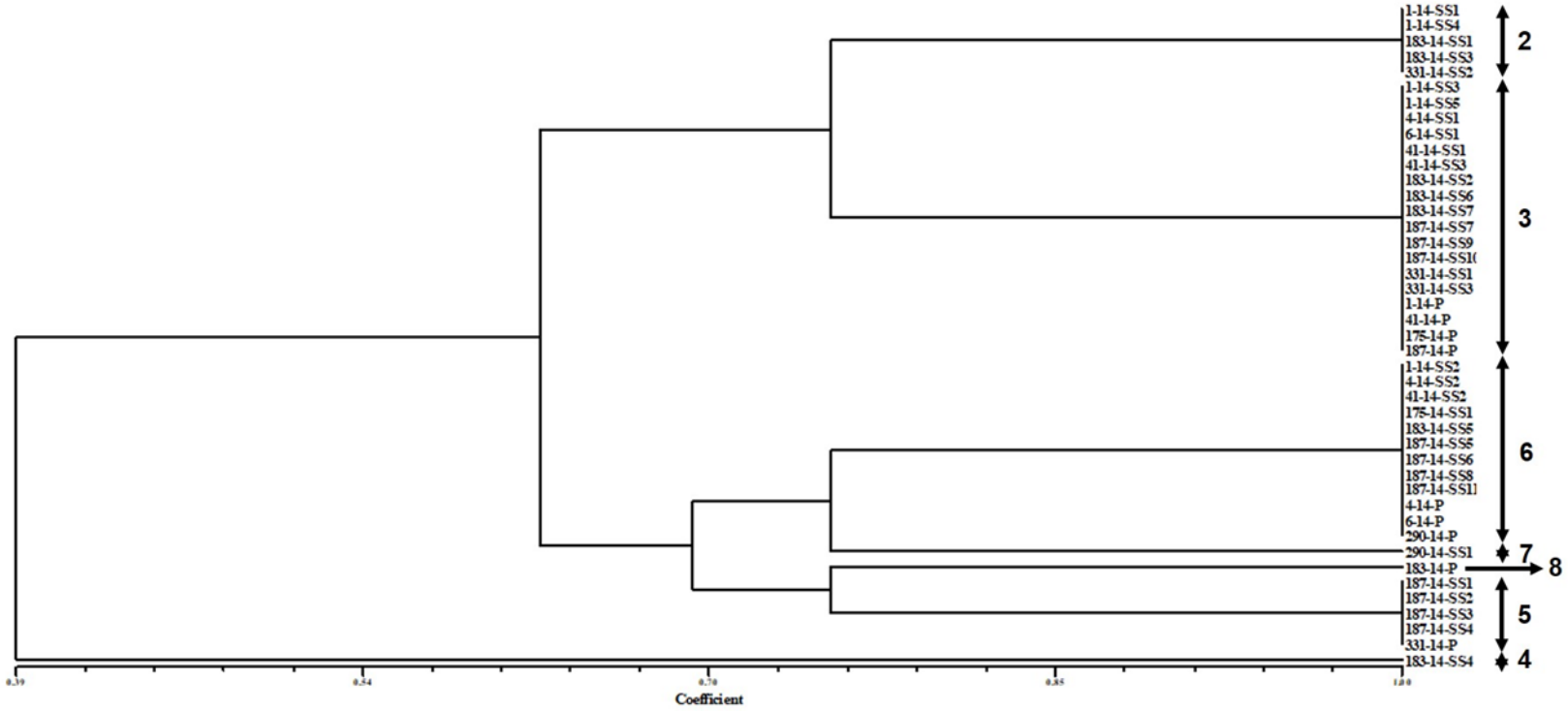

Figure 2.3. Pathogenic similarity of *Plasmodiophora brassicae* isolates on differential hosts of Williams (1966) based on a threshold index of disease (ID) = 50%. The dendrogram was constructed employing the unweighted pair group method with arithmetic mean procedure and simple similarity coefficient with NTSys-pc ver. 2.2. Seven pathotypes 2, 3, 4, 5, 6, 7 and 8 were identified among the isolates tested, with pathotype 3 found to be predominant.

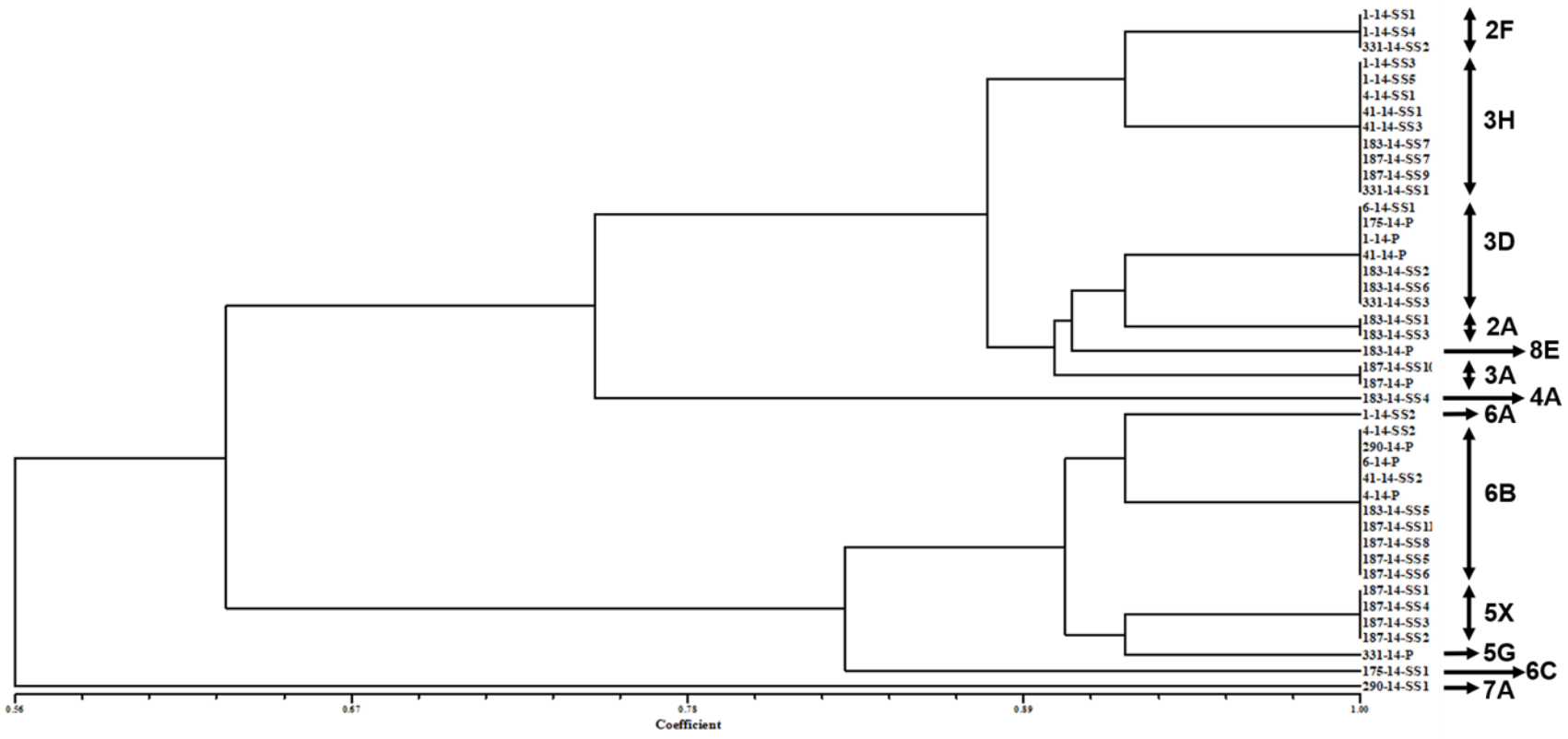

Figure 2.4. Pathogenic similarity of *Plasmodiophora brassicae* isolates on of Canadian Clubroot Differential (CCD) hosts based on a threshold index of disease (ID) = 50%. The dendrogram was constructed employing the unweighted pair group method with arithmetic mean procedure and simple similarity coefficient with NTSys-pc ver. 2.2. Thirteen different pathotypes 2A, 2F, 3A, 3D, 3H, 4A, 5G, 5X, 6A, 6B, 6C, 7A and 8E were identified, with pathotype 6B found to be predominant and followed by 3H.

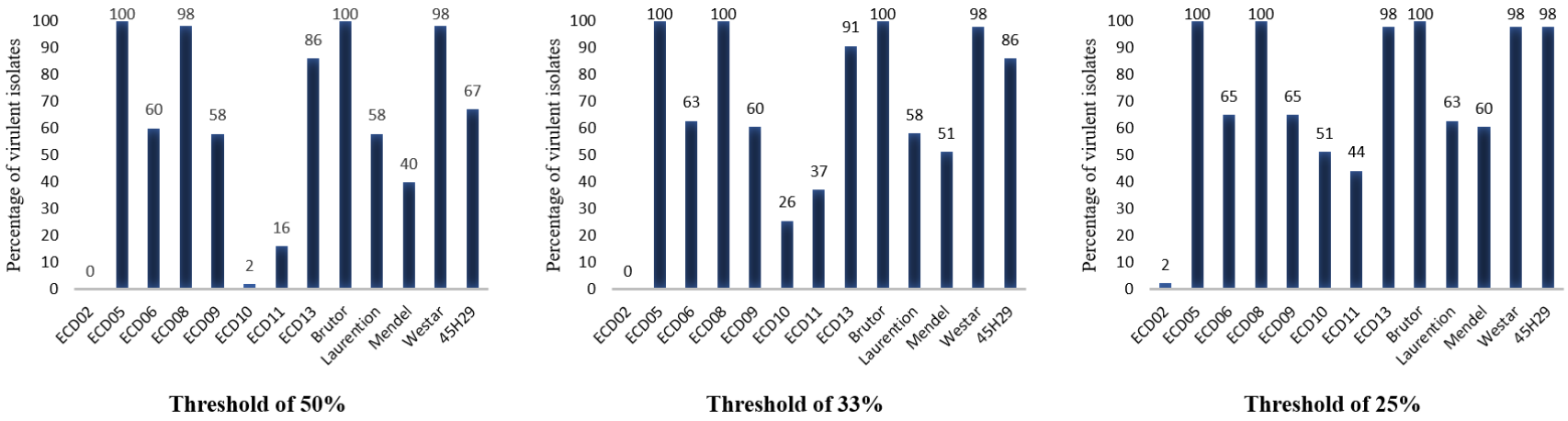

Figure 2.5. Percentage of isolates scored as virulent on the 13 host genotypes of Canadian Clubroot Differential set employing thresholds of 50%, 33% and 25%. For each host genotype-pathogen combination, mean IDs and 95% confidence intervals (CI) were calculated. Three thresholds were compared for the classification of resistant and susceptible reactions. These included ID <50% (host reaction classified as resistant if the ID < 50% and the 95% CI does not overlap 50%; LeBoldus et al. 2012), ID < 33% (host reaction classified as resistant if the ID < 33% and the 95% CI does not overlap 33%), and ID <25% (host reaction classified as resistant if ID < 25% and 95% CI does not overlap 25%; Somé et al. 1996; LeBoldus et al. 2012).

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Chapter 3. Genetic structure of *Plasmodiophora brassicae* populations virulent on clubroot resistant canola (*Brassica napus* L.)

3. 1 Introduction

Plasmodiophora brassicae Woronin, the causal agent of clubroot of crucifers, poses a significant threat to the Canadian canola (*Brassica napus* L.) crop. The life cycle of *P. brassicae* is complex and not completely understood. An obligate parasite, *P. brassicae* forms intracellular plasmodia in infected roots, which eventually develop into large numbers of resting spores (Ludwig-Muller et al. 2015). While traditionally classified as a fungus, *P. brassicae* has more recently been placed in the phylum Cercozoa of the Rhizaria supergroup (Bulman and Braselton 2014) based on phylogenetic analyses. On infected plants, the clubroot parasite causes root malformations known as galls or 'clubs', which are the result of cellular hyperplasia and hypertrophy (Kageyama and Asano 2009; Voorrips 1995). Infected roots are not effective in absorbing water and nutrients from the soil, resulting in foliar wilting even under minor moisture stress. Disease progression eventually results in foliar chlorosis and necrosis, plant stunting, accelerated flowering, and ultimately major yield losses (Dixon 2009; Voorrips 1995).

In Canada, clubroot has been a constraint to the production of *Brassica* vegetables for over a century (Howard et al. 2010), but the disease was not identified on canola in the Prairies region until 2003, when it was found in 12 fields in the province of Alberta (Tewari et al. 2005). By 2018, clubroot infestations had been confirmed in more than 3,000 fields in Alberta (Strelkov et al. 2019b), with additional infestations reported from canola crops in Saskatchewan and Manitoba. Clubroot management is challenging and many strategies have been proposed to control the disease. Most cultural or chemical management methods, however, are either not practical or too

expensive to implement in canola production systems (Hwang et al. 2014). The deployment of clubroot resistant (CR) canola cultivars represents the most effective and convenient approach to manage this disease, but the identification of *P. brassicae* strains capable of overcoming the resistance in canola is a significant challenge for growers (Strelkov et al. 2016, 2018).

An up-to-date understanding of the diversity in *P. brassicae* populations is important for the judicious and effective deployment of resistance genes (Manzanares-Dauleux et al. 2000). Diversity in the virulence of field and single-spore isolates of the clubroot parasite has been examined extensively in Canada, with pathotypes 2, 3, 4, 5, 6, 7, 8 and P_1, P_2, P_3 identified on the differentials of Williams (1966) and Somé et al. (1996), respectively (Chapter 2; Cao et al. 2009; Strelkov et al. 2007, 2016 and 2018; Xue et al. 2008). The recent development of the Canadian Clubroot Differential (CCD) set has enabled detection of many additional pathotypes, further indicating a high level of diversity in the virulence of *P. brassicae* populations (Chapter 2; Strelkov et al. 2018). In addition to knowledge of the virulence of a parasite population; however, information on the genetic structure of the parasite is also helpful for the development of durable plant resistance (McDonald and Linde 2002). In earlier studies, random amplified polymorphic DNA (RAPD) markers were used to investigate genetic diversity among isolates of *P. brassicae*, which revealed a high level of genetic diversity; however, no correlation between molecular groupings and virulence were found (Manzanares-Dauleux et al. 2001; Osaki et al. 2008). Klewer et al. (2001) examined the genetic diversity of four single-spore isolates originating from a German field and reported a reliable differentiation of isolates with restriction fragment length polymorphism (RFLP) markers. Strehlow et al. (2014) found moderately high genetic diversity in *P. brassicae* field isolates from Germany based on an amplified fragment length polymorphism (AFLP) analysis, suggesting that the parasite is a genetically diverse species. In contrast, low levels

of polymorphism were reported among *P. brassicae* isolates from Japan in a study with simple sequence repeat (SSR) markers (Kubo et al. 2017). Based on whole genome single nucleotide polymorphism (SNP) profiles, Canadian *P. brassicae* isolates classified as pathotypes 2, 3, 5 and 8 were found to be highly similar, while the SNP profile of pathotype 6 was more divergent (Rolfe et al. 2016). Holtz et al. (2018), employing restriction site-associated DNA sequencing (RADseq), identified two distinct *P. brassicae* populations in Canada, which were either virulent or avirulent on clubroot resistant canola (CR) cultivars. Most recently, Sedaghatkish et al. (2019) reported a high level of genetic diversity in Canadian *P. brassicae* populations by comparing their whole genome sequences.

Simple sequence repeats have been widely employed as markers in the study of population genetic structures, since they possess high mutation rates with relatively high levels of potential polymorphisms among and within the populations (Goldstein and Schlotterer 1999). These markers consist of tandem repeats of short nucleotide motifs (1-6 bp) (Metz et al. 2016) that can occur throughout the genome, including in coding and non-coding DNA (Li et al. 2004). Variation in SSRs results mainly from errors during the replication process, by DNA polymerase slippage that generates nucleotide deletions or insertions, resulting in smaller or larger regions, respectively (Ellegren 2004). In the current study, SSR markers were used to evaluate the genetic structure of the *P. brassicae* population in Alberta, with the relationships between DNA polymorphisms and virulence compared to identify possible pathotype-specific markers.

3. 2 Materials and methods

3. 2. 1 Field and single-spore isolates

Fifty isolates (38 single-spore and 12 field isolates) were included in an evaluation of the genetic structure of *P. brassicae* in Alberta (Table 3.1). Thirty-four of the single-spores were

isolated from nine field isolates and characterized for pathotype designation on the hosts of Somé et al. (1996), Williams (1966) and the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018) as described in Chapter 2. The field isolates had been selected randomly from clubbed roots collected from commercial fields sown to clubroot resistant (CR) canola cultivars ('74-47 CR', '45H29', '74-54 RR', 'L135C' or '1960'). Seven of these field isolates originated from central Alberta, where clubroot is most prevalent, while one came from southern Alberta and another from northern Alberta (Fig. 3.1). An additional three field isolates were recovered from stored soil and root samples (collected from 2005-2014) specifically for this study, to examine genetic differentiation in *P. brassicae* before and after the cultivation of CR canola (Fig. 3.1). Four single-spore isolates were obtained from these field isolates following the methods described in Chapter 2, and both the field and single-spores were tested for pathotype designation on the CCD set (Table 3.1). All of the isolates were stored as frozen root galls (-20°C) until further processing. The field isolates from Chapter 2 were maintained on galls of the same cultivar on which they were originally identified, whereas all single-spores and field isolates recovered from the stored collections were maintained on Chinese cabbage (*Brassica rapa* L. var. *pekinensis*) 'Granaat'.

3. 2. 2 Preparation of resting spores for DNA isolation

Frozen galls (20 g) were washed three times with sterile distilled (SD) water to remove any soil particles or other debris. The galls were surface-disinfected with 2% sodium hypochlorite (Clorox, USA) for 3 min, washed six times with SD water, and homogenized in SD water with an electronic hand blender for 6 min. The resulting homogenate was filtered through eight layers of cheesecloth (American Fiber & Finishing Inc., Albemarle, NC) and centrifuged for 15 min at 2000×g in 50 mL Falcon conical centrifuge tubes (Fisher Scientific, Rochester, NY). The supernatant was removed and 7.5 mL of 50% sucrose w/v (Fisher Scientific) was added to the

spore pellet, which was resuspended by agitating with a vortex for 2 min. After centrifugation at 2000×g for 10 min, the supernatant was transferred to a new 50 mL Falcon tube, diluted with 42 mL SD water, then vortexed again. The spores were collected by centrifugation at 2000×g for 10 min, and the last three steps were repeated by adding another 7.5 mL of 50% sucrose. Finally, the spores were resuspended in SD water and centrifuged (10 min at 2000×g) twice to remove the remaining sucrose. The spore pellet was resuspended in 1 mL SD water and surface-disinfected by incubating with 1 μg/mL colistin sulfate (Sigma Aldrich, St. Louis, MO) and 1 μg/mL vancomycin hydrochloride (Alfa Aesar, Tewksbury, MA) overnight at 12°C. The spores were collected by centrifugation at 2000×g for 10 min, the supernatant was removed, and the spores were washed twice in SD water to remove the antibiotics.

The washed spore pellet was resuspended in 1 mL of SD water and treated with 10 units/mL of DNase I (Qiagen, Germany) for 3 h at 37°C to remove bacterial, fungal or plant DNA contamination. Ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 5 mM and the solution incubated for 15 min at 70°C to inactivate the DNase I. The resting spores were washed three times with SD water, re-suspended in 4.5 mL SD water, divided into three 2 mL safe-lock centrifuge tubes (Eppendorf, Hamburg, Germany), and centrifuged at 15000×g for 5 min. The supernatant was removed and the spore pellet lyophilized and stored at -20°C until subsequent use.

3. 2. 3 DNA extraction

Genomic DNA was extracted from the lyophilized resting spores following Sambrook et al. (2001) with several modifications. Briefly, 20-30 mg of the lyophilized spores was disrupted in a TissueLyser II (Qiagen, Hilden, Germany) for 1.5 min at 30 Hz using one 5-mm-diam. stainless steel bead (Qiagen) per well. The pulverized spores was suspended in 800 μL preheated (65°C)

extraction buffer (20 mM EDTA, 1.4 M NaCl, 100 mM Tris-HCl pH:8, 2% CTAB, plus 1% β-mercaptoethanol added just before use) and incubated at 65°C for 30 min with gentle mixing by inversion every 10 min. The samples were allowed to cool to room temperature and 1.5 µL RNase A (Qiagen) was added, after which the samples were vortexed vigorously and incubated at 37°C for 30 min. Following centrifugation at 15000×g for 7 min, the supernatant was transferred to a new 1.7 mL microcentrifuge tube (Midwest Scientific, St. Louis, MO) and 750 µL chloroform-isoamyl alcohol (24:1, 10 mL) was added. After mixing the emulsion intensely, the suspension was centrifuged at 15000×g for 10 min. Five hundred µL of the supernatant was transferred to a new 1.7 mL microcentrifuge tube and 650 µL of cold isopropanol added, followed by gentle mixing by inversion and centrifugation at 15000×g for 10 min (4°C). The supernatant was decanted, and the DNA pellet washed once with 90% (v/v) ethanol and twice with 70% (v/v) ethanol, vacuum-dried (Eppendorf) at 45°C for 5 min and then re-suspended in 40 µL nuclease-free water. The quality and quantity of DNA were assessed by gel electrophoresis and with a NanoDrop 2000c Spectrophotometer (Thermo Scientific) and the suspension was diluted to a final concentration of 10 ng/µl prior to PCR analysis. Samples of DNA were also extracted in the same manner from lyophilized healthy roots of the Chinese cabbage host and mycelia of the fungi *Leptosphaeria maculans* (Sowebery) P. Karst. (blackleg of canola) and *Sclerotinia sclerotiorum* (Lib.) de Bary (stem rot of canola) which were served as controls.

3. 2. 4 Simple sequence repeat (SSR) markers

The whole genome sequence of *P. brassicae* single-spore isolate e3 (Schwelm et al. 2015) (GenBank assembly accession: GCA_001049375.1) was inspected for candidate SSR loci. The SSRs were identified and primers designed using WebSat (http://purl.oclc.org/NET/websat/) (Martins et al. 2009). SSRs consisting of a minimum of 10 uninterrupted mononucleotide repeats

and a minimum of 6 uninterrupted repeats of di-, tri-, tetra-, penta- and hexanucleotide motifs were identified across the 25.5 Mb genome sequence. Primers were designed based on the following parameters: primer length between 18-27 bp with optimal length of 22 bp, GC content between 40-80%, melting temperature (Tm) between 57-68°C with an optimum of 60°C and < 1°C difference in the Tm of the forward and reverse primers, amplified product 100-500 bp in size. The specificity of the primers was evaluated in batch using command line BLAST v. 2.6.0, against the *P. brassicae* genome and the non-redundant (nr) GenBank database. Primers specific for *P. brassicae* with only one target site in the genome were selected and a 19 bp universal M13 sequence (CACGACGTTGTAAAACGAC) was added to the 5′-end of all forward primers to facilitate downstream fluorescent labeling (Schuelke 2000).

3. 2. 5 PCR conditions

PCR analyses were performed in 96-well plates in a 12.5 µL reaction volume containing 1X reaction buffer without $MgCl_2$ (Invitrogen, Carlsbad, CA), 0.2 mM dNTPs (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.5 µM of each forward and reverse primer, 0.5 µM of FAM-labeled M13 primer (Applied Biosystems, Foster City, CA), 0.5 U Platinum Taq DNA polymerase (Invitrogen) and 2 µL (20 ng) genomic DNA as a template. All reactions were run in a Mastercycler Nexus Thermal Cycler v. 3.4.0.0 (Eppendorf) under the following conditions: 7 min at 95 °C, followed by 3 cycles of 30 s at 95 °C, 30 s at 52-56 °C (6 °C lower than the Tm of each primer set) and 80 s at 72 °C, then 33 cycles of 15 s at 94 °C, 15 s at 54-58 °C (4°C lower than the Tm of each primer set) and 45 s at 72 °C, with a final extension step of 10 min at 72 °C. Samples were held at 4 °C. Following each amplification, 3 µL of the PCR product from 20 randomly selected samples was run on a 2% agarose gel to confirm proper amplification and product intensity. Contingent on the product intensity, 0.5-1 µL of PCR product was added to a final volume of 10 µL with a mixture

of highly deionized (Hi-Di) formamide (Applied Biosystems, Warrington, UK) and GeneScan 500 LIZ size standards (Applied Biosystems). The amplicons were resolved on an ABI 3730 capillary electrophoresis system (Applied Biosystems), and GeneMapper v. 3.7 (Applied Biosystems) was used to determine the SSR allele sizes by comparison of the amplicons with the size standards. In all PCR amplifications, DNA of the host, *S. sclerotiorum* and *L. maculans* was included as controls.

3. 2. 6 Analysis

Cluster analysis was performed with NTSys-pc v 2.21q (Exeter Software, New York, NY) using simple similarity coefficient and employing the unweighted pair-group method using the arithmetic means (UPGMA) procedure. The input file was a similarity matrix prepared by NTedit 2.21i with binary data assigning a '1' or a '0' for the presence or absence, respectively, of each specific amplified band for the various alleles of each locus. The corresponding association coefficients for qualitative data were calculated using the SIMQUAL computational module in NTSys-pc. The UPGMA analysis was conducted with SAHN in NTSys-pc, and dendrograms were visualized utilizing the Tree Plot program of the same software package (Rohlf 2009). The samples corresponding to Chinese cabbage, *L. maculans* and *S. sclerotiorum* were not used in the calculations with GenAlEx 6.51 b2, but were included in the NTSys-pc cluster analysis.

The frequency- and distance-based statistical procedures of GenAlEx 6.51b2 were employed to perform genetic structure analysis for both single and multiple populations. For single population analysis, the frequencies of alleles at each locus, haploid diversity indices including the number of samples (N), number of alleles (N_a), the effective number of alleles [N_e= No. of Effective Alleles = 1 / (Sum pi^2)], Shannon's Information Index [I = -1× Sum (pi × Ln (pi))], diversity (h= 1 - Sum pi^2, where pi is the frequency of the i^{th} allele for the population and Sum pi^2

is the sum of the squared population allele frequencies), unbiased diversity [uh= (N / (N-1)) × h] for each locus, their means over loci, and the percentage of polymorphic loci were calculated using the 'Frequency' option under 'Frequency-Based' statistical procedures with an input haploid data file with alleles coded numerically from 1 to 5. The significance of haploid disequilibrium was also assessed using the 'Frequency' menu with 999 randomizations. The index of haploid linkage disequilibrium was computed as V_o/V_e, where V_e is the expected variance of K, the number of loci for which two individuals vary. Individuals with matching multilocus genotypes (clones) were also identified using the 'Multilocus' option under the same procedures. Genetic distance and principal coordinates analysis (PCoA) were performed using the 'Distance' and 'PcoA' options, respectively, under 'Distance-Based' statistical procedures. For PCoA analysis, a standard covariance matrix based on genetic distance was used as the input file (Peakall and Smouse 2006, 2012).

For analysis of multiple populations, the isolates were grouped into different populations based on their virulence patterns (pathotype designations) on the differential hosts of Somé et al., Williams and the CCD set. Following the exclusion of pathotype populations with sample sizes < 4, frequencies of alleles at each locus for each population and haploid diversity by population indices, including the number of samples (N), number of alleles (N_a), the effective number of alleles (N_e), the information index (I), diversity (h), and unbiased diversity (uh) for each locus per population, the mean over loci per population, the grand mean and the percentage of polymorphic loci for each population were calculated using similar approaches as described above for the single population analysis, except that a list of the private alleles by population was also generated. Shannon's mutual information index ${}^{S}H_{UA}$, a pairwise measure of differentiation, was computed using the 'Pairwise Pops' sub-menu of the 'Shannon' menu under 'Frequency-Based' statistical

procedures using the natural logarithm. The pairwise mean genetic distance between populations was calculated using the 'Genetic by Pop' sub-menu of 'Distance' menu under the 'Distance-Based' statistical procedures. Analysis of molecular variance was conducted and degrees of genetic differentiation between populations were calculated from the genetic distance summed over all loci, giving PhiPT (PhiPT = AP / (WP + AP) = AP / TOT, where AP = estimated variance among populations, WP = estimated variance within populations, and TOT = total estimated variance), a measure that assumes a stepwise mutation model and estimates variation among individuals within populations relative to the total (Peakall and Smouse 2006, 2012). Gene flow expressed as Nm (number of migrants) was calculated as N_m (Haploid) = [(1 / PhiPT) - 1] / 2. A PCoA analysis was performed for populations with the covariance matrix method using the 'PcoA' option under the 'Distance-Based' statistical procedures (Peakall and Smouse 2006, 2012). The correlation analysis between matrices derived from SSR and virulence data were conducted using a Mantel test in GenAlEx 6.51 b2 with 999 permutations.

3. 3 Results

3. 3. 1 Single-spore isolation and pathotype designation

In addition to the 34 single-spore isolates isolated previously (Chapter 2), four single-spores, representing three field isolates (30-05-P, 139-07-P and 341-12-P), were recovered from 150 'Granaat' seedlings inoculated in the current study. Two plants (4%) with clubroot symptoms were obtained following inoculation with single-spores from field isolate 30-05-P. One out of 50 (2%) seedlings inoculated with spores from each of the field isolates 139-07-P and 341-12-P also developed galls. Taking an ID of 50% as the threshold between a resistant and susceptible host reaction (LeBoldus et al. 2012), pathotypes P_2 (6 isolates, 86%) and P_3 (1 isolate, 14%), pathotypes 2 (5 isolates, 71%), 3 (1 isolate, 14%), 6 (1 isolate, 14%) and pathotypes 3D (1 isolate, 14%), 2F

(4 isolates, 57%), 6B (1 isolate, 14%), 2A (1 isolate, 14%) were identified on the differentials of Somé et al., Williams and the CCD set, respectively.

3. 3. 2 Development of SSR markers

Seven hundred eighty-three SSRs were identified across the 25.5 Mb *P. brassicae* genome sequence, at a density of 1 SSR per 32.6 kb. Trinucleotide repeats were the most prevalent repeat type, accounting for 68% (536 repeats) of SSRs, followed by dinucleotide (21%, 161 repeats) and mononucleotide repeats (9%, 73 repeats). Tetra- and pentanucleotide repeats were the least frequent (<1%), while hexanucleotide repeats were not detected. The length of SSRs ranged from 10-39 bp for mononucleotide, 12-54 bp for dinucleotides, 18-99 bp for trinucleotides, 24-32 bp for tetranucleotides and 30-40 bp for pentanucleotide repeats. Among the mononucleotides, polyG/C (97%) were far more abundant than polyA/T (3%). Ten different combinations of dinucleotides (AC, AG, CA, CG, CT, GA, GC, GT, TC, TG) were found among 161 repeats, among which GT (20%) and AC (19%) were the most frequent (Supplementary Table 3.1). Among the 536 trinucleotide repeats, 41 different trimeric motifs were detected; the most common were CGT and CGA with frequencies of 10.5% and 10%, respectively. Five tetranucleotide (AGGC, CGTC, CTGT, GCAC, GTCT) and four pentanucleotide (AACTG, CCGAC, CGGTC, TAAAC) motifs were detected (Supplementary Table 3.1). Blasting of the primers against non-redundant GenBank database indicated that 747 of 783 were specific to the clubroot pathogen. However, blasting of the primers against the *P. brassicae* genome assembly showed that only 72 primer pairs had a single binding site in the genome and then were evaluated for their suitability in the genotyping analysis; of these, 20 primers pairs amplified either no or several PCR products and, therefore, were excluded from further testing.

3. 3. 3 Population genetic structure

Fifty-two loci (Supplementary Table 3.2) were amplified successfully, with the mean percentage of polymorphic loci over the entire population calculated as 61.5%. The maximum numbers of mismatching loci between two single-spore isolates were 27 and 29, respectively, ignoring and considering missing data (Supplementary Tables 3.3 and 3.4). A single allele per locus was detected in all single-spore and field isolates with the exception of two single-spore (183-14-SS5 and 187-14-SS10) and two field isolates (1-14-P and 30-05-P), for which two alleles were found in 12 loci. These two single-spores and all the field isolates were removed for further analyses to meet the assumptions of SSR data analysis of haploid data in GenAlEx.

Ninety-three distinct alleles were detected with a range of 1-5 alleles per locus. The mean number of alleles (Na) was 1.788, with only one allele found in 20 loci (38%), and 2, 3 or 5 alleles observed in 24 (46%), 7 (13%) and 1 (2%) loci, respectively. The highest number of effective alleles (N_e) was found in locus H21, followed by loci H327 and J21 (Table 3.2). The maximum Shannon's information index (I=1.099), diversity (h=0.667) and unbiased diversity (uh=0.686) were observed in locus H642. In contrast, the minimum of these values (I=0.127, h=0.054, uh=0.056) were observed in loci H325, H522, H547 and H627 (Table 3.2). The mean effective number of alleles and Shannon's information index over all loci for the entire population of single-spore isolates were 1.369 and 0.311, respectively. For the entire population, haploid linkage disequilibrium with 999 permutations was significant (V_e = 5.727, V_o = 87.967, V_o/V_e = 15.359, P ($V_r >= V_o$) = 0.001). The maximum genetic distance was observed between isolate 175-14-SS1 and either of the isolates 41-14-SS2 or 187-14-SS2. The pairwise genetic distance (GD) matrix is presented in Supplementary Table 3.5. Twenty multilocus haplotypes were identified among the 36 single-spore isolates with overall (h) and unbiased (uh) diversities of 0.197 and 0.203, respectively.

The UPGMA cluster analysis using the simple similarity coefficient indicated two main clusters (A and B) at a similarity coefficient of 0.57 among the single-spore isolates (Fig. 3.2). Cluster A further divided into two subclusters (A.1 and A.2) at a similarity coefficient of 0.63. Subcluster A.1 consisted of seven haplotypes, from which the only single-spore isolate from southern Alberta (290-14-SS1) was further separated from the others at a similarity coefficient of 0.79. The single-spore isolate 175-14-SS1, derived from a field isolate collected in northern Alberta, formed subcluster A.2. Five haplotypes were found in cluster B.

Principal coordinates analysis (PCoA) also separated 32 of the single-spore isolates in two discrete groups. These 32 single-spores were derived from field isolates collected in central Alberta. The remaining four single-spore isolates were distinct from one another and also from the two main groups; these included three isolates (175-14-SS1, 290-14-SS1 and 341-12-SS1) from northern and southern Alberta. Isolate 175-14-SS1 had eight private alleles, while only one private allele was identified in 290-14-SS1. Four alleles were not detected in any isolate with the exception of 175-14-SS1 and 290-14-SS1. The variation explained by the first 3 axes was 73.53%, 9.71% and 4.11%, respectively (Fig. 3.3).

3. 3. 4 Relationships between genetic and virulence structure

The results of the cluster analysis were compared with the virulence patterns of the isolates on the hosts of Somé et al. (1996), Williams (1966) and the CCD set (Strelkov et al. 2018). A relatively clear relationship was observed between DNA polymorphisms and pathotype (P_1, P_2 and P_3) classification on the differentials of Somé et al. All single-spore isolates classified as P_2 (23 isolates, 7 haplotypes) grouped in subcluster A.1. Eleven (five haplotypes) of the 12 isolates classified as pathotype P_3 also grouped together, forming cluster B, while the isolate 175-14-SS1 (with eight unique alleles) was distinct and formed subcluster A.2. The only single-spore isolate

(183-14-SS4) classified as pathotype P_1 clustered with the P_3 isolates in cluster B (Fig. 3.2). Four of five field isolates classified as P_2 and four of five field isolates classified as P_3 grouped with the single-spore isolates of the same respective pathotypes. There were just two exceptions: field isolate 331-14-P clustered with the P_2 isolates although it was classified as pathotype P_3, while isolate 41-14-P clustered with the P_3 isolates although it was as P_2 (Supplementary Fig. 3.1).

When treating single-spore isolates classified as pathotypes P_2 and P_3 as two populations (P_2 Pop and P_3 Pop), the percentage of polymorphic loci was 25.00% and 51.92 %, respectively. The number of alleles, effective alleles and Shannon's information index were higher in P_3 Pop than P_2 Pop (Table 3.3). Three private alleles were found in each of the two populations. Furthermore, with the exception of isolates 175-14-SS1 and 290-14-SS1, alleles unique to each population were observed in another 15 loci. Eleven distinct multilocus haplotypes were identified among the 23 isolates in P_2 Pop, while eight haplotypes were found among the 12 isolates in P_3 Pop. The same multilocus haplotypes were detected among the single-spore isolates derived from field isolates collected from the same field in different years. Higher overall diversity and unbiased diversity were found in P_3 (0.099, 0.108) vs. P_2 isolates (0.032, 0.033). The Nei genetic distance and Shannon's mutual information index ($^{S}H_{UA}$) between P_2 Pop and P_3 Pop were 0.399 and 0.206, respectively (Table 3.4). Significant genetic differentiation (PhiPT = 0.809, P = 0.001) was observed between the two populations, with 81% of the genetic variation occurring between populations and 19% within populations (Table 3.5). Principal coordinates analysis also indicated that, with the exception of three P_2 isolates and one P_3 isolate, most isolates classified as P_2 or P_3 formed two distinct groups (Fig. 3.4). Most of the variation (82.66%) was explained by the first 2 axes, with 72.53% for coordinate 1 and 10.13% for coordinate 2.

The single-spore isolates in this study represented pathotypes 2, 3, 4, 5, 6 and 7 on the system of Williams. Isolates classified as pathotypes 2 and 3 grouped together to form subcluster A.1.1, while most of those classified as pathotypes 5 and 6 formed cluster B (Fig. 3.2). The only isolate of pathotype 4 (183-14-SS4) also grouped with pathotypes 5 and 6 in cluster B. One isolate (175-14-SS1) classified as pathotype 6 was the only haplotype in subcluster A.2, while the only isolate of pathotype 7 (290-14-SS1) constituted subcluster A.1.2 (Fig. 3.2). The field isolates included in the analysis represented Williams' pathotypes 2, 3, 5, 6 and 8. No clear relationship, however, was observed between DNA polymorphisms and the virulence patterns of any of the field isolates with the exception of those classified as pathotype 6, which grouped with the single-spore isolates of the same pathotype (Supplementary Fig. 3.1).

When single-spore isolates classified as pathotypes 2, 3, 5 and 6 were regarded as populations (2Pop, 3Pop, 5Pop and 6Pop) (pathotypes 4 and 7 were excluded because they contained < 4 isolates each), the greatest percentage of polymorphic loci was found in 6Pop (51.92%), followed by 2Pop (9.62%), 5Pop (3.85%) and 3Pop (1.92%). The highest and lowest mean of number of alleles (1.596, 1.019), effective alleles (1.181, 1.019) and Shannon's information index (0.146, 0.010) were detected in 6Pop and 3Pop, respectively (Table 3.3). Six multilocus haplotypes were identified in each of 3Pop (14 isolates), 2Pop (8 isolates) and 6Pop (8 isolates), while four were found in 5Pop (4 isolates). The greatest overall diversity and unbiased diversity were found among pathotype 6 isolates (0.128, 0.146), while the lowest was observed among pathotype 3 isolates (0.01, 0.01) (Table 3.3). The highest Nei genetic distance (0.461) and Shannon's mutual information index (0.236) were found for 2Pop and 5Pop; the lowest (0.002, 0.005) was observed for 2Pop and 3Pop (Table 3.4). Analysis of molecular variance indicated significant genetic differentiation (PhiPT=0.764, P=0.001) among populations, with 76% and 24%

of variance coming from among and within populations, respectively (Table 3.5). Two distinct groups were detected by PCoA, with pathotypes 2 and 3 forming one group and pathotypes 5 and 6 another (Fig. 3.5). One each of isolates classified as pathotypes 2 (1-14-SS1) and 6 (175-14-SS1) were found to be distinct from the two main groups. The variation explained by the first 3 axes was 75.61%, 9.92% and 3.29%, respectively.

Single-spore isolates classified as pathotypes 2A, 2F, 3D and 3H on the CCD set clustered together in subcluster A.1.1, while isolates classified as pathotypes 4A, 5X, 6A and 6B formed cluster B (Fig. 3.2). However, the pathotype 5X isolates, along with an isolate (4-14-SS2) of pathotype 6B, were clearly distinct from the other isolates in cluster B. Isolate 1-14-SS2, classified as pathotype 6A and having two specific alleles in loci H21 and H332, formed a discrete subcluster. The single-spore isolates 290-14-SS1 and 175-14-SS1, designated as pathotypes 7A and 6C, respectively, also were distinct and formed subclusters A.1.2 and A.2 (Fig. 3.2). Field isolates representing different CCD pathotypes were distributed randomly through the dendrogram except for isolates of pathotype 6B (Supplementary Fig. 3.1).

The five CCD pathotypes containing ≥ 4 isolates each were regarded as populations (2FPop, 3DPop, 3HPop, 5XPop and 6BPop) and subjected to GenAlEx analysis. The highest percentage of polymorphic loci was detected in 2FPop (9.62%) followed by 5XPop (3.85%) and 3DPop, 3HPop and 6BPop (1.92% in each). The highest and lowest effective alleles (1.063, 1.009) and Shannon's information index (0.055, 0.010) were found in 2FPop and 6BPop, respectively (Table 3.3). Five multilocus haplotypes were identified among isolates in each of the 2FPop and 6BPop, but four were observed in each of the four other populations. The greatest overall diversity and unbiased diversity were found among isolates in pathotype 2F (0.037, 0.046), while the lowest were observed among isolates classified as pathotype 6B (0.006, 0.008) (Table 3.3). The highest

Nei genetic distance (0.465) was found between 2FPop and 5XPop, while there was zero distance between 3DPop and 3HPop (Table 3.4). Significant genetic variation (PhiPT = 0.857, P = 0.001) was observed, with 86% and 14% of the variation occurring among and within populations, respectively (Table 3.5). Principal coordinates analysis also indicated two distinct groups, one that consisted of isolates in pathotypes 2F, 3D and 3H, and another composed of isolates in pathotypes 5X and 6B. Two isolates from each group were distinct from the others (Fig. 3.6). The first two axes explained most of the variation (97%), with 82.38% for coordinate 1 and 4.78% for coordinate 2.

Mantel test results indicated a significant and strong correlation between each combination of threshold ID/host differential matrices and the matrix derived from genetic data of the isolates (Table 3.6). The highest correlation (83%) was found between matrices produced with genetic data and threshold IDs of 50% on the CCD set. Pairwise comparisons of associated matrices generated with genetic data and threshold IDs of 33% and 25% on the hosts of CCD set also indicated high correlations of 79% and 78%, respectively. The lowest, but still significant, correlation (56%) was observed between matrices related to genetic data and a threshold ID of 25% on the differentials of Somé et al. Among the three threshold IDs evaluated for distinguishing between resistant and susceptible host reactions on the hosts of the CCD set and Somé et al., the virulence matrix based on the 50% threshold showed the highest correlation with the genetic data matrix, followed by the matrices based on 33% and 25%. For the hosts of Williams, however, virulence matrices based on threshold IDs of 33% and 50% were the most and the least correlated with the genetic data, respectively.

3. 4 Discussion

The emergence of *P. brassicae* strains capable of overcoming host resistance represents a significant challenge to the management of clubroot on canola (Strelkov et al. 2016, 2018). Knowledge of the genetic structure of a pathogen population is important for the development of durable plant resistance (McDonald and Linde 2002), and in the current study, SSR markers were identified and used to study the genetic structure of *P. brassicae* in Alberta. A cluster analysis was also conducted to evaluate the relationship between the genetic and virulence structures of the pathogen population.

Among the 36 single-spore isolates tested, polymorphism was found in 32 loci (61.5%), and 93 distinct alleles were detected with a range of 1-5 alleles per locus. While significant diversity in the virulence of single-spore isolates from Alberta has been reported (Chapter 2), the level of genetic diversity found in the current study was relatively low (0.197). Holtz et al. (2018) also detected a low level of genetic variation in a collection of 21 *P. brassicae* field and single-spore isolates from Canada. Similarly, Sedaghatkish et al. (2019) reported low genetic variability within isolates collected from Alberta, but noted a high level of genetic diversity between the isolates from Alberta and those collected from other regions. Changes in virulence may reflect small changes in the pathogen genome, for example in a gene related to an effector (Chen et al. 2019; Perez-Lopez et al. 2020), and hence, there is not always a strong relationship between virulence and overall genetic diversity.

Relatively little is known regarding the molecular basis of variation in *P. brassicae*, although mutation, genetic drift, gene flow and migration may contribute to diversity. The longevity of *P. brassicae* resting spores (Wallenhammar 1996), together with a high reproduction rate in each generation (Diederichsen et al. 2009), may result in a large effective population size,

contributing to the development of a highly diverse pathogen population (Strehlow et al. 2014). Sedaghatkish et al. (2019) suggested, however, that given the large numbers of long-lived resting spores in the soil, the role of random genetic drift is small in the genetic diversity of *P. brassicae*. Sexual reproduction also can influence diversity, but there is uncertainty regarding sexual recombination in the life cycle of *P. brassicae*. Some authors have indicated the possibility of sexual reproduction (Manzanares-Dauleux et al. 2001; Tommerup and Ingram 1971), while others disagree with this suggestion (Fähling et al. 2004; Heo et al. 2009). Recently, Stjelja et al. (2019) identified seven active gene candidates encoding proteins associated with the synaptonemal complex, indicating potential genetic recombination events during meiotic division. In the current study, haploid disequilibrium analysis suggested that recombination was rare or absent, implying the occurrence of clonal reproduction. Holtz et al. (2018) also suggested that *P. brassicae* populations from Canada might be clonal, while Sedaghatkish et al. (2019) reported a high frequency of recombination in populations of the pathogen, although higher disequilibrium (low genetic changes) was observed in Prairies populations compared with populations from the rest of Canada. Linkage disequilibrium can be influenced by linkage, migration (population admixture), random genetic drift and selection (Slatkin 2008). Therefore, these contrasting results may reflect differences in the range of pathogen origins and hosts used in these studies. In the current analysis, isolates were collected from canola in Alberta, much like the report of Holtz et al. (2018) who also evaluated mostly Alberta isolates (15 out of 21 isolates) from canola. However, Sedaghatkish et al. (2019) analysed linkage disequilibrium among a collection of isolates from across Canada and from the USA and China, originating on a variety of cole crops and canola. The presence of migration (population admixture) reported by Sedaghatkish et al. (2019) could also influence the results.

Most of the single-spore isolates (33) in this study were from collections made in central Alberta, with only two single-spores derived from northern (Athabasca) and one from southern (Newell) Alberta. It is difficult, therefore, to make any strong conclusions regarding the relationship between pathogen genetic structure and geographic origins. Nonetheless, the isolates from northern and southern Alberta were distinct from each other and from the two main groups of isolates from central Alberta, which represents the heart of the outbreak. In most previous studies, no relationship was found between genetic grouping and the geographic origin of *P. brassicae* isolates (Manzanares-Dauleux et.al. 2001; Osaki et al. 2008; Sedaghatkish et al. 2019; Strehlow et al. 2014). Using whole genome SNP profiles, however, Rolfe et al. (2016) reported a correlation between haplotype clustering, host range and geographic distribution in an analysis of *P. brassicae* isolates representing five pathotypes from Canada. Isolates belonging to pathotypes 2, 3, 5 and 8 (*sensu* Williams), which were collected from the Prairies and are virulent on *B. napus*, were highly similar and grouped together, while pathotype 6, which is virulent on vegetable Brassicas and prevalent in British Columbia and eastern Canada, was more divergent (Rolfe et al. 2016). While there are no other studies on the genetic diversity of *P. brassicae* across Alberta, field and single-spore isolates from northern and southern regions of the province have distinct virulence patterns relative to those from central Alberta (Chapter 2; Cao et al. 2009; Strelkov et al. 2018).

While the isolates in this study represented 3-10 pathotypes, depending on the classification system used, 20 multilocus haplotypes were detected. Hence, multiple isolates with matching multilocus genotypes (five clones each consisting of 2-7 isolates) were observed in Alberta. Similarly, in another SSR-based study from Japan, one clone represented 15 of 24 *P. brassicae* isolates analyzed (Kubo et al. 2017). In contrast, in some earlier reports, each isolate appeared to

represent a unique genotype, based on analyses with AFLP and RAPD-PCR markers (Manzanares-Dauleux et al. 2001; Strehlow et al. 2014). *Plasmodiophora brassicae* is a biotrophic parasite and extracting pure DNA not contaminated with DNA of the host or soil organisms can be challenging. In contrast to SSR markers, both RAPD and AFLP markers are neither locus- nor species-specific; therefore, all DNA present in a sample could be amplified and introduce inaccuracy in downstream genotyping process (Milgroom 2015).

A relatively clear relationship was found between DNA polymorphisms and the pathotype classification of the single-spore isolates based on the system of Somé et al. (1996). Pathotypes P_2 and P_3 were clearly distinct from each other, although the single P_1 isolate tested also grouped with the P_3 isolates. The classification of isolates on the system of Somé et al. is based on the reaction of three differential hosts, ECD 06 (*Brassica napus* L. var. *napus* 'Nevin'), ECD 10 (*Brassica napus* L. var. *napus* 'Wilmesburger') and 'Brutor' (*Brassica napus* L.). This system has not been used widely in Canada because it lacks differentiating capacity, at least with respect to *P. brassicae* isolates recovered from canola (Strelkov et al. 2018; Strelkov and Hwang 2014). Indeed, only the pathotypes included in this study (P_1, P_2 and P_3) have been reported from Canada to date. Nonetheless, the groupings in the current analyses suggest that development of a molecular assay to distinguish these pathotypes is possible, and may facilitate more widespread testing of *P. brassicae* collections based on the system of Somé et al. (1996). Manzanares-Dauleux et al. (2001) reported the separation of P_1 from other pathotypes using RAPD-PCR, and developed a sequence characterized amplified region (SCAR)-marker specific for this pathotype.

The SSR markers in this study also indicated differentiation of pathotypes 2 and 3, as defined on the differentials of Williams (1966), from those isolates classified as pathotypes 5 and 6. A single isolate of pathotype 7 was also distinct from the rest. The only isolate classified as

pathotype 4, however, grouped with the pathotype 6 isolates. Pathotype classifications according to the system of Williams have been widely used in Canada (Strelkov et al. 2018; Strelkov and Hwang 2014), and a method to distinguish these pathotypes based on a molecular analysis would be of significant interest in efforts to monitor clubroot. Nonetheless, more work is required to develop pathotype-specific markers. While the cluster analysis separated pathotypes 5 and 6, pathotypes 2 and 3 could not be differentiated genetically. On the differential hosts, the latter two pathotypes are distinguished based solely on the reaction of the cabbage (*Brassica oleracea* L. var. *capitata*) 'Badger Shipper'. This is an older, open-pollinated cultivar, which often gives intermediate and fluctuating IDs following inoculation with *P. brassicae* (Chapter 2; Strelkov et al. 2006, 2007; Toxopeus et al. 1986; Voorrips 1995; Xue et al. 2008). It is possible therefore, that the classification of some isolates as pathotype 2 or 3 based on this virulence phenotype may not have been as clear as if they had been distinguished by the reaction of another host. Alternatively, and perhaps more likely, the SSR markers employed may not have been sufficient to achieve complete separation of these two pathotypes (or pathotype 4). In an independent study from Japan, pathotypes 2 and 4 of *P. brassicae* also could not be distinguished with SSR markers (Kubo et al. 2017).

Additional variants of the Williams pathotypes can be distinguished on the CCD set (Strelkov et al. 2018), which was developed to better capture the diversity in the virulence of *P. brassicae* populations from Canada. In the current analysis, no specific genetic differentiation pattern was observed for the CCD pathotypes 2F, 2A, 3D and 3H, which clustered together. However, these were distinct from pathotypes 5X, 6A, 6B, 6C and 7A, which generally also grouped independently of each other. The only isolate classified as pathotype 4A grouped with pathotype 6B. Pathotypes 2A, 3D and 6A are virulent on the clubroot resistant (CR) canola

‘45H29’, while pathotypes 6B and 5X are virulent on both ‘45H29’ and the CR oilseed rape (*B. napus*) ‘Mendel’, which is considered a resistance source for Canadian canola (Fredua-Agyeman et al. 2018). In the current analysis, pathotypes 6B and 5X differed at only one locus, while on the CCD set, they are distinguished by their virulence on a single differential ECD 13 (*Brassica oleracea* L. var. *capitata* ‘Jersey Queen’). In other genomic studies from Canada, pathotype 5X also was distinct from single-spore and field isolates avirulent on CR canola (Holtz et al. 2018; Sedaghatkish et al. 2019), while this is the first genetic analysis of pathotype 6B. Interestingly, while 3D is virulent on the CR canola ‘45H29’ and 3H is not (Strelkov et al. 2018), no genetic distance was observed between these two pathotypes in the SSR analysis. It is possible that they differ from each other only at one effector or virulence/avirulence gene, which was not reflected in the current study.

The number of migrants (gene flow) was 0.118, 0.154 and 0.084 among populations (pathotypes) defined on the hosts of Somé et al., Williams and the CCD set, respectively, indicating that gene exchange was low. Similarly, Strehlow et al. (2014) detected no genotype flow between fields with highly diverse *P. brassicae* populations. In contrast, Sedaghatkish et al. (2019) suggested that migration and selection were the cause of the admixture found in their analysis. Since *P. brassicae* is a soil-borne pathogen, low gene flow is expected. Nonetheless, gene flow can be influenced by human activities. Transmission of *P. brassicae* was shown on farm machinery, boots, the hooves of grazing animals, infected transplants, and in surface floodwater in Australia (Donald and Porter 2009). The movement of *P. brassicae*-infested soil on farm machinery seems to be the primary mechanism of pathogen dispersal in western Canada (Cao et al. 2009). Rennie et al. (2015) also found *P. brassicae* resting spores in windblown dust from infested fields, presumably contributing to the pathogen local dispersal.

The same multilocus haplotypes were observed among the single-spores derived from field isolates collected from the same fields in different years (183-14-P and 30-05-P, 187-14-P and 139-07-P). Balancing selection was suggested to occur in the *P. brassicae* population (Sedaghatkish et al. 2019; Strelkov et al. 2018, 2019a), which helps the pathogen to maintain variation and adapt to its environment by preventing loss of low-frequency alleles or those that are less fit. The only single-spore isolate (341-12-SS1) derived from field isolate 341-12-P from northern Alberta in 2012 was genetically distinct from other isolates in the same subcluster. Two (30-05-SS1, 139-04-SS1) of four single-spores derived from field isolates collected prior to the introduction of CR canola (in 2005, 2007 and 2012) were virulent on the CR cultivar '45H29'. Interestingly, 30-05-SS1, which was classified as pathotype 3D and is virulent on '45H29', was derived from a field isolate avirulent on this host. Therefore, its presence may have been be masked by the occurrence of other, avirulent pathotypes found at higher frequencies (Jones et al. 1982). One of three field isolates (139-07-P) collected before the cropping of CR canola also was virulent on both '45H29' and 'Mendel' (pathotype 6B). Collectively, these results highlight the presence of virulent genotypes on CR cultivars in commercial fields in Alberta prior to the introduction of CR cultivars. This finding is consistent with recent genomic analyses that also detected the presence of virulent *P. brassicae* strains in pathogen populations prior to exposure to CR canola (Sedaghatkish et al. 2019; Strelkov et al. 2019a), and which likely rose to pre-eminence by the selection pressure imposed by resistant hosts (Strelkov et al. 2018).

The Mantel test suggested that the highest correlation (83%) is present between the matrices generated using genetic data and a threshold ID of 50% to distinguish host reactions on the CCD set. Significant diversity was previously reported in the virulence of *P. brassicae* single-spore and field isolates from Alberta, which was identified most clearly with the CCD set

employing threshold of 50% (Chapter 2, Strelkov et al. 2018). The current study also supports that use of the CCD set with a 50% threshold for differentiation of resistance and susceptibility is suitable for the evaluation of *P. brassicae* pathotype composition in Alberta.

The amplification of one allele per locus was detected in most of the examined isolates, with the exception of two single-spore and two field isolates in which more than one allele was amplified. Field isolates often consist of heterogeneous mixtures of pathotypes (Chapter 2; Jones et al. 1982; Tinggal and Webster 1981; Xue et al. 2008). Hence, the amplification of two alleles per locus in some field populations may be explained by their heterogeneous nature. For example, Kubo et al. (2017) found two alleles in eight SSR loci in a study of *P. brassicae* field isolates from Japan, and suggested the presence of at least two genotypes in those isolates. The occurrence of more than one allele in two single-spore isolates was less expected. Descendants of a single resting spore should be genetically homogeneous, except in the case of mutations (Voorrips 1995, 1996), since the resting spores of *P. brassicae* are haploid (Tommerup and Ingram 1971). In the current study, it is possible that mutations occurred during the propagation of these two single-spore isolates, given the increase from one to billions of resting spores over one cycle of infection. It is also possible that they became contaminated at some point during their propagation in the greenhouse. Re-isolation of single-spore from these isolates to evaluate their genetic and virulence structure may clarify this matter. Nonetheless, for the purposes of this study, the two single-spore isolates in question (183-14-SS5 and 187-14-SS10) along with all field isolates were excluded from the analysis of *P. brassicae* genetic structure.

The current study indicates that the *P. brassicae* population virulent on CR canola in Alberta has a low level of genetic diversity and may be mainly clonal. Single-spore isolates more clearly reflected the pathogen population genetic structure, and relationships were detected

between the genetic and virulence patterns for some of the pathotypes. This suggests some promise for the future development of pathotype-specific primers. Nonetheless, while genetic variability within each pathotype group, as defined on the differentials of Somé et al., Williams and the CCD set was low, significant genetic differentiation was observed among the pathotypes. Isolation of additional single-spore isolates representing more pathotypes, together with characterization of the current isolates by whole genome sequencing, may provide further insights into *P. brassicae* biology and population genetic structure.

3. 5 Tables

Table 3.1. Field isolates and single-spores included in the current study of the genetic structure of *Plasmodiophora brassicae* in Alberta.

Field isolate[b]	Single-spore isolate	Origin	Year	Pathotype[a]		
				Somé et al.	Williams	CCD
1-14-P		City of Edmonton	2014	P_2	3	3D
	1-14-SS1			P_2	2	2F
	1-14-SS2			P_3	6	6A
	1-14-SS3			P_2	3	3H
	1-14-SS4			P_2	2	2F
	1-14-SS5			P_2	3	3H
4-14-P		Westlock	2014	P_3	6	6B
	4-14-SS1			P_2	3	3H
	4-14-SS2			P_3	6	6B
6-14-P		Westlock	2014	P_3	6	6B
	6-14-SS1			P_2	3	3D
41-14-P		Red Deer	2014	P_2	3	3D
	41-14-SS1			P_2	3	3H
	41-14-SS2			P_3	6	6B
	41-14-SS3			P_2	3	3H
175-14-P		Athabasca	2014	P_2	3	3D
	175-14-SS1			P_3	6	6C
183-14-P		Sturgeon	2014	P_2	8	8E
	183-14-SS1			P_2	2	2A
	183-14-SS2			P_2	3	3D
	183-14-SS3			P_2	2	2A
	183-14-SS4			P_1	4	4A
	183-14-SS5			P_3	6	6B
	183-14-SS6			P_2	3	3D
	183-14-SS7			P_2	3	3H
187-14-P		Sturgeon	2014	P_2	3	3A
	187-14-SS1			P_3	5	5X
	187-14-SS2			P_3	5	5X
	187-14-SS3			P_3	5	5X
	187-14-SS4			P_3	5	5X
	187-14-SS5			P_3	6	6B
	187-14-SS6			P_3	6	6B
	187-14-SS7			P_2	3	3H
	187-14-SS8			P_3	6	6B
	187-14-SS9			P_2	3	3H
	187-14-SS10			P_2	3	3A
	187-14-SS11			P_3	6	6B

Field isolate[b]	Single-spore isolate	Origin	Year	Pathotype[a]		
				Somé et al.	Williams	CCD
290-14-P		Newell	2014	P_3	6	6B
	290-14-SS1			P_2	7	7A
331-14-P		Leduc	2014	P_3	5	5G
	331-14-SS1			P_2	3	3H
	331-14SS2			P_2	2	2F
	331-14-SS3			P_2	3	3D
30-05-P		Sturgeon	2005	P_2	2	2F
	30-05-SS1			P_2	3	3D
	30-05-SS2			P_2	2	2F
139-07-P		Sturgeon	2007	P_3	6	6B
	139-07-SS1			P_2	2	2A
341-12-P		Athabasca	2012	P_2	2	2F
	341-12-SS1			P_2	2	2F

[a] Each field and single-spore isolate was classified into pathotypes based on their virulence on the differential hosts of Somé et al. (1996), Williams (1966) and the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018). An index of disease (ID) of 50% (with the 95% confidence interval not overlapping 50%; LeBoldus et al. 2012) was regarded as the threshold between a resistant and susceptible host reaction for pathotype classification purposes.

[b] Field isolates 30-05-P, 139-07-P and 341-12-P were recovered from stored soil and root samples (collected from 2005-2014) specifically for this study; however, remaining nine field isolates were described in Chapter 2.

Table 3.2. Analysis of allele frequency and haploid diversity by locus in the entire population of *Plasmodiophora brassicae* single-spore isolates included in the current study.

Locus	N[a]	N_a[b]	Allele frequencies	N_e[c]	I[d]	h[e]	uh[f]
H4	36	2	24, 12	1.800	0.637	0.444	0.457
H21	36	5	23, 1, 5, 6, 1	2.189	1.058	0.543	0.559
H57	35	2	1, 34	1.059	0.130	0.056	0.057
H95	36	2	13, 23	1.857	0.654	0.461	0.475
H107	36	3	12, 1, 23	1.923	0.752	0.480	0.494
H118	34	2	12, 22	1.841	0.649	0.457	0.471
H184	35	3	2, 1, 32	1.190	0.347	0.160	0.165
H229	36	2	22, 14	1.906	0.668	0.475	0.489
H268	36	2	23, 13	1.857	0.654	0.461	0.475
H315	36	2	24, 12	1.800	0.637	0.444	0.457
H325	36	2	1, 35	1.057	0.127	0.054	0.056
H327	36	3	12, 2, 22	2.051	0.828	0.512	0.527
H332	35	3	5, 29, 1	1.413	0.535	0.292	0.301
H335	36	2	2, 34	1.117	0.215	0.105	0.108
H349	34	2	1, 33	1.061	0.133	0.057	0.059
H362	35	2	2, 33	1.121	0.219	0.108	0.111
H367	36	2	13, 23	1.857	0.654	0.461	0.475
H454	36	2	24, 12	1.800	0.637	0.444	0.457
H518	35	2	1, 34	1.059	0.130	0.056	0.057
H522	36	2	1, 35	1.057	0.127	0.054	0.056
H525	36	2	2, 34	1.117	0.215	0.105	0.108
H547	36	2	35, 1	1.057	0.127	0.054	0.056
H556	36	2	24, 12	1.800	0.637	0.444	0.457
H581	36	2	12, 24	1.800	0.637	0.444	0.457
H627	36	2	35, 1	1.057	0.127	0.054	0.056
H642	36	3	12, 12, 12	3.000	1.099	0.667	0.686
H668	36	2	23, 13	1.857	0.654	0.461	0.475
H724	36	2	12, 24	1.800	0.637	0.444	0.457
J13	36	2	12, 24	1.800	0.637	0.444	0.457
J21	36	3	2, 12, 22	2.051	0.828	0.512	0.527
J23	35	2	1, 34	1.059	0.130	0.056	0.057
J31	36	2	24, 12	1.800	0.637	0.444	0.457

[a] Sample size; [b] Number of Different Alleles; [c] Number of Effective Alleles; [d] Shannon's Information Index [e] Diversity; [f] Unbiased Diversity

Table 3.3. Analysis of haploid diversity by population among *Plasmodiophora brassicae* single-spore isolates distinguished by their virulence patterns on hosts of Somé et al. (1996), Williams (1966) and Canadian Clubroot Differential set (CCD) (Strelkov et al. 2018).

		Mean of					
Differential set	Population	N[a]	N_a[b]	N_e[c]	I[d]	h[e]	uh[f]
Somé et al.	P_2 Pop	23	1.250	1.044	0.059	0.032	0.033
	P_3 Pop	12	1.596	1.145	0.183	0.099	0.108
Williams	2Pop	8	1.096	1.047	0.046	0.029	0.034
	3Pop	14	1.019	1.019	0.013	0.010	0.010
	5Pop	4	1.038	1.027	0.023	0.016	0.022
	6Pop	8	1.596	1.181	0.223	0.128	0.146
CCD	2FPop	5	1.096	1.063	0.055	0.037	0.046
	3DPop	5	1.019	1.018	0.013	0.009	0.012
	3HPop	9	1.019	1.019	0.013	0.009	0.011
	5XPop	4	1.038	1.027	0.023	0.016	0.022
	6BPop	5	1.019	1.009	0.010	0.006	0.008

[a] Sample size; [b] Number of Different Alleles; [c] Number of Effective Alleles; [d] Shannon's Information Index [e] Diversity; [f] Unbiased Diversity

Table 3.4. Pairwise Nei genetic distance and Shannon's mutual information index ($^{S}H_{UA}$) of *Plasmodiophora brassicae* populations distinguished based on their virulence patterns on hosts of Somé et al. (1996), Williams (1966) and Canadian Clubroot Differential set (CCD) (Strelkov et al. 2018).

Differential set	Population (Pop)	Nei genetic distance	$^{S}H_{UA}$
Somé et al.	P_2 Pop, P_3 Pop	0.399	0.206
Williams	2Pop, 3Pop	0.002	0.005
	2Pop, 5Pop	0.461	0.236
	2Pop, 6Pop	0.386	0.216
	3Pop,5Pop	0.460	0.198
	3Pop, 6Pop	0.384	0.211
	5Pop, 6Pop	0.020	0.027
CCD	2FPop, 3DPop	0.005	0.007
	2FPop, 3HPop	0.006	0.008
	2FPop, 5XPop	0.465	0.256
	2FPop, 6BPop	0.457	0.259
	3DPop, 3HPop	0.000	0.000
	3DPop, 5XPop	0.460	0.256
	3DPop, 6BPop	0.447	0.253
	3HPop, 5XPop	0.460	0.230
	3HPop, 6BPop	0.447	0.238
	5XPop, 6BPop	0.016	0.012

Table 3.5. Analysis of molecular variance (AMOVA) among and within populations distinguished based on their virulence patterns on hosts of Somé et al. (1996), Williams (1966) and Canadian Clubroot Differential set (CCD) (Strelkov et al. 2018).

Differential set	Population	Source	df[a]	SS[b]	MS[c]	Est. Var.	Variation %	PhiPT[d]	P
Somé et al.	P_2 Pop	Among Pops	1	125.794	125.794	7.859	81	0.809	0.001
	P_3 Pop	Within Pops	33	61.178	1.854	1.854	19		
		Total	34	186.971		9.712	100		
Williams	2Pop	Among Pops	3	130.500	43.500	5.235	76	0.764	0.001
	3Pop	Within Pops	30	48.500	1.617	1.617	24		
	5Pop	Total	33	179.000		6.852	100		
	6Pop								
CCD	2FPop	Among Pops	4	116.800	29.200	5.185	86	0.857	0.001
	3DPop	Within Pops	23	19.950	0.867	0.867	14		
	3HPop	Total	27	136.750		6.052	100		
	5XPop								
	6BPop								

[a] Degree of freedom; [b] Sum of squares; [c] Mean squares

[d] PhiPT (PhiPT = AP / (WP + AP) = AP / TOT, AP = estimated variance among populations, WP = estimated variance within populations) denotes the proportion of the total genetic variance that is due to the variance among populations (P = 0.05).

Table 3.6. Correlation between genetic structure and virulence dataset on based index of disease (ID) thresholds of 25%, 33% and 50% to distinguish between resistant and susceptible reactions on the hosts of the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018), Williams (1966) and Somé et al. (1996).

Differential set	Thresholds	Correlation
CCD	25%	78%
	33%	79%
	50%	83%
Williams	25%	58%
	33%	60%
	50%	57%
Somé et al.	25%	56%
	33%	62%
	50%	79%

3. 6 Figures

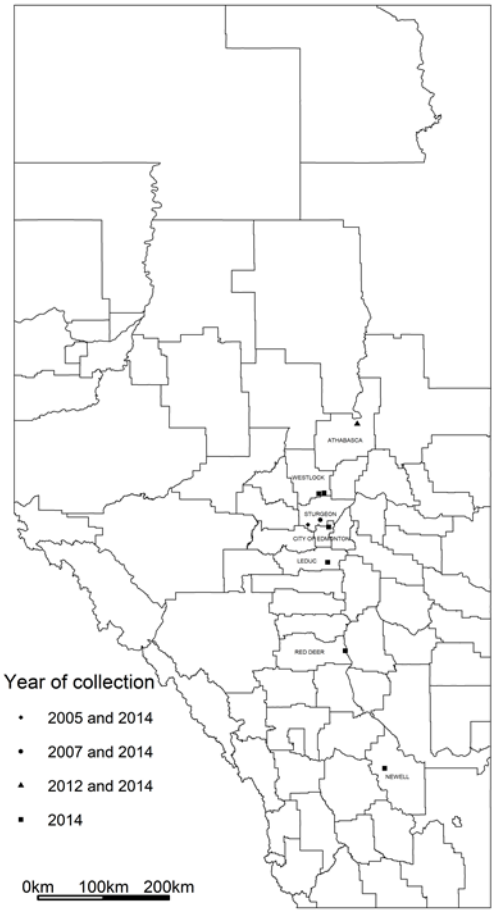

Figure 3.1. Distribution of fields in Alberta, Canada, from which field and single-spore isolates of *Plasmodiophora brassicae* were collected for the current study.

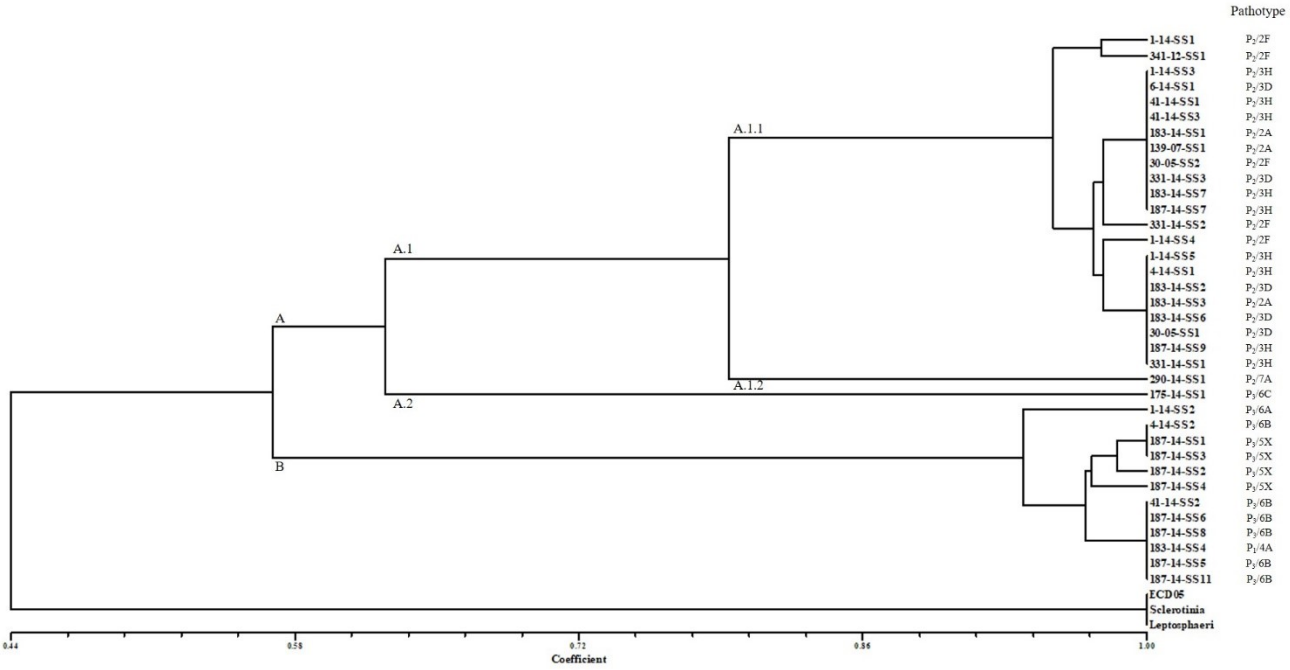

Figure 3.2. Genetic similarity of single-spore isolates of *Plasmodiophora brassicae*. The dendrogram was constructed employing the unweighted pair group method with arithmetic mean procedure and simple similarity coefficient with NTSys-pc ver. 2.2. Cluster analysis indicated that all isolates clustered in two distinct main groups (A and B). The three entries at the bottom of the dendrogram include Chinese cabbage (*Brassica rapa* var. *pekinenses*) 'Granaat' (European Clubroot Differential (ECD 05), *Sclerotinia sclerotiorum* and *Leptosphaeria maculans*, which were served as controls. Pathotype designations follow Somé et al. (1996) and Williams (1966)/Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018).

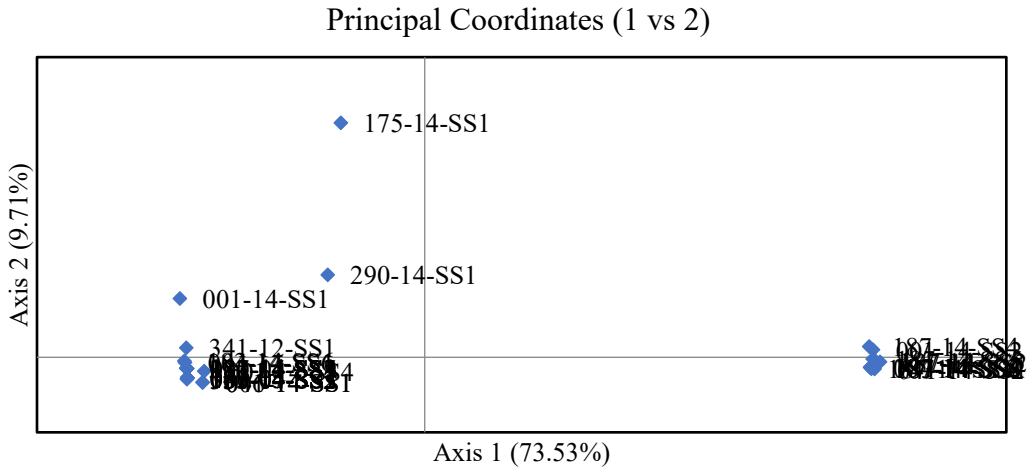

Figure 3.3. Scatter plot of the first and second coordinates in principal coordinate analysis (PCoA) for whole populations (all single-spore isolates) of *Plasmodiophora brassicae*, determined with the covariance matrix method using the "PcoA" option under "Distance-Based" statistical procedures of GenAlEx 6.51 b2. The percentage variation explained by the first three axes was 73.53, 9.71 and 4.11, respectively.

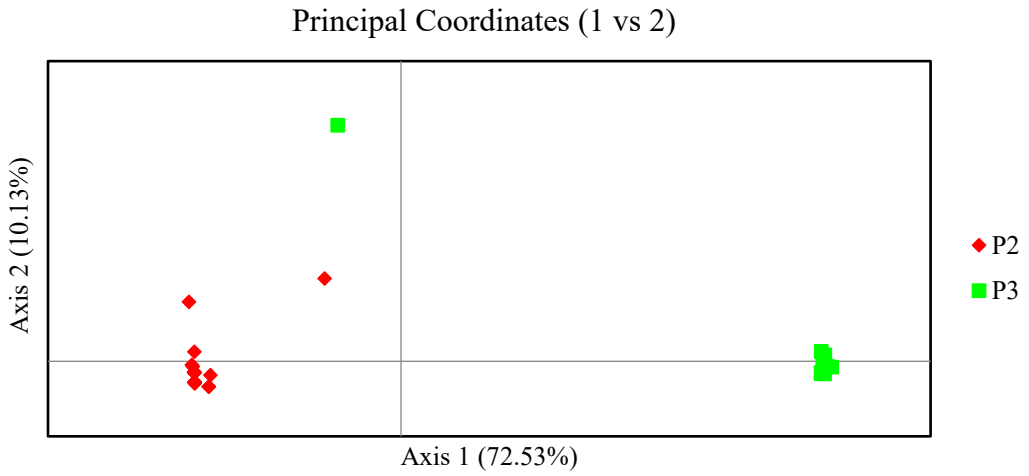

Figure 3.4. Scatter plot of the first and second coordinates in principal coordinate analysis (PCoA) for populations of *Plasmodiophora brassicae* distinguished by their virulence patterns on the differential hosts of Somé et al. (1996), determined with the covariance matrix method using the "PcoA" option under "Distance-Based" statistical procedures of GenAlEx 6.51 b2. Most of the variation (82.66%) was explained by the first two axes with 72.53% for coordinate 1 and 10.13 % for coordinate 2.

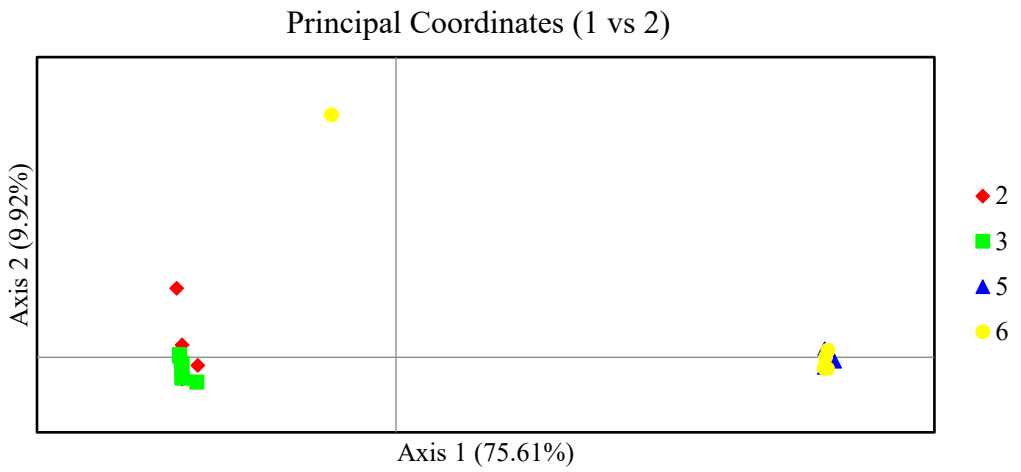

Figure 3.5. Scatter plot of the first and second coordinates in principal coordinate analysis (PCoA) for populations of Plasmodiophora brassicae distinguished by their virulence patterns on the differential hosts of Williams (1966), determined with the covariance matrix method using the "PcoA" option under "Distance-Based" statistical procedures of GenAlEx 6.51 b2. The percentage variation explained by the first three axes was 75.61, 9.92 and 3.29, respectively.

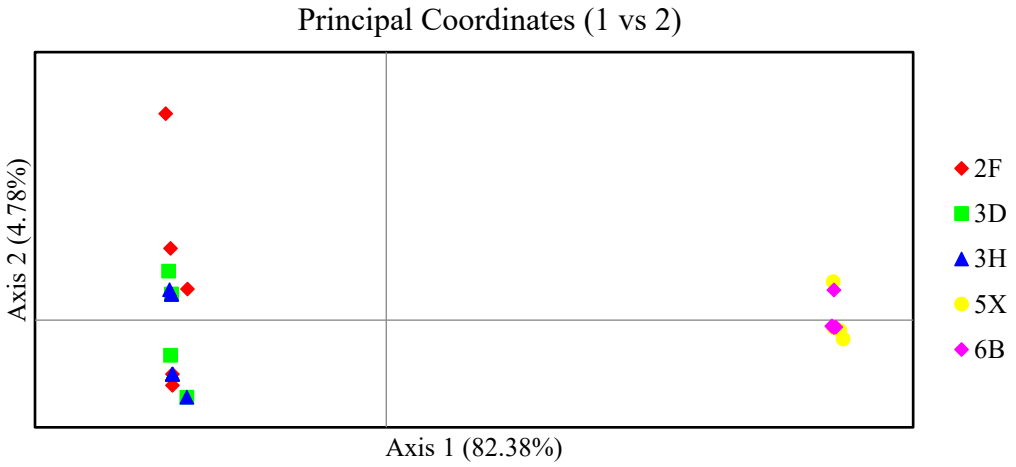

Figure 3.6. Scatter plot of the first and second coordinates in principal coordinate analysis (PCoA) for populations of *Plasmodiophora brassicae* distinguished by their virulence patterns on the hosts of the Canadian Clubroot Differential set (Strelkov et al. 2018), determined with the covariance matrix method using "PcoA" option under "Distance-Based" statistical procedures of GenAlEx 6.51 b2. The first two axes explained the main variation (97%), with 82.38 % for coordinate 1 and 4.78 % for coordinate 2.

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Chapter 4. Variation in the virulence of *Plasmodiophora brassicae* on clubroot resistant canola (*Brassica napus* L.)

4. 1 Introduction

Clubroot, caused by *Plasmodiophora brassicae* Wor., is one of the most significant soilborne diseases of the Brassicaceae (Dixon 2009; Karling 1968) and a major threat to the economically important canola (*Brassica napus* L.) industry in Canada. The disease was first identified on canola in western Canada in 2003 in a dozen crops near Edmonton, Alberta (Tewari et al. 2005), and has since spread to more than 3,000 fields across the province (Strelkov et al. 2019b). Yield losses due to clubroot can be as high as 30-100% in severely infected canola crops (Strelkov and Hwang 2014). The most characteristic symptom of clubroot development is the formation of large galls on infected roots, which impair the capacity for water and nutrient uptake (Kageyama and Asano 2009; Voorrips 1995). Resting spores serve as the primary survival structures of *P. brassicae* and can persist in the soil for many years (Wallenhammar 1999). While their half-life was estimated to be 3.6 years based on European research (Wallenhammar 1999), substantial declines in soil inoculum levels were reported in the first two years following a canola crop under Canadian conditions (Ernst et al. 2019; Peng et al. 2015).

The persistence of *P. brassicae* resting spores in the soil makes the management of clubroot challenging. Cultural strategies such as long rotations out of susceptible hosts can be effective, but have not been widely adopted in western Canada where canola remains one of the most lucrative crops (Howard et al. 2010; Hwang et al. 2014; Strelkov et al. 2011). Liming of the soil to increase its alkalinity is among the oldest methods for clubroot control (Donald and Porter 2009; Karling 1942), but is not always sufficient to keep crops free of the disease (Gossen et al. 2013; Murakami

et al. 2002). Similarly, strategies such as biological control (Lahlali et al. 2011, 2013; Peng et al. 2011) and the planting of bait crops (Ahmed et al. 2011; Friberg et al. 2006) have not been successful at significantly reducing resting spore populations under field conditions. Soil-applied fungicides are prohibitively expensive in the large-scale canola production systems of western Canada and can have associated environmental concerns (Hwang et al. 2014). Given these limitations to cultural and chemical approaches for clubroot control, the deployment of resistant cultivars represents the most effective and practical strategy to manage the disease (Peng et al. 2014a, b; Rahman et al. 2014).

Most of the sources of resistance to *P. brassicae* are race- or pathotype specific, apart from some *B. oleracea* pathotype-independent resistant accessions (Diederichsen et al. 2009). In western Canada, the first CR canola cultivar ('45H29') was released in 2009, followed by multiple other CR cultivars from various suppliers. Rahman et al. (2014) noted that some resistant host genotypes, including the European winter oilseed rape (*B. napus*) 'Mendel', rutabaga (*B. napus*) and Pak Choi (*Brassica rapa* L.), were utilized in efforts to breed clubroot resistant (CR) spring canola cultivars for the Canadian market. The full genetic basis of the resistance in these cultivars, however, is not publicly available due to proprietary concerns. Nonetheless, there is strong evidence that this resistance, at least in most cultivars, was derived from 'Mendel' (Fredua-Agyeman et al. 2018). The effectiveness and convenience of CR canola resulted in its rapid adoption for clubroot management in western Canada (Strelkov et al. 2016, 2018). Although there were suggestions that this resistance was not durable and should be managed cautiously (LeBoldus et al. 2012), CR canola has continued to be grown in tight rotations in many fields throughout clubroot-infested regions. In 2013, the first cases of severe symptoms of clubroot on CR canola were identified in central Alberta (Strelkov et al. 2016), soon followed by the emergence of

multiple ‘novel’ pathotypes of *P. brassicae* capable of overcoming genetic resistance in many fields across the province (Chapter 2; Strelkov et al. 2018, 2020). These resistance-breaking pathotypes pose one of the biggest challenges to sustainable canola production on the Canadian prairies (Strelkov et al. 2018).

Field isolates of *P. brassicae* represent a mixture of pathogen genotypes (Fähling et al. 2003; Jones et al. 1982; Somé et al. 1996), which can proliferate selectively on resistant hosts. Thus, when assessing host resistance using field isolates, it is possible that pathogen genotypes present at higher frequencies mask other genotypes occurring at lower frequencies (Jones et al. 1982). Additional factors may also confound the interpretation of host reactions obtained with field isolates of *P. brassicae*, including potential competition between virulent and avirulent pathogen genotypes for infection sites on the roots, and the possible impact of avirulent genotypes on host resistance (Jones et al. 1982; Voorrips 1996). Therefore, the use of genetically homogeneous single-spore isolates may be advisable in resistance screening programs, at least for validation of the results obtained with field isolates. Moreover, collections of single-spore isolates may better reflect the presence of rare or emerging pathotypes of *P. brassicae*, enabling proactive approaches to maintaining effective host resistance.

The objectives of this study were to evaluate the virulence spectrum of a collection of *P. brassicae* single-spore and field isolates from Alberta on a suite of CR canola cultivars, determine if all single-spores derived from a single field isolate caused similar reactions on the resistant hosts, and evaluate the relationships between DNA polymorphisms and virulence patterns observed.

4. 2 Materials and methods

4. 2. 1 Field and single-spore isolates

Twelve field and 38 single-spore isolates of *P. brassicae*, which had been previously evaluated for pathotype classification and included in a genetic structure analysis (Chapters 2, 3), were included in the present study (Table 4.1). Most of the field isolates were collected from the roots of the CR canola cultivars '74-47 CR', '45H29', '74-54 RR', 'L135 C' and '1960' during an annual clubroot survey conducted in Alberta in 2014. The remaining field isolates were recovered from three fields surveyed in 2005, 2007 and 2012, from which samples also were obtained in 2014. The single-spore isolates were derived from the field isolates as described in Chapters 2 and 3. All single-spores and field isolates collected in 2005, 2007 and 2012 were increased and maintained on the European Clubroot Differential (ECD) 05 (*Brassica rapa* L. var. *pekinensis* 'Granaat') (Buczacki et al. 1975), whereas field isolates collected in 2014 were increased and maintained on the same cultivar from which each was originally recovered.

4. 2. 2 Virulence on CR canola

The virulence of the *P. brassicae* isolates was assessed on seven CR canola cultivars ('45H29', '6056CR', '74-54RR', '9558C', 'D3152', 'L135C'and '1960') plus the Chinese cabbage ECD 05, which was included as a universally susceptible check. Seed of the commercial canola cultivars was supplied by producing companies or local retailers. Experiments were performed with four replicates for each host genotype-pathogen isolate combination, with 12 plants per replicate (experimental unit) employing a split-plot experimental design with isolates as main plots and differential host genotypes as subplots. Galls (20 g) of each isolate were homogenized in 200 mL of SD water using a hand blender (KitchenAid, Michigan, USA). The homogenate was filtered through six layers of cheesecloth (American Fiber & Finishing Inc., Albemarle, NC), and the resting spore concentration estimated with a haemocytometer (VWR, Mississauga, ON) and adjusted to 10^7 spores per mL in SD water. Seven-day-old seedlings were

inoculated via the root dip method following Strelkov et al. (2006). The inoculated seedlings were planted in potting mix (Sunshine Mix LA7, Sunshine Growers) in (6 cm × 6 cm × 6 cm) plastic pots, and then grown for 6 weeks in a greenhouse maintained at ca. 24°C/18°C (day/night) under natural light supplemented with artificial lighting (16 h photoperiod). The potting mix was kept saturated with water for the first 2 weeks after inoculation, and then watered and fertilized as required. To ensure the stability of the reactions, the entire experiment was repeated with one single-spore isolate of each pathotype.

4. 2. 3 Disease evaluation and data analysis

The seedlings were carefully uprooted 6-weeks after inoculation, with the roots washed with tap water and assessed for clubroot symptom development. Roots were scored on a 0-to-3 scale (Kuginuki et al. 1999), where: 0 = no galling, 1 = a few small galls, 2 = moderate galling and 3 = severe galling. An index of disease (ID) severity was determined for each replicate based on the formula of Horiuchi and Hori (1980) as modified by Strelkov et al. (2006):

$$ID\ (\%) = \{[\sum (n \times 0) + (n \times 1) + (n \times 2) + (n \times 3)]/ N \times 3\} \times 100\ \%$$

Where n is the number of plants in each class, N is the total number of plants, and 0, 1, 2 and 3 represent the symptom severity classes.

A host reaction was regarded as resistant if the ID < 50% and the 95% confidence interval (CI) did not overlap 50% (LeBoldus et al. 2012). Cluster analysis was conducted using NTSys-pc v 2.21q (Exeter Software, New York, NY) with the simple similarity coefficient and the unweighted pair-group method using the arithmetic means (UPGMA) procedure (Rohlf 2009). A similarity matrix generated with NTedit 2.21i was used as the input file, with binary data allotting '1' for a susceptible reaction and '0' for a resistant reaction. The corresponding association

coefficients for qualitative data were generated with the SIMQUAL computational module of NTSys-pc. The UPGMA analysis was carried out using the SAHN program of NTSys-pc and dendrograms were created with Tree Plot (Rohlf 2009).

4. 2. 4 Genetic structure and its relationship to virulence patterns on CR canola cultivars

Isolates of *P. brassicae* were grouped into two different populations based on their virulence pattern on the CR canola cultivars. The two populations consisted of isolates that were either virulent or avirulent on all CR canola cultivars. Genetic structure analysis for these two populations was conducted with the frequency- and distance-based statistical procedures of GenAlEx 6.51b2. Frequencies of alleles at each locus for each population and indices of haploid diversity by population were calculated for each locus per population, the mean over loci per population, the grand mean and the percentage of polymorphic loci for each population. Indices of haploid diversity consisted of the number of samples (N), number of alleles (N_a), the effective number of alleles [N_e = No. of Effective Alleles = 1 / (Sum pi^2)], the Shannon's Information Index [I = -1× Sum (pi × Ln (pi))], diversity (h = 1 - Sum pi^2, where pi is the frequency of the i^{th} allele for the population and Sum pi^2 is the sum of the squared population allele frequencies), diversity (h), and unbiased diversity [uh = (N / (N-1)) × h]. A list of the private alleles by population was also generated. Shannon's mutual information index ${}^{S}H_{UA}$, which is a pairwise measure of differentiation, was calculated with the 'Pairwise Pops' sub-menu of the 'Shannon' menu under 'Frequency-Based' statistical procedures applying the natural Log. This option also was used to conduct a convenient chi-square-based statistical test for allele frequency differences between the two populations, through the conversion of ${}^{S}H_{UA}$ to the log-likelihood contingency test G statistic (Peakall and Smouse 2006, 2012). The pairwise mean genetic distance between populations was

calculated with the 'Genetic by Pop' sub-menu of the 'Distance' menu under 'Distance-Based' statistical procedures. Using the same package, the analysis of molecular variance and the degrees of genetic differentiation between populations were computed from the genetic distance summed over all loci, yielding PhiPT (PhiPT = AP / (WP + AP) = AP / TOT, where AP = estimated variance among populations, WP = estimated variance within populations, and TOT = total estimated variance). This is a convenient parameter that assumes a stepwise mutation model, and which measures variation among individuals within populations relative to the total (Peakall and Smouse 2006, 2012). Gene flow shown as Nm (number of migrants) was calculated as Nm (Haploid) = [(1 / PhiPT) - 1] / 2. PCoA analysis was performed for populations using the covariance matrix method in the 'PCoA' option under 'Distance-Based' statistical procedures (Peakall and Smouse 2006, 2012). To explore the relationship between virulence phenotypes and molecular polymorphism, the cluster analysis using virulence data was compared with that of a similar analysis using simple sequence repeat (SSR) DNA markers, which was performed previously on the same set of isolates (Chapter 3). A correlation analysis between matrices derived from SSR (Chapter 3) and virulence data was conducted using a Mantel test in GenAlEx 6.51 b2 with 999 permutations.

4. 3 Results

4. 3. 1 Virulence of the isolates on CR canola

The mean IDs obtained on the seven CR canola cultivars and susceptible check (ECD 05) and the lower and upper limits of the associated 95% CI are summarized in Table 4.2 for each of the isolates. As expected, all single-spore and field isolates were highly virulent on ECD 05 (IDs between 92% and 100%). Thirty-two (64%) of the isolates were virulent on '45H29', 29 (58%) were virulent on 'D3152', 26 (52%) were virulent on '1960', 25 (50%) were virulent on each of 'L135C' and '6056CR', 23 (46%) were virulent on '74-54RR' and 22 (44%) were virulent on

‘9558C’ (Fig. 4.1). In cases where all host genotypes were susceptible, the lowest mean ID was observed on ‘9558C’ (75.3%), while a range of 91.1% to 95% was found on the other hosts.

The UPGMA cluster analysis indicated nine groups of isolates with distinct virulence patterns on the CR cultivars (Fig. 4.2). The two main distinct groups consisted of isolates that were virulent (14 single-spore and 6 field isolates) or avirulent (13 single-spore and 3 field isolates) on the CR canola cultivars. A third group consisted of six single-spore isolates (6-14-SS1, 183-14-SS1, 183-14-SS6, 331-14-SS3, 30-05-SS1 and 139-07-SS1) that were virulent on ‘45H29’ and avirulent on all of the other cultivars, and a fourth group included two single-spore isolates (1-14-SS1 and 331-14-SS1) virulent on ‘D3152’ and avirulent on the other hosts. One field isolate (331-14-P) and one single-spore isolate (1-14-SS2), both of which were virulent on all hosts except ‘9558C’ and ‘74-54RR’, clustered together. Another cluster consisted of field isolate 1-14-P and single-spore isolate 183-14-SS2, which were virulent on all hosts except ‘9558C’. Field isolates 41-14-P (virulent on ‘45H29’, ‘6056CR’, ‘D3152’ and ‘1960’) and 175-14-P (virulent on all except ‘6056CR’), and the single-spore isolate 175-14-SS1 (virulent on ‘9558C’ and ‘D3152’) possessed unique virulence patterns and did not cluster with any other isolates.

4. 3. 2 Comparisons of the virulence of field isolates and associated single-spores

Five single-spore isolates (1-14-SS1, 1-14-SS2, 1-14-SS3, 1-14-SS4 and 1-14-SS5) derived from the field isolate 1-14-P were included in the analysis. While 1-14-P was virulent on all the CR canola cultivars tested except for ‘9558C’, three of the single-spores (1-14-SS3, 1-14-SS4 and 1-14-SS5) were avirulent on all of these hosts. The single-spore isolate 1-14-SS1 also exhibited generally distinct virulence patterns from 1-14-P, causing the same reaction on only two canola cultivars (‘9558C’ and ‘D3152’). In contrast, 1-14-SS2 exhibited a virulence similar to 1-14-P on all of the cultivars except ‘74-54RR’.

Two single-spore isolates (4-14-SS1 and 4-14-SS2) were derived from the field isolate 4-14-P. While all of the CR cultivars were susceptible to 4-14-P and one of its single-spores (4-14-SS2), they were resistant to another single-spore (4-14-SS1). Only one single-spore isolate (6-14-SS1) was obtained from field isolate 6-14-P, and while this field isolate was highly virulent on all CR cultivars, the single-spore was virulent only on '45H29'.

Three single-spore isolates (41-14-SS1, 41-14-SS2 and 41-14-SS3) were obtained from field isolate 41-14-P. However, while the hosts '9558C', '74-54RR' and 'L135C' were resistant to this field isolate, the single-spore (41-14-SS2) was highly virulent on these hosts as well as others. The two other single-spores (41-14-SS1 and 41-14-SS3) were avirulent on all of the CR canola cultivars.

Seven single-spores were derived from field isolate 183-14-P. Three of the single-spores (183-14-SS3, 183-14-SS4 and 183-14-SS5) shared the same virulence pattern as the original field isolate, which was virulent on all of the CR canola cultivars, while the single-spore 183-14-SS2 was virulent on all hosts except '9558C'. In contrast, the single-spore (183-14-SS7) was avirulent on all of the CR canola cultivars, and 183-14-SS1 and 183-14-SS6 were virulent only on '45H29'.

The greatest number (11) of single-spores was recovered from field isolate 187-14-P. All hosts were susceptible to this field isolate and to nine of its single-spores (187-14-SS1-6, 187-14-SS8, 187-14-SS10 and 187-14-SS11). Conversely, two of the single-spores (187-14-SS7 and 187-14-SS9) were avirulent on all of the CR canola cultivars.

Only one single-spore (290-14-SS1) was derived from field isolate 290-14-P. While this field isolate was virulent on all the CR cultivars, the single-spore was avirulent. Three single-spores (331-14-SS1, 331-14-SS2 and 331-14-SS3) were obtained from field isolate 331-14-P, which was virulent on all the CR canola cultivars tested except for '9558C' and '74-54RR'. All

single-spores exhibited generally distinct virulence patterns from 331-14-P. The single-spore 331-14-SS2 was avirulent on all CR cultivars, while isolates 331-14-SS1 and 331-14-SS3 were virulent only on ‘D3152’ and ‘45H29’, respectively.

Two field isolates, 30-05-P and 341-12-P, which were collected in 2005 and 2012 prior to the first reports of resistance breakdown in 2013, were avirulent on all of the CR canola cultivars. The single-spore isolates 30-05-SS2 and 341-12-SS1, derived from these field isolates, also were avirulent on the CR canola. In contrast, the single-spore 30-05-SS1 was virulent on ‘45H29’. Another field isolate, 139-07-P, which was collected in 2007, was virulent on all of the hosts, and its associated single-spore 139-07-SS1 was virulent on ‘45H29’.

4. 3. 3 Genetic structure and its relationship to virulence on CR canola cultivars

Most (83%) of the virulent *P. brassicae* isolates (11 single-spore, 4 field isolates) grouped in the main cluster B (Chapter 3, Fig 3.2). Cluster A, meanwhile, consisted of all of the avirulent isolates along with isolates virulent on some of the CR cultivars, with no specific pattern for their differentiation (Chapter 3, Fig 3.2). The single-spore 183-14-SS3 and two field isolates 183-14-P and 187-14-P, which were virulent on all of the CR cultivars, also grouped in cluster A.

When considering virulent (12 single-spores) and avirulent (13 single-spores) isolates as two populations, namely V Pop and AV Pop, respectively, the percentage of polymorphic loci was 40.38% in V Pop and 23.08% in AV Pop. The number of alleles, effective alleles and Shannon's information index were higher in V Pop (1.423, 1.095 and 0.128, respectively) than in AV Pop (1.231, 1.056 and 0.073). The two populations differed at 17 loci with the exception of isolate 183-14-SS3, which shared the same alleles as the avirulent isolates. No private alleles were detected in either population. Higher overall diversity and unbiased diversity were found among isolates in V Pop (0.070, 0.076) than in AV Pop (0.041, 0.045). Eight distinct multilocus haplotypes were

identified in each of AV Pop and V Pop. Isolate 183-14-SS3 in V Pop and two isolates in AV Pop (1-14-SS5 and 187-14-SS9) were of the same multilocus haplotype. The Nei genetic distance and Shannon's mutual information index ($^{S}H_{UA}$) between V Pop and AV Pop were 0.365 and 0.196, respectively. Shannon analysis over loci for the pairwise population combinations indicated that the two populations were significantly different at 19 loci. Significant genetic differentiation (PhiPT = 0.804, P = 0.001) was observed between the two populations, with 80% of the genetic variation occurring between populations and 20% within populations (Table 4.3). The number of migrants (gene flow) between virulent and avirulent populations was low (0.122).

Principal coordinates analysis also indicated two distinct groups consisting of virulent and avirulent isolates. However, one of the virulent isolates (183-14-SS3) grouped with the avirulent isolates. The avirulent isolate 290-14-SS1, derived from a field isolate collected in southern Alberta, also was distinct from the others. Most of the variation (88.41%) was explained by the first two axes, including 82.36% for coordinate 1 and 6.05% for coordinate 2 (Fig. 4.3).

The Mantel test results indicated a significant and strong correlation (72%) between the matrices generated from the genetic and virulence datasets in the current study.

4. 4 Discussion

The recent emergence in western Canada of *P. brassicae* strains able to overcome genetic resistance represents an important challenge to clubroot management in canola (Strelkov et al. 2016, 2018). The identification of novel and effective sources of genetic resistance depends on an accurate assessment of the composition of *P. brassicae* populations in the region. Field isolates can represent heterogeneous mixtures of virulent and avirulent genotypes, which can confound the results from resistance screening experiments. In the current study, the virulence patterns of 38 single-spore and 12 field isolates of *P. brassicae* were compared on a suite of CR canola cultivars,

and a cluster analysis was conducted to evaluate the relationships between the genetic and virulence patterns of the pathogen.

While nine distinct virulence patterns were detected, most isolates were either virulent (40%) or avirulent (30%) on all of the CR canola cultivars. The remaining isolates (30%) were virulent on only some of the tested host genotypes. The heterogeneity of the field isolates was evident, with all field isolates collected in 2014 found to be mixtures of virulent and avirulent *P. brassicae* genotypes. Distinct virulence patterns were observed among single-spore isolates derived from the same field isolate and between some field isolates and their single-spores. In some cases, avirulent single-spore isolates were obtained from field isolates that were virulent on all the CR canola cultivars, a phenomenon that has been reported previously (Jones et al. 1982; Scott 1985; Tinggal and Webster 1981; Voorrips 1995; Xue et al. 2008) but not with respect to CR canola. Voorrips (1995) suggested that the recovery of avirulent single-spore isolates from a virulent field isolate could reflect the segregation of virulence genes in the pathogen population. In the current study, the inverse situation also occurred, with some hosts found to be susceptible to single-spore isolates derived from avirulent field isolates. The simultaneous presence of spores representing different *P. brassicae* genotypes within the same field isolates may influence the infection dynamics on different host genotypes (Jones et al. 1982).

One field isolate (139-07-P) collected in 2007 prior to the introduction of CR canola was virulent on all of the cultivars tested. The identification of a virulent field isolate before the clubroot resistance trait was introduced suggests the loss of resistance that first occurred in 2013 was a consequence of the selective propagation of virulent *P. brassicae* genotypes on CR canola cultivars. This finding is consistent with previous genomic analyses that indicated the presence of virulent strains of the clubroot pathogen in western Canada prior to the cultivation of CR canola

(Sedaghatkish et al. 2019; Strelkov et al., 2019a). The hypothesis that resistance-breaking *P. brassicae* isolates were already present at low frequencies (Holtz et al. 2018; Seaman et al. 1963, Strelkov et al. 2016, 2018) but proliferated because of the selection pressure imposed by the cultivation of CR cultivars is strengthened further by the identification of single-spore isolate 30-05-SS1. This isolate was virulent on the clubroot resistant '45H29', but was derived from an avirulent field isolate (30-05-P) collected prior to the introduction of resistance. The presence of virulent isolates at low frequencies prior to the cropping of CR canola does not exclude the possibility that some virulent strains were introduced separately or more recently, but this was clearly not the only mechanism for the emergence of new pathogen strains.

The most rapid evolutionary response of a pathogen to the introduction of host resistance is an alteration in the frequency of the pathogen strains, without any changes in genome structure (Anderson et al. 2010). This type of evolutionary change occurs regularly in the clubroot pathosystem (Crute and Pink 1989; Jones et al. 1982; Seaman et al. 1963; Strelkov et al. 2016 and 2018; Tanaka and Ito 2013). Balancing selection in the *P. brassicae* population may allow it to adjust to its environment by maintaining low-frequency alleles and alleles that are less fit (Sedaghatkish et al. 2019; Strelkov et al. 2018). LeBoldus et al. (2012) demonstrated the rapid adaptation of *P. brassicae* isolates to various host genotypes under controlled conditions. Similarly, the continuous cultivation of CR canola results in a swift increase in virulent pathotypes, ultimately leading to a loss of resistance (Cao et al. 2020; Hwang et al. 2017; Tanaka and Ito 2013). The loss or erosion of resistance to *P. brassicae* is not unique to canola and has been observed in cabbage (Seaman et al. 1963), Chinese cabbage (Hatakeyama et al. 2006; Osaki et al. 2004) and the oilseed rape 'Mendel' (Diederichsen et al. 2014; Oxley 2007).

In all but one case, if an isolate could induce an ID ≥ 68 on at least one of the CR cultivars, then it was virulent on them all. In contrast, isolates that caused an ID < 68% were virulent on only one or some of the CR hosts. This was consistent with the earlier report of Strelkov et al. (2018), who found that *P. brassicae* field isolates able to induce IDs > 75% on at least one cultivar were virulent on all of the CR cultivars in their study. In cases where all CR canola cultivars were susceptible to an isolate, most of the hosts developed similar IDs with the exception of '9558C'. Clubroot severity was generally lower in '9558C' versus the other CR canola cultivars, a finding consistent with previous reports and suggestive of some difference in the basis of its resistance (Strelkov et al. 2016, 2018). Nevertheless, the almost identical reaction of most of the other CR cultivars to each isolate indicates that these hosts may share the same source of resistance, consistent with the previous suggestion that this resistance was derived from the winter oilseed rape 'Mendel' (Fredua-Agyeman et al. 2018).

Although the focus of this chapter was not on the pathotype composition of *P. brassicae*, pathotype designations were available for all of the isolates evaluated (Chapter 2). The CR canola cultivars were found to be susceptible to all isolates classified as pathotypes P_2/3A, P_3/5X, P_3/6B and P_2/8E, as defined on the systems of Somé et al. (1996) and Williams (1966)/Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018), as well as to the lone isolates classified as pathotype P_1/4A. All of the CR cultivars also were susceptible to one of the single-spore isolates classified as pathotype P_2/2A. On the other hand, isolates designated as pathotypes P_2/2F and P_2/3H were generally avirulent, with the exception of two single-spores on the CR canola 'D3152'. Various virulence patterns were observed on the CR canola hosts among isolates classified as pathotype P_2/3D, although more variation was detected among field than single-spore isolates. No genetic distance was observed between the single-spore isolates classified as pathotypes 3H and 3D, which

are distinguished by their virulence on '45H29' in the CCD set (Chapter 3). These pathotypes may differ only at one effector or virulence/avirulence gene, which would not necessarily be reflected in the SSR data.

Previous research indicated that pathotype 6, as defined on the differentials of Williams, is rare in Alberta (Strelkov et al. 2014) and not generally aggressive on canola (Adhikari et al. 2012; Al-Daoud et al. 2018). In the current study, however, the pathotype 6 variant 6B was predominant among the isolates recovered from five fields in central Alberta (Westlock, Red Deer and Sturgeon) and from one field in southern Alberta (Newell) in 2014. This pathotype was also detected in one of the collections made from Sturgeon County in 2007. Other variants of pathotype 6 (6A and 6C) found to be virulent on some of the CR canola cultivars also were detected among single-spore isolates from Edmonton and Athabasca. The increased virulence of pathotype 6 on *B. napus* canola may reflect the selection pressure imposed by the cultivation of CR hosts. Similar shifts were observed with Williams' pathotype 9 in Japan, isolates of which showed increased virulence on CR cultivars of Chinese cabbage (Tanaka and Ito, 2013).

Twelve virulent and 13 avirulent single-spore isolates of *P. brassicae* were analyzed as two distinct populations, V Pop and AV Pop, respectively. The population V Pop, which consisted of virulent isolates, was more diverse than AV Pop. Significant genetic differentiation also was observed between the two populations, with 80% of the genetic variation occurring between populations and 20% within populations. A recent analysis employing restriction site-associated DNA sequencing (RAD-seq) also found two distinct *P. brassicae* populations, corresponding to virulent and avirulent isolates, as well as a low level of genetic diversity within each population (Holtz et al. 2018). Similarly, another study found that pathotype 5X (virulent on CR canola) was

distinct from single-spore and field isolates avirulent on CR cultivars in Canada, based on single nucleotide polymorphism (SNP) profiles (Sedaghatkish et al. 2019).

Relatively little is known regarding the molecular basis of variation among different strains of *P. brassicae*, although mutation, genetic drift, gene flow and migration may play roles. McDonald and Linde (2002) suggested that new virulent or more aggressive strains of a pathogen could result from mutations that overcome major resistance genes or erode the effectiveness of minor genes, and noted that more mutants are likely present in pathogens with a larger population size. The longevity of *P. brassicae* resting spores (Wallenhammar 1996) combined with the rapid increases in inoculum possible with each cycle of infection (Diederichsen et al. 2009) may lead to a large effective population size and high mutation rates (Strehlow et al. 2014). Nonetheless, Yang et al. (2018) found a high level of polymorphism in avirulent and virulent isolates of *P. brassicae*, and suggested that the emergence of virulent pathotypes is not as simple as losing an avirulence gene or the occurrence of mutations in a few genes. As such, changes in the virulence phenotype of *P. brassicae* may reflect a lengthy coevolution with its hosts (Yang et al. 2018).

The number of migrants (gene flow) was calculated as 0.122 between the virulent and avirulent populations, suggesting a low level of gene exchange. A low level of genetic exchange also was observed among populations representing different pathotypes of *P. brassicae* (Chapter 3). As a soil-borne pathogen, the number of *P. brassicae* migrants is expected to be low; however, dispersal of the pathogen by human activities (Cao et al. 2009; Donald and Porter 2009) and/or wind (Rennie et al. 2015) could increase migration rates. Virulent strains of the pathogen emerging in one field could be transferred to other fields through the movement of farm machinery or in windborne dust (Strelkov et al. 2016). 'New' virulent strains of *P. brassicae* could also arise independently of each other via the selection pressure imposed in each field (Strelkov et al. 2016).

The current study indicated that *P. brassicae* populations in Alberta consist of mixtures of virulent and avirulent isolates, which are genetically distinct. The presence of novel, highly virulent genotypes among the single-spore isolates underscores the need for ongoing monitoring of this pathogen. It also underscores the potential vulnerability of genetic resistance to clubroot in canola. Therefore, for the sustainable management of clubroot, it will be prudent to identify novel sources of resistance, and to use these in conjunction with other management strategies, such as longer rotations out of susceptible hosts.

4. 5 Tables

Table 4.1. *Plasmodiophora brassicae* field and single-spore isolates evaluated for their virulence on clubroot resistant canola (*Brassica napus*).

Field isolate	Single-spore isolate	Origin	Year collected	Pathotype[a]	Virulence[b]
1-14-P		City of Edmonton	2014	P_2/3D	Mixed
	1-14-SS1			P_2/2F	Mixed
	1-14-SS2			P_3/6A	Mixed
	1-14-SS3			P2/3H	Avirulent
	1-14-SS4			P_2/2F	Avirulent
	1-14-SS5			P_2/3H	Avirulent
4-14-P		Westlock	2014	P_3/6B	Virulent
	4-14-SS1			P_2/3H	Avirulent
	4-14-SS2			P_3/6B	Virulent
6-14-P		Westlock	2014	P_3/6B	Virulent
	6-14-SS1			P_2/3D	Mixed
41-14-P		Red Deer	2014	P_2/3D	Mixed
	41-14-SS1			P_2/3H	Avirulent
	41-14-SS2			P_3/6B	Virulent
	41-14-SS3			P_2/3H	Avirulent
175-14-P		Athabasca	2014	P_2/3D	Mixed
	175-14-SS1			P_3/6C	Mixed
183-14-P		Sturgeon	2014	P_2/8E	Virulent
	183-14-SS1			P_2/2A	Mixed
	183-14-SS2			P_2/3D	Mixed
	183-14-SS3			P_2/2A	Virulent
	183-14-SS4			P_1/4A	Virulent
	183-14-SS5			P_3/6B	Virulent
	183-14-SS6			P_2/3D	Mixed
	183-14-SS7			P_2/3H	Avirulent
187-14-P		Sturgeon	2014	P_2/3A	Virulent
	187-14-SS1			P_3/5X	Virulent
	187-14-SS2			P_3/5X	Virulent
	187-14-SS3			P_3/5X	Virulent
	187-14-SS4			P_3/5X	Virulent
	187-14-SS5			P_3/6B	Virulent
	187-14-SS6			P_3/6B	Virulent
	187-14-SS7			P_2/3H	Avirulent
	187-14-SS8			P_3/6B	Virulent
	187-14-SS9			P_2/3H	Avirulent
	187-14-SS10			P_2/3A	Virulent
	187-14-SS11			P_3/6B	Virulent

Field isolate	Single-spore isolate	Origin	Year collected	Pathotype[a]	Virulence[b]
290-14-P		Newell	2014	P_3/6B	Virulent
	290-14-SS1			P_2/7A	Avirulent
331-14-P		Leduc	2014	P_3/5G	Mixed
	331-14-SS1			P_2/3H	Mixed
	331-14SS2			P_2/2F	Avirulent
	331-14-SS3			P_2/3D	Mixed
30-05-P		Sturgeon	2005	P_2/2F	Avirulent
	30-05-SS1			P_2/3D	Mixed
	30-05-SS2			P_2/2F	Avirulent
139-07-P		Sturgeon	2007	P_3/6B	Virulent
	139-07-SS1			P_2/2A	Mixed
341-12-P		Athabasca	2012	P_2/2F	Avirulent
	341-12-SS1			P_2/2F	Avirulent

[a] Pathotype designations are based on Somé et al. (1996) and Williams (1966)/Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018) and were obtained in Chapters 2 and 3.

[b] Virulence was assessed on seven CR canola cultivars ('45H29', '6056CR', '74–54RR', '9558C', 'D3152', 'L135C' and '1960'), with the Chinese cabbage 'Granaat' included as a susceptible check. Avirulent: the isolate was avirulent on all CR canola cultivars, Virulent: the isolate was virulent on all CR canola cultivars, and Mixed: the isolate was virulent on one or some of the CR canola cultivars and avirulent on others. For the purposes of classification of isolates as virulent/avirulent, a host reaction was resistant if the index of disease < 50% and the 95% confidence interval did not overlap 50% (LeBoldus et al. 2012).

Table 4.2. Reaction of clubroot resistant canola cultivars and the universally susceptible Chinese cabbage 'Granaat' (European Clubroot Differential (ECD) 05) to inoculation with field and single-spore isolates of *Plasmodiophora brassicae*, shown as mean indices of disease (%) with the lower and upper limits of the associated 95% confidence interval (in parentheses).

	Host genotype							
Isolate ID[a]	ECD 05	45H29	9558C	D3152	74-54RR	1960	L135C	6056CR
1-14-SS1	99 (98,101)	22 (12,31)	26 (23,29)	31 (6,56)	11 (7,15)	10 (-1,21)	22 (16,28)	21 (14,28)
1-14-SS2	100 (100,100)	59 (41,77)	40 (35,46)	56 (46,67)	32 (17,47)	45 (38,52)	48 (34,62)	45 (33,57)
1-14-SS3	99 (98,101)	27 (23,31)	9 (2,15)	21 (10,31)	0 (0,0)	6 (-6,18)	0 (0,0)	10 (-5,26)
1-14-SS4	100 (100,100)	21 (17,24)	26 (16,36)	6 (2,10)	0 (0,0)	4 (-4,12)	0 (0,0)	15 (7,22)
1-14-SS5	97 (94,100)	26 (19,33)	13 (8,19)	30 (15,45)	1 (-1,4)	6 (2,10)	0 (0,0)	11 (6,17)
4-14-SS1	100 (100,100)	27 (21,33)	15 (8,22)	35 (25,44)	0 (0,0)	15 (4,27)	1 (-1,2)	23 (13,33)
4-14-SS2	100 (100,100)	100 (100,100)	99 (98,101)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)
6-14-SS1	100 (100,100)	38 (19,58)	13 (2,23)	10 (6,14)	1 (-1,2)	0 (0,0)	2 (-1,5)	24 (15,33)
41-14-SS1	97 (94,100)	22 (12,32)	13 (2,24)	29 (19,40)	0 (0,0)	10 (6,14)	0 (0,0)	5 (-1,11)
41-14-SS2	96 (89,102)	99 (96,101)	74 (66,82)	100 (100,100)	99 (97,100)	99 (96,101)	99 (98,101)	100 (100,100)
41-14-SS3	100 (100,100)	18 (7,29)	13 (3,22)	26 (18,34)	0 (0,0)	10 (-2,23)	1 (0,3)	9 (1,16)
175-14-SS1	99 (96,101)	38 (34,41)	33 (13,53)	52 (44,60)	19 (15,23)	19 (7,32)	24 (13,34)	30 (20,39)
183-14-SS1	100 (100,100)	38 (16,61)	11 (3,19)	0 (0,0)	0 (0,0)	6 (2,10)	0 (0,0)	14 (4,23)
183-14-SS2	100 (100,100)	53 (34,72)	24 (10,37)	51 (29,74)	68 (47,89)	63 (36,89)	52 (34,70)	57 (27,87)
183-14-SS3	100 (100,100)	82 (59,105)	51 (29,74)	65 (42,88)	82 (58,105)	70 (35,104)	79 (55,104)	83 (63,103)
183-14-SS4	100 (100,100)	100 (100,100)	84 (73,96)	76 (56,96)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)
183-14-SS5	100 (100,100)	100 (100,100)	66 (49,84)	99 (98,101	100 (100,100)	100 (100,100)	99 (96,101)	100 (100,100)
183-14-SS6	100 (100,100)	50 (33,67)	13 (2,23)	19 (15,23)	13 (-2,29)	13 (4,21)	15 (3,26)	17 (6,28)
183-14-SS7	98 (94,102)	36 (26,47)	14 (3,25)	21 (15,27)	0 (0,0)	13 (4,21)	0 (0,0)	4 (-1,9)
187-14-SS1	100 (100,100)	100 (100,100)	76 (59,94)	100 (100,100)	100 (100,100)	99 (98,101)	100 (100,100)	100 (100,100)
187-14-SS2	92 (83,102)	94 (90,98)	45 (38,52)	90 (83,97)	85 (76,94)	95 (91,99)	89 (81,97)	92 (87,97)
187-14-SS3	100 (100,100)	100 (100,100)	65 (42,88)	99 (96,101)	99 (8,101)	100 (100,100)	96 (91,101)	100 (100,100)

	Host genotype							
Isolate ID[a]	ECD 05	45H29	9558C	D3152	74-54RR	1960	L135C	6056CR
187-14-SS4	98 (94,102)	100 (100,100)	67 (59,75)	99 (98,101)	99 (98,101)	99 (98,101)	99 (98,101)	100 (100,100)
187-14-SS5	100 (100,100)	100 (100,100)	98 (94,102)	98 (95,103)	100 (100,100)	100 (100,100)	99 (98,101)	100 (100,100)
187-14-SS6	100 (100,100)	100 (100,100)	94 (89,98)	100 (100,100)	100 (100,100)	98 (94,102)	100 (100,100)	100 (100,100)
187-14-SS7	99 (98,101)	24 (12,35)	13 (2,23)	21 (16,26)	0 (0,0)	6 (2,10)	0 (0,0)	18 (9,27)
187-14-SS8	100 (100,100)	99 (98,101)	88 (84,92)	100 (100,100)	98 (94,102)	100 (100,100)	99 (98,101)	100 (100,100)
187-14-SS9	100 (100,100)	33 (24,42)	24 (18,31)	24 (17,32)	3 (0,7)	6 (2,9)	0 (0,0)	19 (7,31)
187-14-SS10	100 (100,100)	95 (87,103)	67 (62,73)	97 (92,101)	96 (91,101)	94 (87,102)	100 (100,100)	100 (100,100)
187-14-SS11	100 (100,100)	100 (100,100)	91 (86,95)	94 (87,102)	100 (100,100)	100 (100,100)	99 (98,101)	100 (100,100)
290-14-SS1	98 (94,102)	29 (16,42)	11 (-2,24)	20 (7,33)	0 (0,0)	2 (-2,6)	10 (-1,22)	0 (0,0)
331-14-SS1	96 (92,101)	23 (6,40)	7 (-2,16)	48 (38,58)	0 (0,0)	2 (-2,6)	2 (-2,6)	20 (15,26)
331-14-SS2	100 (100,100)	21 (16,26)	14 (6,22)	38 (29,46)	2 (-1,5)	10 (-1,22)	2 (-1,5)	15 (2,28)
331-14-SS3	98 (94,102)	100 (100,100)	19 (9,28)	31 (23,38)	3 (-1,7)	8 (-1,16)	0 (0,0)	11 (3,20)
1-14-P	97 (90,103)	67 (52,82)	10 (5,15)	60 (47,72)	74 (54,95)	91 (90,92)	63 (56,71)	69 (52,86)
4-14-P	100 (100,100)	94 (91,98)	55 (32,78)	99 (98,101)	100 (100,100)	100 (100,100)	92 (75,103)	100 (100,100)
6-14-P	100 (100,100)	100 (100,100)	100 (100,100)	91 (90,92	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)
41-14-P	100 (100,100)	60 (48,72)	21 (14,29)	46 (30,62)	31 (21,41)	49 (45,54)	36 (30,42)	50 (36,64)
175-14-P	100 (100,100)	57 (32,82)	34 (14,54)	60 (47,72)	54 (43,66)	47 (38,57)	53 (38,69)	41 (35,47)
183-14-P	96 (88,104)	88 (80,96)	69 (58,80)	80 (59,101)	79 (60,99)	81 (63,99)	65 (52,78)	73 (56,89)
187-14-P	93 (84,101)	99 (96,101)	73 (56,90)	100 (100,100)	96 (91,101)	100 (100,100)	99 (96,101)	98 (94,102)
290-14-P	100 (100,100)	91 (80,102)	100 (100,100)	92 (75,103)	98 (94,102)	100 (100,100)	94 (82,106)	92 (80,103)
331-14-P	92 (76,107)	56 (32,79)	29 (11,47)	42 (32,53)	29 (16,42)	51 (34,69)	44 (34,69)	64 (43,85)
30-05-SS1	92 (88,96)	34 (16,51)	18 (1,35)	10 (6,14)	6 (-2,14)	4 (-1,9)	4 (-4,12)	13 (8,17)
30-05-SS2	99 (96,101)	39 (37,41)	11 (3,20)	8 (-1,16)	0 (0,0)	10 (6,14)	6 (-2,13)	14 (2,25)
139-07-SS1	100 (100,100)	43 (35,52)	20 (17,24)	23 (3,43)	4 (-1,9)	17 (6,27)	13 (5,21)	19 (6,31)
341-12-SS1	96 (88,104)	26 (12,40)	19 (9,28)	31 (23,38)	3 (-1,7)	8 (-1,16)	0 (0,0)	11 (3,20)
30-05-P	100 (100,100)	39 (32,46)	27 (10,43)	0 (0,0)	2 (-2,6)	4 (-4,12)	2 (-2,6)	13 (-3,28)

	Host genotype							
Isolate ID[a]	ECD 05	45H29	9558C	D3152	74-54RR	1960	L135C	6056CR
139-07-P	92 (75,108)	59 (28,90)	44 (27,62)	43 (35,52)	58 (17,100)	63 (20,102)	74 (34,107)	56 (21,92)
341-12-P	96 (88,104)	18 (12,22)	14 (8,19)	30 (24,39)	3 (-1,7)	5 (2,8)	0 (0,0)	9 (3,15)

[a] isolate IDs that include '-P' denote field isolates, while those followed by '-SSx' denote single-spore isolates

Table 4.3. Analysis of molecular variance (AMOVA) between and within populations of *Plasmodiophora brassicae* based on their virulence patterns on clubroot resistant canola cultivars.

Population[a]	Source	df[b]	SS[c]	MS[d]	Est. Var.	Variation %	PhiPT[e]	P
V Pop	Among Pops	1	125.794	125.794	7.859	81	0.809	0.001
AV Pop	Within Pops	33	61.178	1.854	1.854	19		
	Total	34	186.971		9.712	100		

[a] V Pop and AV Pop denote population of single-spore isolates virulent or avirulent on all CR canola cultivars, respectively.

[b]Degree of freedom; [c] Sum of squares; [d] Mean squares

[e] PhiPT (PhiPT = AP / (WP + AP) = AP / TOT, AP = estimated variance among populations, WP = estimated variance within populations) denotes the proportion of the total genetic variance that is due to the variance among populations (P = 0.05).

4. 6 Figures

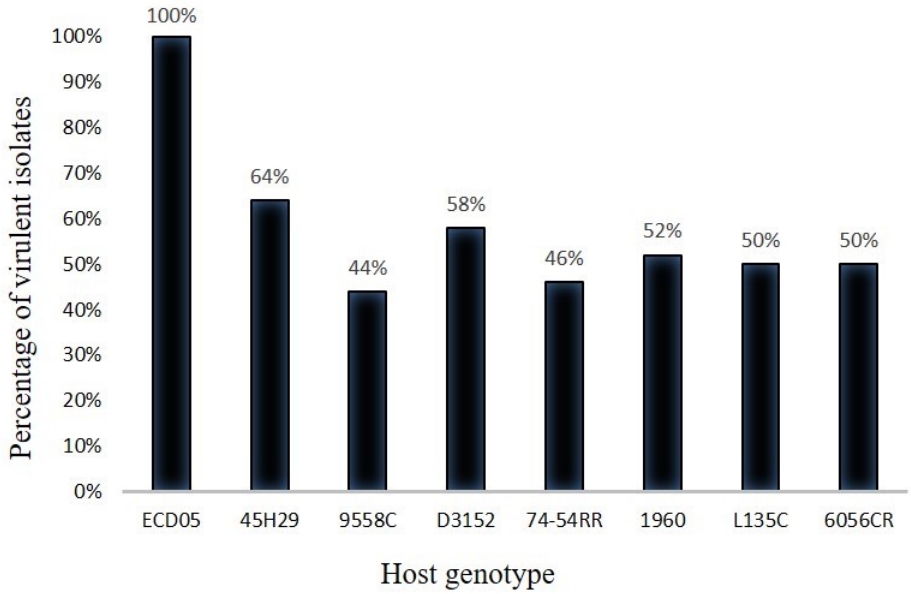

Figure 4.1. Percentage of *Plasmodiophora brassicae* isolates found to be virulent on the clubroot resistant canola cultivars '45H29', '6056CR', '74-54RR', '9558C', 'D3152', 'L135C' and '1960' and the universally susceptible cultivar Chinese cabbage 'Granaat' (European Clubroot Differential (ECD) 05). A host was regarded as resistant to an isolate if the index of disease < 50% and the 95% confidence interval did not overlap 50% (LeBoldus et al. 2012).

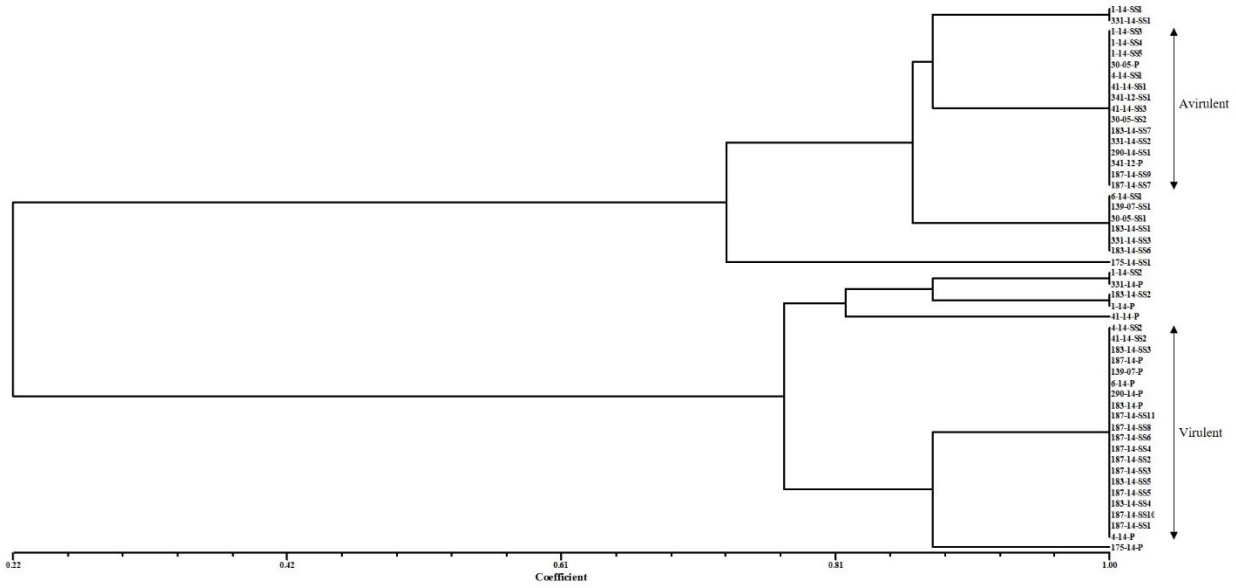

Figure 4.2. Pathogenic similarity of *Plasmodiophora brassicae* field and single-spore isolates on a suite of seven clubroot resistant (CR) canola cultivars. Isolates denoted as "Avirulent" were avirulent on all CR cultivars, while those denoted as "Virulent" were virulent on all CR cultivars. All others showed mixed virulence patterns (virulent on some hosts, avirulent on others). The dendrogram was constructed employing the unweighted pair group method with arithmetic mean procedure and simple similarity coefficient with NTSys-pc ver. 2.2.

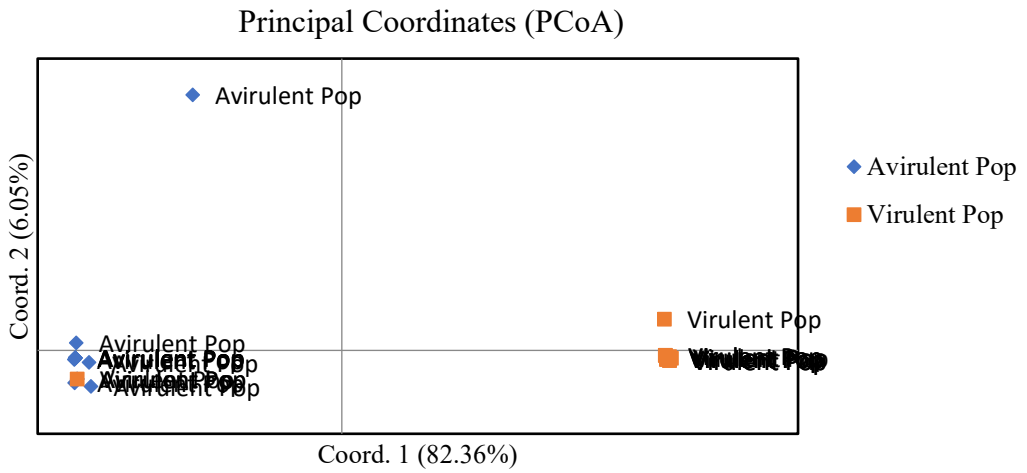

Figure 4.3. Scatter plot of the first and second coordinates in a principal coordinate analysis (PCoA) of *Plasmodiophora brassicae* populations from Alberta, defined based on their virulence or avirulence on a suite of clubroot resistant canola cultivars. The percentage of variation explained by the first three axes was 82.36, 6.05 and 2.80, respectively.

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Chapter 5. General discussion

In western Canada, clubroot is one of the most important diseases of canola and is managed primarily by planting clubroot resistant (CR) cultivars. Unfortunately, genetic resistance has been overcome in an increasing number of fields by isolates of *Plasmodiophora brassicae* representing multiple 'novel' pathotypes (Strelkov et al. 2016, 2018). An understanding of the genetic and virulence structure of a parasite population is important for predicting its evolutionary potential and helpful in the development of durable plant resistance (McDonald and Linde 2002). Given the difficulties in obtaining single-spore isolates of *P. brassicae*; however, studies of clubroot resistance are often conducted using field isolates. These field isolates may consist of a mixture of different pathotypes, confounding results and hindering efforts to characterize the genetics of the host-pathogen interaction. As such, the purification and study of *P. brassicae* single-spore isolates are valuable in resistance breeding and for improving knowledge of the clubroot pathosystem.

Single-spore isolates from *P. brassicae* collections made in Canada were first obtained prior to the introduction of CR canola cultivars (Xue et al. 2008). Subsequent testing indicated that these isolates were avirulent on the first generation of CR canola cultivars (Strelkov et al. 2016); this observation was expected, since many of these isolates had been used to screen for resistance during the development of these cultivars. A primary objective of this dissertation was to obtain a new set of single-spore isolates from collections made after the introduction of resistance, with an emphasis on *P. brassicae* recovered from CR canola. Thirty-eight single-spores were obtained as described in Chapter 2. These isolates may serve as an important resource in future studies, and also could be used by canola breeders for clubroot resistance screening.

The success of single-spore isolation depends on the viability and maturity of the resting spores and technical differences between procedures (Buczacki 1977; Voorrips 1996). Given recent reports that highlight methods to evaluate resting spore vitality (Al-Daoud et al. 2017; Harding et al. 2019), it may be useful to assess the viability of a field isolate or pathogen collection before using it to attempt the isolation of single-spores. Any such approach, however, would have to ensure that the effect of the chemicals used to evaluate spore vitality does not itself reduce this viability. Nonetheless, in the current thesis, the recovery rate for single-spore isolates was favourably comparable with previously published reports (Buczacki 1977; Diederichsen et al. 2016; Jones et al. 1982; Manzanares-Dauleux et al. 1994; Scott 1985; Somé et al. 1996; Tinggal and Webster 1981; Voorrips 1996; Xue et al. 2008). The efficiency of single-spore isolation may be increased further; however, by using root hairs containing a single zoosporangium in place of a single resting spore as the inoculum source. This could help to overcome the challenges associated with the manipulation of a single resting spore.

Recent studies have indicated a fair amount of diversity in the virulence of *P. brassicae* populations from Canada. Prior to completion of this dissertation, two pathotypes had been identified on the differentials of Somé et al. (1996), five on the hosts of Williams (1966) and 17 on the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018). In Chapter 2, many of these same pathotypes were found, along with several new pathotypes not reported previously. These included pathotype P_1 as defined by Somé et al., pathotypes 4 and 7 as per Williams, and the CCD pathotypes 2A, 4A, 6A, 6B, 6C and 7A. The large number of pathotypes detected, particularly on the CCD set, further highlights the diversity in the virulence of *P. brassicae*, particularly when considering that all of the isolates originated from Alberta. Several of these novel

pathotypes could overcome resistance, and it is clear that additional monitoring will be required in the coming years to track changes in the pathogen population.

Most of the single-spore isolates in this study came from central Alberta, the historic heart of the clubroot outbreak (Strelkov and Hwang 2014). Nonetheless, a few originated from northern and southern regions of the province, and these isolates possessed distinct virulence profiles on the host differential sets (Chapter 2). Similarly, previous reports also noted distinct virulence patterns in some isolates from regions beyond central Alberta (Cao et al. 2009; Strelkov et al. 2018). Unlike in these earlier studies, however, in this dissertation an analysis of genetic diversity was also conducted, which showed that the isolates from southern and northern Alberta also were genetically distinct (Chapter 3). It may be helpful, in future studies, to evaluate these differences within the context of agricultural practices, as well as climatic conditions, to better understand their origin. Moreover, given the relatively small number of isolates tested in the current and previous reports, a larger collection of single-spore and field isolates is needed to better explore the relationships between geographic origin and the virulence and genetic structure of *P. brassicae* from canola.

In this dissertation, three indices of disease (IDs, 25%, 33% and 50% +/- 95% confidence interval) were compared as thresholds to distinguish between resistant and susceptible host reactions. Significant and strong correlations were found between the virulence matrices obtained with each of these thresholds on the host differential sets. The strongest correlations between the three threshold IDs, however, were observed on the CCD set (Chapter 2). Moreover, significant and strong correlations were found between virulence matrices generated with the different thresholds and matrices generated with the genetic data (Chapter 3). The strongest of these correlations was obtained between the genetic data and a threshold ID of 50% on the CCD set

(Chapter 3). As has been previously suggested (Strelkov et al. 2006, 2007), it appears that a threshold ID of 50% is appropriate to classify host reactions as resistant or susceptible for the purposes of pathotype classification. For resistance breeding; however, a threshold of 50% may be too high, and more stringent criteria, such as ID < 20% or ID < 10%, may be preferable.

The old, open-pollinated canola cultivar 'Westar' has often been regarded as universally susceptible to *P. brassicae* (Strelkov et al. 2016, 2018). In Chapter 2, however, this genotype was resistant to one of the single-spore isolates (290-14-SS1) evaluated. Interestingly, using simple sequence repeat (SSR) markers, one private allele was identified in this isolate (Chapter 3). Therefore, it may be worthwhile to investigate the possible relationship between this specific genetic marker and the observed avirulent phenotype of this isolate on 'Westar'. Conversely, the differential host *Brassica rapa* L. subsp. *rapifera* (ECD 02) was highly resistant to all of the isolates in this as well as earlier studies from Canada (Cao et al. 2009; Strelkov et al. 2007, 2016, 2018; Xue et al. 2008), and may serve as a possible source of resistance in Canadian breeding programs.

A low level of genetic diversity was detected among the single-spore isolates in Chapter 3. This finding was consistent with the results of Holtz et al. (2018) and Sedaghatkish et al. (2019), who also reported a low level of genetic variation in populations of *P. brassicae* from Alberta. Milgroom (2015) noted that while mutations may contribute to genetic variation by producing new alleles, they may have minimal impact quantitatively on overall genetic diversity. Sedaghatkish et al. (2019) suggested that random genetic drift also has little effect on the genetic diversity of *P. brassicae*. Calculation of linkage disequilibrium in the current dissertation indicated that recombination was rare or perhaps absent in the tested isolates (Chapter 3). Furthermore, a low level of gene exchange (gene flow) was found (Chapter 3). As such, genetic variation among *P.*

brassicae in Alberta may not be greatly influenced by mutation, random genetic drift, recombination or migration.

Selection is the other major process that contributes to diversity; however, it generally lowers the diversity by surging the frequencies of alleles linked to the highest fitness and removal of deleterious mutations (Milgroom 2015). Since the field isolates analyzed in this study were collected from CR canola cultivars (the resistance of which is likely derived from the same source; Fredua-Agyeman et al. 2018), they probably were subjected to similar selection pressure by the same or similar resistance genes. This may help to explain the low level of genetic diversity observed in Chapter 3. It would be useful to study the population genetics of *P. brassicae* further by comparing the whole genome sequences of this collection. Isolation and analysis of additional single-spores, particularly from samples collected before the introduction of CR canola, may also provide better understanding of pathogen population genetics and evolution in Alberta. Given the uncertainty regarding sexual recombination in the life cycle of *P. brassicae*, study of the occurrence of sexual recombination in *P. brassicae* may also be of interest in future studies.

In Chapter 4, the virulence of the *P. brassicae* field and single-spore isolates was compared on a suite of CR canola cultivars. All of the field isolates were found to consist of a mixture of virulent and avirulent pathogen genotypes. Furthermore, high genetic differentiation was observed between virulent and avirulent pathogen populations (Chapter 4). The detection of a virulent isolate in field samples collected before the cultivation of CR canola indicated that virulent strains were present in Alberta prior to the introduction of resistance, and increased in recent years by selective propagation on the resistant hosts. While earlier studies had suggested the presence of virulent isolates in old collections of *P. brassicae* based on genomic analyses (Sedaghatkish et al. 2019; Strelkov et al. 2019), this is the first time that this virulence has been demonstrated phenotypically

by inoculation of host cultivars. As new sources of resistance are introduced, it will be helpful to consider that isolates able to overcome this resistance are most likely already present, and will increase as a component of the pathogen population if proper resistance stewardship is not employed.

Clubroot represents one of the most significant challenges facing Canadian canola farmers. The work in this dissertation has provided new resources (single-spore isolates) for resistance screening, along with an improved understanding of the virulence and genetic structure of *P. brassicae* populations in Alberta. It is clear, as the pathogen continues to spread and resistance is broken in an ever-increasing number of fields, that effective and sustainable clubroot management will require an integrated approach. Part of such an approach will include the identification of novel and diverse sources of resistance, and the stacking of resistance genes in canola cultivars. More importantly, however, it will also require that resistance be deployed together with other strategies including longer rotations out of canola. In this way, the production of this highly profitable crop will be able to continue.

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Appendix

Supplementary Tables

Supplementary Table 2.1. The mean index of disease (%) and lower and upper limits of the associated 95% confidence interval of each differential host genotype and pathogen isolate combination evaluated in this study.

	Host genotype [a]												
Isolate ID	ECD02	ECD05	ECD06	ECD08	ECD09	ECD10	ECD11	ECD13	Brutor	Laurentian	Mendel	Westar	45H29
1-14-SS1	0 [b]	99	100	100	100	18	49	77	100	97	21	99	22
	(0,0)	(98,101)	(100,100)	(100,100)	(100,100)	(5,31)	(46,53)	(64,91)	(100,100)	(95,99)	(5,36)	(98,101)	(12,31)
1-14-SS2	1	100	6	99	9	12	0	40	92	6	17	99	59
	(-1,2)	(100,100)	(-2,13)	(96,101)	(4,14)	(6,18)	(0,0)	(29,51)	(86,98)	(5,8)	(13,22)	(96,101)	(41,77)
1-14-SS3	0	99	88	100	93	16	7	47	99	92	6	100	27
	(0,0)	(98,101)	(81,95)	(100,100)	(87,99)	(7,25)	(2,12)	(27,67)	(96,101)	(86,98)	(-1,12)	(100,100)	(23,31)
1-14-SS4	1	100	97	100	99	23	39	83	100	97	17	100	21
	(-1,2)	(100,100)	(92,101)	(100,100)	(96,101)	(8,38)	(15,62)	(71,95)	(100,100)	(91,102)	(7,26)	(100,100)	(17,24)
1-14-SS5	6	97	98	99	94	9	7	67	99	90	5	99	26
	(-2,14)	(94,100)	(95,100)	(98,101)	(87,102)	(1,16)	(3,10)	(59,75)	(98,101)	(84,95)	(-1,10)	(96,101)	(19,33)
4-14-SS1	5	100	97	100	100	18	24	81	88	100	13	100	27
	(-1,10)	(100,100)	(95,99)	(100,100)	(100,100)	(11,25)	(20,28)	(77,84)	(80,97)	(100,100)	(9,18)	(100,100)	(21,33)
4-14-SS2	8	100	6	85	17	30	3	71	99	0	95	100	100
	(8,8)	(100,100)	(2,10)	(66,105)	(3,31)	(24,36)	(1,6)	(59,84)	(98,101)	(0,0)	(90,100)	(100,100)	(100,100)
6-14-SS1	0	100	92	100	100	18	42	94	100	91	10	100	38
	(0,0)	(100,100)	(82,103)	(100,100)	(100,100)	(7,29)	(35,48)	(91,98)	(100,100)	(84,98)	(-1,20)	(100,100)	(19,58)
41-14-SS1	3	97	92	99	91	8	6	69	94	85	9	97	22
	(-1,7)	(94,100)	(87,98)	(96,101)	(82,101)	(1,14)	(3,10)	(54,84)	(86,102)	(77,93)	(-1,19)	(92,101)	(12,32)
41-14-SS2	4	96	4	99	7	14	0	36	100	0	76	99	99
	(-1,9)	(89,102)	(-1,9)	(96,101)	(2,11)	(4,24)	(0,0)	(22,51)	(100,100)	(0,0)	(64,89)	(98,101)	(96,101)
41-14-SS3	2	100	96	100	93	15	13	58	96	72	13	98	18
	(-2,6)	(100,100)	(88,104)	(100,100)	(83,103)	(1,29)	(5,21)	(37,79)	(91,101)	(52,93)	(3,23)	(95,101)	(7,29)
175-14-SS1	0	99	23	90	4	0	26	85	99	1	21	100	38
	(0,0)	(96,101)	(18,28)	(83,97)	(-1,9)	(0,0)	(15,36)	(78,93)	(98,101)	(-1,2)	(8,34)	(100,100)	(34,41)
183-14-SS1	0	100	97	99	100	23	42	73	100	94	4	100	38
	(0,0)	(100,100)	(93,101)	(98,101)	(100,100)	(13,33)	(24,61)	(55,90)	(100,100)	(88,100)	(-1,9)	(100,100)	(16,61)
183-14-SS2	0	100	86	100	99	20	39	90	100	96	13	100	53
	(0,0)	(100,100)	(76,96)	(100,100)	(96,101)	(14,25)	(30,48)	(82,98)	(100,100)	(92,101)	(-8,34)	(100,100)	(34,72)

	Host genotype [a]												
Isolate ID	ECD02	ECD05	ECD06	ECD08	ECD09	ECD10	ECD11	ECD13	Brutor	Laurentian	Mendel	Westar	45H29
183-14-SS3	2 (-2,6)	100 (100,100)	99 (96,101)	100 (100,100)	100 (100,100)	26 (15,38)	37 (12,62)	85 (79,92)	100 (100,100)	97 (91,103)	13 (2,23)	100 (100,100)	82 (59,105)
183-14-SS4	3 (-2,9)	100 (100,100)	90 (81,99)	100 (100,100)	91 (78,104)	59 (55,63)	35 (20,50)	65 (55,76)	100 (100,100)	89 (84,95)	81 (74,88)	100 (100,100)	100 (100,100)
183-14-SS5	9 (-7,27)	100 (100,100)	4 (-4,12)	99 (98,101)	6 (-2,14)	17 (13,21)	4 (-2,11)	57 (35,79)	100 (100,100)	2 (-2,6)	88 (75,101)	98 (94,102)	100 (100,100)
183-14-SS6	2 (-2,6)	100 (100,100)	100 (100,100)	100 (100,100)	100 (100,100)	10 (5,14)	29 (16,42)	67 (55,78)	100 (100,100)	95 (90,101)	8 (8,8)	100 (100,100)	50 (33,67)
183-14-SS7	0 (0,0)	98 (94,102)	94 (89,98)	97 (92,103)	100 (100,100)	16 (4,28)	24 (14,34)	74 (61,87)	100 (100,100)	92 (85,98)	10 (3,18)	99 (97,100)	36 (26,47)
187-14-SS1	8 (-1,18)	100 (100,100)	5 (-2,11)	100 (100,100)	13 (8,17)	9 (3,15)	0 (0,0)	17 (6,28)	96 (91,101)	13 (6,19)	76 (57,95)	98 (95,101)	100 (100,100)
187-14-SS2	6 (-1,12)	92 (83,102)	4 (-1,9)	81 (61,100)	10 (-2,23)	1 (-1,2)	0 (0,0)	26 (9,42)	80 (64,96)	0 (0,0)	40 (20,61)	90 (81,99)	94 (90,98)
187-14-SS3	3 (0,7)	100 (100,100)	4 (-1,9)	100 (100,100)	4 (-1,9)	3 (-1,8)	0 (0,0)	20 (15,25)	90 (76,104)	1 (-1,2)	63 (42,84)	93 (85,101)	100 (100,100)
187-14-SS4	1 (-1,2)	98 (94,102)	5 (-5,14)	100 (100,100)	10 (7,12)	3 (-2,9)	0 (0,0)	24 (11,38)	99 (98,101)	0 (0,0)	77 (52,102)	99 (97,100)	100 (100,100)
187-14-SS5	6 (2,10)	100 (100,100)	8 (2,15)	100 (100,100)	4 (1,8)	30 (23,37)	15 (10,21)	72 (58,85)	100 (100,100)	0 (0,0)	94 (89,98)	100 (100,100)	100 (100,100)
187-14-SS6	2 (-2,7)	100 (100,100)	0 (0,0)	100 (100,100)	9 (-3,20)	25 (14,35)	0 (0,0)	34 (16,53)	100 (100,100)	0 (0,0)	83 (72,94)	99 (98,101)	100 (100,100)
187-14-SS7	1 (-1,2)	99 (98,101)	99 (98,101)	100 (100,100)	99 (96,101)	24 (19,28)	12 (2,22)	78 (65,91)	97 (92,103)	98 (94,102)	9 (1,16)	98 (94,102)	24 (12,35)
187-14-SS8	1 (0,3)	100 (100,100)	6 (0,11)	95 (89,101)	3 (-1,8)	19 (15,23)	1 (-1,4)	51 (37,65)	100 (100,100)	0 (0,0)	78 (71,86)	100 (100,100)	99 (98,101)
187-14-SS9	2 (-2,6)	100 (100,100)	91 (80,102)	100 (100,100)	96 (91,101)	8 (2,14)	32 (21,42)	78 (68,89)	100 (100,100)	95 (90,101)	10 (0,21)	99 (98,101)	33 (24,42)
187-14-SS10	5 (-3,13)	100 (100,100)	90 (82,98)	98 (95,101)	99 (96,101)	22 (11,33)	8 (3,13)	55 (34,75)	99 (96,101)	72 (63,81)	50 (44,55)	100 (100,100)	95 (87,103)
187-14-SS11	0 (0,0)	100 (100,100)	6 (-2,14)	94 (84,105)	6 (-2,14)	18 (5,30)	5 (2,8)	50 (35,65)	99 (98,101)	1 (-1,2)	97 (93,101)	99 (98,101)	100 (100,100)
290-14-SS1	0 (0,0)	98 (94,102)	100 (100,100)	33 (28,37)	44 (40,48)	1 (-1,2)	68 (60,75)	100 (100,100)	100 (100,100)	16 (7,25)	2 (-2,6)	8 (2,15)	29 (16,42)
331-14-SS1	3 (0,6)	96 (92,101)	91 (82,100)	98 (95,101)	91 (90,92)	18 (15,21)	11 (4,18)	43 (25,61)	88 (85,92)	77 (61,93)	15 (5,26)	96 (91,101)	23 (6,40)
331-14-SS2	0 (0,0)	100 (100,100)	98 (94,102)	99 (98,101)	99 (96,101)	24 (18,29)	53 (42,63)	92 (85,100)	100 (100,100)	100 (100,100)	10 (3,18)	100 (100,100)	21 (16,26)
331-14-SS3	1 (-1,2)	98 (94,102)	96 (91,101)	100 (100,100)	100 (100,100)	7 (2,12)	31 (17,46)	96 (91,101)	100 (100,100)	99 (98,101)	8 (2,15)	100 (100,100)	100 (100,100)
1-14-P	0 (0,0)	97 (90,103)	68 (58,78)	75 (62,89)	71 (53,90)	11 (5,18)	16 (2,29)	55 (41,69)	72 (47,96)	68 (46,89)	30 (15,45)	92 (84,101)	67 (52,82)

	Host genotype [a]												
Isolate ID	ECD02	ECD05	ECD06	ECD08	ECD09	ECD10	ECD11	ECD13	Brutor	Laurentian	Mendel	Westar	45H29
4-14-P	6 (-1,12)	100 (100,100)	2 (-2,6)	94 (90,98)	5 (1,9)	23 (17, 28)	10 (6,14)	60 (46,74)	100 (100,100)	1 (-1,2)	89 (78,100)	100 (100,100)	94 (91,98)
6-14-P	5 (-3,13)	100 (100,100)	2 (-2,6)	98 (94,102)	6 (2,10)	21 (19,22)	4 (-4,12)	55 (47,63)	96 (88,104)	0 (0,0)	77 (42,112)	100 (100,100)	100 (100,100)
41-14-P	0 (0,0)	100 (100,100)	96 (93,99)	100 (100,100)	98 (94,102)	27 (17,36)	21 (5,37)	60 (47,74)	100 (100,100)	99 (96,101)	10 (6,14)	100 (100,100)	60 (48,72)
175-14-P	2 (-2,6)	100 (100,100)	86 (70,102)	100 (100,100)	94 (86,102)	31 (5,47)	19 (10,29)	48 (33,64)	92 (85,98)	91 (85,98)	22 (13,31)	94 (86,102)	57 (32,82)
183-14-P	2 (-1,5)	96 (88,104)	91 (77,105)	90 (70,110)	72 (62,81)	1 (-1,4)	6 (1,11)	26 (23,30)	81 (58,105)	79 (48,111)	34 (22,46)	79 (62,96)	88 (80,96)
187-14-P	1 (0,3)	93 (84,101)	75 (63,87)	90 (76,104)	79 (63,94)	21 (9,33)	16 (9,23)	64 (59,70)	73 (61,85)	86 (77,96)	83 (78,87)	100 (100,100)	99 (96,101)
290-14-P	1 (-1,4)	100 (100,100)	2 (-2,6)	97 (93,101)	3 (0,6)	15 (13,17)	19 (4,35)	65 (48,83)	100 (100,100)	0 (0,0)	94 (88,101)	100 (100,100)	91 (80,102)
331-14-P	1 (-1,2)	93 (84,101)	28 (10,47)	75 (62,89)	18 (7,28)	7 (2,12)	2 (-2,6)	8 (4,13)	73 (61,85)	10 (-6,25)	14 (-1,29)	83 (78,87)	56 (32,79)

[a] Host genotypes of the Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018), which includes the differentials of Williams (1966) and Somé et al. (1996). European Clubroot Differential (ECD) 02: *Brassica rapa* ssp. *rapifera* line *AAbbCC*; ECD 05: *Brassica rapa* var. *pekinensis* 'Granaat'; ECD 06: *Brassica napus* var. *napus* 'Nevin'; ECD 08: *Brassica napus* var. *napus* 'Giant Rape' selection; ECD 09: *Brassica napus* var. *napus* New Zealand resistant rape; ECD 10: *Brassica napus* var. *napus* 'Wilmesburger'; ECD 11: *Brassica oleracea* var. *capitata* 'Badger Shipper'; ECD 13: *Brassica oleracea* var. *capitata* 'Jersey Queen'; Brutor: *Brassica napus* 'Brutor'; Laurentian: *Brassica napus* var. *napobrassica* 'Laurentian'; Westar: *Brassica napus* 'Westar'; 45H29: *Brassica napus* '45H29'; and Mendel: *Brassica napus* 'Mendel'.

[b] The mean index of disease (%) for each host genotype-pathogen combination, with the numbers in brackets indicating the lower and upper limits of the associated 95 % confidence interval (mean ID ± CI).

Supplementary Table 3.1. Types of mono-, di-, tri-, tetra-and penta-nucleotides repeats identified in across the whole genome sequence of *Plasmodiophora brassicae* (Schwelm et al. 2015; GenBank assembly accession: GCA_001049375.1).

Repeats	Motifs (frequency)	Total number
Mononucleotide	C (28), G (43), T (2)	73
Dinucleotide	AC (31), AG (9), CA (25), CG (9), CT (10), GA (5), GC (13), GT (32), TC (3), TG (24)	161
Trinucleotide	AAC (2), AAG (1), ACA (2), ACC (2), ACG (21), AGA (3), AGC (11), ATC (1), ATG (1), CAA (1), CAC (3), CAG (49), CAT (3), CCA (3), CCG (1), CCT (1), CGA (54), CGC (13), CGG (10), CGT (56), CTC (1), CTG (31), GAC (47), GAG (5), GAT (4), GCA (29), GCC (9), GCG (8), GCT (25), GGC (11), GGT (4), GTA (1), GTC (48), GTG (2), GTT (3), TCC (1), TCG (32), TGA (2), TGC (29), TGG (5), TGT (1)	536
Tetranucleotide	AGGC (1), CGTC (1), CTGT (2), GCAC (1), GTCT (1)	6
Pentanucleotide	AACTG (1), CCGAC (3), CGGTC (1), TAAAC (2)	7

Supplementary Table 3.2. Characteristics of 52 primer pairs developed from the whole genome sequence of *Plasmodiophora brassicae* (Schwelm et al. 2015; GenBank assembly accession: GCA_001049375.1).

SSR marker	Primer sequence	Motif	T_m (°C)	Target size (bp)	Number of alleles and size (bp)
H4	F: GAATCATCCGAATCCCTGTTAG R: GCTATGAGCAGCAAGAAGAGGT	$(GTC)_{10}$	60	255	2 (269, 275)
H7	F: AAACCCACTAACCAACGTGAAT R: AGCCACGACAACACATCGT	$(TG)_{10}$	60	338	1 (357)
H21	F: TATAGTCACACGAAGCCACCAC R: ACGGGAACCACAATGACAAC	$(G)_{20}$	60	304	5 (318, 319, 320, 321, 322)
H36	F: CCAGACTCGGTTAGTGTGTCGT R: TATCGACGGTGACATAGGTGC	$(GCA)_7$	61	352	1 (155)
H57	F: ATCAACTCATGCTGTCAACTCC R: TATTGCTACCGTTTCCGTCTG	$(CTG)_9$	60	305	2 (305, 337)
H95	F: AACACACAAGTCTGGGACAGC R: TATAGTCGTTCGTCCTGAGTGC	$(CA)_{16}$	60	210	2 (205, 226)
H107	F: GATTCAAACCCGAACACTGC R: CGTTGACCTACCGAACTTTCTC	$(AG)_{22}$	60	264	3 (270, 285, 300)
H118	F: TCACGACATTCCACCAAGG R: CCCATTGAGCACTAACTTTTCC	$(GA)_{14}$	60	390	2 (403, 405)
H184	F: ATTTGTGTACCGTGCTGCC R: TCTCCTTCCCAATATCGACAAC	$(GT)_{10}$	60	376	3 (378, 385, 393)
H229	F: GGTTTTCTTCATTCCCACTGC R: ATTGTCCTGCCGTTAGATTCAC	$(GA)_{23}$	60	368	2 (386, 500)
H265	F: CTTCAGGGACTAAAAGGCGAAT R: GCACCAACCAACCGTGTC	$(C)_{16}$	60	310	1 (302)
H268	F: AGCATCTGGCTACCTGTGTCTT R: GTAATGAATGTGAACGCCCA	$(G)_{21}$	60	328	2 (331, 335)
H315	F: CTGGACCGATGAGCAGCA R: AGAAGTGGAGCAGGGACTGG	$(GAC)_9$	62	383	2 (386, 398)
H325	F: TGCCGAATAAAACACGAGC R: GAAGGTCATACAGGTTGCGAT	$(GT)_{11}$	59	161	2 (176, 181)
H327	F: TACGCTGTCACCAAGTCCAA R: TATTAGTCAGGATGTGCGAGGA	$(CGA)_9$	60	399	3 (403, 407, 413)
H332	F: CCATGCTGTACCCAGTCAGTAA R: CCGAACAGAACCAATGTTGA	$(G)_{39}$	60	399	3 (288, 289, 290)
H335	F: CCGCCTTGATGGATTGTATT R: AATTGTCAGAGACGAACGACC	$(CA)_{11}$	59	154	2 (169, 172)

SSR marker	Primer sequence	Motif	T_m (°C)	Target size (bp)	Number of alleles and size (bp)
H345	F: GTTTGCCGAATACCCATCC R: CCCTCTGCTACCTAACTCCAAC	$(GTC)_9$	60	201	1 (220)
H349	F: GTCGAACTCCTGTCGTGTGTCT R: CTGAAGCAGCCGGACAAGT	$(TCG)_{10}$	61	348	2 (296, 364)
H362	F: CGACTTTTACTGTGCCGATTG R: CAGGACCCGATAGTTCTCCC	$(GGC)_8$	60	337	2 (340, 343)
H366	F: ACGGAAATCACTCTTCAGCAAC R: CTCAACATACCACCAACGCAT	$(CAG)_8$	61	371	1 (385)
H367	F: ATCCTGGGTATCAACCGTAGTG R: CTGCTATGCTGGGAACGAATA	$(CAG)_8$	60	211	2 (220, 228)
H373	F: TACGACCTACCCACACGCA R: CGAAACCTATCTGACGCAATGT	$(GCA)_8$	61	305	1 (318)
H374	F: GCTGAATGTAACGGAGCCTG R: AGCAACTCACCAACCAATACCT	$(GCT)_9$	60	325	1 (345)
H397	F: CGTGAAGATGGGGTGGAA R: AGTTGGTGTGTGAGTGTGGG	$(C)_{26}$	60	359	1 (338)
H424	F: AGCGTCCGAGTGGCTAAACTAT R: GAAATGACAGTGCCTTCCAGAC	$(AC)_{15}$	61	260	1 (277)
H441	F: CAACACGACCCAGCAGAAGT R: CCGCATCAGAGCACGAAG	$(AGC)_{10}$	61	400	1 (412)
H454	F: CCGCCTGACATACATTGCC R: GCCCTCACGGACAAGACC	$(AC)_{12}$	62	387	2 (396, 398)
H459	F: GATGGAGTGTCCGCTTGAAT R: CAAATCTCTCGCAGCAGGT	$(CGG)_9$	60	292	1 (308)
H464	F: CGCTCGTGTTAGTTGCGTG R: CGTTGGTGGATGATTGCCT	$(CA)_{27}$	62	337	1 (307)
H518	F: GGTTCACAGTTCGCATCACA R: AGACACCGTTCGCCTCTATTC	$(GA)_{13}$	60	241	2 (250, 252)
H522	F: ACGAATGCCCAGTGCCTAT R: TGCGTGTCCAAGTCCTGA	$(AC)_{13}$	60	306	2 (286, 320)
H523	F: CCTTCGTGGTCCCTCTGC R: GAACTCCTCCGTGTCGCTC	$(CGT)_{10}$	61	305	1 (323)
H525	F: CCATTCGCCTGTTCTCCA R: GGGTCGTTTGGTTCCCAG	$(TGC)_8$	61	324	2 (324, 343)
H534	F: GTTTCCTCTTCTCCCGCC R: GATGTCCCTGTCCTGTGTCTG	$(CGT)_8$	60	391	1 (405)
H547	F: GAGGTGACGATGCTCCATTC R: TCTGAGTAAGCGGGCAGG	$(GT)_{16}$	60	114	2 (117, 132)

SSR marker	Primer sequence	Motif	T_m (°C)	Target size (bp)	Number of alleles and size (bp)
H556	F: GATGTACCCCTGTTCGGTCA R: GAAGAGCGCCTACCTTCGAC	$(TG)_{12}$	61	344	2 (360, 362)
H581	F: AGGAACCGACAGAATGACCTC R: GACACACCCAACCCATAATCAC	$(G)_{18}$	61	355	2 (371, 383)
H627	F: ATGCGACAGGTGCCAAAT R: CCACAGTAAACGACACGATACG	$(GA)_{18}$	60	341	2 (355, 357)
H640	F: CGGGAACGACTAAAGAGGCTA R: GGGGTGGACAGAAAGTGCT	$(CT)_{11}$	60	106	1 (122)
H642	F: CATCCGCTTCCAAAGATACG R: ACTGATAGGTAATCCCGTGCC	$(G)_{19}$	60	334	3 (353, 354, 355)
H666	F: ATGAGACAAGAGGCACGGAT R: CGCACCTACAAGCGATACC	$(CTG)_9$	59	393	1 (409)
H668	F: GAGAACTGCGGGACAAGGTA R: TCGTCTACAAGCGACCGAT	$(TG)_{19}$	60	340	2 (339, 359)
H700	F: CCGAGTTGCTGCGTATGTT R: GGTAGAAGTGCTGGACGAGTTC	$(TG)_{17}$	60	392	1 (409)
H724	F: AAAGACCCTCAAAAGGTGGC R: AGGAAACCGTCAGCACGA	$(G)_{27}$	60	400	3 (390, 391, 392)
H734	F: CTATTGTCTCCCACGGTTGTC R: ATCTGTCCTTTTCTGCCCAC	$(AGC)_{15}$	59	395	1 (409)
J13	F: GAAGCCTCGTGTCGTTCATT R: CCCATTGAGCACTAACTTTTCC	$(GA)_{14}$	60	290	2 (304, 306)
J21	F: CTGCTCGTTTTCTTGTTGCTG R: AACTTACGTCAATGGCCGATAC	$(CTG)_{20}$	61	361	3 (343, 373, 381)
J23	F: AGAAGTTGATATAGTTCGGGGC R: GAATCGTCGCATCCGTATC	$(TGG)_9$	59	329	2 (333, 348)
J24	F: GACCGACATGACAGGGTTG R: ACGATAGAGTTCGACCGGC	$(GAC)_8$	60	224	1 (242)
J25	F: AGCATGGCACAGTAGGTAGGTT R: CAGTGTATTGGCACAGCGAG	$(GAC)_{12}$	60	110	1 (126)
J31	F: GACGTTGTAGTCCGTTATCGTG R: GTCGAGTCTGTGCTGGATCA	$(AC)_{13}$	59	189	2 (205, 207)

Supplementary Table 3.3. The numbers of mismatching loci among single-spore isolates ignoring missing data

Isolate	001-14-SS1	001-14-SS2	001-14-SS3	001-14-SS4	001-14-SS5	004-14-SS1	004-14-SS2	006-14-SS1	041-14-SS1	041-14-SS2	041-14-SS3	175-14-SS1	183-14-SS1	183-14-SS2	183-14-SS3	183-14-SS4	183-14-SS6	183-14-SS7	187-14-SS1	187-14-SS2	187-14-SS3	187-14-SS4	187-14-SS5	187-14-SS6	187-14-SS7	187-14-SS8	187-14-SS9	187-14-SS11	290-14-SS1	331-14-SS1	331-14-SS2	331-14-SS3	030-05-SS1	030-05-SS2	139-07-SS1	341-12-SS1
001-14-SS1	0	22	3	3	2	2	21	3	3	21	3	13	3	2	2	21	1	3	21	22	21	20	21	21	3	21	2	21	10	2	4	2	2	3	3	1
001-14-SS2	22	0	21	21	21	21	3	20	20	3	21	27	21	21	21	3	21	21	3	2	2	3	3	3	21	3	21	3	21	21	22	20	21	21	21	21
001-14-SS3	3	21	0	2	1	1	19	0	0	19	0	18	0	1	1	19	1	0	19	20	19	20	19	19	0	19	1	19	9	1	1	0	1	0	0	2
001-14-SS4	3	21	2	0	1	1	19	2	2	19	2	18	2	1	1	19	1	2	19	20	19	20	19	19	2	19	1	19	11	1	3	2	1	2	2	2
001-14-SS5	2	21	1	1	0	0	19	1	1	19	1	17	1	0	0	19	0	1	19	20	19	20	19	19	1	19	0	19	10	0	2	1	0	1	1	1
004-14-SS1	2	21	1	1	0	0	19	1	1	19	1	16	1	0	0	19	0	1	19	20	19	20	19	19	1	19	0	19	10	0	2	1	0	1	1	1
004-14-SS2	21	3	19	19	19	19	0	18	18	1	19	27	19	19	19	1	19	19	0	1	0	1	1	1	19	1	19	1	20	19	20	19	19	19	19	20
006-14-SS1	3	20	0	2	1	1	18	0	0	18	0	18	0	1	1	18	1	0	18	19	18	19	18	18	0	18	1	18	9	1	1	0	1	0	0	2
041-14-SS1	3	20	0	2	1	1	18	0	0	18	0	18	0	1	1	18	1	0	18	19	18	19	18	18	0	18	1	18	9	1	1	0	1	0	0	2
041-14-SS2	21	3	19	19	19	19	1	18	18	0	19	27	19	19	19	0	19	19	1	2	1	2	0	0	19	0	19	0	20	19	20	19	19	19	19	20
041-14-SS3	3	21	0	2	1	1	19	0	0	19	0	18	0	1	1	19	1	0	19	20	19	20	19	19	0	19	1	19	9	1	1	0	1	0	0	2
175-14-SS1	13	27	18	18	17	16	27	18	18	27	18	0	18	17	17	27	16	18	27	27	26	26	26	27	18	27	17	27	15	17	19	17	17	18	18	16
183-14-SS1	3	21	0	2	1	1	19	0	0	19	0	18	0	1	1	19	1	0	19	20	19	20	19	19	0	19	1	19	9	1	1	0	1	0	0	2
183-14-SS2	2	21	1	1	0	0	19	1	1	19	1	17	1	0	0	19	0	1	19	20	19	20	19	19	1	19	0	19	10	0	2	1	0	1	1	1
183-14-SS3	2	21	1	1	0	0	19	1	1	19	1	17	1	0	0	19	0	1	19	20	19	20	19	19	1	19	0	19	10	0	2	1	0	1	1	1
183-14-SS4	21	3	19	19	19	19	1	18	18	0	19	27	19	19	19	0	19	19	1	2	1	2	0	0	19	0	19	0	20	19	20	19	19	19	19	20
183-14-SS6	1	21	1	1	0	0	19	1	1	19	1	16	1	0	0	19	0	1	19	20	19	20	19	19	1	19	0	19	10	0	2	1	0	1	1	1
183-14-SS7	3	21	0	2	1	1	19	0	0	19	0	18	0	1	1	19	1	0	19	20	19	20	19	19	0	19	1	19	9	1	1	0	1	0	0	2
187-14-SS1	21	3	19	19	19	19	0	18	18	1	19	27	19	19	19	1	19	19	0	1	0	1	1	1	19	1	19	1	20	19	20	19	19	19	19	20
187-14-SS2	22	2	20	20	20	20	1	19	19	2	20	27	20	20	20	2	20	20	1	0	0	2	2	2	20	2	20	2	21	20	20	20	20	20	20	21
187-14-SS3	21	2	19	19	19	19	0	18	18	1	19	26	19	19	19	1	19	19	0	0	0	1	1	1	19	1	19	1	20	19	20	19	19	19	19	20
187-14-SS4	20	3	20	20	20	20	1	19	19	2	20	26	20	20	20	2	20	20	1	2	1	0	2	2	20	2	20	2	19	20	21	19	20	20	20	19
187-14-SS5	21	3	19	19	19	19	1	18	18	0	19	26	19	19	19	0	19	19	1	2	1	2	0	0	19	0	19	0	20	19	20	19	19	19	19	20
187-14-SS6	21	3	19	19	19	19	1	18	18	0	19	27	19	19	19	0	19	19	1	2	1	2	0	0	19	0	19	0	20	19	20	19	19	19	19	20
187-14-SS7	3	21	0	2	1	1	19	0	0	19	0	18	0	1	1	19	1	0	19	20	19	20	19	19	0	19	1	19	9	1	1	0	1	0	0	2
187-14-SS8	21	3	19	19	19	19	1	18	18	0	19	27	19	19	19	0	19	19	1	2	1	2	0	0	19	0	19	0	20	19	20	19	19	19	19	20
187-14-SS9	2	21	1	1	0	0	19	1	1	19	1	17	1	0	0	19	0	1	19	20	19	20	19	19	1	19	0	19	10	0	2	1	0	1	1	1
187-14-SS11	21	3	19	19	19	19	1	18	18	0	19	27	19	19	19	0	19	19	1	2	1	2	0	0	19	0	19	0	20	19	20	19	19	19	19	20
290-14-SS1	10	21	9	11	10	10	20	9	9	20	9	15	9	10	10	20	10	9	20	21	20	19	20	20	9	20	10	20	0	10	10	8	10	9	9	9
331-14-SS1	2	21	1	1	0	0	19	1	1	19	1	17	1	0	0	19	0	1	19	20	19	20	19	19	1	19	0	19	10	0	2	1	0	1	1	1
331-14-SS2	4	22	1	3	2	2	20	1	1	20	1	19	1	2	2	20	2	1	20	20	20	21	20	20	1	20	2	20	10	2	0	1	2	1	1	3
331-14-SS3	2	20	0	2	1	1	19	0	0	19	0	17	0	1	1	19	1	0	19	20	19	19	19	19	0	19	1	19	8	1	1	0	1	0	0	1
030-05-SS1	2	21	1	1	0	0	19	1	1	19	1	17	1	0	0	19	0	1	19	20	19	20	19	19	1	19	0	19	10	0	2	1	0	1	1	1
030-05-SS2	3	21	0	2	1	1	19	0	0	19	0	18	0	1	1	19	1	0	19	20	19	20	19	19	0	19	1	19	9	1	1	0	1	0	0	2
139-07-SS1	3	21	0	2	1	1	19	0	0	19	0	18	0	1	1	19	1	0	19	20	19	20	19	19	0	19	1	19	9	1	1	0	1	0	0	2
341-12-SS1	1	21	2	2	1	1	20	2	2	20	2	16	2	1	1	20	1	2	20	21	20	19	20	20	2	20	1	20	9	1	3	1	1	2	2	0

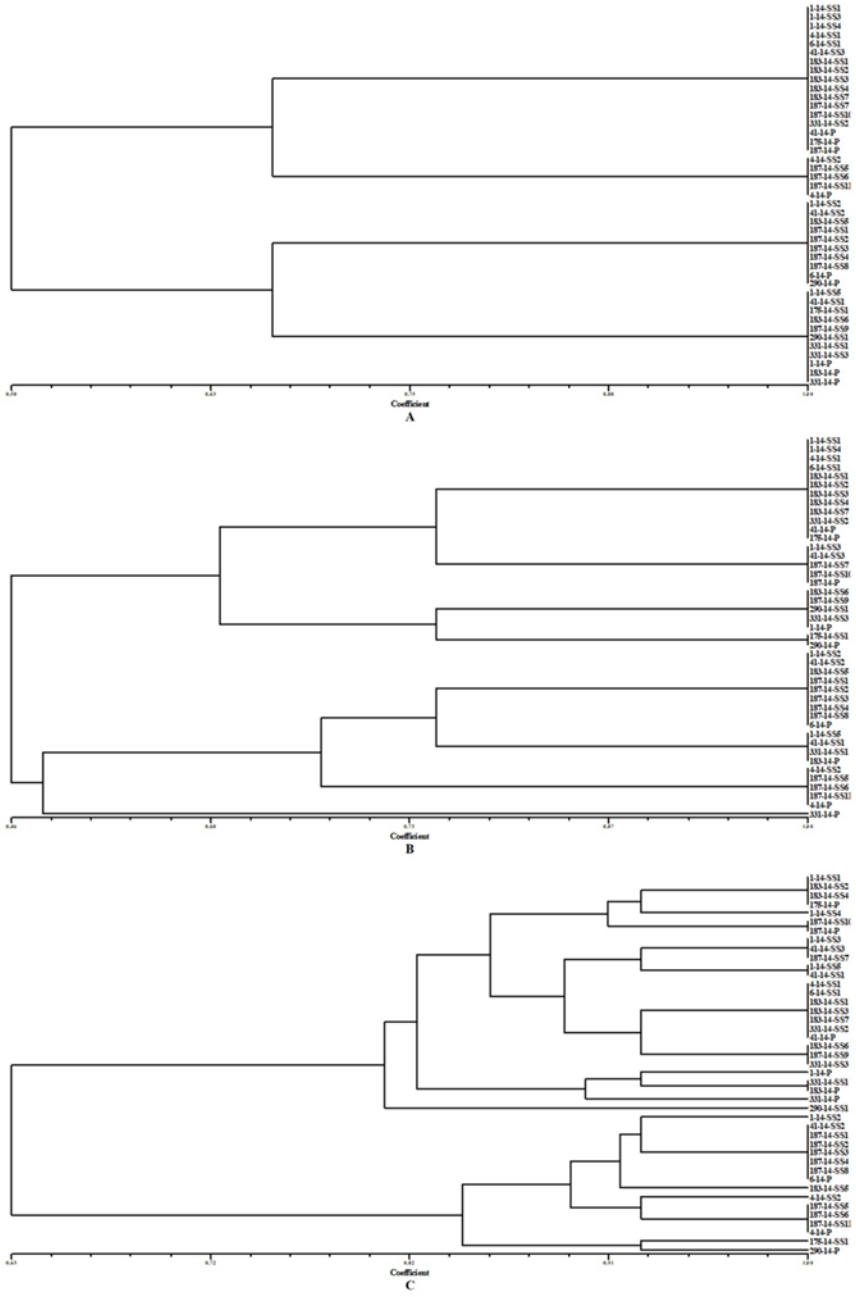
Supplementary Table 3.4. The number of mismatching loci among single-spore isolates considering missing data

Isolate	001-14-SS1	001-14-SS2	001-14-SS3	001-14-SS4	001-14-SS5	004-14-SS1	004-14-SS2	006-14-SS1	041-14-SS1	041-14-SS2	041-14-SS3	175-14-SS1	183-14-SS1	183-14-SS2	183-14-SS3	183-14-SS4	183-14-SS6	183-14-SS7	187-14-SS1	187-14-SS2	187-14-SS3	187-14-SS4	187-14-SS5	187-14-SS6	187-14-SS7	187-14-SS8	187-14-SS9	187-14-SS11	290-14-SS1	331-14-SS1	331-14-SS2	331-14-SS3	030-05-SS1	030-05-SS2	139-07-SS1	341-12-SS1
001-14-SS1	0	25	6	6	5	6	24	7	7	25	6	15	6	5	5	24	5	6	24	26	25	23	23	24	6	24	5	24	13	5	7	6	5	6	6	4
001-14-SS2	25	0	21	21	21	22	3	21	21	4	21	28	21	21	21	3	22	21	3	3	3	3	4	3	21	3	21	3	21	21	22	21	21	21	21	21
001-14-SS3	6	21	0	2	1	2	19	1	1	20	0	19	0	1	1	19	2	0	19	21	20	20	20	19	0	19	1	19	9	1	1	1	1	0	0	2
001-14-SS4	6	21	2	0	1	2	19	3	3	20	2	19	2	1	1	19	2	2	19	21	20	20	20	19	2	19	1	19	11	1	3	3	1	2	2	2
001-14-SS5	5	21	1	1	0	1	19	2	2	20	1	18	1	0	0	19	1	1	19	21	20	20	20	19	1	19	0	19	10	0	2	2	0	1	1	1
004-14-SS1	6	22	2	2	1	0	20	3	3	21	2	18	2	1	1	20	2	2	20	22	21	21	21	20	2	20	1	20	11	1	3	3	1	2	2	2
004-14-SS2	24	3	19	19	19	20	0	19	19	2	19	28	19	19	19	1	20	19	0	2	1	1	2	1	19	1	19	1	20	19	20	20	19	19	19	20
006-14-SS1	7	21	1	3	2	3	19	0	0	20	1	20	1	2	2	19	3	1	19	21	20	20	20	19	1	19	2	19	10	2	2	2	2	1	1	3
041-14-SS1	7	21	1	3	2	3	19	0	0	20	1	20	1	2	2	19	3	1	19	21	20	20	20	19	1	19	2	19	10	2	2	2	2	1	1	3
041-14-SS2	25	4	20	20	20	21	2	20	20	0	20	29	20	20	20	1	21	20	2	4	3	3	2	1	20	1	20	1	21	20	21	21	20	20	20	21
041-14-SS3	6	21	0	2	1	2	19	1	1	20	0	19	0	1	1	19	2	0	19	21	20	20	20	19	0	19	1	19	9	1	1	1	1	0	0	2
175-14-SS1	15	28	19	19	18	18	28	20	20	29	19	0	19	18	18	28	18	19	28	29	28	27	28	28	19	28	18	28	16	18	20	19	18	19	19	17
183-14-SS1	6	21	0	2	1	2	19	1	1	20	0	19	0	1	1	19	2	0	19	21	20	20	20	19	0	19	1	19	9	1	1	1	1	0	0	2
183-14-SS2	5	21	1	1	0	1	19	2	2	20	1	18	1	0	0	19	1	1	19	21	20	20	20	19	1	19	0	19	10	0	2	2	0	1	1	1
183-14-SS3	5	21	1	1	0	1	19	2	2	20	1	18	1	0	0	19	1	1	19	21	20	20	20	19	1	19	0	19	10	0	2	2	0	1	1	1
183-14-SS4	24	3	19	19	19	20	1	19	19	1	19	28	19	19	19	0	20	19	1	3	2	2	1	0	19	0	19	0	20	19	20	20	19	19	19	20
183-14-SS6	5	22	2	2	1	2	20	3	3	21	2	18	2	1	1	20	0	2	20	22	21	21	21	20	2	20	1	20	11	1	3	3	1	2	2	2
183-14-SS7	6	21	0	2	1	2	19	1	1	20	0	19	0	1	1	19	2	0	19	21	20	20	20	19	0	19	1	19	9	1	1	1	1	0	0	2
187-14-SS1	24	3	19	19	19	20	0	19	19	2	19	28	19	19	19	1	20	19	0	2	1	1	2	1	19	1	19	1	20	19	20	20	19	19	19	20
187-14-SS2	26	3	21	21	21	22	2	21	21	4	21	29	21	21	21	3	22	21	2	0	2	3	4	3	21	3	21	3	22	21	21	22	21	21	21	22
187-14-SS3	25	3	20	20	20	21	1	20	20	3	20	28	20	20	20	2	21	20	1	2	0	2	3	2	20	2	20	2	21	20	21	21	20	20	20	21
187-14-SS4	23	3	20	20	20	21	1	20	20	3	20	27	20	20	20	2	21	20	1	3	2	0	3	2	20	2	20	2	19	20	21	20	20	20	20	19
187-14-SS5	23	4	20	20	20	21	2	20	20	2	20	28	20	20	20	1	21	20	2	4	3	3	0	1	20	1	20	1	21	20	21	21	20	20	20	21
187-14-SS6	24	3	19	19	19	20	1	19	19	1	19	28	19	19	19	0	20	19	1	3	2	2	1	0	19	0	19	0	20	19	20	20	19	19	19	20
187-14-SS7	6	21	0	2	1	2	19	1	1	20	0	19	0	1	1	19	2	0	19	21	20	20	20	19	0	19	1	19	9	1	1	1	1	0	0	2
187-14-SS8	24	3	19	19	19	20	1	19	19	1	19	28	19	19	19	0	20	19	1	3	2	2	1	0	19	0	19	0	20	19	20	20	19	19	19	20
187-14-SS9	5	21	1	1	0	1	19	2	2	20	1	18	1	0	0	19	1	1	19	21	20	20	20	19	1	19	0	19	10	0	2	2	0	1	1	1
187-14-SS11	24	3	19	19	19	20	1	19	19	1	19	28	19	19	19	0	20	19	1	3	2	2	1	0	19	0	19	0	20	19	20	20	19	19	19	20
290-14-SS1	13	21	9	11	10	11	20	10	10	21	9	16	9	10	10	20	11	9	20	22	21	19	21	20	9	20	10	20	0	10	10	9	10	9	9	9
331-14-SS1	5	21	1	1	0	1	19	2	2	20	1	18	1	0	0	19	1	1	19	21	20	20	20	19	1	19	0	19	10	0	2	2	0	1	1	1
331-14-SS2	7	22	1	3	2	3	20	2	2	21	1	20	1	2	2	20	3	1	20	21	21	21	21	20	1	20	2	20	10	2	0	2	2	1	1	3
331-14-SS3	6	21	1	3	2	3	20	2	2	21	1	19	1	2	2	20	3	1	20	22	21	20	21	20	1	20	2	20	9	2	2	0	2	1	1	2
030-05-SS1	5	21	1	1	0	1	19	2	2	20	1	18	1	0	0	19	1	1	19	21	20	20	20	19	1	19	0	19	10	0	2	2	0	1	1	1
030-05-SS2	6	21	0	2	1	2	19	1	1	20	0	19	0	1	1	19	2	0	19	21	20	20	20	19	0	19	1	19	9	1	1	1	1	0	0	2
139-07-SS1	6	21	0	2	1	2	19	1	1	20	0	19	0	1	1	19	2	0	19	21	20	20	20	19	0	19	1	19	9	1	1	1	1	0	0	2
341-12-SS1	4	21	2	2	1	2	20	3	3	21	2	17	2	1	1	20	2	2	20	22	21	19	21	20	2	20	1	20	9	1	3	2	1	2	2	0

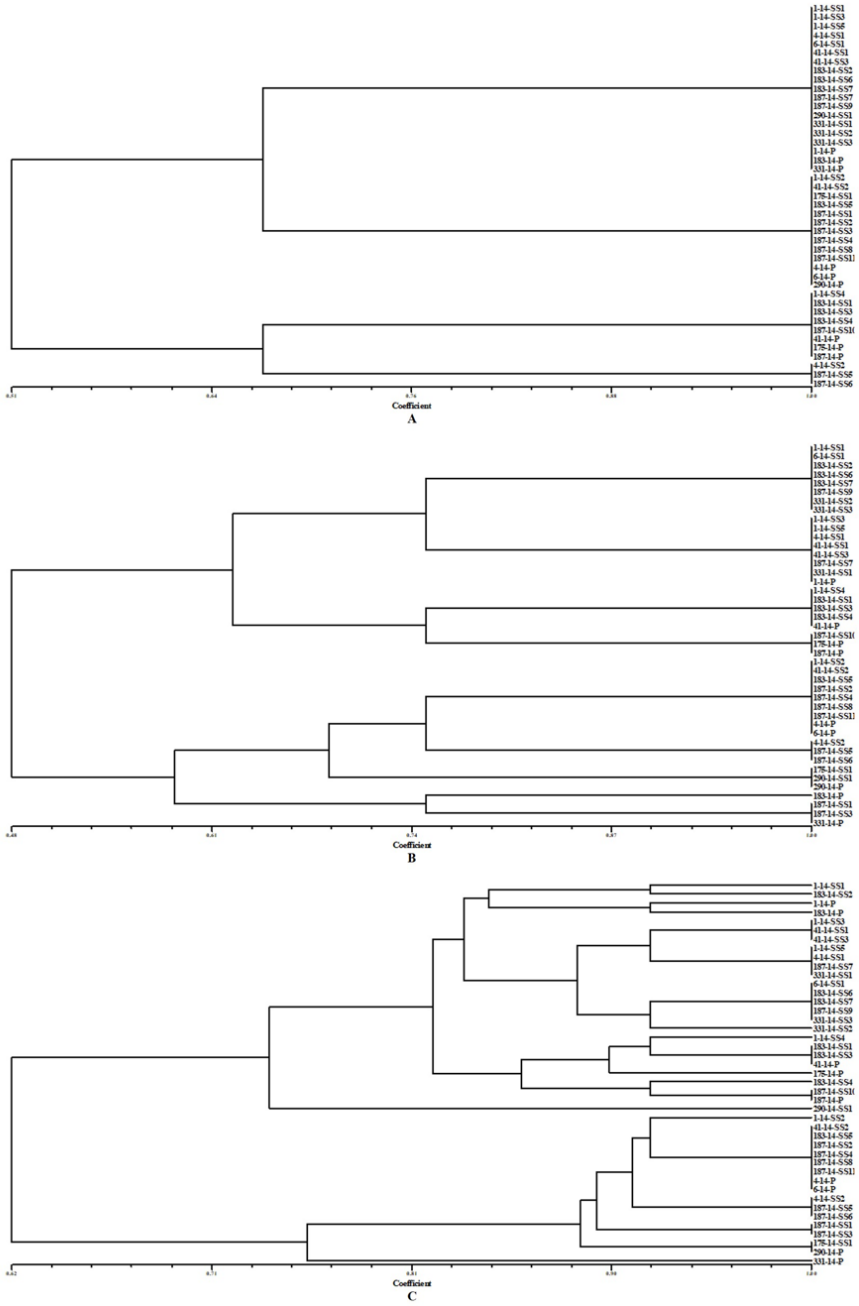
Supplementary Table 3.5. The pairwise genetic distance (GD) matrix of single-spore isolates

Isolate	001-14-SS1	001-14-SS2	001-14-SS3	001-14-SS4	001-14-SS5	004-14-SS1	004-14-SS2	006-14-SS1	041-14-SS1	041-14-SS2	041-14-SS3	175-14-SS1	183-14-SS1	183-14-SS2	183-14-SS3	183-14-SS4	183-14-SS6	183-14-SS7	187-14-SS1	187-14-SS2	187-14-SS3	187-14-SS4	187-14-SS5	187-14-SS6	187-14-SS7	187-14-SS8	187-14-SS9	187-14-SS11	290-14-SS1	331-14-SS1	331-14-SS2	331-14-SS3	030-05-SS1	030-05-SS2	139-07-SS1	341-12-SS1
001-14-SS1	0																																	
001-14-SS2	25	0																																
001-14-SS3	6	21	0																															
001-14-SS4	6	21	2	0																														
001-14-SS5	5	21	1	1	0																													
004-14-SS1	6	22	2	2	1	0																												
004-14-SS2	24	3	19	19	19	20	0																											
006-14-SS1	7	21	1	3	2	3	19	0																										
041-14-SS1	7	21	1	3	2	3	19	0	0																									
041-14-SS2	25	4	20	20	20	21	2	20	20	0																								
041-14-SS3	6	21	0	2	1	2	19	1	1	20	0																							
175-14-SS1	15	28	19	19	18	18	28	20	20	29	19	0																						
183-14-SS1	6	21	0	2	1	2	19	1	1	20	0	19	0																					
183-14-SS2	5	21	1	1	0	1	19	2	2	20	1	18	1	0																				
183-14-SS3	5	21	1	1	0	1	19	2	2	20	1	18	1	0	0																			
183-14-SS4	24	3	19	19	19	20	1	19	19	1	19	28	19	19	19	0																		
183-14-SS6	5	22	2	2	1	2	20	3	3	21	2	18	2	1	1	20	0																	
183-14-SS7	6	21	0	2	1	2	19	1	1	20	0	19	0	1	1	19	2	0																
187-14-SS1	24	3	19	19	19	20	0	19	19	2	19	28	19	19	19	1	20	19	0															
187-14-SS2	26	3	21	21	21	22	2	21	21	4	21	29	21	21	21	3	22	21	2	0														
187-14-SS3	25	3	20	20	20	21	1	20	20	3	20	28	20	20	20	2	21	20	1	2	0													
187-14-SS4	23	3	20	20	20	21	1	20	20	3	20	27	20	20	20	2	21	20	1	3	2	0												
187-14-SS5	23	4	20	20	20	21	2	20	20	2	20	28	20	20	20	1	21	20	2	4	3	3	0											
187-14-SS6	24	3	19	19	19	20	1	19	19	1	19	28	19	19	19	0	20	19	1	3	2	2	1	0										
187-14-SS7	6	21	0	2	1	2	19	1	1	20	0	19	0	1	1	19	2	0	19	21	20	20	20	19	0									
187-14-SS8	24	3	19	19	19	20	1	19	19	1	19	28	19	19	19	0	20	19	1	3	2	2	1	0	19	0								
187-14-SS9	5	21	1	1	0	1	19	2	2	20	1	18	1	0	0	19	1	1	19	21	20	20	20	19	1	19	0							
187-14-SS11	24	3	19	19	19	20	1	19	19	1	19	28	19	19	19	0	20	19	1	3	2	2	1	0	19	0	19	0						
290-14-SS1	13	21	9	11	10	11	20	10	10	21	9	16	9	10	10	20	11	9	20	22	21	19	21	20	9	20	10	20	0					
331-14-SS1	5	21	1	1	0	1	19	2	2	20	1	18	1	0	0	19	1	1	19	21	20	20	20	19	1	19	0	19	10	0				
331-14-SS2	7	22	1	3	2	3	20	2	2	21	1	20	1	2	2	20	3	1	20	21	21	21	21	20	1	20	2	20	10	2	0			
331-14-SS3	6	21	1	3	2	3	20	2	2	21	1	19	1	2	2	20	3	1	20	22	21	20	21	20	1	20	2	20	9	2	2	0		
030-05-SS1	5	21	1	1	0	1	19	2	2	20	1	18	1	0	0	19	1	1	19	21	20	20	20	19	1	19	0	19	10	0	2	2	0	
030-05-SS2	6	21	0	2	1	2	19	1	1	20	0	19	0	1	1	19	2	0	19	21	20	20	20	19	0	19	1	19	9	1	1	1	1	0
139-07-SS1	6	21	0	2	1	2	19	1	1	20	0	19	0	1	1	19	2	0	19	21	20	20	20	19	0	19	1	19	9	1	1	1	1	0
341-12-SS1	4	21	2	2	1	2	20	3	3	21	2	17	2	1	1	20	2	2	20	22	21	19	21	20	2	20	1	20	9	1	3	2	1	2

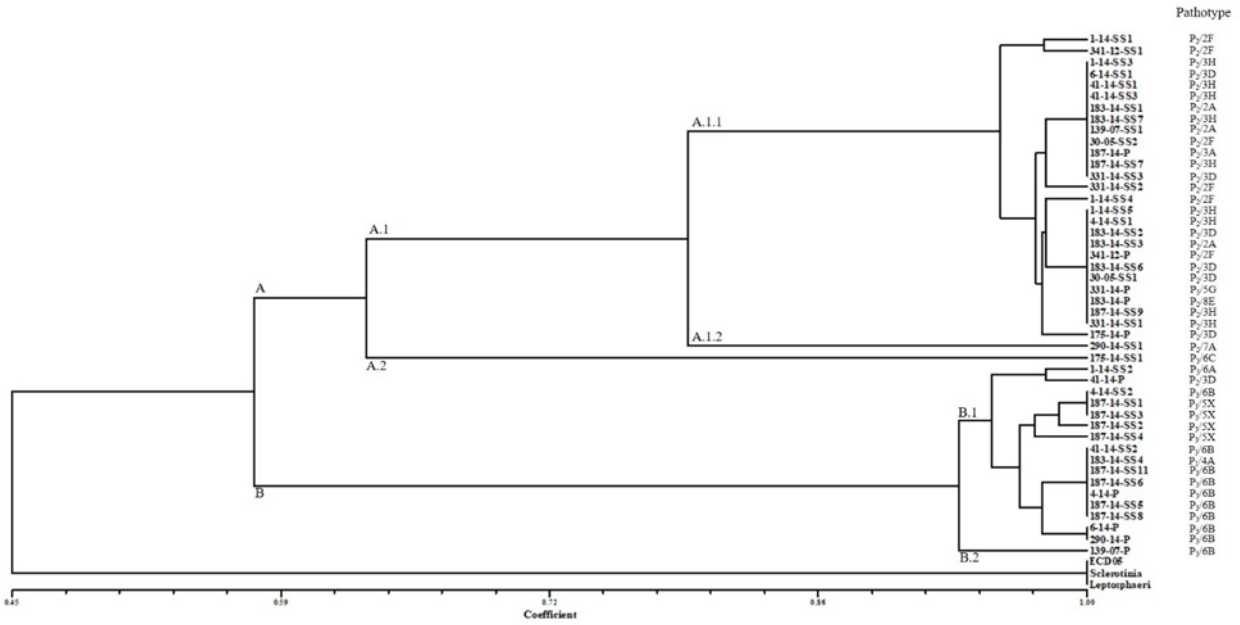
Supplementary Figures



Supplementary Figure 2.1. Pathogenic similarity of *Plasmodiophora brassicae* isolates on the differential hosts of Somé et al. (A), Williams (B) and the CCD set (C), based on a threshold index of disease (ID) = 25%. The dendrograms were constructed employing the unweighted pair group method with arithmetic mean procedure and simple similarity coefficient with NTSys-pc ver. 2.2. Four, eight and 18 pathotypes were identified, respectively, with each set of differential hosts.



Supplementary Figure 2.2. Pathogenic similarity of *Plasmodiophora brassicae* isolates on the differential hosts of Somé et al. (A), Williams (B) and the CCD set (C), based on a threshold index of disease (ID) =33%. The dendrograms were constructed employing the unweighted pair group method with arithmetic mean procedure and simple similarity coefficient with NTSys-pc ver. 2.2. Four, nine and 20 pathotypes were identified, respectively, with each set of differential hosts.



Supplementary Figure 3.1. Genetic similarity of single-spore and field isolates of *Plasmodiophora brassicae*. The dendrogram was constructed using he unweighted pair group method with arithmetic mean procedure and simple similarity coefficient with NTSys-pc ver. 2.2. Cluster analysis indicated that all isolates clustered in two distinct main groups (A and B). The three entries at the bottom of the dendrogram include Chinese cabbage (*Brassica rapa* L.var. *pekinenses*) 'Granaat' (European Clubroot Differential (ECD) 05), *Sclerotinia sclerotiorum* and *Leptosphaeria maculans*, which were served as controls. Pathotype designations follow Somé et al. (1996) and Williams (1966)/Canadian Clubroot Differential (CCD) set (Strelkov et al. 2018).