Available online at www.sciencedirect.com
ScienceDirect
Journal of Invertebrate Pathology 98 (2008) 101–113
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Genetic diversity of *Metarhizium anisopliae* var. *anisopliae* in southwestern British Columbia

G. Douglas Inglis [a,*], Grant M. Duke [a], Mark S. Goettel [a], J. Todd Kabaluk [b]

[a] *Agriculture and Agri-Food Canada, Lethbridge Research Centre, 5403-1st Avenue S, Lethbridge, Canada AB T1J 4B1*
[b] *Agriculture and Agri-Food Canada, Agassiz Research Centre, Agassiz, Canada BC V0M 1A0*

Received 30 August 2007; accepted 2 December 2007
Available online 8 December 2007

Abstract

The abundance and genetic diversity of the entomopathogenic fungus, *Metarhizium anisopliae* var. *anisopliae*, in southwestern British Columbia (BC) and southern Alberta was examined. The fungus was found to be widespread in soil throughout southwestern BC, and was recovered from 56% of 85 sample sites. In contrast to southwestern BC, no *M. anisopliae* isolates were recovered in southern Alberta. An automated fluorescent amplified fragment length polymorphism (AFLP) method was used to examine genetic diversity. In excess of 200 isolates were characterized. The method identified 211 polymorphic amplicons, ranging in size from ≈92 to 400 base pairs, and it was found to be reproducible with a resolution limit of 86.2% similarity. The AFLP method distinguished *Metarhizium* from other entomopathogenic fungal genera, and demonstrated considerable genetic diversity (25 genotypes) among the reference strains of *M. anisopliae* isolates examined (i.e. recovered from various substrates and geographical locations). Although 13 genotypes of *M. anisopliae* var. *anisopliae* were recovered from southwestern BC soils, the vast majority of isolates (91%) belonged to one of two closely-related genotypes. Furthermore, these two genotypes predominated in urban, agricultural and forest soils. The reasons for the limited diversity of *M. anisopliae* var. *anisopliae* in southwestern BC are uncertain. However, findings of this study are consistent with island biogeography theory, and have significant implications for the development of this fungus for microbial control of pest insects.

Keywords: Mitosporic fungi; *Metarhizium anisopliae*; British Columbia; Wireworms; AFLP; Genetic diversity

1. Introduction

Members of the genus, *Metarhizium*, are entomopathogenic fungi that reproduce exclusively asexually (i.e. mitosporic fungi). The genus currently contains a number of morphologically distinct species including *M. album*, *M. anisopliae*, *M. cylindrosporae*, *M. flavoviride*, *M. guizhouense*, and *M. pingshaense* (Driver et al., 2000; Guo et al., 1986; Petch, 1931; Rath et al., 1995). *M. anisopliae* is a cosmopolitan taxon whose members fall into one of four primary genetic groupings, and include *M. anisopliae* var. *acridum*, *M. anisopliae* var. *anisopliae*, *M. anisopliae* var. *frigidum*, and *M. anisopliae* var. *lepidiotum* (Driver et al., 2000). Evidence has been obtained to indicate that these subspecies are non-interbreeding (Bridge et al., 1993; Driver et al., 2000; St. Leger et al., 1992). In selecting efficacious genotypes for microbial control programs and understanding factors that modulate epizootics, it is important to have an understanding of the population genetics represented within a particular ecosystem. Enkerli et al. (2005) examined the genetic diversity of *M. anisopliae* in Switzerland using microsatellite markers, but the population genetics of these fungi within defined geographical regions and in specific habitats has largely been ignored. However, Bidochka et al. (2001) examined the genetic diversity of 83 isolates of *M. anisopliae* collected from soil in southern Ontario using randomly amplified polymorphic DNA, restriction fragment length polymorphism of the *pr1* gene, and allozyme analysis (six enzymes). They found considerable genetic variability, and that the isolates fell into

* Corresponding author. Fax: +1 403 382 3156.
E-mail address: inglisd@agr.gc.ca (G.D. Inglis).

0022-2011/$ - see front matter
doi:10.1016/j.jip.2007.12.001

one of two major lineages. Based on these findings they concluded that habitat selection, not host selection drives the population structure of this group of fungi. This possibility has not yet been explored in other geographical regions. Elucidation of factors that control the occurrence and abundance of entomopathogenic fungi from an ecological perspective will provide new insights for risk evaluation of microbial control strains intended for release into the environment, and may also lead to new strategies for exploiting natural populations in conservation biological control. In the current study we erected the following hypotheses: *M. anisopliae* var. *anisopliae* is endemic in southwestern British Columbia (BC) and Alberta, Canada; considerable genetic diversity will occur amongst populations of this taxon in both locales; and habitat will influence the population genetics of this fungus. To test these hypotheses, the objectives of the study were to: (i) recover *M. anisopliae* isolates from soils in two geographical regions (i.e. southwestern BC and southern Alberta); (ii) determine the relative prevalence of subspecies in these regions; (iii) develop and utilize an automated whole genome typing method; and (iv) measure the genetic diversity represented by *M. anisopliae* isolates recovered from soils from a diversity of habitats (e.g. urban, agricultural, and forest) from each geographical location. A subsidiary aim of the project was to obtain new knowledge of the relationship of soil dwelling isolates of *M. anisopliae* in BC to those from culture collections with activity against wireworms, since these are a major pest and focus of microbial control efforts in the Province (Kabaluk et al., in press).

2. Materials and methods

2.1. Sampling locations

In southwestern BC (Pacific Maritime Ecozone), a equidistant sampling grid was established; the grid covered an area of approximately 114 km east to west, and 24 km north to south (2736 km^2) (Fig. 1). The grid consisted of 100 sample sites (five rows of 20 sites) established at 6-km-intervals, and were located using a hand-held global positioning system device. The sites represented a variety of habitats including urban, agricultural, and forest ecosystems (Table 1). Soils samples were collected in February, 2004 from the A-horizon (≈5 to 10 cm deep) using a trowel surface-sanitized by immersion in 0.525% sodium hypochlorite followed by a rinse in 70% ethanol. Soil collected from each site was pooled into a single composite sample, and samples were stored on ice in plastic bags until processed using the *Galleria* baiting method (three larvae per sample) (Zimmerman, 1986).

In southern Alberta (Prairie Ecozone), soil samples were collected from Elkwater to Burmis (east to west) and from Medicine Hat to Del Bonita (north to south), an area of ≈300 km by 110 km (33,000 km^2). All samples were taken from grassland (i.e. short-grass prairie) or cultivated agroecosystems. Sample sites were situated approximately 20 km apart, and composite samples per site were collected in March 2005 ($n = 68$). Fungi were isolated using the *Galleria* baiting method (three larvae per sample).

To examine inter- and intra-field diversity, two mixed-grass pastures (Field A and B) were intensively sampled near Agassiz, BC in December 2003, using two diagonal transects (10-m-intervals). Although no *M. anisopliae* var. *anisopliae* had been released in the areas sampled, the fungus had been applied to soil in experimental field plots located adjacent to the sample areas (Figs. 2 and 3). In Field A, LRC 112 and LRC 145 were released in 2002 and 2003, whereas in Field B, LRC 145 was released in 2002. Soil samples were obtained from the A-horizon along the two transects using a 2-cm-diameter soil probe; between samples, the probe was cleaned of soil with water, and subsequently sanitized by immersion in sodium hypochlorite followed by ethanol. A total of 54 locations were sampled in Field A, and 28 locations were sampled in Field B. All soil samples were placed in plastic bags, and stored at 5 °C until processed.

Selected experimental plots located adjacent to Field A that were previously inoculated with LRC 112 or LRC 145 also were sampled. Soil samples were obtained centrally from experimental plots 42, 50, and 51; plots 42 and 50 had been previously treated with LRC 112, whereas plot 51 was left untreated (i.e. control treatment). Soil samples also were obtained at 1-m-intervals (up to 10 m) moving away from experimental plots 27 and 52 (Fig. 2). Plot 27 had been treated with LRC 145, whereas, plot 52 had been treated with LRC 112.

2.2. Isolation

Within ca. 5 to 7 days of collection, soil samples were thoroughly mixed by hand. For all samples collected, the *Galleria* baiting method was applied. Briefly, three larvae (7th or 8th instar) were placed on the soil surface of each sample in a plastic bag. The bag was filled with air, the top was sealed with a twist tie to prevent moisture loss, and bags were placed in a controlled environment at 22 °C with a 16 h light and a 8 h dark period. Bags were checked daily for moribund larvae, and cadavers were aseptically removed and placed in a sterile petri dish containing a moistened filter paper sealed with Parafilm. Conidia from cadavers colonized by fungi characteristic of *M. anisopliae* (i.e. producing bright green cylindrical conidia in tall basipetal chains from phialides branched in a candelabra-like fashion with or without stroma) were transferred onto potato dextrose agar (PDA), subsequently purified, and examined microscopically for conidiogenesis characteristic of *M. anisopliae*.

For soil samples collected at Field A and B near Agassiz, *M. anisopliae* also was isolated on semi-selective medium described by Veen and Ferron (1966). The medium consisted of glucose (10 g), peptone (10 g), bile (15 g; Sigma), rose bengal (1:15,000), and agar (30 g) in 1 L of deionized water. The medium was amended with 0.002 g

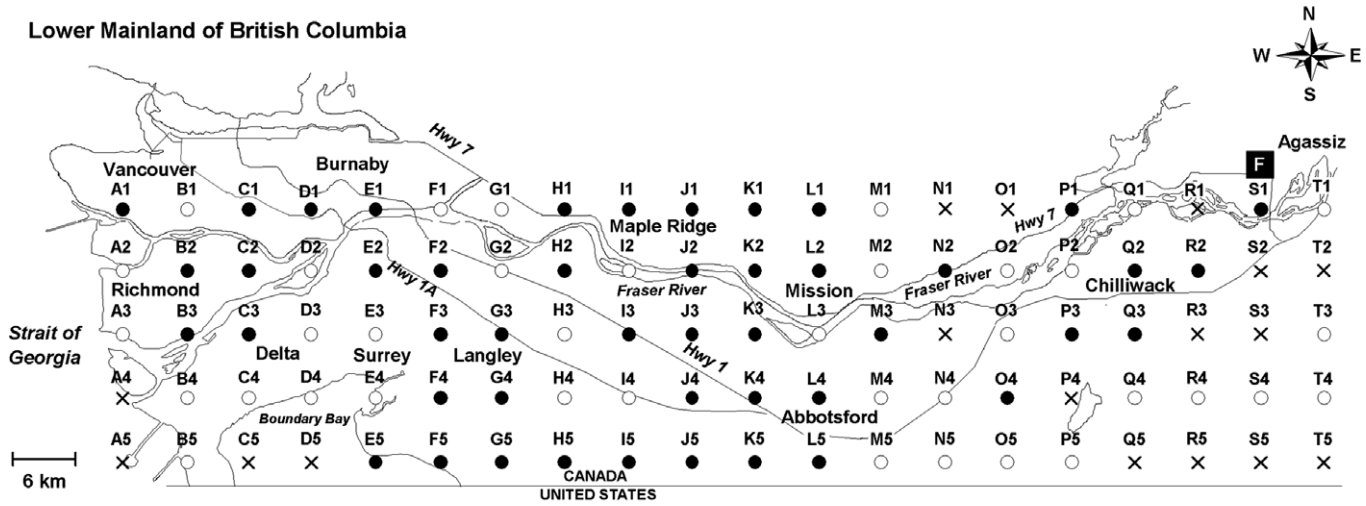


Fig. 1. Sample sites and isolation frequency of *Metarhizium anisopliae* var. *anisopliae* in southwestern British Columbia. The sample grid was arranged in five by 10 lattice covering an area of approximately 114 by 24 km (2736 km^2). Sample sites marked by an "X" were inaccessible (ocean or mountains). Sample sites marked with a closed circle were positive for *M. anisopliae* var. *anisopliae*, whereas the fungus was not recovered from sites marked with an open circle. The closed squares with a "F" at the north-east area of the map indicates the site of the two mixed-grass pastures that we intensively sampled.

L^{-1} dodine (*N*-dodecylguanidine monoacetate; American Cyanamid), 0.12 g L^{-1} cycloheximide (Sigma) and 0.25 g L^{-1} chloramphenicol (Sigma). A 10 g sub-sample of soil was placed into 90 ml of sterile water, and the sample was homogenized using a surface-sanitized Waring blender. The homogenate was diluted three times in a 10-fold dilution series in sterile phosphate buffer (100 mM, pH 7.0), 100 μl from each dilution was spread onto the semi-selective medium, and cultures were maintained at 25 °C in the dark. Cultures were examined after 4 days and daily thereafter for 3 additional days. Conidia characteristic of *Metarhizium* were transferred onto PDA, the cultures were purified, and examined microscopically for characteristic conidiogenesis.

All isolates were subsequently propagated from a single conidium. To accomplish this, 100 μl of a suspension containing a low density of conidia in phosphate buffer was spread onto PDA. After 24 h growth at 25 °C, a single germinated conidium that was separate from other conidia was identified using a dissecting microscope (20 times magnification), a piece of the medium encompassing only the target conidium was aseptically removed and transferred to PDA, and the culture was maintained at room temperature. After ca. 5 days, morphological characteristics of *Metarhizium* were confirmed microscopically, and conidia were collected and stored in sterile 30% glycerol at −80 °C until required.

2.3. Reference isolates

A number of reference strains of *M. anisopliae* var. *anisopliae* ($n = 25$) along with isolates representing six other entomopathogenic fungal taxa were included in the study. The isolates of *M. anisopliae* var. *anisopliae* were isolated from diverse geographical locations and hosts. Where possible, isolates recovered from wireworms were included given the subsidiary objective of the study. The reference isolates were used to validate the genotyping method applied.

2.4. Genomic DNA

Conidia were aseptically removed from PDA cultures and suspended in 100 ml of Sabouraud's dextrose broth (Difco) amended with 1% yeast extract (Difco) in 250 ml Erlenmeyer flasks, and cultures were grown at 22 °C for 3 days on a rotary shaker (200 revolutions per minute). The growth medium was removed from mycelium by filtration (Whatman #1, 90-mm-diameter). The resulting mycelial mat was aseptically removed from the filter paper and stored at −20 °C.

To extract genomic DNA, mycelium from each isolate was frozen in liquid nitrogen and ground into a powder using a mortar and pestle. DNA was extracted from the mycelial powder using the Qiagen DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol for the isolation of total DNA from plant tissue. Genomic DNA was electrophoresed in a 1% TAE–agarose gel (Invitrogen Corp., Burlington, ON), stained with ethidium bromide, and visualized under UV light.

2.5. Variety identification

Sequence data for the entire 5.8S gene, and the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) were obtained from representative isolates and reference strains. The primers, SSZ (5′-ATA ACA GGT CTG TGA TG-3′) and LSU4 (5′-TTG TGC GCT ATC GGT CTC-3′) were used to amplify the partial large and small rRNA genes, the ITS regions, and the 5.8S rRNA gene (Hausner et al.,

Table 1
Metarhizium anisopliae var. *anisopliae* genotype distribution by habitat in southwestern British Columbia

Grid[a]	Habitat	Genotype	Grid[a]	Habitat	Genotype
A1	Urban	1.2	J3	Agricultural	1.2
A2	Urban (riparian)[b]	0[c]	J4	Agriculture–forest interface	1.2
A3	Urban	0	J5	Forest	1.2
B1	Urban	0	K1	Agriculture–forest interface	1.2
B2	Agricultural	1.2	K2	Agriculture–forest interface	1.2
B3	Agricultural	1.2	K3	Agriculture–forest interface	1.1, 1.2
B4	Agricultural	0	K4	Agricultural	1.2
B5	Urban	0	K5	Agricultural	6.3
C1	Urban woodlands	1.1, 1.2	L1	Forest	1.2, 6.3
C2	Agricultural	1.1, 1.2	L2	Agriculture–forest interface	1.2
C3	Agricultural (bog)[e]	1.2	L3	Urban woodlands	0
C4	Agricultural	0	L4	Agriculture–forest interface	1.2
D1	Urban woodlands	1.1	L5	Agricultural	1.10
D2	Urban	0	M1	Industrial	0
D3	Forest	0	M2	Agricultural	0
D4	Agricultural (ocean)	0	M3	Agricultural	1.9
E1	Industrial (riparian)	1.2	M4	Urban	0
E2	Industrial	1.2	M5	Agricultural	0
E3	Urban	0	N2	Agricultural	1.9
E4	Urban woodlands	0	N4	Highway ditch	0
E5	Urban–woodland interface	1.2	N5	Agricultural	0
F1	Industrial	0	O2	Agricultural	0
F2	Industrial	1.1	O3	Agricultural	0
F3	Agricultural	1.2	O4	Agricultural	1.2
F4	Agricultural	1.2	O5	Agricultural (forest)	0
F5	Urban	1.1	P1	Forest	1.2
G1	Urban	0	P2	Agriculture	0
G2	Agriculture–forest interface	0	P3	Agriculture	10.1
G3	Agriculture–forest interface	1.1, 1.2	P5	Agriculture–forest interface	0
G4	Agriculture–forest interface	1.2	Q1	Agriculture–riparian interface	0
G5	Agricultural	1.2	Q2	Agricultural	1.9
H1	Urban	1.1	Q3	Agricultural	1.2
H2	Agricultural (forest)	1.1, 1.2	Q4	Forest	0
H3	Agricultural	0	R2	Agricultural	1.2
H4	Agricultural	0	R4	Forest	0
H5	Agricultural	1.1	S1	Agricultural	1.2
I1	Agricultural	1.1, 1.2	S4	Forest	0
I2	Urban	0	T1	Forest	0
I3	Agricultural	1.2	T3	Forest	0
I4	Urban park	0	T4	Forest	0
I5	Forest	1.2	Field A[d]	Agricultural	1.1, 1.2, 1.7, 1.8, 1.12, 6.2
J1	Agriculture–forest interface	1.1, 1.2	Field B[d]	Agricultural	1.1, 1.2, 1.3, 1.5, 1.11
J2	Forest	1.2			

[a] For the grid location in southwestern BC see Fig. 1.
[b] The description in parentheses indicates situations in which there was a prominent adjacent habitat.
[c] "0" indicates that *M. anisopliae* var. *anisopliae* was not isolated.
[d] Field A and B were both mixed-grass pasture located near Agassiz, BC.
[e] Text in parentheses indicates the presence of minor habitats within the major habitat type.

1993). The conditions for amplification were 1 cycle at 95 °C for 3 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C, ending with an extension cycle of 10 min at 72 °C. The mixtures consisted of a total volume of 20 μl containing 1× reaction buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.5 μM of each primer (Sigma-Genosys, Oakville, ON), and 1 U *Taq* polymerase (Gibco/BRL, Burlington, ON). Each PCR reaction was performed with a total of 2 μl of genomic DNA (≈50 ng/μl) and the negative control consisted of optima water instead of template. The resulting PCR products (10 μl) were electrophoresed in a 2% TAE–agarose gel, and a 100 bp ladder (Promega) was used to size products. The PCR amplicons were purified using the Qiagen QIAquick kit. To obtain sequences, the primers, SSU3 (5′-GTC GTA ACA AGG GTC TCC G-3′), LSU2 (5′-GAT ATG CTT AAG TTC AGC G-3′), and 5.8SB (5′-TGT ACA CAC CGC CCG TC-3′) were used with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The same reaction concentrations as described above were used, but conditions for amplification were 30 cycles of 30 s at 95 °C,

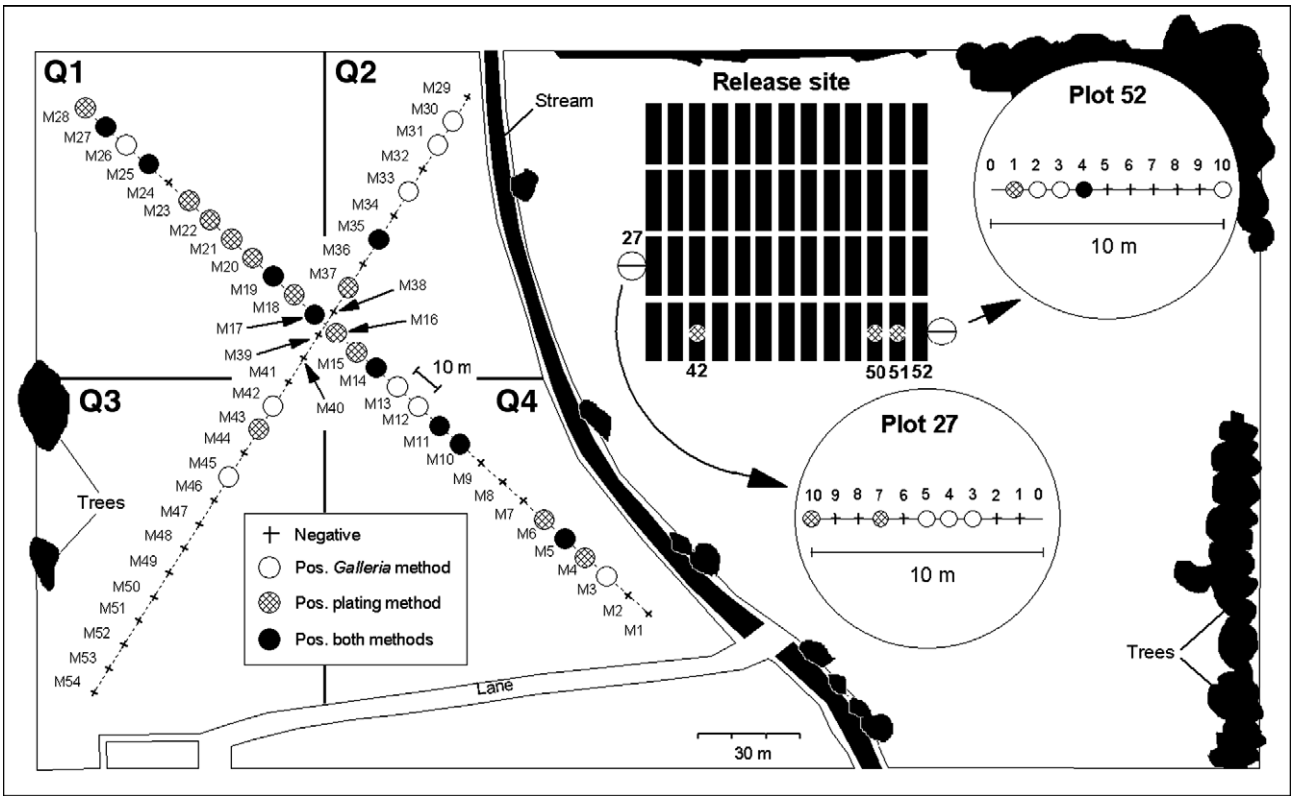


Fig. 2. Sample transect sites and isolation frequency of *Metarhizium anisopliae* var. *anisopliae* in an organic mixed-grass pasture near Agassiz, British Columbia (Field A). The field was divided into four quadrants (Q1, Q2, Q3, and Q4). LRC 112 and LRC 145 were released at a site adjacent to the transect area (''release site''). In addition, *M. anisopliae* var. *anisopliae* was isolated from release plots 42, 50, 51, and 52, and at various distances (1-m-intervals) adjacent to plots 27 and 52.

15 s at 50 °C, and 4 min at 60 °C. Prior to sequencing, excess dye was removed with the Qiagen DyeEx Spin Kit. Sequences were obtained with an ABI PRISM 377 Automated DNA sequencer (Applied Biosystems), and contigs were constructed using Sequencher (Gene Codes Corporation, Ann Arbor, MI).

Nucleotide sequences for isolates were aligned with selected sequences obtained from NCBI using the multialignment program ClustalW, and the alignments were refined visually using GeneDoc (Nicholas and Nicholas, 1997). Aligned sequence data were analyzed using programs contained within they PHYLIP package (Felsenstein, 2002). Phylogenetic estimates were obtained based on neighbor-joining distance methods. Divergence (or distance) of each pair of sequences was calculated by DNADIST using Kimuras two- parameter model. The NEIGHBOR program was used to carry out the neighbor-joining method for estimating phylogenies from the distance matrices. Support for the internal branches within the resulting trees was obtained by bootstrap analysis; 1000 bootstrap replicates were generated by SEQBOOT, majority-rule consensus trees were constructed by the CONSENSE program, and the tree was visualized using TreeView (Page, 2001). Sequences for the ITS1 and ITS2 regions, and 5.8s rRNA gene for 47 strains (including 25 reference strains) were conducted, and sequences were deposited in GenBank under the Accession Nos. EU307885–EU307931.

2.6. Genotyping

Isolates were subjected to fluorescent amplified fragment length polymorphism (AFLP) analysis using the basic procedure described by Kokotovic and On (1999). DNA was quantified using a Synergy HT microplate reader (Bio-Tek Instruments, Inc.) using 96-well black plates and the fluorescent dye, Hoechst 33258 (Molecular Probes, Inc., Eugene, OR). The wave lengths used were, excitation, 360/40 and emission, 460/40. Genomic DNA (250 ng) was restricted for 2 h at 37 °C with 10 U of *Csp*6I, and for additional 2 h at 37 °C with 10 U of *Bgl*II. The digested DNA (5 μl) was ligated with 2 pM (final concentration) of *Bgl*II adaptors (5′-CGG ACT AGA GTA CAC TGT C-3′ and 5′-GAT CGA CAG TGT ACT CTA GTC-3′), and 20 pM (final concentration) of *Csp*6I adaptors (5′-AAT TCC AAG AGC TCT CCA GTA C-3′ and 5′-TAG TAC TGG AGA GCT CTT GG-3′), using 1 U of T4 DNA ligase (New England Biolabs, Ipswich, MA) and 10× ligase buffer. The total reaction volume was 20 μl. The reaction was performed at 24 °C for 4 h. The ligated DNA was heated to 80 °C for 10 min to inactivate T4 enzyme, and a 10× dilution of the ligated DNA was used

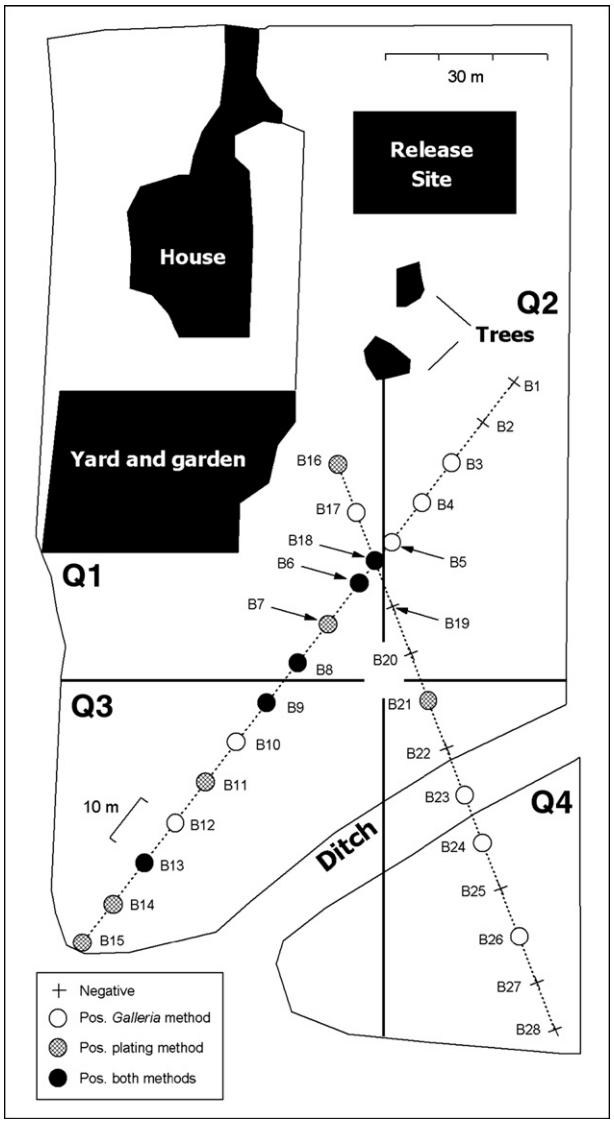


Fig. 3. Sample sites and isolation frequency of *Metarhizium anisopliae* var. *anisopliae* in a mixed-grass pasture near Agassiz, British Columbia (Field B). The field was divided into four quadrants (Q1, Q2, Q3, and Q4). LRC 145 was released at a site adjacent to the transect area (“release site”).

as template for PCR reactions. PCR was conducted using primers, BLG2 F-0 (6-fam 5′-GAG TAC AAG AGC TCT CCA GTA C-3′) and CSP61-A (5′-GAG CTC TCC AGT ACT ACA-3′). The PCR reaction mixture (25 µl total volume) consisted of 2.5 µl of 10× buffer, 4 µl of 1.25 mM dNTPs, 0.5 µl of 10 µM primer, 0.12 µl of 1.25 U Hot Star Taq (Qiagen Inc., Mississauga, ON), 1 µl of 25 mM $MgCl_2$, and 1 µl of diluted ligation product. The conditions for amplification were 1 cycle at 95 °C for 15 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 54 °C, and 1.5 min at 72 °C, ending with an extension cycle of 10 min at 72 °C.

Amplified DNA products (2 µl of a 1:20 dilution) were mixed with 2 µl of deionized formamide, 0.3 µl of internal lane size standard (Genescan-ROX 500; Applied Biosystems), and 0.7 µl of loading buffer (supplied with size standard). The mixture was heated at 94 °C for 2 min, cooled on ice, and samples were electrophoresed on a 6% denaturing polyacrylamide gels using an ABI PRISM 377 Automated DNA sequencer (Applied Biosystems). Data collection, preprocessing and fragment analysis were done using the Genescan 3.7 fragment analysis software (Applied Biosystems). GeneScan-processed data files comprising both fungal AFLP profiles and the internal molecular mass standard were imported into the program BioNumerics 4.01 (Applied Maths, Inc., Austin, TX). After registration of strain details, profiles were normalised within and between runs by linking profiles for analysis to their respective molecular mass standard, as described in the software manufacturer’s instructions. Normalised AFLP profiles were compared using the Dice coefficient and clustered by the unweighted pair group with mathematical average (UPGMA) method.

2.7. Resolution determination

To ascertain the resolution limit for distinguishing between genotypes, AFLP profiles run on separate occasions were obtained for 21 arbitrarily-selected reference and field isolates. The isolates selected were LRC 42 ($n = 2$), LRC 112 ($n = 2$), LRC 145 ($n = 2$), LRC 148 ($n = 2$), LRC 171 ($n = 2$), LRC 172 ($n = 2$), LRC 180 ($n = 8$), LRC 181 ($n = 6$), LRC 182 ($n = 2$), LRC 187 ($n = 2$), LRC 190 ($n = 2$), LRC 199 ($n = 2$), LRC 200 ($n = 2$), LRC 202 ($n = 2$), LRC 206 ($n = 2$), SBX14 ($n = 4$), SBX21 ($n = 2$), SBX6 ($n = 4$), SMX16 ($n = 3$), SMX43 ($n = 2$), and SMX5 ($n = 2$). AFLP profiles for a total of 57 samples were analysed. The level of strain resolution provided by the AFLP method was chosen as the minimum similarity level distinguishing unique genotypes.

2.8. Genetic diversity

To obtain a measure of genetic diversity, the similarity of AFLP profiles between pairs of clones was calculated using the basic similarity index described by Lynch (1988, 1990); only polymorphic peaks were considered in the index calculation. The univariate procedures of SAS (1999) was used to calculate the similarity index.

3. Results

3.1. Reproducibility of AFLP

The fluorescent AFLP method developed provided 211 bands (i.e. loci) ranging in size from ≈92 to 400 bp, and 118 of these loci were polymorphic for the isolates of *M. anisopliae* var. *anisopliae* recovered from southwestern BC. The method also provided robust and consistent results. Of the 21 isolates run on multiple occasions (2 to 8 times), the AFLP patterns were identical for 11 of the isolates (data not presented). For the other 10 isolates, some

variability in AFLP band patterns were observed. However, variability within this group was less than or equal to 13.8%, and this was chosen as the resolution limit distinguishing unique genotypes (i.e. 86.2% similarity). Major clades were defined as genotypes that were less than 64.0% similar, and minor clades were genotypes between 64.0 and 86.2% similarity.

3.2. Reference strains

A total of 35 reference strains were included in the analysis. The ITS1 and ITS2 regions along with the 5.8S rRNA gene were sequenced for the 25 *Metarhizium* strains that had not been assigned to a variety (Fig. 4). Two reference strains (LRC 111 and LRC 208) formed a robust clade with known strains of *M. anisopliae* var. *acridum*; LRC 111 was isolated from an acridid in Brazil and LRC 208 was isolated from an unknown source. All other reference strains that we sequenced clustered with *M. anisopliae* var. *anisopliae*, including strains previously identified as *M. flavoviride* var. *minus* (LRC 42) and *M. brunneum* (LRC 182) (Table 2).

Analysis of AFLP patterns separated the 35 reference isolates, representing eight taxa from various substrates and isolation locations, into distinct clusters based at the genus and species level (Table 2; Fig. 5). Strains of *Beauveria bassiana*, *Hirsutella* spp., *Lecanicillium longisporum*, and *Tolypocladium cylindrosporum* clustered separately from *Metarhizium* strains. With the exception of LRC 42, all of the *M. anisopliae* reference strains grouped together. Three of the four strains of *M. anisopliae* var. *acridum* analyzed grouped together separate from *M. anisopliae* var. *anisopliae*. The one exception was LRC 208. We observed considerable variability in AFLP patterns within the 25 reference isolates of *M. anisopliae* var. *anisopliae* that we examined; these isolates belonged to one of 21 genotypes. Given the current interest in using *M. anisopliae* var. *anisopliae* to control wireworms, we included 15 reference strains of *M. anisopliae* isolated from wireworms in various geographical locations (four countries representing three continents). These isolates belonged to one of 11 genotypes. Surprisingly, four of the five reference isolates previously recovered from wireworms in southwestern BC were an identical genotype (genotype 1.1); these four isolates (LRC 112, LRC 145, LRC 180, and LRC 181) were recovered from disparate locations within BC, whereas the genetically distinct strain LRC 142, was isolated from the same location and substrate as LRC 112 and LRC 145. The only other two reference isolates that belonged to an identical clade (i.e.

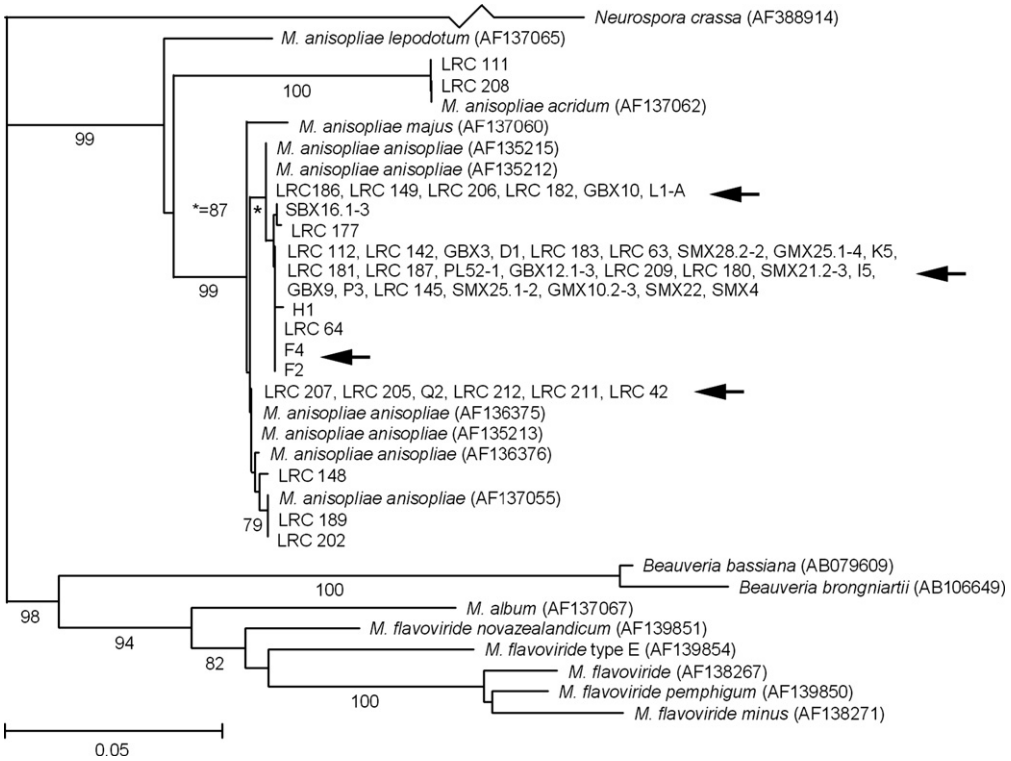


Fig. 4. A dendrogram based on a majority-rule consensus tree obtained by analyzing the internal transcribed spacer regions (ITS) 1 and 2, and the 5.8S ribosomal gene of *Metarhizium anisopliae* isolates recovered from British Columbia soils (arrows). NCBI accession numbers of reference strains are presented in parentheses, and the outgroup strain used was *Neurospora crassa*. The bar at the bottom of the dendrogram represents 0.05 nucleotide substitutions per base, and numbers at nodes indicate support for the internal branches within the tree obtained by bootstrap analysis (% of 1000 bootstraps); only values >75% are shown.

Table 2
Reference strain information

No.	Taxon	Incoming Id	Identifier	Substrate	Location	Genotype[b]
LRC 26	*Beauveria bassiana*	Same	ARSEF 201	Corn rootworm	Oregon, USA	20.1
LRC 42	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium flavoviride* var. *minus*	ARSEF 3341	Grasshopper	Niger	21.1
LRC 63	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	92141	Japanese beetle	Azores	1.4
LRC 64	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	MA-605	Japanese beetle	Azores	4.2
LRC 111	*Metarhizium anisopliae* var. *acridum*[a]	*Metarhizium anisopliae*	CG 325	Acridid	Brazil	13.1
LRC 112	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	DAOM 231489	Wireworm	Agassiz, BC, Canada	1.1
LRC 113	*Metarhizium anisopliae* var. *acridum*	Same	IMI 330189	Locust	Niger	13.2
LRC 142	*Metarhizium anisopliae* var. *anisopliae*[a]	—	—	Wireworm	Agassiz, BC, Canada	7.1
LRC 145	*Metarhizium anisopliae* var. *anisopliae*[a]	—	Cadaver E	Wireworm	Agassiz, BC, Canada	1.1
LRC 148	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	FI-522	Peanut scarab	Australia	12.1
LRC 149	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	ARSEF 1377	Wireworm	Switzerland	4.1
LRC 171	*Beauveria bassiana*	—	Bcmu BB01	Wireworm	Agassiz, BC, Canada	19.1
LRC 172	*Tolypocladium cylindrosporum*	—	—	Wireworm	Agassiz, BC, Canada	22.1
LRC 177	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	Keller 714	Wireworm	Switzerland	3.1
LRC 180	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	180A	Wireworm	Pender Island, BC, Canada	1.1
LRC 181	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	181A	Wireworm	Vancouver, BC, Canada	1.1
LRC 182	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium brunneum*	CABI 014746	Wireworm	Oregon, USA	6.4
LRC183	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	UAMH 421	Unknown insect	West Virginia, USA	1.6
LRC 184	*Metarhizium anisopliae* var. *acridum*	Same	ARSEF 324	Acridid	Australia	14.1
LRC 186	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	UAMH 4450	Soil	Alberta, Canada	5.1
LRC 187	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	ATCC 90448, F52, BIO1020	Codling moth	Austria	1.6
LRC 189	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	ARSEF 2518	Wireworm	New Zealand	3.2
LRC 190	*Lecanicillium longisporum*	Same	1.72, ATCC 46578	Aphid	United Kingdom	18.1
LRC 199	*Hirsutella necatrix*	Same	49.81, IMI 2552317	Mite	United Kingdom	17.1
LRC 200	*Hirsutella thompsonii*	Same	34.79, ARSEF 257, CBS 451.78	Mite	Ivory Coast	16.1
LRC 202	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	MA1200, ATCC 26176	Nematode	Illinois, USA	2.1
LRC 204	*Metarhizium anisopliae* var. *anisopliae*	Same	ARSEF 23	Wireworm	North Carolina, USA	15.1
LRC 205	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	ARSEF 1903	Wireworm	USA	9.1
LRC 206	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	ARSEF 2107	Wireworm	Oregon, USA	6.1
LRC 207	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	ARSEF 1897	Wireworm	California, USA	4.3
LRC 208	*Metarhizium anisopliae* var. *acridum*[a]	*Metarhizium anisopliae*	—	Unknown	Unknown	4.4
LRC 209	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	TM109, ARSEF5520	Soil	Norway	4.5
LRC 211	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	ARSEF 2949	Termite	Brazil	8.1
LRC 212	*Metarhizium anisopliae* var. *anisopliae*[a]	*Metarhizium anisopliae*	ARSEF 2951	Termite	Brazil	11.1
LRC 213	*Metarhizium anisopliae* var. *anisopliae*	Same	ARSEF 4556	Tick	Florida, USA	9.2

[a] Variety identifications determined by sequencing the internal transcribed spacer regions 1 and 2 and the entire 5.8S rRNA gene.

[b] Genetic diversity of isolates was examined using fluorescent amplified fragment length polymorphism analysis. For genotype designations, the first number indicates the major clade assignment, and the decimal number indicates the minor clade (see text for a more detailed description).

genotype 1.6) were LRC 183 (isolated for an unidentified insect in the USA) and LRC 187 (isolated from a codling moth in Austria).

3.3. Abundance and diversity in southwestern British Columbia

Soil samples were obtained from 83 of the 100 sample sites (Fig. 1); the other 17 grid sites were deemed inaccessible and/or were inappropriate (e.g. the site occurred in a body water or on a mountain). Using the *Galleria* baiting method, we observed that *M. anisopliae* was widely distributed throughout southwestern BC. The fungus was recovered from 46 locations (55.4% of the sites sampled), and a total of 63 isolates were recovered. Sequencing of the ITS1 and ITS2 regions of representative isolates from AFLP clades (i.e. D1, F2, F4, H1, K5, L1-A, L5, P3, Q2) indicated that isolates were *M. anisopliae* var. *anisopliae*.

Most of the isolates (88.9%, $n = 56$) belonged to one of two closely-related genotypes; 20.6% and 68.3% of the isolates recovered were genotypes 1.1 and 1.2, respectively (Fig. 5). These two genotypes were isolated from urban, agricultural and forest soils (Table 1). The mean similarity between pairs of clones was 0.88 (standard deviation = 0.083) with a range from 0.60 to 1.0, and the most frequent similarity index was 0.92. (Fig. 6). Seven isolates were genetically distinct from genotypes 1.1 and 1.2, and belonged to one of four genotypes (i.e. 1.9, 1.10, 6.3, and 10.1) (Table 1). These isolates were all recovered in the east-central area of the sample grid (i.e. K5, L1, L5, M3, N2, P3, and Q2); six of the sites were agricultural, whereas one site (L1) was forest. Genotype 6.3 (L1-A and K5) was most closely grouped with LRC 182 (genotype 6.4) and LRC 206 (genotype 6.1), strains isolated from wireworms in Oregon. From 12 sites, multiple isolates were recovered from different *Galleria* cadavers, and different genotypes were detected for eight of these sites.

3.4. Abundance and diversity in southern Alberta

No *M. anisopliae* isolates were recovered from the 68 soil samples. However, the entomopathogenic fungus, *B bassiana* was isolated using the *Galleria* baiting method at six sites (near Del Bonita, Manyberries, Medicine Hat, Pincher Creek, and Spring Coulee).

3.5. Inter- and intra-field diversity

In total, 120 isolates were recovered from the transects within the two intensively sampled field sites, which included 75 isolates from Field A and 45 isolates from Field B. *M. anisopliae* was recovered from 55.6% of the sample sites along the transects in Field A (Fig. 2), and from 71.4% of the sample sites in Field B (Fig. 3). The *Galleria* baiting method was found to be conspicuously more effective than the plating method (i.e. using a semi-selective medium) for isolating *M. anisopliae*; 82 isolates were recovered using *Galleria* baiting, whereas only 15 isolates were recovered by plating. In both fields, isolates appeared aggregated. A lower prevalence of sample sites were positive for *M. anisopliae* in quadrant 3 (21%) than in quadrants 1 (79%), 2 (62%), and 4 (62%) in field A (Fig. 2). In field B, 29% and 50% of the sites were positive for *M. anisopliae* in quadrants 2 and 4, respectively, whereas all of the sites were positive for the fungus in quadrants 1 and 3.

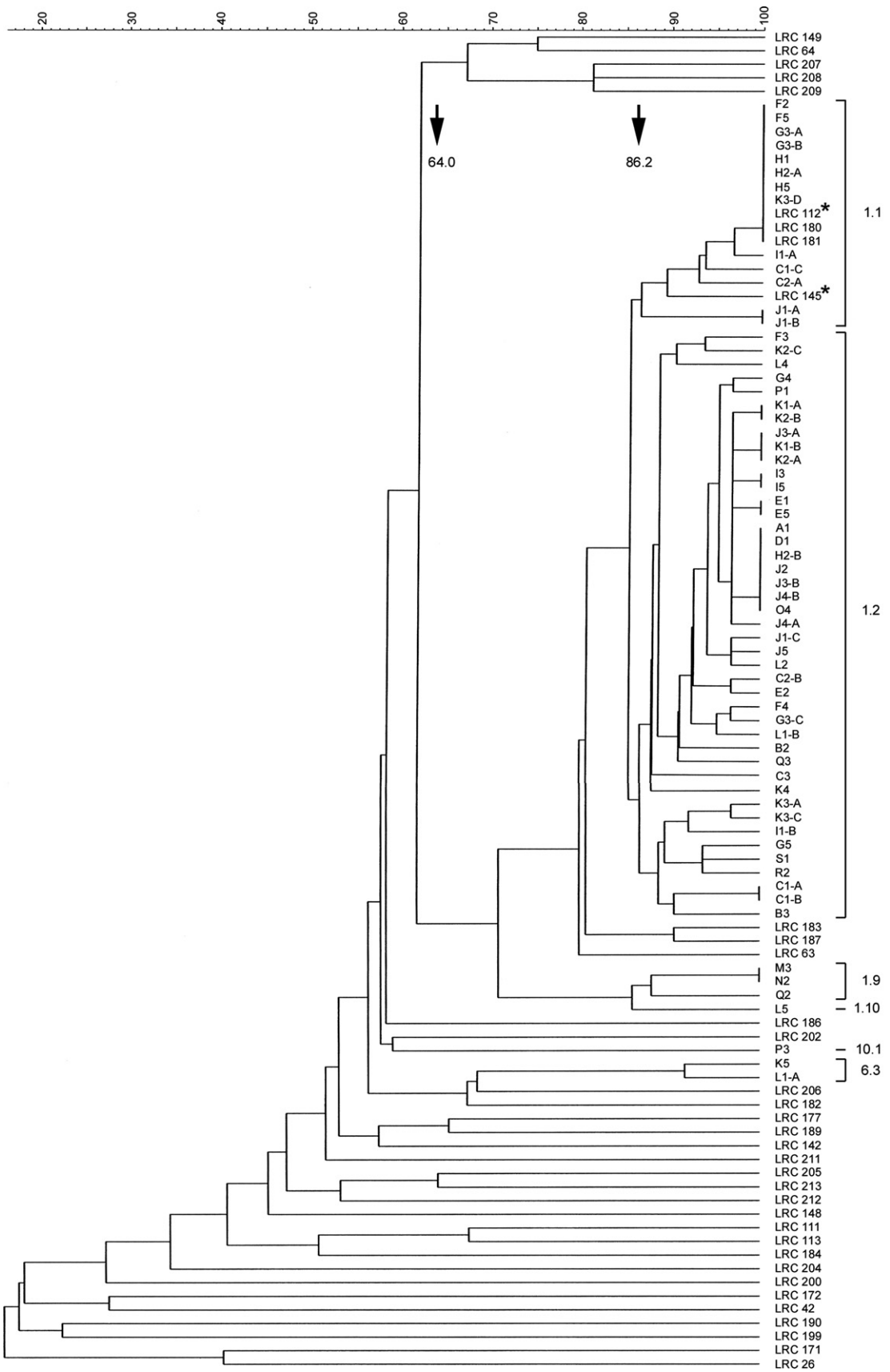
AFLP analyses indicated that the vast majority of isolates (83–84%) recovered from fields A and B belonged to genotype 1.1 (data not presented). A low frequency of other genotypes also was detected in both fields. From Field A, genotypes 1.2 ($n = 10$), 1.7 ($n = 1$), 1.8 ($n = 1$), 1.12 ($n = 1$), and 6.2 ($n = 1$) were recovered, and from Field B, genotypes 1.2 ($n = 1$), 1.3 ($n = 2$), 1.5 ($n = 3$), and 1.11 ($n = 1$) were isolated. These genotypes were distributed throughout the fields. Sequencing of the ITS1 and ITS2 regions of representative isolates from AFLP genotypes (i.e. GBX10, GBX12.1-3, GBX3, GBX9, GMX10.2-3, GMX25.1-4, PL52-1, SMX4, SBX16.1-3, SMX21.2-3, SMX22, SMX25.1-2, and SMX28.2-2) indicated that isolates were *M. anisopliae* var. *anisopliae* (Fig. 4). Multiple isolates were recovered from 19 of the 30 positive sample sites in Field A, whereas more than one isolate was detected at 11 of 20 positive sites in Field B. Multiple genotypes were recovered from 19 (63.3%) and three (27.3%) sites in Field A and B, respectively.

3.6. Overall genotypic diversity

In total, 201 isolates of *M. anisopliae* var. *anisopliae* were recovered from urban, agricultural, and forest soils throughout southwestern BC and were subjected to AFLP analysis. The overall mean similarity between pairs of clones was 0.94 (standard deviation = 0.055). Two peaks of similarity index were observed, one at 1.0 and one at 0.94. A total of 14 genotypes were detected in the isolates recovered, but 91.0% of the isolates belonged to either genotype 1.1 (63.2%) or genotype 1.2 (27.9%). In contrast, genotypes 1.3, 1.5, 1.7, 1.8, 1.9, 1.10, 1.11, 1.12, 6.2, 6.3, 7.1, and 10.1 occurred at relatively low frequencies (0.5–1.5%).

4. Discussion

We examined the prevalence and genetic variability of the entomopathogenic fungus, *M. anisopliae* var. *anisopliae* in various habitats in southwestern BC and southern Alberta. *M. anisopliae* is a cosmopolitan fungus, and it has been isolated from a variety of diverse habitats and geographical locations (St. Leger et al., 1992). We found that the fungus was endemic in soils throughout southwestern BC. Isolates of *M. anisopliae* var. *anisopliae* were recovered from 56% of the sample sites including soils from urban, agricultural, and forest habitats. In contrast, no isolates of *M. anisopliae* var. *anisopliae* were recovered from


20
30
40
50
60
70
80
90
100
64.0
86.2
LRC 149
LRC 64
LRC 207
LRC 208
LRC 209
F2
F5
G3-A
G3-B
H1
H2-A
H5
K3-D
LRC 112*
LRC 180
LRC 181
I1-A
C1-C
C2-A
LRC 145*
J1-A
J1-B
1.1
F3
K2-C
L4
G4
P1
K1-A
K2-B
J3-A
K1-B
K2-A
I3
I5
E1
E5
A1
D1
H2-B
J2
J3-B
J4-B
O4
J4-A
J1-C
J5
L2
C2-B
E2
F4
G3-C
L1-B
B2
Q3
C3
K4
K3-A
K3-C
I1-B
G5
S1
R2
C1-A
C1-B
B3
1.2
LRC 183
LRC 187
LRC 63
M3
N2
Q2
1.9
L5
1.10
LRC 186
LRC 202
P3
10.1
K5
L1-A
6.3
LRC 206
LRC 182
LRC 177
LRC 189
LRC 142
LRC 211
LRC 205
LRC 213
LRC 212
LRC 148
LRC 111
LRC 113
LRC 184
LRC 204
LRC 200
LRC 172
LRC 42
LRC 190
LRC 199
LRC 171
LRC 26

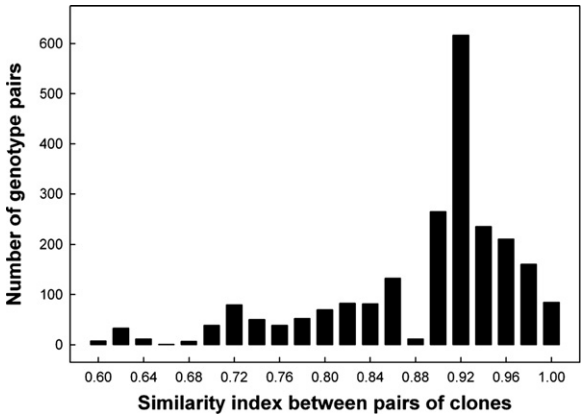


Fig. 6. Frequency distribution of pairwise distances, calculated assuming an infinite alleles model, for AFLP-data from 68 isolates of *Metarhizium anisopliae* var. *anisopliae* recovered from southwestern British Columbia.

any of the sites sampled in southern Alberta. Although this fungus has previously been reported from Alberta (Table 2) (Entz et al., 2005), our results show that *M. anisopliae* is rare in southern Alberta soils. Southern Alberta and southwestern BC occur in conspicuously different ecozones (http://www.ccea.org/ecozones/), and are separated by a series of mountain ranges. Southern Alberta is a semi-arid (250–500 mm/year) grassland ecozone dominated by short-grass prairie. Winters are cold (the mean temperature in the coldest month $\leqslant -8$ °C) and summers are short and warm. Although dry, arctic air predominates in winter, periodic warm westerly chinook winds bring spring-like conditions to the region rapidly reducing snow cover and removing moisture from soil. In contrast, southwestern BC occurs in the pacific maritime ecozone dominated by mountains and valleys. This ecozone possesses the warmest (mean temperatures range from ≈16 °C in the summer to ≈3 °C in winter) and wettest (1500–3000 mm/year) climate found in Canada.

To assess the genetic diversity of *M. anisopliae* var. *anisopliae* in southern BC, we utilized a fluorescent AFLP method. While the AFLP method has been applied to study the diversity of other fungi (e.g. Majer et al., 1996; Mueller et al., 1996; Rosendahl and Taylor, 1997) including entomopathogenic taxa (e.g. Aquino de Muro et al., 2003; Boucias et al., 2000), to our knowledge it has not been applied to *M. anisopliae*. We found that the AFLP method provided highly reproducible results (resolution limit of 86%), and showed a high level of genotypic diversity (i.e. 211 loci) among the reference fungi we examined. The method distinguished *M. anisopliae* isolates from other fungal taxa, and separated genotypes of *M. anisopliae*. For the 25 reference strains of *M. anisopliae* var. *anisopliae* that we examined, 21 genotypes were determined; many of these strains were isolated from wireworms. Surprisingly, the four reference isolates recovered from disparate locations in southwestern BC belonged to a single genotype (i.e. 1.1). The AFLP methods has a number of advantages over other genotyping method including that the library can be created from very small amounts of DNA, the method can be automated, and it typically provides very reproducible results (Taylor et al., 1999). The variable fragments (i.e. loci) have two alleles, and similarly to other methods (e.g. RFLP and RAPD), the occurrence of null alleles is a possibility. However, the large number of reproducible fragments that are produced by AFLP analysis largely limit this concern.

We observed very minimal genetic variability among the 63 isolates of *M. anisopliae* var. *anisopliae* that were isolated throughout southwestern BC. The similarity index values between clone pairs showed a high degree of relatedness, and 89% of the isolates belonged to one of two closely-related genotypes (i.e. 1.1 and 1.2). Our results contrast with other geographical areas such as Ontario, Canada and Switzerland where considerable genetic variability was observed among isolates of *M. anisopliae* var. *anisopliae* (Bidochka et al., 2001; Enkerli et al., 2005). In Ontario, two distinct lineages of strains beyond the resolution of ITS sequence were identified. Furthermore, evidence indicated that they represented two non-recombining populations of *M. anisopliae* var. *anisopliae* which were termed "crytpic species" (Bidochka et al., 2005). *M. anisopliae* is a haploid, mitosporic entomopathogenic fungus with no known teleomorphic stage (Driver et al., 2000). Parasexuality has been reported for this fungus, but evidence to date indicates that the hyphal anastomosis only occurs in closely related strains (Bidochka et al., 2000; Leal-Bertioli et al., 2000; St. Leger et al., 1992). The lack of a sexual state and segregation based on anastomotic grouping are both consistent with the occurrence of sympatric and non-reproducing lineages of *M. anisopliae* var. *anisopliae*. Bidochka et al. (2001, 2005) observed that strains in the two lineages tended to occupy different habitats (i.e. agriculture versus forest ecosystems). However, the habitat associations that they observed most likely represent different niche requirements of the two lineages, and speciation may have occurred by vicariance associated with glaciation or other events rather than sympatric speciation driven by habitat. The predominance of two closely-related genotypes across habitat in southwestern BC suggests that sympatric but non-recombining lineages as was observed in

Fig. 5. Unweighted Pair-Group Method using Arithmetic Averages (UPGMA) dendrogram of fluorescent amplified fragment length polymorphism fingerprints from selected entomopathogenic fungal taxa. The analysis included 35 reference isolates (identification number proceeded by LRC) (see Table 2 for isolate information), and 63 isolates of *Metarhizium anisopliae* var. *anisopliae* isolated from various locations in southwestern British Columbia (BC). These isolates are identified by the site at which they were recovered (see Fig. 1 for sample grid information). The bar at the bottom of the graph indicates genetic similarity (%). Isolates that are $\geqslant$86.2% (arrow) similar in banding patterns are deemed to belong to the same genotype. Major clades are defined as genotypes that were less than 64.0% similar (arrow), and minor clades are defined as genotypes between 64.0 and 86.2% similarity. Isolates (LRC 112 and LRC 145) marked with an "*" were applied in adjacent areas to the two field plots intensively sampled at Agassiz, BC.

Ontario are uncommon. However, the area we sampled was relatively restricted, particularly latitudinally, due to mountainous terrain. Of interest, we did recover a small number of isolates ($n = 7$) that belonged to different genotypes from 1.1 and 1.2. Four of the isolates belonged to genotype 1.5, and the remaining three isolates belonged to the disparate genotypes, 5.3 and 8.2. The distribution of these two latter genotypes, and whether they represent non-reproducing lineages of *M. anisopliae* var. *anisopliae* in southwestern BC requires further investigation.

To examine the extent of genetic diversity within a specific habitat, we intensively and spatially sampled two mixed-grass pastures that were known to possess high populations of wireworms. Furthermore, *M. anisopliae* var. *anisopliae* (genotype 1.1) had been previously applied in areas adjacent to the sample locations. A large number of isolates ($n = 120$) were recovered from soil in these two fields, but the fungus was not uniformly distributed, and aggregations of the fungus were not in proximity to release sites. This observation suggests that the isolates did not disseminate from the release sites. The spatial distribution of soil organisms is uneven and often aggregated, but reasons for the apparent aggregation of *M. anisopliae* var. *anisopliae* in the two pastures we sampled are not clear and warrant investigation. Although both genotype 1.1 and 1.2 were isolated from both field plots, most of the isolates recovered (83%) belonged to genotype 1.1. This contrasts with the 21% frequency for this genotype observed throughout southwestern BC. The relative fitness of genotype 1.1 versus genotype 1.2, and the factors that influence their relative prevalence requires additional study. Interestingly, although occurring at low frequencies, nine additional genotypes of *M. anisopliae* var. *anisopliae* (i.e. genotypes 1.3, 1.6, 1.7, 1.8, 1.9, 1.10, 1.11, 1.12, and 15.1) were recovered from these two pastures. Whether they represent non-recombining lineages (i.e. from genotypes 1.1 and 1.2) is currently unknown. However, the high prevalence of wireworms in these fields coupled with the low frequency of occurrence of these genotypes would suggest that they are less virulent against wireworms than genotype 1.1, and this possibility warrants investigation.

Reasons for the cosmopolitan predominance of *M. anisopliae* var. *anisopliae* of genotypes 1.1 and 1.2 in southwestern BC are uncertain. One possibility is that these genotypes possess superior ecological fitness to other genotypes across all habitats (i.e. urban, agriculture, and forest). This seems improbable as considerable genetic diversity has been reported for mitosporic fungi, including the entomopathogenic taxa *M. anisopliae* var. *anisopliae* and *B. bassiana* in contiguous habitats (Aquino de Muro et al., 2003; Bidochka et al., 2001). A more plausible explanation for the limited diversity of *M. anisopliae* var. *anisopliae* in southwestern BC may be due to unique geography of this region. Southwestern BC is relatively segregated, bordered by the Pacific Ocean on the west, and mountain ranges to the north and east. Furthermore, southwestern BC was completely covered by the Fraser Glacier which reached its height ca. 23 to 18 thousand years ago, but ice free regions occurred to the north (i.e. Yukon and Alaska) and to the south (i.e. southern Washington) of BC (Smith et al., 2001). Island biogeography theory indicates that large, continuous zones have more species than small, fragmented zones (MacArthur and Wilson, 1967). One of the current hypotheses of biogeography theory indicates that local species richness is affected by past events (e.g. glaciation) and contemporary environmental conditions (Hughes Martiny et al., 2006). Furthermore, the biogeography theory is applicable to genetically distinct taxonomic units within a morphologically-based microbial species (de Vargas et al., 1999). In the case of *M. anisopliae* var. *anisopliae* in southwestern BC, the limited genetic diversity of strains is consistent with island biogeography theory. We suggest that following glaciation, southwestern BC was colonized by *M. anisopliae* var. *anisopliae* via immigration, but geological barriers limit the extent of emigration that occur. However, the relative influence of contemporary environmental conditions versus historical contingencies is unknown. For example, no *M. anisopliae* was isolated in the adjacent region of southern Alberta. To address the issue of island biogeography further, we plan to examine the genetic diversity of *M. anisopliae* var. *anisopliae* in regions adjacent to southwestern BC that were (i.e. interior regions of BC, northern Washington) and were not (i.e. southern Washington and northern Oregon) covered by the Fraser Glacier. Furthermore, the interior regions of BC are geographically isolated from southwestern BC by mountain ranges. Interestingly, the four reference strains of *M. anisopliae* var. *anisopliae* that we examined from California and Oregon represented four genotypes, all disparate from genotypes 1.1 and 1.2.

In conclusion, we observed that two closely-related genotypes of *M. anisopliae* var. *anisopliae* predominated in urban, agricultural, and forest soils of southwestern BC. This contrasts with a previous finding in southern Ontario in which considerable genetic variability occurred with *M. anisopliae* var. *anisopliae*, and two non-recombining lineages were found; one lineage typically occurred in agricultural soils while the other was most common in forest soils (Bidochka et al., 2001). In southwestern BC, this does not appear to be the case. Southwestern BC was covered by the Fraser glacier, a period in which most soil fungi would have been eliminated. Furthermore, this region is geographically isolated thereby restricting microbial emigration, particularly for mitosporic fungi such as *M. anisopliae* var. *anisopliae* that produce conidia that are not adapted for long-distance dissemination in air. The predominance of two closely-related genotypes of *M. anisopliae* var. *anisopliae* across habitats is consistent with island biogeography theory, and has implications for the efficacious development of this fungus as a microbial control agent of insect pests in BC and adjacent ecozones.

Acknowledgments

We wish to thank the following individuals at Agriculture and Agri-Food Canada, Lethbridge for their invaluable assistance with aspects of this project: Jenny Gusse for her assistance with all molecular aspects of the project; Kaarina Benkel and Edith Valle for sequencing support; and Toby Entz for determining the similarity index between pairs of clones. We also wish to thank Jerry Ericsson for his assistance with the collection of soil samples at Agassiz, Viola Lam for her help with the collection of soil samples in BC, and the two anonymous reviewers for their comments. This research was supported by a grant from the Agriculture and Agri-Food Canada, Improving Farming Systems and Practices Initiative (IFSP) Pesticide Risk Reduction Program. This manuscript is contribution 060632 from the Agriculture and Agri-Food Canada Research Centre, Lethbridge, Alberta, Canada.

References

Aquino de Muro, M., Mehta, S., Moore, D., 2003. The use of amplified fragment length polymorphism for molecular analysis of *Beauveria bassiana* isolates from Kenya and other countries, and their correlation with host and geographical origin. FEMS Microbiol. Lett. 229, 249–257.

Bidochka, M.J., Kamp, A.M., Lavender, T.M., Dekoning, J., De Croos, J.N.A., 2001. Habitat association in two genetic groups of the insect-pathogenic fungus *Metarhizium anisopliae*: uncovering cryptic species? Appl. Environ. Microbiol. 67, 1335–1342.

Bidochka, M.J., Melzer, M.J., Lavender, T.M., Kamp, A.M., 2000. Genetically related isolates for the entomopathogenic fungus *Metarhizium anisopliae* harbour homologous dsRNA viruses. Mycol. Res. 104, 1094–1097.

Bidochka, M.J., Small, C.L.N., Spironello, M., 2005. Recombination within sympatric cryptic species of the insect pathogenic fungus *Metarhizium anisopliae*. Environ. Microbiol. 7, 1361–1368.

Boucias, D.G., Tigano, M.S., Sosa-Gomez, D.R., Glare, T.R., Inglis, P.W., 2000. Genotypic properties of the entomopathogenic fungus *Nomuraea rileyi*. Biol. Contr. 19, 124–138.

Bridge, P.D., Williams, M.A.J., Prior, C., Paterson, R.R.M., 1993. Morphological, biochemical and molecular characteristics of *Metarhizium anisopliae* and *M. flavoviride*. J. Gen. Microbiol. 139, 1163–1169.

de Vargas, C., Norris, R., Zaninetti, L., Gibb, S.W., Pawlowski, J., 1999. Molecular evidence of cryptic speciation in planktonic foraminifers and their relation to oceanic provinces. Proc. Natl. Acad. Sci. USA 96, 2864–2868.

Driver, F., Milner, R.J., Trueman, J.W.H., 2000. A taxonomic revision of *Metarhizium* based on phylogenetic analysis of rDNA sequence data. Mycol. Res. 104, 134–150.

Enkerli, J., Kölliker, R., Keller, S., Widmer, R., 2005. Isolation and characterization of microsatellite markers from the entomopathogenic fungus *Metarhizium anisopliae*. Mol. Ecol. Notes 5, 384–386.

Entz, S.C., Johnson, D.L., Kawchuk, L.M., 2005. Development of a PCR-based diagnositic assy for specific detection of the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum*. Mycol. Res. 109, 1302–1312.

Felsenstein, J., 2002. PHYLIP (Phylogenetic Inference Package). Department of Genetics, University of Washington, Seattle, WA.

Guo, H. -L., Ye, B. -L., Yue, Y. -Y., Chen, Q. -T., Fu, C. -S., 1986. Three new species of *Metarhizium*. Acta Mycol. Sin. 5, 177–184, in Chinese.

Hausner, G., Reid, J., Klassen, G.R., 1993. ;On the subdivision of *Ceratocystis s.l.*, based on partial ribosomal DNA sequences. Can. J. Bot. 71, 52–63.

Hughes Martiny, J.B., Bohannan, B.J.M., Brown, J.H., Colwell, R.K., Fuhrman, J.A., Green, J.L., Horner-Devine, M.C., Kane, M., Adams Krumins, J., Kuske, C.R., Morin, P.J., Naeem, S., Øvreås, L., Reysenback, A.L., Smith, V.H., Staley, J.T., 2006. Microbial biogeography: putting microorganisms on the map. Nature Rev. Microbiol. 4, 102–112.

Kabaluk, J.T., Vernon, R.S., Goettel, M.S. Field infection of wireworms (Coleoptera: Elateridae) with inundative applications of *Metarhizium anisopliae*. Phytoprotection (in press).

Kokotovic, B., On, S.L., 1999. High-resolution genomic fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* by analysis of amplified fragment length polymorphisms. FEMS Microbiol. Lett. 173, 77–84.

Leal-Bertioli, S.C.M., Butt, T.M., Peberdy, J.F., Bertioli, D.J., 2000. Genetic exchange in *Metarhizium anisopliae* strains co-infecting *Phaedon cochleariae* is revealed by molecular markers. Mycol. Res. 104, 409–414.

Lynch, M., 1988. Estimation of relatedness by DNA fingerprinting. Mol. Biol. Evol. 5, 584–599.

Lynch, M., 1990. The similarity index and DNA fingerprinting. Mol. Biol. Evol. 7, 478–484.

MacArthur, R.H., Wilson, E.O., 1967. The Theory of Island Biogeography. Princeton Univ. Press, Princeton, N.J.

Majer, D., Mithen, R., Lewis, B.G., Vos, P., Oliver, R.P., 1996. The use of AFLP fingerprinting for the detection of genetic variation in fungi. Mycol. Res. 100, 1107–1111.

Mueller, U.G., Lipari, S.E., Milgroom, M.G., 1996. Amplified fragment length polymorphism (AFLP) fingerprinting of symbiotic fungi cultured by the fungus-growing ant *Cyphomyrmex minutus*. Mol. Ecol. 5, 119–122.

Nicholas, K.B., Nicholas, H.B., 1997. GeneDoc: a tool for editing and annotating multiple sequence alignments. <http://www.psc.edu/biomed/genedoc/>.

Page, R.D.M., 2001. TreeView.<http://taxonomy.zoology.gla.ac.uk/rod/rod.html/>.

Petch, T., 1931. Notes on entomogenous fungi. Trans. Br. Mycol. Soc. 15, 67–71.

Rath, A.C., Carr, C.J., Graham, B.R., 1995. Characterization of *Metarhizium anisopliae* strains by carbohydrate utilization (API50CH). J. Invertebr. Pathol. 65, 12–16.

Rosendahl, S., Taylor, J.W., 1997. Development of multiple genetic markers for studies of genetic variation in arbuscular mycorrhizal fungi using AFLP. Mol. Ecol. 6, 821–830.

SAS Institute Inc. 1999. SAS/STAT user's guide, version 8.0. SAS Institute, Inc., Cary, NC.

Smith, C.T., Nelson, R.J., Wood, C.C., Koop, B.F., 2001. Glacial biogeography of North American coho salmon (Oncorhynchus kisutch). Mol. Ecol. 10, 2775–2785.

St. Leger, R.J., May, B., Allee, L.L., Frank, D.C., Staples, R.C., Roberts, D.W., 1992. Genetic differences in allozymes and in formation of infection structures among isolates of the entomopathogenic fungus *Metarhizium anisopliae*. J. Invertebr. Pathol. 60, 89–101.

Taylor, J.W., Geiser, D.M., Burt, A., Koufopanou, V., 1999. The evolutionary biology and population genetics underlying fungal strain typing. Clin. Microbiol. Rev. 12, 126–146.

Veen, K.H., Ferron, P., 1966. A selective medium for the isolation of *Beauveria tenella* and of *Metarhizium anisopliae*. J. Invertebr. Pathol. 8, 268–269.

Zimmerman, G., 1986. The '*Galleria* bait method' for detection of entomopathogenic fungi in soil. J. Appl. Entomol. 102, 213–215.