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Molecular and genetic analyses of geographic variation in isolates of *Phoma macrostoma* used for biological weed control

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Abstract: Molecular and genetic approaches were used to evaluate the genetic relatedness among isolates of the fungus *Phoma macrostoma* Montagne originating from Canada and Europe and to other species in the genus *Phoma*. Distinct differences were observed in genetic variation among nine species of the genus *Phoma*. Randomly amplified polymorphic DNA (RAPD) revealed the presence of intraspecific genetic variation among the isolates of *P. macrostoma,* with the isolates being used for biological weed control being distributed in a distinct phylogenetic cluster. Additional variation within the biocontrol isolate cluster in *P. macrostoma* was revealed by pulsed field gel electrophoresis (PFGE), which showed that biocontrol isolates generated two different chromosomal profiles, however the profiles did not relate to their Canadian ecozone origin. Mating studies showed that biocontrol isolates of *P. macrostoma* from Canada did not produce sexual reproductive structures and were incapable of crossing. These studies also confirmed that no obvious differentiation exists among the biocontrol isolates of *P. macrostoma* from Canadian Ecozones 3 and 4.

Key words: biocontrol, genetic variation, mating, PFGE, *Phoma macrostoma,* RAPD

INTRODUCTION

The application of micro-organisms for biological pest control is an approach that has gained acceptance as a useful tool for integrated pest management programs. However applications using large quantities of specific micro-organisms to a defined area of release need to be studied to ensure environmental safety (Charudattan 1988, Cook et al 1996, Hintz et al 2001, Tebeest et al 1992, Teng and Yang 1993, Templeton 1992, Wapshere 1974). One aspect of the biosafety assessment is to determine the genetic variability of the biocontrol strain in relation to other similar isolates and species that may exist in the intended area of release. For the purposes of registering a microbial pest control product in Canada, the Pest Management Regulatory Agency has designated five ecozones across the country that are based on large, ecologically distinctive areas resulting from generalized criteria involving landform, soil, water, climate, flora, fauna and human factors (FIG. 1). To avoid or minimize the risk of introducing new alleles into an area through asexual or sexual interactions, it should be known that the biocontrol strain is similar genetically to the local populations before releasing it across ecozones (Hintz et al 2001, Slatkin 1987, Taylor et al 1995, Templeton et al 1979).

Various broadleaf weed pests including Canada thistle (*Cirsium arvense* L. [Scop.]), dandelion (*Taraxacum officinale* Weber ex F.H. Wigg.), chickweed (*Stellaria media* [L.] Vill.) and scentless chamomile (*Matricaria perforata* Mérat) are economically important in western Canada (Van Aker et al 2000). Ten isolates of *Phoma macrostoma* Montagne recently were discovered to have bioherbicidal effects on these weeds (Bailey and Derby 2001). Application of these isolates to soil resulted in chlorosis and seedling death of several broadleaf weed species but had no effect on monocotyledonous plants. The 10 isolates originated in two Canadian ecozones and their biological and morphological characteristics appeared similar, but the genetic variation occurring in them was unknown.

Randomly amplified polymorphic DNA (RAPD) is an effective molecular fingerprinting tool that identifies genetic variation at the species level and higher analyses (Arisan-Atac et al 1995, Chang et al 1996, Gosselin et al 1996, Hintz et al 2001, McDermott et al 1994, Nicholson and Rezanoor 1994, Zimand et al 1994). Pulsed field gel electrophoresis (PFGE) also has been used widely and proved to be a reliable tool in studies of genetic variation in *Phoma lingam* Tode ex Fr. (sexual state *Leptosphaeria maculans* [Desm.] Ces & de Not.) (Chen et al 1996; Howlett 1997; Koch et al 1991; Lim and Howlett 1994; Morales et al 1993; Moreno-Rico et al 2002; Plummer and Howlett 1993,

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FIG. 1. Five microbial pesticide ecozones of Canada. (Source: Pest Management Regulatory Agency, 2001).

1995). More conventional studies on genetic variation within a species may be evaluated by observing mating between fungal isolates (Cozijnsen et al 2000, Mengistu et al 1995, Moreno-Rico et al 2002). These molecular tools and mating studies presumably would be useful to determine the genetic variability in the biocontrol isolates of *P. macrostoma*.

The objectives of the present study were to evaluate the genetic variation occurring among isolates *P. macrostoma* with different geographic origins and to show the genetic relatedness of *P. macrostoma* with other species of *Phoma*.

MATERIALS AND METHODS

Fungal strains and growth conditions.—Ten biocontrol isolates of *P. macrostoma* and 18 reference isolates comprising nine *Phoma* spp. were used. The biocontrol isolates came from Canada thistle plants found in Canadian Ecozone 3 (94-44B, 85-24B, 95-268B, 89-25A2, 94-359A, 97-12B and 97-15B2) and Ecozone 4 (95-54A1, 94-26 and 94-134). They are on deposit with the International Depositary Authority of Canada, Winnipeg, Manitoba. The other isolates originated in North America and Europe. The host plant and geographic origins of all the fungal isolates are listed (TABLE I). All fungal isolates were derived from single-spore cultures and grown on V-8 juice agar at room temperature (20–24 C) under a 16 h photoperiod at 100–150 $\mu E/m^2/s$.

DNA extraction.—Mycelial tufts (100 mg) were scraped from the surface of 14 d old colonies growing on V-8 juice agar, weighed and transferred into an 1.5 mL microcentrifuge tube. The sample was ground into fine powder with liquid nitrogen and a plastic minipestle before being used immediately for DNA extraction with the DNeasy® Plant Mini Kit (QIAGEN) according to the supplier's protocols.

RAPD analyses.—The primers used were 10-mer oligonucleotides purchased from the Biotechnology Laboratory (University of British Columbia, Vancouver, British Columbia) (TABLE II). DNA amplification was performed in 25 μL reaction mixtures, each containing 20 ng template DNA, 1 unit AmpliTaq Gold Polymerase (5U/μL, Applied Biosystems), 1/10 volume (2.5 μL) of GeneAmp® 10×PCR Buffer II, 2.0 mM $MgCl_2$, 0.2 μM primer, and 100 μM of each dNTPs (MBI Fermentas). The PCR program was initiated at 94 C for 10 min, followed by 45 cycles of 94 C 1 min, 35 C 1 min, 72 C 2 min, at 72 C 10 min and held at 10 C. All amplification reactions were performed in an Alpha Unit™ Block Assembly for PTC DNA Engine™ Systems (MJ Research Inc.). Amplification products were resolved by electrophoresis in 1.5% agarose gels. Each amplified DNA fragment was scored as either present or absent for each isolate. To check reproducibility of amplicons, the amplification of the DNA templates was repeated twice; there were no problems with reproducibility.

PFGE analysis.—The chromosomal DNA of the biocontrol isolates of *P. macrostoma* from Ecozones 3 and 4, with one isolate of *P. medicaginis* and three isolates of *P. herbarum,* was compared by pulsed field gel electrophoresis (PFGE) analysis. Single-spore cultures of the isolates were maintained by subculture on 10% V8-juice agar and incubated at room temperature (20–24 C) with a 16 h photoperiod at 100–150 $\mu E/m^2/s$. Pycnidiospores of 14 d old cultures were harvested by scraping the surface with 0.05% Tween 80 and filtering the spore suspension through two layers of Miracloth. Chromosome inserts were prepared as described by Plummer and Howlett (1993). Electrokaryotyping was performed on a contour-clamped homogeneous electric field (CHEF) DR II system (Bio-Rad, Mississauga, Ontario) with 0.5× TBE buffer at 11 C according to Chen and Séguin-Swartz (1999), with minor modifications: The initial switch time was 600 s and final switch time was 600 s at 100 V for 72 h, followed by initial 400 s and final 400 s at 100 V for 46 h. Gels were stained in 0.1 μg/mL ethidium bromide solution and photographed. The chromosomal DNA bands were recorded for each isolate as present or absent.

Genetic mating.—Five biocontrol isolates of *P. macrostoma* from Ecozones 3 and 4 were crossed among themselves and to tester isolates of *P. lingam* with different mating types (Mat+ or Mat−). Although no sexual state for *P. macrostoma* has been reported, it was important to confirm that crossing could not occur among the biocontrol isolates. *Phoma lingam* was used because it has known mating types, can reproduce sexually under laboratory conditions, is an important plant pathogen of canola (*Brassica napus* and *B. rapa*) in Ecozones 3 and 4 and might have some genetic relatedness to *P. macrostoma*. All fungal isolates were cul-

TABLE I. *Phoma* isolates used in the studies

Species	Isolate	Host of origin	Place of origin[a]	Source[b]
Phoma macrostoma	85-24B	*Cirsium arvense* (L.) Scop.	Saskatchewan, Canada [3]	SRC
P. macrostoma	89-25A2	*Cirsium arvense*	Saskatchewan, Canada [3]	SRC
P. macrostoma	94-26	*Cirsium arvense*	Ontario, Canada [4]	SRC
P. macrostoma	94-44B	*Cirsium arvense*	Saskatchewan, Canada [3]	SRC
P. macrostoma	94-134	*Cirsium arvense*	New Brunswick, Canada [4]	SRC
P. macrostoma	94-359A	*Cirsium arvense*	Saskatchewan, Canada [3]	SRC
P. macrostoma	95-54A1	*Cirsium arvense*	Nova Scotia, Canada [4]	SRC
P. macrostoma	95-268B	*Cirsium arvense*	Saskatchewan, Canada [3]	SRC
P. macrostoma	97-12B	*Cirsium arvense*	Alberta, Canada [3]	SRC
P. macrostoma	97-15B2	*Cirsium arvense*	Alberta, Canada [3]	SRC
P. macrostoma var. *incolorata*	CBS 839.84	*Hordeum vulgare* L.	Monheim, Germany	CBS
P. macrostoma var. *macrostoma*	CBS 154.83	*Philadelphus coronaries* L.	Baarn, Netherlands	CBS
P. macrostoma var. *macrostoma*	CBS 482.95	*Larix decidua* Mill.	München, Germany	CBS
P. macrostoma var. *macrostoma*	CBS 488.94	*Forsythia* sp.	Baarn, Netherlands	CBS
P. macrostoma var. *macrostoma*	CBS 837.84	*Triticum aestivum* L.	Monheim, Germany	CBS
P. dennisii var. *dennisii*	CBS 135.96	*Solidago canadensis* L.	Ontario, Canada [4]	CBS
P. lingam	Leroy	*Brassica napus* L.	Saskatchewan, Canada [3]	SRC
P. lingam	Peace-3	*Brassica napus*	British Columbia, Canada [1]	SRC
P. lingam	Pl 86-12	*Brassica napus*	Manitoba, Canada [3]	SRC
P. lingam	Pl 89-19	*Brassica sp.*	Unknown, Australia	SRC
P. lingam	Pl 89-21	*Brassica napus*	Mt Barker, Australia	SRC
P. herbarum	Al	*Taraxacum officinale* Webber ex F.H. Wigg.	Ontario, Canada [4]	G. Boland
P. herbarum	AIV	*Taraxacum officinale*	Ontario, Canada [4]	G. Boland
P. herbarum	G/5/2	*Taraxacum officinale*	Ontario, Canada [4]	G. Boland
P. chrysanthemicola	90-64	*Ambrosia artemisifolia* L.	Ontario, Canada [4]	SRC
P. chrysanthemicola	91-27I	*Ambrosia artemisifolia*	Ontario, Canada [4]	SRC
P. exigua	92-180-1	*Cirsium arvense*	Manitoba, Canada [3]	SRC
P. medicaginis	94-335A1	*Medicago lupulina* L.	Saskatchewan, Canada [3]	SRC
P. nebulosa	92-74	*Cirsium arvense*	Saskatchewan, Canada [3]	SRC
P. pomorum	91-177	*Ambrosia artemisifolia*	Iowa, USA	SRC

[a] The number in parentheses refers to the Canadian ecozone.

[b] SRC = Saskatoon Research Centre, Agriculture and Agri-Food Canada, Saskatoon, Canada; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

tured on 2% V-8 juice agar medium for 14 d. On a fresh agar plate, a 10 mm mycelial plug from each isolate in the mating pair combination was placed ca. 7.5 cm apart. The cultures were incubated at 16 h photoperiod under cool white fluorescent light (100–150 $\mu E/m^2/s$) at 22 C for 3 d or until it was possible to determine the growth rate of each isolate. A sterile wooden toothpick was placed between the two isolates at the junction where they would meet and merge (Mengistu et al 1993, 1995) and incubated at 10 C with the same light conditions 4–7 wk. The toothpick and the culture media were examined at 5, 6 and 7 wk for mature pseudothecia under a binocular microscope at 40×

TABLE II. The 10-mer oligonucleotide primers selected for RAPD analyses

Primer No.	Sequence	GC %	Source
308	5′AGCGGCTAGG3′	70	University of British Columbia
350	5′TGACGCGCTC3′	70	University of British Columbia
356	5′GCGGCCCTCT3′	80	University of British Columbia
357	5′AGGCCAAATG3′	50	University of British Columbia
382	5′ATACACCAGC3′	50	University of British Columbia
734	5′GGAGAGGGAG3′	70	University of British Columbia
736	5′GAGGGAGGAG3′	70	University of British Columbia
740	5′GGAGGGAGGA3′	70	University of British Columbia

magnification. Pseudothecia that formed on toothpicks or culture medium in the plates were extracted and crushed on a glass slide in a drop of water. The slide was examined for asci containing ascospores with a light microscope at 40–100× magnification. The presence or absence of mature pseudothecia, asci and ascospores was recorded.

Phylogenetic data analyses.—The presence (1) or absence (0) of DNA bands in agarose gel were converted as 0–1 matrix. The data were analyzed by the phylogenetic software package TREECON® for Windows (Version 1.3b, Van de Peer and de Wachter 1994). The evolutionary distance estimation was performed according to Nei and Li (1979). An unweighted pair group cluster method with arithmetic averages (UPGMA; Benzécri 1973) was used to infer tree topology. Bootstrap analyses were included in the distance estimation and tree topology to place confidence intervals on phylogenies (Efron and Gong 1983, Felsenstein 1985, Sworfford et al 1996).

RESULTS

Genetic variation in Phoma *isolates as revealed by RAPD.*—A total of 96 polymorphic DNA bands were produced by amplification with eight random primers, and a phylogenetic tree was generated from the data showing only the results from two primers (FIG. 2A, B). With primers 734 and 736, banding patterns were detected for all isolates of *P. macrostoma, P. lingam* and one isolate of *P. chrysanthemicola.* Other primers (not shown) detected *P. exigua, P. medicaginis, P. nebulosa* or *P. pomorum,* but not *P. macrostoma,* which was the species of prime interest. Both inter- and intraspecific genetic variation in the *Phoma* species and isolates was observed for all primers.

All 10 biocontrol isolates of *P. macrostoma* were related closely and clustered within the group designated as ''*P. macrostoma* Biocontrol Isolates'' (FIG. 2B). The isolates were somewhat variable from each other, to a limited extent within this group. For example 97-15B2 had a banding pattern slightly different than the other biocontrol isolates using Primer 734 (FIG. 2A). However the biocontrol isolates did not form distinct subgroups based on their geographic origins because the isolates from Ecozone 3 (94-44B, 85-24B, 95-268B, 89-25A2, 94-359A and 97-12B) and Ecozone 4 (95-54A1, 94-26 and 94-134) were distributed within the same cluster.

The RAPD fragment patterns from the biocontrol isolates were different when compared to the other isolates of *P. macrostoma* var. *macrostoma, P. macrostoma* var. *incolorata* and the other isolates of various *Phoma* species (FIG. 2A, B). The isolates of *P. macrostoma* var. *macrostoma* originating from *Larix, Forsythia, Philadelphus* and *Triticum* were more similar to *P. dennisii* from *Solidago* than to the biocontrol isolates from Canada thistle (FIG. 2B). Also the isolates of *P. herbarum* were genetically distinct from the biocontrol isolates (FIG. 2B). The primers did not detect genetic variation among *P. lingam* Leroy, *P. nebulosa* 92-74, *P. pomorum* 91-177, *P. chrysanthemicola* 90-64, *P. exigua* 92-180-1 and *P. medicaginis* 94-335A1. These latter species were more distantly related to the biocontrol isolates.

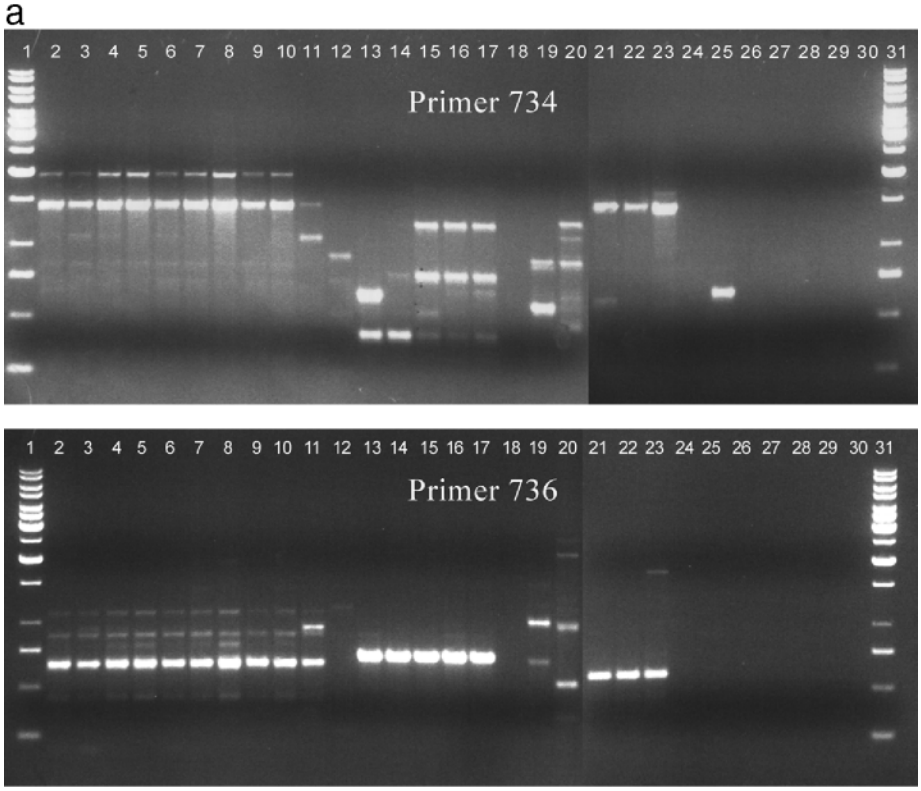
Genetic variation in Phoma *isolates as revealed by PFGE.*—Twenty-seven polymorphic chromosomal DNA bands were generated with the CHEF analysis. The chromosomal profiles of *P. medicaginis* and *P. herbarum* were different from those of *P. macrostoma* (FIG. 3A) but exhibited some similarity to *P. macrostoma.* However the biocontrol isolates of *P. macrostoma* separated into two different categories of chromosomal profiles (Types I and II). Type I included isolates 94-44B, 85-24B, 94-26, 95-268B and 95-54A1, while the other five biocontrol isolates belonged to Type II (FIG. 3B). Similar to that revealed by RAPD analysis, isolates from Ecozone 3 (94-44B, 85-24B, 95-268B, 89-25A2, 94-359A, 97-12B and 97-15B2) and Ecozone 4 (95-54A1, 94-26 and 94-134) were distributed randomly in these two chromosome profile types. The phylogenetic tree also showed that the isolates of *P. herbarum* separated into two chromosome profiles (FIG. 3B).

Genetic variation in Phoma *isolates as revealed by crossing studies.*—The crossing pair combinations and formation of asexual or sexual reproductive structures are presented (TABLE III). Only mycelial growth and asexual sporulation from pycnidia were observed when all *P. macrostoma* isolates were crossed with themselves, among each other or with the different mating types of *P. lingam.* Pseudothecia containing ascospores were formed only by crossing different mating type isolates of *P. lingam.*

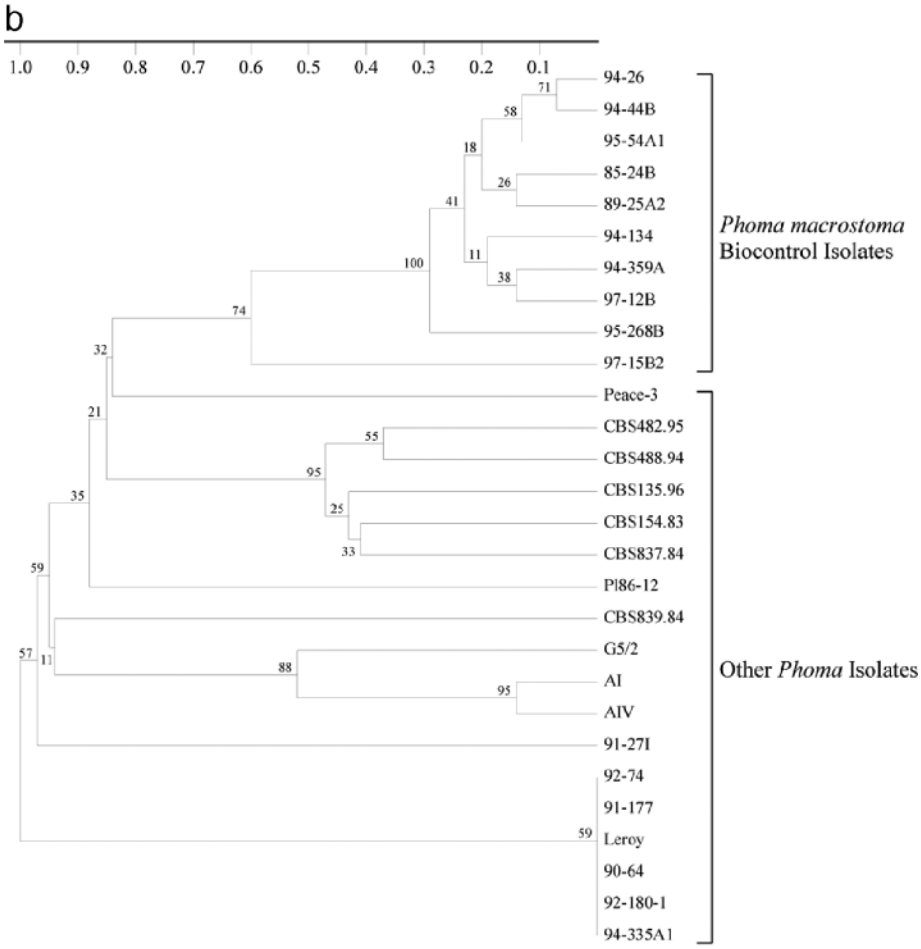
DISCUSSION

Genetic variation in the population of a biocontrol fungus must be assessed to understand the affect of a specific strain on the natural population after the release (Hintz et al 2001). Concerning the release of a bioherbicide across ecozones, the biocontrol isolate should be genetically similar to local populations to reduce the potential unexpected affects, such as introducing new alleles that could alter host range or pathogen growth and survival. Various approaches have been adopted for such assessment. Recent reports on the mycoherbicide *Chondrostereum purpureum* (Pers. : Fr.) Pouzar by biochemical and molecular marker analyses showed no host or geographic specialization in this fungus and that the introduction of rare pathogenicity alleles from isolates used

a


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
Primer 734
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
Primer 736

b


1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1
94-26
94-44B
95-54A1
85-24B
89-25A2
94-134
94-359A
97-12B
95-268B
97-15B2
Peace-3
CBS482.95
CBS488.94
CBS135.96
CBS154.83
CBS837.84
P186-12
CBS839.84
G5/2
AI
AIV
91-27I
92-74
91-177
Leroy
90-64
92-180-1
94-335A1
Phoma macrostoma Biocontrol Isolates
Other Phoma Isolates

as biocontrol agents was unlikely (Shamoun and Wall 1996; Ramesfield et al 1996, 1999; Gosselin et al 1999; Hintz et al 2001). In our present study molecular and genetic tools were used to investigate the genetic variation of a potential broadleaf biocontrol fungus, *P. macrostoma*.

RAPD has been used widely as an effective molecular tool to evaluate genetic variance at or below the species level. In our RAPD analyses different species and isolates of the genus *Phoma* produced different DNA profiles on the gels denoting significant inter- and intraspecific genetic variation. The biocontrol isolates of *P. macrostoma* were most similar to each other but uniquely different than other isolates within the same species (*P. macrostoma* var. *macrostoma* and *P. macrostoma* var. *incolorata*) and quite different from other *Phoma* species, including *P. lingam, P. herbarum, P. denisii* var. *denisii, P. medicaginis, P. nebulosa, P. pomorum, P. chrysanthemicola* and *P. exigua*. A related study on molecular monitoring of the biocontrol isolates of *P. macrostoma* in plant and soil environments also supported our findings. In that study a pair of PCR primers designed from the genomic DNA of the biocontrol isolates was found to be specific to only the biocontrol isolates and no bands were generated from other common soil fungi such as *Cochliobolus, Epicoccum, Fusarium, Penicillium, Pythium, Sclerotinia* and *Septoria* (Zhou et al 2004). The intraspecific variation occurring in *P. macrostoma* may be due to either geographic origin (Europe vs. Canada) or a unique trait (i.e. bioherbicidal activity) that evolved with a host-pathogen interaction. Although some variation occurred within the 10 biocontrol isolates, their distribution in the phylogenetic tree generated from the RAPD data were not based on geographic origin. This suggested that the *P. macrostoma* biological control isolates, which were isolated from Ecozone 3 or Ecozone 4, are genetically similar, so the use of those isolates across these two ecozones has little risk of introducing novel alleles into a local population. Genetic variation in fungal populations is common as illustrated by *P. lingam* (Johnson and Lewis 1990, McGee and Petrie 1978, Petrie 1969, Hassan et al 1991, Kuusk et al 2002). Our RAPD study also showed that the genetic distances among three *P. lingam* isolates were significantly different based on the confidence intervals used for bootstrap analyses. This demonstrated that these *P. lingam* populations were genetically diverse and movement of these populations likely would introduce new alleles into an area. With *P. macrostoma* there was obvious genetic variation among isolates from different continents, although the isolates from the two Canadian ecozones were relatively similar.

PFGE also was effective in distinguishing among different species and isolates according to chromosomal profiles. The phylogenetic analysis indicated that *P. macrostoma* may be more closely related to *P. herbarum* than other *Phoma* spp. The present study showed two distinctly different chromosomal profiles within the species *P. macrostoma*. However it is interesting to note that the 10 biocontrol isolates of *P. macrostoma* orginating from two different ecozones were distributed into the two chromosomal subgroups. At this time no other biological traits (such as host range or growth characteristics) have been associated with these two subgroups (personal observation based on unpublished data).

Studies of mating types of fungal isolates also may be useful for subspecies classification and evaluation of genetic variation. In the case of *P. macrostoma* it was demonstrated that the biocontrol isolates did not produce a sexual state in mating studies among themselves or when paired with known mating types from another *Phoma* species possessing the capability of sexual reproduction. Other researchers also have noted that *P. macrostoma* has no known teleomorph (Boerema et al 2004). The use of a crossing strategy was used to demonstrate to regulators that Canadian isolates of *P. macrostoma* were unable to reproduce

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FIG. 2. A. RAPD analyses of genetic variations among *Phoma* isolates with two 10-mer oligonucleotide primers (734 on top and 736 on bottom). Lanes 1 and 31, GeneRuler™ 1kb DNA Ladder (MBI Fermentas); Lanes 2–11, *Phoma macrostoma* isolates 85-24B, 89-25A2, 94-26, 94-44B, 94-134, 94-359A, 95-54A1, 95-268B, 97-12B, and 97-15B2; Lane 12, *P. dennisii* var. *dennisii* CBS135.96; Lanes 13–16, *P. macrostoma* var. *macrostoma* isolates CBS154.83, CBS482.95, CBS488.94 and CBS837.84; Lane 17, *P. macrostoma* var. *incolorata* CBS839.84; Lanes 18–20, *P. lingam* isolates Leroy, Peace-3 and Pl86-12; Lanes 21–23, *P. herbarum* isolates AI, AIV and G/5/2; Lanes 24–25, *P. chrysanthemicola* 90-64 and 91-27I; Lanes 26–29, *P. exigua* 92-180-1, *P. medicaginis* 94-335A1, *P. nebulosa* 92-74, and *P. pomorum* 91-177; Lane 30, Blank. B. Phylogenetic relationship among the biocontrol isolates of 10 *Phoma macrostoma* and 18 reference *Phoma* isolates generated by TREECON® for Windows with RAPD data. The biocontrol isolates were clustered together and genetically distant from the reference isolates. Within the cluster of biocontrol isolates, the isolates originating from two Canadian ecozones were scattered randomly. The evolutionary distance scale was placed on the top of the figure and a bootstrap value was presented on each node of the tree.

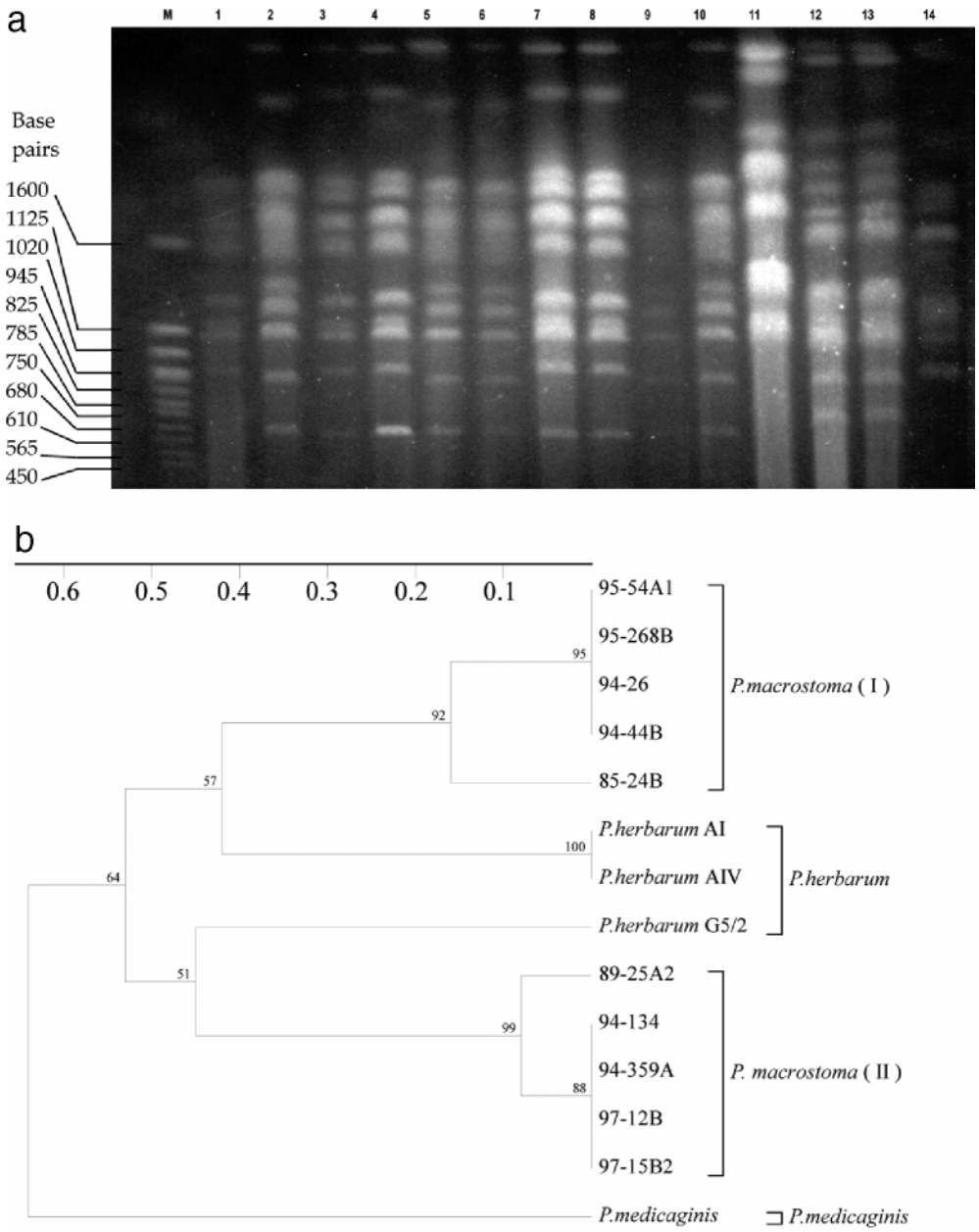

FIG. 3. A. Electrokaryotypes of *P. macrostoma* and several other *Phoma* isolates separated by pulsed field gel electrophoresis. Lane M corresponds to *Saccaromyces cerevisiae* marker; Lanes 1–10 to the *P. macrostoma* isolates 85-24B, 89-25A2, 94-26, 94-44B, 94-134, 94-359A, 95-54A1, 95-268B, 97-12B, and 97-15B2, respectively; Lane 11 to *P. medicaginis* 94-335 A1; and Lanes 12–14 to *P. herbarum* isolates AI, AIV and G5/5/2, respectively. B. Phylogenetic relationship among several *Phoma* isolates revealed by CHEF profiles. Two distinct subgroups were clustered within which limited variation was found. Biocontrol agents 94-44B, 85-24B, 94-26, 95-268B and 95-54A1 were clustered in subgroup I (Type I profile), while isolates 89-25A2, 94-134, 94-359A, 97-12B, and 97-15B2 in subgroup II (Type II profile). The evolutionary distance scale (placed on the top of the figure) and bootstrap values (presented on nodes of the tree) were calculated with TREECON® for Windows software.

sexually thus reducing the risk of developing new alleles through recombination and to demonstrate to plant breeders that there was no exchange of genetic material with an economically important plant pathogen, *P. lingam*. However the crossing approach did not consider the occurrence of other mechanisms of genetic exchange such as mutation or the parasexual cycle. This would require the use of a visible marker strategy, such as deploying antibiotic resistance in one isolate and the green fluorescent protein marker

TABLE III. Presence (+) or absence (0) of pseudothecia and ascospores after crossing biocontrol isolates of *Phoma macrostoma* (PM) among themselves and with *P. lingam* (PL) tester isolates with different mating types (Mat+ or Mat−)

Phoma species and isolate	PM 94-44B	PM 85-24B	PM 95-54A1	PM 94-26	PM 97-15B2	PM 94-134	PL 186-12 (Mat−)
P. macrostoma 94-44B	0	0	0	0	0	0	0
P. macrostoma 85-24B	0	0	0	0	0	0	0
P. macrostoma 95-54A1	0	0	0	0	0	0	0
P. macrostoma 94-26	0	0	0	0	0	0	0
P. macrostoma 97-15B2	0	0	0	0	0	0	0
P. lingam PL189-21 (Mat+)	0	0	0	0	0	0	+
P. lingam PL189-19 (Mat+)	0	0	0	0	0	0	+
P. lingam PL186-12 (Mat−)	0	0	0	0	0	0	0

in another isolate, and then subculturing the intermingled colonies to determine which clones now possessed both marker traits.

In conclusion *P. macrostoma* may be distinguished genetically from other *Phoma* spp. Ecological zones have not attributed to the genetic variation in the biocontrol isolates of *P. macrostoma,* as determined by molecular and genetic approaches. Therefore any *P. macrostoma* weed biocontrol isolates originating from Ecozone 3 and Ecozone 4 could be considered by the regulators for release across these ecozones as a biopesticide with low risk of gene flow from asexual or sexual interactions.

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LITERATURE CITED

Arisan-Atac I, Heidenreich E, Kubicek CP. 1995. Randomly amplified polymorphic DNA fingerprinting identifies subgroups of *Trichoderma viride* and other *Trichoderma* sp. capable of chestnut blight control. FEMS Microbiol Lett 126:249–255.

Bailey KL, Derby J. 2001. Fungal isolates and biological control compositions for the control of weeds. US Patent Application Serial No. 60/294,475. Filed 20 May 2001.

Benzécri JP. 1973. L'analyse des données. Tome I. La taxonomie. Paris, France: Dunod.

Boerema GH, de Gruyter J, Noordeloos ME, Hamers MEC. 2004. *Phoma* identification manual: differentiation of specific and infra-specific taxa in culture. Wallingford, UK: CABI Publishing.

Chang TT, Yang WW, Wang WY. 1996. Use of random amplified polymorphic DNA markers for the detection of genetic variation in *Phytophthora cinnamomi* in Taiwan. Bot Bull Acad Sin 37:165–171.

Charudattan R. 1988. Inundative control of weeds with indigenous fungal pathogens. In: Burge MN, ed. Fungi in biological control systems. New York: Manchester University Press. 269 p.

Chen CY, Séguin-Swartz G. 1999. Reaction of wild crucifers to *Leptosphaeria maculans,* the causal agent of blackleg of crucifers. Can J Plant Pathol 21:361–367.

———, Plummer KM, Howlett BJ. 1996. Ability of a *Leptosphaeria maculans* isolate to form stem cankers on Indian mustard segregates as a single locus. Eur J Plant Pathol 102:349–352.

Cook RJ, Bruckart WL, Coulson JR, Goettel MS, Humber RA, Lumsden RD, Maddox JV, McManus ML, Moore L, Meyer SF, Quimby PC Jr, Stack JP, Vaughn JL. 1996. Safety of microorganisms intended for pest and plant disease control: a framework for scientific evaluation. Biol Control 7:333–351.

Cozijnsen AJ, Popa KM, Purwantara A, Rolls BD, Howlett BJ. 2000. Genome analysis of the plant pathogenic ascomycete *Leptosphaeria maculans*; mapping mating type and host-specificity loci. Mol Plant Pathol 1:293–302.

Efron B, Gong G. 1983. A leisurely look at the bootstrap, the jacknife, and cross-validation. Am Stat 37:36–48.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Gosselin L, Jobidon R, Bernier L. 1996. Assessment of genetic variation with *Chondrostereum purpureum* from Québec by random amplified polymorphic DNA analysis. Mycol Res 100:151–158.

Hassan AK, Schulz C, Sacristan MD, Wöstemeyer J. 1991. Biochemical and molecular tools for the differentiation of aggressive and non-aggressive isolates of the oilseed rape pathogen, *Phoma lingam*. J Phytopathol 131:120–136.

Hintz WE, Becker EM, Shamoun SF. 2001. Development of genetic markers for risk assessment of biological control agents. Can J Plant Pathol 23:13–18.

Howlett BJ. 1997. Genome analysis of the fungal plant pathogen, *Leptosphaeria maculans* using pulsed field gel electrophoresis. Electrophoresis 18:1544–1547.

Johnson RD, Lewis BG. 1990. DNA polymorphism in *Leptosphaeria maculans*. Physiol Mol Plant Pathol 37:417–424.

Koch E, Song K, Osborn TC, Williams PH. 1991. Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphism in *Leptosphaeria maculans*. Mol Plant–Microbe Interact 4:341–349.

Kuusk A-K, Happstadius I, Zhou L, Steventon LA, Griese H, Dixelius C. 2002. Presence of *Leptosphaeria maculans* group A and group B isolates in Sweden. J Phytopathol 150:349–356.

Lim L, Howlett BJ. 1994. Linear plasmids, pLm9 and pLm10, can be isolated from the phytopathogenic ascomycete *Leptosphaeria maculans* by pulsed-field gel electrophoresis. Curr Genet 26:276–280.

McDermott JM, Brandle U, Dutly F, Haemmerli UA, Keller S, Muller KE, Wolfe MS. 1994. Genetic variation in powdery mildew of barley: development of RAPD, SCAR, and VNTR markers. Phytopathology 84:1316–1321.

McGee DC, Petrie GA. 1978. Variability of *Leptosphaeria maculans* in relation to blackleg of oilseed rape. Phytopathology 68:625–630.

Mengistu A, Hill CB, Williams PH. 1995. A toothpick method for mating *Leptosphaeria maculans*, the causal agent of blackleg of crucifers. Plant Dis 79:755–756.

———, Rimmer SR, Williams PH. 1993. Protocols for in vitro sporulation, ascospore release, sexual mating and fertility in crosses of *Leptosphaeria maculans*. Plant Dis 77:538–540.

Morales VM, Séguin-Swartz G, Taylor JL. 1993. Chromosome size polymorphism in *Leptosphaeria maculans*. Phytopathology 83:503–509.

Moreno-Rico O, Séguin-Swartz G, Nettleton JA, Luna-Ruiz JJ, Frias-Treviño AG, Romero-Cova S. 2002. Mexican isolates of *Leptosphaeria maculans* belong to the aggressive strain of the fungus. Can J Plant Pathol 24:69–73.

Nei M, Li WH. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273.

Nicholson P, Rezanoor HN. 1994. The use of random amplified polymorphic DNA to identify pathotype and detect variation in *Pseudocercosporella herpotrichoides*. Mycol Res 98:13–21.

Pest Management Regulatory Agency 2001. Regulatory Directive DIR 2002–02 Guidelines for the registration of microbial pest control agents and products. Website: www.hc-sc.gc.ca

Petrie GA. 1969. Variability of *Leptosphaeria maculans* (Desm.) Ces. & de Not., the cause of blackleg of rape [Doctoral thesis]. Saskatoon, Saskatchewan: University of Saskatchewan.

Plummer KM, Howlett BJ. 1993. Major chromosomal length polymorphisms are evident after meiosis in the phytopathogenic fungus *Leptosphaeria maculans*. Curr Genet 24:107–113.

———, Howlett BJ. 1995. Inheritance of chromosomal length polymorphisms in the ascomycete *Leptosphaeria maculans*. Mol Gen Genet 247:416–422.

Ramsfield TD, Becker EM, Rathlef SM, Tang Y, Vrain TC, Shamoun SF, Hintz WE. 1996. Geographic variation of *Chondrostereum purpureum* detected by polymorphisms in the ribosomal DNA. Can J Bot 74:1919–1929.

———, Shamoun SF, Punja ZK, Hintz WE. 1999. Variation in mitochondrial DNA of the potential biological control agent *Chondrostereum purpureum*. Can J Bot 77: 1490–1498.

Shamoun SF, Wall RE. 1996. Characterization of Canadian isolates of *Chondrostereum purpureum* by protein content, APIZYM and isozyme analysis. Eur J For Pathol 26:333–342.

Slatkin M. 1987. Gene flow and the geographic structure of natural populations. Science 236:787–792.

Swofford DL, Olsen GJ, Waddell PJ, Hillis DM. 1996. Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK, eds. Molecular Systematics. Sinauer Associates, Sunderland, USA. p 407–514.

Taylor JL, Pedras MSC, Morales VM. 1995. Horizontal transfer in the phytopathogenic fungal genus *Leptosphaeria* and host-range expansion. Trends Biotechnol 3:202–206.

TeBeest DO, Yang XB, Cisar CR. 1992. The status of biological control of weeds with fungal pathogens. Annu Rev Phytopathol 30:637–657.

Templeton GE. 1992. Regulations and guidelines: critical issues in biological control. In: Proceedings of the USDA–CSRS National Workshop. 10–12 Jun 1991, Vienna, Virginia. p 63.

———, TeBeest DO, Smith RJ Jr. 1979. Biological weed control with mycoherbicides. Annu Rev Phytopathol 17: 301–310.

Teng PS, Yang XB. 1993. Biological impact and risk assessment in plant pathology. Annu Rev Phytopathol 31: 495–521.

Van Acker RC, Thomas AG, Leeson JY, Knezevic SZ, Frick BL. 2000. Comparison of weed communities in Manitoba ecoregions and crops. Can J Plant Sci 80:963–972.

Van de Peer Y, de Wachter R. 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput Applic Biosci 10:569–570.

Wapshere AJ. 1974. A strategy for evaluating the safety of organisms for biological weed control. Ann Appl Biol 77:201–211.

Zhou L, Bailey KL, Derby J. 2004. Plant colonization and environmental fate of the biocontrol fungus, *Phoma macrostoma*. Bio Control 20:634–644.

Zimand G, Valinsky L, Elad Y, Chet I, Manulis S. 1994. Use of RAPD procedure for the identification of *Trichoderma* strains. Mycol Res 98:531–534.