Virulence and Genetic Variability Among Isolates of *Mycosphaerella pinodes*

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ABSTRACT

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Fifty-eight isolates of *Mycosphaerella pinodes*, collected from western Canada, New Zealand, France, Australia, the United Kingdom, and Ireland, were analyzed for pathogenic and genetic variation according to their virulence on six differential cultivars of field pea (AC Tamor, Bohatyre, Danto, Majoret, Miko, and Radley) and amplified fragment length polymorphism markers. The 56 isolates were classified into 15 pathotypes. Pathotype 1 consisted of 31 isolates that were virulent on all six pea differential cultivars. Pathotypes 14 and 15 consisted of eight isolates that were avirulent on all six differential cultivars or virulent on one of six differential cultivars. The analysis of molecular variance showed that 57.2% of the total variation was caused by differences among populations, and 42.8% was due to molecular diversity within populations. Phylogenetic analysis of molecular variation of isolates showed that most of the Canadian isolates and four Australian isolates formed two clustered groups, respectively, regardless of virulence on the six differential cultivars. Isolates from New Zealand were geographically clustered into two groups. However, the isolates from France, Ireland, and the United Kingdom were clustered with the Canadian isolates.

Additional keywords: *Mycosphaerella* blight

Mycosphaerella blight, caused by *Mycosphaerella pinodes* (Berk. & Bloxam) Vestergr., is the most important foliar disease of field pea (*Pisum sativum* L.) worldwide (2,7,16,17,23,30,31). In Canada, average dry seed yield losses caused by this disease have been estimated at 10%, and losses of over 50% have been reported in field trials (28,34).

Management for Mycosphaerella blight is limited to fungicidal seed treatment, crop rotation (18), and chemical sprays on a preventive and systematic schedule (2). However, each method has deficiencies. The use of resistant cultivars would be the most effective and economical method. Unfortunately, cultivars with high levels of resistance to Mycosphaerella blight are not commercially available. Some promising resistance sources have been identified from pea cultivars and accessions tested in Canada (32,34), New Zealand (17), and the United Kingdom (7). Studies on genetic analysis of resistance to *M. pinodes* showed that resistance is conferred by one, two, or multiple genes (7,25).

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A number of pathotypes of *M. pinodes* have been reported based on their reactions on differential hosts in different countries. For example, 22 pathotypes were identified in Canada (33), 6 in West Germany (22), 6 in Poland (10), and 15 in Australia (1). In these studies, resistance or susceptibility of differential cultivars were determined by host reactions, via a disease severity scale. These contrasting results are most likely the results of not using uniform differential hosts, inoculum concentrations, environmental conditions, inoculation methods, or disease assessment scales.

Molecular markers, in combination with pathogenicity tests, have been used to identify pathogen biotypes or races (5,8,24). Pathogen population dynamics, evolutionary relationships between pathogen races (6), geographical distribution and genetic diversity of pathogens (11), and differentiation of pathogen species with similar phenotypic characters (12,14,21) also have been studied using molecular markers. There are many molecular techniques that can be used for assessment of molecular variation in plants and pathogens. Amplified fragment length polymorphism (AFLP) DNA is more efficient in rapidly regenerating genotype data for large numbers of individuals, including plants (13), bacteria (15), and fungi (24). González et al. (11) demonstrated that four AFLP combinations could generate band numbers four times higher than 10 primers of random amplified polymorphic DNA (RAPD), and the number of polymorphic bands was three times higher than that produced by the RAPD primers.

Molecular markers have been used in the studies of Ascochyta blight complex on field pea caused by three related species, *Ascochyta pisi* Lib., *M. pinodes*, and *Phoma medicaginis* var. *pinodella* (Jones) Boerema (23). *A. pisi* was clearly distinguished from *M. pinodes* and *P. medicaginis* var. *pinodella* using RAPD markers (3) and restriction fragment length polymorphism (RFLP) of rDNA spacers (9). Onfroy et al. (23) distinguished *M. pinodes* from *P. medicaginis* var. *pinodella* using RAPD markers and demonstrated that the two species had low intraspecific genetic variability. Information concerning pathogenicity and genetic variation among isolates of *M. pinodes* is lacking. Therefore, the objectives of this study were to (i) test virulence of *M. pinodes* isolates on six differential hosts to determine pathotypes of the isolates tested and (ii) analyze the relationship between virulence and genetic variation among isolates of *M. pinodes*.

MATERIALS AND METHODS

Collection and culture of fungal isolates. A total of 58 isolates of *M. pinodes* collected from diseased field pea plants were used in this study (Table 1). Of these isolates, 33 were collected from fields in Manitoba (MB), Saskatchewan (SK), and Alberta (AB), Canada. Because *M. pinodes*, *A. pisi*, and *P. medicaginis* var. *pinodella* all can cause blights on pea, *M. pinodes* was distinguished from *A. pisi* and *P. medicaginis* var. *pinodella* according to the characteristics of pathogens and symptoms described by Lawyer (18). Canadian isolates were identified and characterized in Allen Xue's laboratory. The 11 isolates from New Zealand (NZ), 2 from the United Kingdom (UK), and 5 from Ireland (IR) were identified and provided by J. Kraft at the United States Department of Agriculture-Agricultural Research Service, Irrigated Agriculture Research and Extension Centre, Prosser, WA in 1999. The three isolates from France (FR) and four isolates from Australia (AU) were identified and provided by B. Tivoli (Institute National de la Recherche Agronomique, Centre de Recherches de Rennes, Domaine de la Motte, France) and J. Davidson (South Australia Research and Development Institute, Adelaide, South Australia), respectively, in 1999. These isolates were cultured, single spored, and isolated at AAFC-Morden Research Centre before

being tested. To obtain single pycnidiospore isolates, pure cultures were generated by isolation of the pathogen from diseased leaves and stems. Approximately 5 mm^2 of diseased leaves or stems were surface sterilized for 1.5 min in 0.05% sodium hypochlorite. The tissues then were rinsed in sterile water three times, placed on sterile filter paper to remove excess water, and cultured on potato dextrose agar (PDA) in petri dishes for 14 days. Pycnidiospores from resulting cultures were spread on water agar and incubated for 24 h. Single germinating pycnidiospores were transferred onto PDA with the aid of a dissecting microscope. All cultures were incubated at room temperature (20 to 24°C) with a 14-h photoperiod under cool white fluorescent lamps. Resultant single-spore cultures were maintained on PDA slants and stored in the dark at 3°C.

Table 1. Geographic origin and collection date of 58 *Mycosphaerella pinodes* isolates[z]

Isolate	Origin	Year of collection
MB01	Underhill, MB	1996
MB02	Baldron, MB	1996
MB03	Elm Creek, MB	1996
MB04	Darlingford, MB	1996
MB05	Osborne, MB	1995
MB06	Foxwaren, MB	1994
MB07	Darlingford, MB	1994
MB08	Dunrea, MB	1994
MB09	Plum Coulee, MB	1994
MB10	Gladstone, MB	1994
MB11	Morden, MB	2000
MB12	Morden, MB	2000
MB13	Morden, MB	2000
MB14	Morden, MB	2000
MB15	Morden, MB	2000
MB16	Morden, MB	2000
MB17	Morden, MB	2000
MB18	Morden, MB	2000
SK01	Melfort, SK	1995
SK02	Valparaiso, SK	1996
SK03	Redvers, SK	1996
SK04	Carlyle, SK	1996
SK05	Saskatoon, SK	1995
SK06	Melfort, SK	1996
SK07	Lashburn, SK	1996
SK08	Bently, SK	1994
AB01	Lacombe, AB	1996
AB02	Three Hills, AB	1996
AB03	Barrhead, AB	1995
AB04	Pine Lake, AB	1995
AB05	Aspelund, AB	1995
AB06	Lacombe, AB	1995
AB07	Rimbey, AB	1994
UK01	Norwich, UK	1991
UK02	Norwich, UK	1991
NZ01	Gore, NZ	1999
NZ02	Gore, NZ	1999
NZ03	Gore, NZ	1999
NZ04	Gore, NZ	1999
NZ05	Gore, NZ	1999
NZ06	Saunders, NZ	1999
NZ07	Saunders, NZ	1999
NZ08	Saunders, NZ	1999
NZ09	Saunders, NZ	1999
NZ10	Saunders, NZ	1999
NZ11	Saunders, NZ	1999
IR01	Ireland	1999
IR02	Ireland	1999
IR03	Ireland	1999
IR04	Ireland	1999
IR05	Ireland	1999
FR01	Rennes, FR	1999
FR02	Rennes, FR	1999
FR03	Rennes, FR	1999
AU01	Australia	1999
AU02	Australia	1999
AU03	Australia	1999
AU04	Australia	1999

[z] MB, SK, and AB represent the provinces Manitoba, Saskatchewan, and Alberta, Canada, respectively; NZ, IR, FR, and AU represent New Zealand, Ireland, France, and Australia, respectively.

Differential pea cultivars. Six cultivars of field pea were used as differentials. Of these, 'AC Tamor' was susceptible to all isolates tested in the previous study (34). 'Danto' was used as a differential host in similar studies in Canada and West Germany, where it was resistant to 10 and 98% of *M. pinodes* isolates, respectively (22,33). 'Majoret' and 'Radley' were moderately resistant to disease in a previous study in western Canada, while 'Miko and Bohatyr' were susceptible differential cultivars in western Canada (32,34). For each cultivar, eight seed were sown in a 20-cm-diameter plastic pot containing a mixture of soil, sand, and peat (2:2:1, vol/vol/vol), with three replicate pots per isolate. Pots were placed in a growth chamber at 20°C for a 14-h photoperiod with a light intensity of 360 $\mu mol\ m^{-2}\ s^{-1}$. Plants were thinned to four plants per pot prior to inoculation.

Inoculum preparation and plant inoculation. Pycnidiospores of *M. pinodes* from 2-week-old single-spore cultures on PDA were used for inoculation. The spores were harvested by flooding the cultures with distilled water containing 0.05% Tween 20 and scraping gently with a sterile glass slide to dislodge spores. The spore suspension was filtered through two layers of cheesecloth and adjusted to approximately 10^6 pycnidiospores/ml with a hemacytometer. The plants were inoculated at a rate of 0.5 ml of inoculum suspension per plant using a De Vilbiss model 15 atomizer (The De Vilbiss Co., Somerset, PA) at the four- to six-node stage approximately 2 weeks after seeding. After allowing the inoculated plants to dry for 30 min, the plants were transferred to a polyethylene-covered humidity chamber. Humidity in the chamber was maintained by continuous operation of an ultrasonic humidifier. Air temperature and humidity in the chamber were 20 ± 1°C and 100% relative humidity. The plants were maintained in the humidity chamber for 48 h in the dark, and subsequently returned to the growth room with a 14-h photoperiod at 20°C. Pea genotypes were arranged in a completely randomized design. Three pots of each cultivar sprayed with 0.05% Tween 20 were included with each inoculation as checks against extraneous airborne inoculum.

Observation of days between inoculation and symptomatic appearance and disease assessment. To calculate the days between inoculation and symptomatic appearance (DISA), symptom development on leaves from the time of inoculation with all the isolates on the six cultivars was observed every day up to 6 days. By the sixth day, all plants were symptomatic. Disease severity was assessed for each plant 10 days after inoculation. Those isolates that showed a specific genotype–isolate interaction, but with lower disease severity on six differential cultivars, were tested twice in each test.

Disease severity on leaves and stems was rated 10 days after inoculation using a 0-to-5 scale modified from Nasir and Hoppe (22) and Xue et al. (33). The disease severity was based on a 0-to-5 scale where 0 = symptomless; 1 = a few small flecks on leaves; 2 = less than 10% of plant surface area covered by lesions, but no symptoms on stem; 3 = numerous necrotic flecks and few large lesions on leaves and stems but covering less than 50% of plant area; 4 = large, coalescing lesions on leaves and stems and covering more than 50% of plant area but plants surviving; and 5 = large, coalescing lesions on leaves and girdling lesions on stems, plant withering and dying. Differentials with scores 0, 1, and 2 were classified as resistant and scores 3, 4, and 5 were considered susceptible responses. The virulence test was repeated twice.

Analysis of variance was conducted to compare the genotypic differences in partial resistance to 56 isolates on six pea genotypes and differences in isolate virulence. The mean DISA or the mean severity of each cultivar to 56 isolates were

Table 2. Analysis of variance of disease severity and days between inoculation and symptomatic appearance (DISA) period caused by 56 *Mycosphaerella pinodes* isolates on six pea genotypes

Source of variance	df	Mean square	*F*	*P* > *F*
Disease severity				
Genotype	5	20.17	134.52	<0.0001
Isolate	55	26.87	178.91	<0.0001
Genotype × isolate	275	1.11	7.41	<0.0001
Error	330	0.15	…	…
DISA				
Genotype	5	4.51	4.47	0.0006
Error	330	1.01	…	…
Isolate	55	4.72	13.12	<0.0001
Error	280	0.36	…	…

separated by the Duncan's multiple range test at $P = 0.05$. Statistical analysis was performed using SAS (SAS Institute Inc., Cary, NC).

DNA preparation. The biomass of each isolate was prepared by using 75 ml of PDA broth, inoculated with a 0.5-ml suspension of pycnidiospores (approximately 106 ml^{-1}) of each isolate and incubated for 4 days with agitation in the laboratory at room temperature. Mycelia were harvested by vacuum filtration through two layers of sterilized Miracloth (Calbiochem, CN Biosciences, Inc. La Jolla, CA) and rinsed with sterile water twice, then stored at –80°C until lyophilized. DNA of isolates was extracted essentially by the method described by Lodhi et al. (20). All DNA extracts were quantified by a spectrophotometer and adjusted to a final concentration of 5 ng/µl for AFLP analysis.

AFLP analysis. AFLP assays were performed with the AFLP Analysis System II (Life Technologies, Inc. Caithersburg, MD) following the manufacturer's instructions. Fungal, genomic DNA was digested by restriction endonucleases *Eco*RI and *Mse*I, ligated to *Eco*RI and *Mse*I adapters, and amplified by polymerase chain reaction (PCR), using primers that contain the common sequences of the adapters and one to two arbitrary nucleotides as selective sequences.

Primary template DNA was prepared in a one-step restriction ligation reaction. Fungal genomic DNA (250 ng) was di-

Table 3. Disease severity of 56 isolates of *Mycosphaerella pinodes* on six differential pea (*Pisum sativum*) cultivars[y]

Isolate	AC Tamor	Bohatyr	Danto	Majoret	Radley	Miko
MB03	5.0 a	5.0 a	5.0 a	5.0 a	5.0 a	5.0 a
SK05	5.0 a	5.0 a	5.0 a	5.0 a	5.0 a	5.0 a
MB17	5.0 a	5.0 a	4.9 ab	5.0 a	5.0 a	5.0 a
MB10	5.0 a	5.0 a	4.8 a–c	4.9 ab	5.0 a	4.8 ab
AB07	5.0 a	4.6 a–c	5.0 a	4.8 ab	5.0 a	4.8 a–c
IR05	5.0 a	4.5 a–d	4.5 a–g	5.0 a	4.8 a	5.0 a
NZ08	5.0 a	4.4 a–d	4.8 a–d	4.8 a–c	4.8 a	5.0 a
MB15	5.0 a	4.8 ab	4.3 b–h	4.3 a–e	5.0 a	5.0 a
SK06	5.0 a	3.8 d–h	4.7 a–e	4.6 a–d	5.0 a	5.0 a
MB02	4.6 a–c	4.7 a–c	4.7 a–f	4.2 a–f	5.0 a	4.8 a–c
MB06	4.0 ef	4.9 a	4.0 f–i	5.0 a	5.0 a	4.9 a
NZ03	5.0 a	4.8 ab	4.9 ab	4.3 a–f	4.5 a–d	4.1 d–h
SK04	5.0 a	4.5 a–d	4.3 c–h	4.4 a–e	4.4 a–d	4.7 a–d
MB18	5.0 a	5.0 a	3.8 g–k	3.4 f–l	4.8 a	4.8 a–c
SK01	5.0 a	5.0 a	3.9 g–j	3.1 h–m	4.6 a–c	5.0 a
UK02	5.0 a	3.9 d–h	3.2 k–n	4.1 b–g	5.0 a	4.9 a
AB04	4.3 b–e	4.8 ab	3.7 h–k	3.8 e–j	4.7 a–c	4.4 a–g
AB01	5.0 a	4.3 a–e	3.7 h–l	3.8 e–k	4.8 a	4.0 e–i
NZ05	4.8 ab	3.9 d–g	3.4 j–m	3.9 c–h	4.7 a–c	4.1 e–i
NZ06	4.5 a–d	3.1 h–k	4.1 e–i	4.8 a–c	4.8 ab	3.4 i–k
MB11	5.0 a	3.8 d–h	2.8 mn	3.6 e–l	4.3 a–d	4.0 e–i
MB09	3.5 gh	4.0 c–e	3.5 i–m	3.9 d–i	4.1 b–d	4.5 a–e
MB07	3.6 f–h	3.8 d–h	4.0 g–i	3.3 g–m	4.0 cd	4.5 a–e
MB16	4.7 ab	3.6 e–j	3.0 mn	3.0 i–m	4.6 a–c	4.0 f–i
MB14	3.6 f–h	3.1 h–k	4.1 d–i	2.7 l–n	4.7 a–c	4.3 b–h
MB01	3.1 g–i	3.9 d–g	3.0 mn	4.0 c–g	5.0 a	3.4 i–k
AB02	4.2 c–e	3.8 d–h	3.1 l–n	3.0 i–m	3.3 ef	4.9 a
NZ04	4.0 d–f	5.0 a	4.2 c–h	3.6 e–l	2.7 f–i	2.5 m–o
SK08	4.0 d–f	4.1 b–e	3.3 j–n	2.7 l–n	2.9 fg	3.8 g–j
MB05	4.3 b–e	3.5 e–k	2.8 mn	3.1 h–m	2.7 f–i	3.8 g–j
MB04	4.5 a–d	3.8 d–h	1.9 p–r	2.5 m–o	3.0 fg	3.3 j–l
FR03	4.3 b–e	3.1 h–k	2.3 op	2.9 k–m	1.9 i–m	4.3 a–g
MB08	4.6 a–c	3.2 f–k	1.5 q–s	1.3 p–r	4.0 cd	4.2 c–h
IR03	3.9 e–g	2.0 mn	2.7 no	3.4 f–l	3.8 de	2.8 l–n
SK03	3.5 gh	3.9 d–g	3.7 h–l	2.6 m–o	1.8 k–n	3.0 k–m
NZ01	3.4 gh	2.8 kl	2.1 pq	3.5 e–l	2.5 h–j	3.8 f–j
SK07	2.8 jk	3.1 h–k	1.5 q–s	1.7 o–q	3.9 cd	4.1 d–h
AB03	3.4 gh	3.9 d–g	1.0 s–u	3.5 f–l	1.4 l–p	3.1 k–m
MB13	4.0 d–f	3.1 h–k	1.2 s–u	1.3 p–r	2.7 f–i	3.1 k–m
IR02	2.8 jk	2.2 lm	1.9 p–r	2.8 k–n	2.0 i–l	3.6 h–k
AB05	3.8 e–g	2.0 mn	2.3 op	2.0 n–p	2.3 h–k	1.8 pq
IR04	2.9 i–k	1.6 m–p	3.0 mn	2.4 m–o	1.5 k–o	2.5 m–o
MB12	3.8 e–g	2.3 lm	1.3 s–u	2.8 k–n	1.2 n–p	2.5 m–o
FR01	2.3 lm	3.0 i–k	1.6 q–s	1.7 pq	2.3 h–k	2.2 n–p
FR02	3.4 gh	2.6 kl	1.3 r–t	2.4 m–o	1.4 l-p	1.9 o–q
NZ07	1.9 mn	2.0 mn	1.1 s–u	1.1 q–s	2.8 f-h	3.7 g–j
AU03	3.9 e–g	2.8 j–l	1.0 s–u	1.0 q–s	1.0 o–q	2.6 m–o
SK02	2.5 kl	1.2 o–r	1.3 r–t	1.0 q–s	2.5 g–j	1.6 p–r
NZ02	2.6 kl	1.4 n–q	1.4 rs	1.0 q–s	0.8 p–r	1.4 qr
NZ11	1.3 op	1.8 m–o	1.0 s–u	1.0 q–s	1.3 m–p	1.8 pq
NZ09	1.7 no	1.7 m–p	1.0 s–u	1.0 q–s	1.0 o–q	1.5 p–r
UK01	1.0 p	1.2 p–r	1.0 s–u	1.0 q–s	1.0 op	1.3 qr
AU01	1.0 p	1.0 p–r	1.0 s–u	1.0 q–s	1.0 o–q	1.0 rs
IR01	2.2 lm	0.5 r	0.7 uv	0.5 r–t	0.9 p–r	1.0 rs
AU02	3.4 gh	1.0 q–s	0.3 vw	0.1 t	0.5 rs	0.5 s
AU04	1.0 p	1.0 p–r	0.8 t–v	0.9 q–s	0.3 s	0.5 s
Mean[z]	3.7 a	3.3 a–c	2.9 c	3.0 bc	3.3 bc	3.4 ab

[y] Disease was scored 10 days after inoculation using a 0-to-5 scale. Disease severity with the same letter in columns are not significantly different at $P = 0.05$ (Duncan's multiple range test).

[z] Mean disease severity in the rows was calculated by averaging disease severity caused by 56 isolates on each differential cultivar.

gested by *Eco*RI and *Mse*I at 37°C for 2 h and heated to 70°C for 15 min to inactivate enzymes. The DNA fragments were ligated to *Eco*RI and *Mse*I adapters for 2 h at 22°C. After terminating the reaction, the ligation mixture was diluted threefold with Tris-EDTA buffer and stored at –20°C. These ligated fragments served as templates in the preamplification reaction. The primers used for the preamplification PCR were *Eco*RI primer 5′-AGACTGCGTACCAATTC-3′ and *Mse*I primer 5′-GACGATGAGTCCTGAGTAA/C-3′ (*Mse*I + C). The preamplification reaction was performed in a 25-µl reaction containing 7.2 µl of template DNA, 30 ng each of primers, 1 U of Taq polymerase (Fisherbrand), 100 mM Tris-HCL (pH 8.0), 500 mM KCL, 1.5 mM $MgCL_2$, and 0.2 mM each of dNTPs. The fragments were preamplified by 20 PCR cycles in a PTC 100TM programmable thermal controller (MJ Research Inc., Boston, MA), using a temperature profile of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s in each cycle, as described by Vos et al. (27). The samples of the preamplified fragments were diluted 10-fold to be used as DNA templates in selective amplification.

For selective amplification of restriction fragments, six *Eco*RI selective primers with two selective nucleotides were used as follows: *Eco*RI +AA, +AC, +AG, + AT, + TA, or +TT. The effectiveness of each of these primers was evaluated in combination with selective primer *Mse*I + C. The selective amplification was performed in a 20-µl reaction containing 2 µl of diluted product of preamplification reaction, 5 ng of *Eco*RI primer, and 30 ng of *Mse*I + C primer, 1 U of Taq polymerase (Fisherbrand), 100 mM Tris-HCL (pH 8.0), 500 mM KCL, 1.5 mM $MgCL_2$, and 0.2 mM each of dNTPs. The temperature profile of the selective amplification PCR was one cycle at 94°C for 60 s, 65°C for 60 s, and 72°C for 90 s; then the annealing temperature was lowered during each cycle by 1°C for 9 cycles. The remainder of the amplification was 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. The 4 µl of the selective amplification PCR product in each shark-tooth comb-formed well was separated by electrophoresis, using a denaturing 5% (wt/vol) polyacrylamide DNA sequencing gel containing 7.5 M urea. Silver staining was used for staining DNA bands. The gel plate was placed in a fixing solution (10% of glacial acetic acid) and shaken for 30 min until the tracking dye disappeared. After rinsing three times for 2 min each time in distilled water, the gel was placed into a staining solution (2 g of silver nitrate and 3 ml of 37% formaldehyde in 2 liters of distilled water) and shaken for 30 min. The gel then was dipped in distilled water for 2 s and placed immediately into developing solution stored at 4°C (102 g of sodium carbonate, 6 ml of 37% formaldehyde, and 800 µl of sodium thiosulphate [10 mg/ml] in 4 liters of distilled water). The gel was agitated in the developing solution until clear bands were visible and the fixing solution was added to stop the developing reaction. The gel was rinsed with distilled water and dried by air for band evaluation. PCR repetitions using the same set of primers and isolates and different DNA preparations of the same isolates were conducted to check the repeatability of results.

The presence and absence of all fragments between molecular sizes of 50 and 500 bp were scored in each of the 58 isolates. Bands representing molecular sizes larger than 500 bp were not scored because the resolution was insufficient to discriminate between bands of various molecular sizes. The data matrix was analyzed based on the assumption that co-migrating bands in AFLP gels were homologous. The phylogenetic tree was built using unweighted pair group means analysis (UPGMA), which was performed by the software NTSYSpc 2.1 (Exeter Software, Setauket, NY). To determine the robustness of the dendrogram, the presence or absence of data were resampled by replacement with 1,000 bootstrap replicates. The analysis of molecular variance (AMOVA; online as Arlequin software, hosted by the Department of Anthropology, University of Geneva, Switzerland) is used for partitioning molecular variance between and within populations.

RESULTS

Virulence test. All isolates caused symptoms on the six differential cultivars. Highly significant differences in disease severity were detected among pea genotypes, fungal isolates, and genotype–isolate interactions (Table 2). The greatest differences were observed among the isolates, showing that virulence of isolates was the major factor that contributed to the variance. Genotype–isolate, although a smaller component of variation than isolate or genotype, also was significant (Table 2). Higher levels of disease severity was observed 10 days after inoculation on the susceptible cvs. AC Tamor and Miko, while the lowest disease severity was observed on Danto (Table 3). Most isolates from Canada were virulent on the six differential hosts. Four isolates from New Zealand (NZ02, NZ07, NZ09, and NZ11), four from Australia (AU01, AU02, AU03, and AU04), one from the United Kingdom (UK01), one from Ireland (IR01), and one from Saskatchewan (SK02) were significantly less virulent than most of the other isolates tested (Table 3). Disease severity caused by each of these 11 isolates on most of the individual differential hosts was ≤2.0. A continuous wide variation in virulence was observed based on disease severity on the individual differential cultivars (Table 3). Of the six differential cultivars,

Table 4. Fifteen pathotypes differentiated from 56 *Mycosphaerella pinodes* isolates based on reactions of six differential cultivars of *Pisum sativum*

		Disease reaction of isolates on six differential hosts[y]					
Pathotype	**Isolate[z]**	**AC Tamor**	**Bohatyr**	**Danto**	**Majoret**	**Radley**	**Miko**
1	MB01–03, MB05–07, MB09–11, MB14–18, SK01, SK04–06, SK08, AB01, AB02, AB04, AB07, NZ01, NZ03–06, NZ08, IR05, UK02	…	…	…	…	…	…
2	MB04	…	…	R	…	…	…
3	FR03	…	…	…	…	R	…
4	MB08, MB13, SK07, FR01	…	…	R	R	…	…
5	IR03	…	R	…	…	…	…
6	SK03	…	…	…	…	R	…
7	AB03, MB12, IR02	…	…	R	…	R	R
8	AB05	…	R	…	R	…	…
9	IR04	…	R	…	…	R	…
10	FR02	…	…	R	…	R	R
11	NZ07	R	R	R	R	…	…
12	AU03	…	…	R	R	R	…
13	SK02	…	R	R	R	…	R
14	AU02, IR01, NZ02	…	R	R	R	R	R
15	NZ09, NZ11, UK01, AU01, AU04	R	R	R	R	R	R

[y] R indicates a resistant reaction (disease severity ≤2.0) and … indicates a susceptible reaction.

[z] MB = Manitoba, Canada; FR = France; SK = Saskatchewan, Canada; IR = Ireland; AB =Alberta, Canada; NZ = New Zealand; AU = Australia; and UK = United Kingdom.

Danto, being less severely affected by the pathogen, was resistant to 20 out of 56 isolates tested (disease severity ≤2.0). AC Tamor was susceptible to 50 out of 56 isolates, being the most susceptible differential host. Other differential hosts were resistant to 11 to 16 isolates.

The severity reaction of 56 isolates to susceptible cv. AC Tamor ranged from 1.0 to 5.0 and to resistant cv. Danto from 0.3 to 5.0. Duncan's multiple range test on disease severity on each differential host showed a continuous wide variation of virulence among the isolates tested (Table 3). The 56 isolates can be grouped into 15 pathotypes based on disease reactions on the six individual differentials (Table 4). Pathotype 1, consisting of 31 isolates (23 from Canada, 6 from New Zealand, 1 from Ireland, and 1 from the United Kingdom), was the most numerous and virulent to all six differential hosts. Pathotype 15, consisting of five isolates (two from Australia, two from New Zealand, and one from the United Kingdom), was the second most numerous and much less virulent to all differentials. Other pathotypes each contained one to four isolates and were virulent to one to five differentials.

Table 5. Number of days between inoculation and symptomatic appearance (DISA) of 56 isolates of *Mycosphaerella pinodes* on six differential cultivars of pea (*Pisum sativum*)

Isolate[x]	AC Tamor	Bohatyr	Danto	Majoret	Radley	Miko	Mean[y]
AU01	4	5	5	5	5	5	4.8 a
UK01	4	5	5	5	5	4	4.7 a
AU04	4	4	5	5	5	4	4.5 ab
AU02	3	4	5	5	5	5	4.5 ab
NZ09	4	4	5	5	4	4	4.3 a–c
SK02	5	4	5	5	3	4	4.3 a–c
NZ02	4	3	4	4	5	5	4.2 a–d
IR01	3	4	5	5	5	3	4.2 a–d
NZ11	4	4	5	4	4	4	4.2 a–d
IR04	3	4	4	5	4	3	3.8 b–e
AU03	2	3	6	5	4	3	3.8 b–e
FR01	3	4	5	4	4	3	3.8 b–e
NZ03	2	2	5	5	5	4	3.8 b–e
NZ04	5	2	2	5	5	4	3.8 b–e
IR02	3	3	5	4	4	3	3.7 c–f
NZ03	2	2	5	5	5	4	3.7 c–f
AB05	3	4	4	4	3	3	3.5 e–d
MB12	3	4	4	3	4	3	3.5 e–d
IR03	3	3	3	3	4	4	3.3 e–g
NZ01	3	3	3	3	4	3	3.2 e–h
FR03	3	3	3	3	4	3	3.2 e–h
MB14	3	3	3	4	3	3	3.2 e–h
NZ07	3	3	4	4	3	2	3.2 e–h
MB07	3	3	3	3	3	3	3.0 f–i
AB02	2	3	3	3	3	2	2.7 g–j
MB16	2	2	3	3	3	3	2.7 g–j
MB13	2	3	4	3	2	2	2.7 g–j
MB15	3	2	3	3	3	2	2.7 g–j
MB18	2	3	3	3	2	2	2.5 h–j
MB08	2	2	3	3	3	2	2.5 h–j
MB17	2	2	3	3	2	3	2.5 h–j
SK08	2	2	3	3	3	2	2.5 h–j
SK03	2	2	2	3	3	3	2.5 h–j
MB11	2	2	3	3	3	2	2.5 h–j
MB04	2	2	3	2	3	2	2.3 h–j
AB03	2	2	3	2	2	3	2.3 h–j
MB09	3	2	3	2	2	2	2.3 h–j
NZ05	2	2	3	2	3	2	2.3 h–j
SK01	2	2	2	3	3	2	2.3 h–j
SK07	2	2	3	3	2	2	2.3 h–j
MB02	2	2	3	2	2	2	2.2 ij
MB06	2	2	3	2	2	2	2.2 ij
MB01	2	2	3	2	2	2	2.2 ij
MB10	2	2	2	2	2	2	2.0 j
IR05	2	2	2	2	2	2	2.0 j
AB04	2	2	2	2	2	2	2.0 j
NZ06	2	2	2	2	2	2	2.0 j
NZ08	2	2	2	2	2	2	2.0 j
AB01	2	2	2	2	2	2	2.0 j
SK04	2	2	2	2	2	2	2.0 j
SK05	2	2	2	2	2	2	2.0 j
SK06	2	2	2	2	2	2	2.0 j
MB03	2	2	2	2	2	2	2.0 j
AB07	2	2	2	2	2	2	2.0 j
MB05	2	2	2	2	2	2	2.0 j
UK02	2	2	2	2	2	2	2.0 j
Mean[z]	2.6 c	2.7 bc	3.3 a	3.2 a	3.1 ab	2.7 bc	…

[x] AU = Australia; UK = United Kingdom; NZ = New Zealand; SK = Saskatchewan Canada; IR = Ireland; FR = France; AB =Alberta, Canada; MB = Manitoba, Canada.

[y] Mean DISA for each isolate on six differential cultivars. Means followed by the same letter in the column were not significantly different at $P = 0.05$ according to Duncan's multiple range test (DMRT).

[z] Mean of DISA of 56 isolates on each differential cultivar. Means followed by the same letter in the row were not significantly different at $P = 0.05$ (DMRT).

DISA. Symptoms of Mycosphaerella blight usually occurred on the most susceptible cv. AC Tamor within 2 to 4 days after inoculation and on the most resistant cv. Danto within 2 to 6 days (Table 5). Significant differences of DISA were observed among the isolates and differential hosts (Table 2). The mean DISA of 56 isolates on AC Tamor, Bohatyre, and Miko were 2.6 to 2.7 days, which was significantly shorter than the 3.3 days of DISA on resistant cv. Danto (Table 5). In all, 33 isolates, the majority of which were from Canada, had a significantly shorter DISA (≤3.0 days) than the 14 isolates from other countries with a longer DISA (≥3.8 days). Four isolates from Australia had a significantly longer DISA (≥3.8 days) than those with DISA of <3.2 days. A continuous wide variation of DISA was observed among the isolates tested.

AFLP analysis. The 86 polymorphic DNA fragments generated by six AFLP primer combinations were scored as discrete character data. AMOVA analysis showed that 57.2% of variation was caused by differences among populations, and 42.8% was due to molecular diversity within populations. The dendrogram produced from combined AFLP data from six primer pair sets was used to assess relatedness among isolates (Fig. 1). The 58 isolates were clustered as two distinct groups using UPGMA with the genetic distance coefficient. The four isolates from Australia were clustered in one group, although the differences in virulence between AU03 and the other three Australian isolates were significant (Table 3) and the mean DISA of AU03 on the six differential hosts also was significantly different from AU01 (Table 5). All isolates from other countries were clustered together in another group, within which five isolates collected from Gore, New Zealand (NZ01, NZ02, NZ03, NZ04, and NZ05) were clustered in one subgroup, while the other six isolates from Saunders, New Zealand were clustered into another distinct subgroup (NZ07, NZ08, NZ09, NZ10, and NZ11). All Canadian isolates in different pathotypes were clustered within one subgroup with a lower genetic distance coefficient (<0.04) except for four isolates (SK05, SK06, AB02, and MB03). Two isolates (UK1 and UK2) from the United Kingdom with significantly different DISA

and virulence in different pathotypes were clustered together. These results showed that the isolates from Canada, New Zealand, Australia, and the United Kingdom in this study were geographically clustered together, respectively. However, the isolates from Ireland and France did not display this character. Three isolates from France were closely clustered with Canadian isolates in genetic relationship, whereas the isolates from Ireland did not show an obvious genetic relationship with other isolates. Significant differences in virulence and DISA of isolates were detected within the same cluster although the isolates in the same cluster had higher genetic similarity, such as isolates in the Australian isolate cluster (Fig. 1; Tables 3 and 5). The isolates in the same pathotype also could fall into different cluster (for example, pathotype 1). Therefore, virulence was not correlated to the genetic variation of the isolates in this study.

DISCUSSION

Fifteen pathotypes were detected in a collection of 56 isolates of *M. pinodes* based on the reaction patterns of the isolates on the six differential dry pea cultivars of *P. sativum*. Pathotype 1, in which all isolates were virulent to each of the six differential hosts, consisted mainly of isolates from Canada and New Zealand. In all, 23 of 32 Canadian isolates (71.9%) and 6 of 10 isolates from New Zealand (60%) were classified as this pathotype. Xue et al. (33) tested the pathogenicity of 275 isolates from Canada on nine differential hosts; 80% of those isolates belonged to pathotype 1, and were virulent to all nine differential hosts used in their experiments. Although the differential hosts used in our study differed from the previous research, our results agree with the previous conclusions, suggesting that the *M. pinodes* population in Canada is composed of virulent isolates and is relatively homogenous in virulence. In addition, the isolates from Canada generally were more virulent than the isolates from other countries. All eight less-virulent isolates, composing pathotypes 14 and 15, were collected from other countries. *M. pinodes* is transmitted readily in seed. In Canada, frequent exchange of breeding materials may facilitate the movement of the pathogen between provinces and thus maintain homogeneity in virulence among isolates of *M. pinodes.*

Danto was considered the most resistant differential cultivar in previous reports (22,33). In the present study, it was resistant to 20 of the 56 isolates. Although Danto was susceptible to 85% of Canadian *M. pinodes* isolates, its overall disease severity was lower than that of other individual differential hosts. The fact that Danto is the most resistant over time and in different countries, as well as its lineage-wide resistance to *M. pinodes* populations, suggests that the resistance of Danto may be relatively stable. This information is important for breeders interested in developing durable resistance to Mycosphaerella blight.

Although a number of pathotypes of *M. pinodes* have been identified in several countries, it is difficult to compare the pathotypes from different studies due to the use of different evaluating criteria, such as different numbers and genotypes of differential hosts, and inconsistent scales of

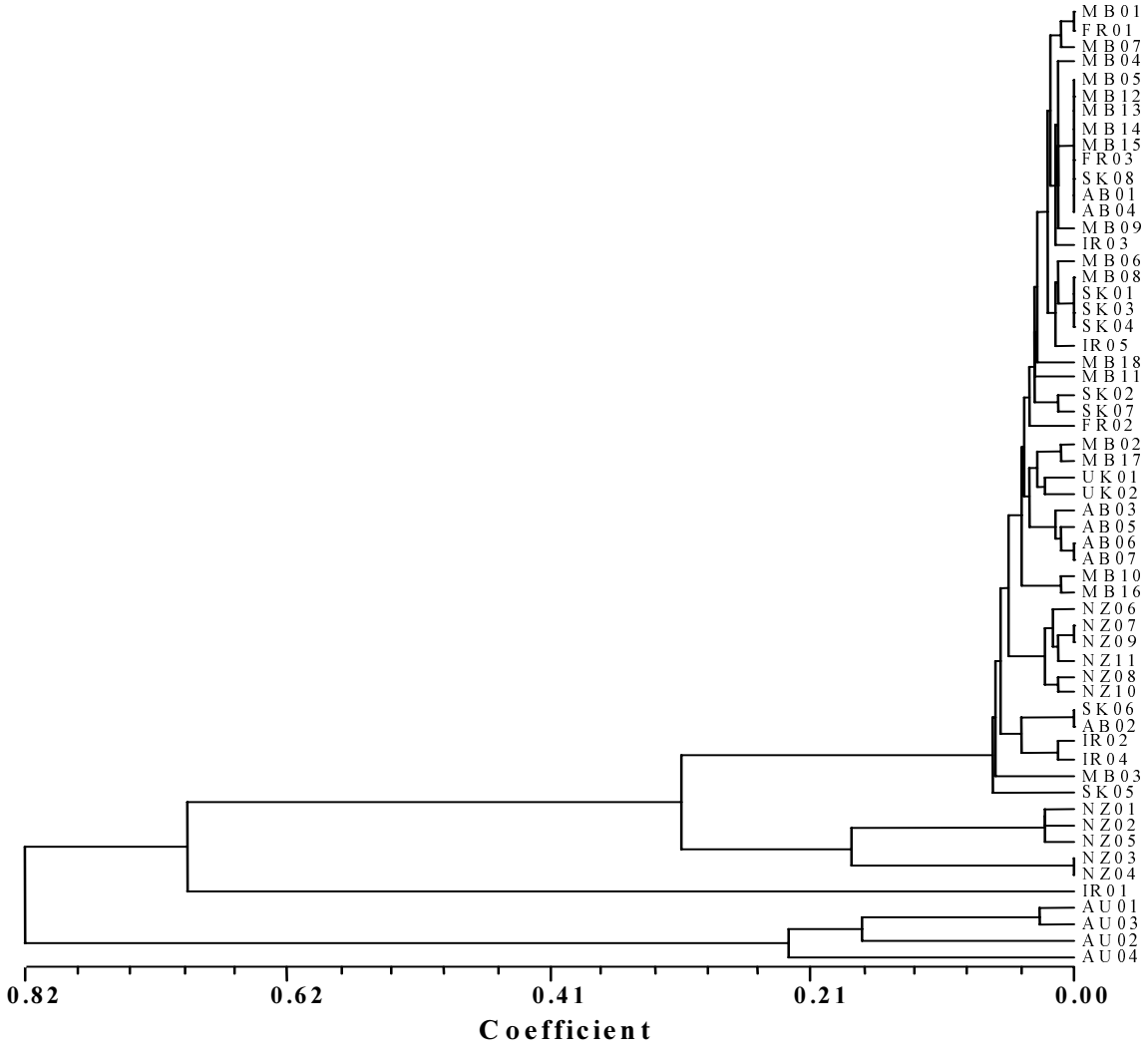

Fig. 1. Estimates of genetic similarity among *Mycosphaerella pinodes* isolates collected from Manitoba (MB), Saskatchewan (SK), Alberta (AB), the United Kingdom (UK), France (FR), Ireland (IR), New Zealand (NZ), and Australia (AU) based on amplified fragment length polymorphisms. Bootstrap values (1,000 replicates) for branch nodes ranged from 10 to 100%.

disease resistant assessments. In addition, it was reported that the level of inoculum pressure (22) significantly affects the expression of symptoms. An excessive inoculum pressure might mask the difference between resistance and susceptibility among pea cultivars (30). Previous research has found no correlation between foot rot and foliar disease scores (22,28), suggesting that pathotypes from the respective assessment on foot and foliar symptoms may be different. In addition, it was reported that foliar, stem, and pod blights were more severe at plant maturity than at the seedling stage, suggesting that the evaluation for seedling resistance does not address adult plant resistance and might lead to an erroneous explanation of adult resistance (1,17). Therefore, there is a substantial need for the development of a set of genetically-defined differential hosts, such as near-isogenic lines with the difference of single resistance genes and a standard procedure for resistance assessment to standardize and compare the results from different studies.

Virulence data alone may not reflect the true genetic variability and evolutionary history of the isolates studied. For example, isolates that are genetically distinct may have similar or identical virulence patterns because they have been subjected to the same selection pressure by a common set of hosts. Molecular markers that are selectively neutral and randomly distributed in a genome can provide additional useful information on genetic variation of pathogen populations (24). The AFLP approach is objective, repeatable, and unaffected by environmental factors, and it provides a direct assessment of genetic relationships of the fungal isolates at the DNA level. This study showed that the AFLP technique was useful for the characterization of intraspecific variation among the *M. pinodes* isolates. Six primer combinations enabled the amplification of more than 500 fragments, of which 17% were polymorphic.

Various studies have shown that it is only on rare occasions that all isolates originating from the same host have been found to be genetically or molecularly similar (29). In these cases, plants with different genotypes may have a biological role in applying selection pressure on the pathogen. For example, González et al. (11) found that a perfect correlation between genotype and pathotype of *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib. on common bean seldom is observed. However, a correlation between the defined cluster and locations where isolates were collected could be established. In their analysis, the host selection may direct geographic distribution of isolates. In the present study, the four isolates from Australia were clustered in a distinct group although there were significant differences detected in virulence among isolates. Similarly, five isolates collected in Gore and six from Saunders, New Zealand were clustered in two separate groups, regardless of diversity of virulence. However, we do not have sufficient data to corroborate the selection pressures that resulted in geographical clusters of the isolates from Australia and New Zealand. Specific pea genotypes growing in these regions also might be one important selection pressure.

Most Canadian isolates were clustered in a subgroup and further divided into subgroups in lineage, but these subgroups were not widely divergent in genotype. Seed contaminated by the pathogen are one of the most important sources of long-distance dissemination of *M. pinodes.* It is possible that the frequent exchanges of field pea breeding lines in western Canada have led to movement of the pathogen so that the isolates in Canada are relatively genetically homogenous. In addition, as reported for other host–pathogen interactions, higher complexity in terms of cluster number in phylogenetic analysis was considered as a consequence of a greater period of crop cultivation. The limited number of these clusters was taken to reflect the limited period of crop cultivation in a production area (26). Canadian isolates of *M. pinodes* were divided into many discrete clusters with small differences in genetic similarity. This status might be caused by the selection of various cultivars on the *M. pinodes* population during a relatively long period of cultivation.

Two isolates from the United Kingdom fell into two distinct pathotypes. However, this could not characterize the pathogenic variation and molecular diversity of *M. pinodes* populations in the United Kingdom due to the insufficient number of isolates tested. On the basis of the results of this study, the lineage of Australian isolates determined by AFLP analysis seemed to be associated with their virulence, where all four Australian isolates were less aggressive on six differential hosts although they were classified into different pathotypes. The isolates from France and the United Kingdom had higher similarity with Canadian isolates and were closely clustered with Canadian isolates, suggesting that transcontinental movement of the pathogen might occur and the pathogen might be dispersed over a large geographical area, perhaps by seed exchange.

Australian isolates and the remainder of isolates tested were clustered in two distinct lineages. The genotypes that were detected among four Australian isolates were different from those of Canadian isolates. We do not have sufficient data to infer the causes of the genotypic differences. Sexual reproductive isolation is one of the most important assumptions for molecular divergence among species (4). The longer the period of isolation of reproduction, the greater the divergence in genotypes because no genetic exchanges have occurred between isolates from isolated regions. Further work needs to be conducted by analyzing more Australian isolates and investigating the pathogen movement with hosts to confirm whether Australian isolates have been genetically isolated from isolates from other countries.

There were a number of cases where a cluster contained two distinct pathotypes, suggesting that both distinct pathotypes had evolved from a recent common ancestor (19). In contrast, a pathotype was composed of more than one cluster, indicating that convergent pathotype evolution can occur from genetically distinct ancestors (19). These two types of patterns also were found in our study. For example, the cluster composed of five isolates from New Zealand contained the isolates from two pathotypes, NZ01, NZ03, NZ04, and N05 from pathotype 1 and NZ02 from pathotype 15, suggesting that five isolates might originate from a common ancestor, but pathogenicity for isolate NZ02 was divergent from the other four isolates. Pathotype 1 contained several clusters composed of isolates from different countries sampled, suggesting that this pathotype might evolve from isolates with different recent ancestors. However, four Australian isolates were relatively correlated between pathotype and molecular similarity, suggesting that they were relatively stable in origin.

Based on virulence test data and AFLP analysis, all isolates from Canada, New Zealand, Australia, France, and Ireland were classified into 15 pathotypes. Isolates were genetically clustered based on geographic locations rather than pathotypes. Most Canadian isolates belonged to pathotype 1 with higher virulence, and remained relatively genetically homogeneous. These results presumably showed that no specific race existed in the field in Canada, and breeding programs would develop local horizontal resistant pea cultivars.

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