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Differentiation of *Phoma foveata* from *P. exigua* using a RAPD generated PCR-RFLP marker

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Abstract

Phoma foveata and *P. exigua* variety *exigua* both infect potatoes and are morphologically very similar. *P. foveata* produces a pigment which allows differentiation from *P. exigua* in culture. Discrimination of the two species based on the production of a secondary metabolite, which is dependent on the growth conditions, is not reliable. Therefore, there is a need to develop nucleic acid based identification markers. A 482 bp random amplified polymorphic DNA (RAPD) fragment from *P. foveata* was isolated and sequenced. Polymerase chain reaction (PCR) primers, developed from the sequence of the RAPD product, amplified a 474 bp fragment for *P. foveata* and *P. exigua* varieties *exigua*, *diversispora*, *inoxydibilis* and *sambuci-nigrae*. The similarity of the PCR fragments was demonstrated by sequence analysis and by using the restriction enzymes *Dde*I and *Dpn*II. *P. foveata* was distinguished from the four varieties of *P. exigua* on the basis of the RFLP patterns of the PCR fragment. Ten isolates of *P. foveata* and nine of *P. exigua* var. *exigua* from different geographic locations were tested and all isolates but one showed the restriction digest pattern of the PCR fragment (PCR-RFLP) specific to each species. One isolate of *P. foveata* demonstrated a PCR-RFLP pattern similar to *P. exigua* var. *exigua* leading to the conclusion that the isolate had been previously misidentified as a strain of *P. foveata* lacking the ability to produce pigment.

Abbreviations: bp – base pairs; CTAB – hexadecyltrimethylammonium bromide; PCR – polymerase chain reaction; RAPD – random amplified polymorphic DNA; Rf – rate of flow; RFLP – restriction fragment length polymorphism; TLC – thin layer chromatography.

Introduction

Phoma foveata Foister is the major cause of potato gangrene which is a dry rot disease of potato tubers (Boerema, 1967; Boyd, 1972). The principal host is potato (*Solanum tuberosum*), but it has been found on other plant species near potato fields (Boerema, 1976). The fungus has been reported mostly in Europe but also in Northern Africa, New Zealand and Australia (EPPO, 1997). In addition, *P. foveata* is found in the South-American Andes where the species is believed to have originated (Otazú et al., 1979).

Potato gangrene caused by *P. foveata* has been often confused with another potato rot caused by a morphologically similar pathogen: *P. exigua* var. *exigua* (Boerema, 1967), which has been isolated from a wide variety of host plants and has wide geographical distribution (Boerema and Höweler, 1967; Sutton, 1980). The two *Phoma* species cannot be distinguished by their pycnidia or conidia (Boerema, 1967). However, *P. foveata* produces an yellowish-brown to reddish-brown anthraquinone pigment which is not produced by *P. exigua*. Since these two *Phoma* species are morphologically similar, it is believed that they are

closely related; *P. foveata* has also been named as a variety of the species *P. exigua*: *P. exigua* var. *foveata* (Boerema, 1967). The Andean origin of *P. foveata* and an extensive study based on physiological and morphological characters of *Phoma* species led to the hypothesis that *P. foveata* and *P. exigua* var. *exigua* are distinct species (Boerema et al., 1987; Montel et al., 1991). It is important to distinguish between the two species because *P. foveata* is more pathogenic and tolerates lower temperatures than *P. exigua* var. *exigua* (Boerema, 1967), resulting in different risks and different national and international regulations. Pigment production by *P. foveata* appears to be a variable character and pigment production in culture is often lost which makes these isolates of *P. foveata* difficult to differentiate from *P. exigua* var. *exigua* (Boerema, 1967; Malcolmson, 1958). To increase confidence in the differentiation, other techniques have to be established. These techniques could involve nucleic acid sequences whose presence in the genome is independent of growth condition or life stages.

Identification techniques involving *Phoma* species and using the PCR technology have been used in the last few years to differentiate between genera or species (Faris-Mokaiesh et al., 1996; Keinath et al., 1995; Rollo et al., 1990). The technique of PCR allows the amplification of DNA fragments of specific or non-specific sequences (Mullis and Faloona, 1987; Saiki et al., 1985). The use of primers homologous to conserved sequences of ribosomal DNA allows the amplification of DNA through the internal transcribed spacers (ITS) which may vary from species to species within a genus (White et al., 1990). We have used these primers to amplify the ITS1 and ITS2 regions from *P. foveata* and *P. exigua* var. *exigua* and analysed the sequences to find differences that would allow the differentiation of the two fungi. The ITS1 sequences of *P. foveata* and *P. exigua* var. *exigua* were identical, as were the ITS2 sequences. It was, therefore, not possible to use these regions for identification (data not shown) (GenBank accession numbers: AF079892, AF079893, AF079894 and AF079895). PCR using random primers, RAPD, has been used to display DNA polymorphisms between genomes (Welsh and McClelland, 1990; Williams et al., 1990). It is fast and does not require sequence information. Generating information from a RAPD fragment for the design of specific primer is a technique used when amplification of conserved sequences, such as the ribosomal ITS primers, is not desirable (Garner and Slavicek, 1996; Ouellet and Seifert, 1993; Paran and Michelmore, 1993).

The objective of this work was to use RAPD fingerprinting to find polymorphisms between *P. foveata* and *P. exigua* var. *exigua* and to use the sequence information to develop a PCR assay that would discriminate between the two pathogens.

Materials and methods

Fungal strains

The *Phoma* strains used and their sources are listed in Table 1. ATCC strains were maintained by the American Type Culture Collection and DAOM strains were maintained by the Canadian Collection of Fungal Cultures (Eastern Cereals and Oilseeds Research Centre, Agriculture and Agri-Food Canada, 960 Carling Ave., Ottawa, Ontario, Canada, K1A 0C6). The other *Phoma* strains were provided by Dr. Hans de Gruyter (Plantenziektenkundige Dienst, Geertjesweg 15, Wageningen, The Netherlands). Potato dextrose agar plates (39 g/l) were inoculated with rehydrated lyophilized cultures. After 4–6 days at room temperature, plugs from the plates were used to inoculate 150 ml (in a 500 ml flask) of media #115 (Dhingra and Sinclair, 1985) for *P. foveata* strains and #113 (Dhingra and Sinclair, 1985) for all other *Phoma* species isolates. Cultures were grown for 2–3 weeks at room temperature on an orbital shaker at 125 rpm. The spheres of mycelium were washed with saline (0.85% NaCl), dried on paper, then sliced open and pressed dry.

Thin layer chromatography

P. foveata anthraquinone pigment was visualized by TLC (Mosch and Mooi, 1979). Cross sections of agar with mycelium were placed in 2 ml of $CHCl_3$ overnight. After removing the agar, the $CHCl_3$ was evaporated on a hot plate at a low setting for about 30 min. The samples were redissolved in 100 µl of $CHCl_3$ and 2–10 µl were applied to a silica plate (Alltech, Deerfield, IL). After drying for 15 min the plates were developed in toluene : acetone (95 : 5) and visualized under ultraviolet light. An orange-yellow spot at Rf 0.79 corresponded to the presence of the pigment.

Table 1. List of the *Phoma* species and their origin

Phoma species	Isolate #	Origin[1]
P. foveata	ATCC 24771	*Solanum tuberosum*, Ireland
	79/139	*Chenopodium quinoa*, Peru
	79/1040	*S. tuberosum*, Peru
	90/480	*S. tuberosum*, unknown
	90/481	*S. tuberosum*, unknown
	94/1394	*S. tuberosum*, Bulgaria
	ATCC 28763	*S. tuberosum*, Switzerland
	ATCC 32160	*S. tuberosum*, Denmark
	ATCC 48902	*S. tuberosum*, Ireland
	ATCC 64355	*S. tuberosum*, France
P. exigua var. *exigua*	94/1396	*S. tuberosum*, Bulgaria
	82/942	*Salix*, Netherlands
	90/482	*S. tuberosum*, unknown
	90/731	*S. tuberosum*, Philippines
	90/835-3	*Digitalis*, Netherlands
	ATCC 28964	*S. tuberosum*, Ireland
	ATCC 28967	*S. tuberosum*, England
	ATCC 28980	*S. tuberosum*, Germany
	ATCC 64336	*S. tuberosum*, France
P. exigua var. *diversispora*	ATCC 58123	*Phaseolus vulgaris*, Kenya
P. exigua var. *inoxydibilis*	ATCC 32161	*Vinca minor*, Netherlands
P. exigua var. *sambuci-nigra*	ATCC 32811	*Sambucus nigra*, Netherlands
P. exigua var. *linicola*	ATCC 32332	*Linum usitatissimum*, Netherlands
P. eupyrena	DAOM 167330	Soil, Alb., Canada
	DAOM 169241	Pine needles, Ont., Canada
P. glomerata	DAOM 169235	*Juniperus*, Que., Canada
	DAOM 214574	*Juniperus*, Que., Canada
	DAOM 214575	*Ulmus pumila*, Que., Canada
P. medicaginis	DAOM 174525	red clover, Que., Canada
P. multirostrata	DAOM 216035	*Lupinus* sp., Que., Canada
	DAOM 216036	*Lupinus* sp., Que., Canada
	DAOM 216038	*Lupinus* sp., Que., Canada
P. pomorum	DAOM 172382	*Brassica napus*, Ont., Canada
	DAOM 191265	*Avena fatus*, Sask., Canada
	DAOM 215170	*Ambrosia artemisiifolia*,Sask., Canada
P. sorghina	DAOM 170848	Corn, Ont., Canada
	DAOM 170849	Corn, Ont., Canada
	DAOM 194920	*Mucuna aterrima*, Zambia
	DAOM 214589	*Atherigona soccata*, Burkina

[1]Plant host/substrate and country of origin.

DNA extraction

DNA was extracted following the method of Möller et al. (1992) with modifications. Mycelia were ground in liquid nitrogen with a mortar and pestle. Approximately 100 mg of powdered mycelia were transferred to a 1.5 ml microcentrifuge tube with 500 µl of extraction buffer (100 mM Tris–HCl, pH 8.0, 10 mM EDTA, 2% SDS); 100 µg of Proteinase K (Boehringer Mannheim, Indianapolis, IN) was added to the mixtures and incubated at 56 °C for 60 min. After the incubation, 143 µl of 5 N NaCl was added followed by 65 µl of 10% CTAB. After 10 min at 65 °C, one volume of chloroform : isoamyl alcohol (24 : 1) was added and the mixtures were placed on ice for 15 min. After 10 min centrifugation at 14 000 × g, the supernatants were transferred to fresh microcentrifuge tubes with 0.55 volume of isopropanol, mixed well and centrifuged for 10 min at 14 000 × g. The DNA pellets were resuspended in 100 µl of 0.1 TE (1 mM Tris–HCl, pH 8.0, 0.1 mM EDTA). DNase-free RNase (Boehringer Mannheim, Indianapolis, IN) was added (1 µg) and the

DNA extracts were incubated at 37 °C for 10–15 min. The DNA concentration was determined on agarose gels by comparison with a DNA mass ladder (Gibco-BRL, Gaithersburg, MD).

DNA amplification

RAPD reactions were performed using the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT). Approximately 100–250 pg of DNA extracts (1 µl of 1 : 40 dilution) were amplified in 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 250 µM of each dNTP, 1.5 mM $MgCl_2$, 1.2 µM 10-mer random primer and 0.6 unit of AmpliTaq (Perkin-Elmer Cetus, Norwalk, CT) for a total volume of 20 µl. The reactions were carried out in a Perkin-Elmer 9600 thermocycler (Perkin-Elmer Cetus, Norwalk, CT) with an initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 5 s, 35 °C for 30 s, a 2 min ramping to 72 °C (0.3 °C/s) and an extension at 72 °C for 2.5 min. After 35 cycles, there was a final extension at 72 °C for 5 min. The random primers for DNA polymorphism screening were purchased from the University of British Columbia (Dr. John Hobbs Nucleic Acid–Protein Service (NAPS) Unit Biotechnology Laboratory). Primers #304, #305, #308– #329 and #357– #365 were tested (Table 2).

PCR reactions using sequence-specific primers were performed using Gibco-BRL reagents (Gaithersburg, MD). Approximately 200–500 pg of DNA extracts (1 µl of 1 : 20 dilution) were amplified in 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 200 µM of each dNTP, 2.5 mM $MgCl_2$, 0.4 µM of each of the specific primers and 0.25 unit of Taq DNA polymerase (Gibco-BRL, Gaithersburg, MD) in a total volume of 10 µl. The reactions were carried out in a PTC-200 DNA engine thermocycler (MJ Research, Watertown, MA), with an initial denaturation at 95 °C for 1 min followed by 30 cycles of 94 °C for 15 s, 62 °C for 30 s, an extension at 72 °C for 1.5 min and, at the end of the last cycle, a final extension at 72 °C for 3 min. The specific primers were synthesized by Gibco-BRL (Gaithersburg, MD) and were: Phoma-2 (forward primer): 5′-GGA CCC CTG TAC TGA CGT C-3′, and the reverse primer Phoma-7: 5′-AGC GGC TAG GAT AGA CAG GCG-3′.

Amplified products were visualized following electrophoresis in 1.5% agarose gels, stained with ethidium bromide and visualized under ultraviolet light.

Cloning and sequencing RAPD or PCR products

Selected amplified DNA products were isolated from agarose gels by electroelution (International Biotechnologies, New Haven, CT), and ligated into the *Eco*RV site of vector pBluescript SK+ (Gibco-BRL, Gaithersburg, MD). After transformation of *Escherichia coli*, white colonies were selected, plasmid DNA was prepared and after restriction digest, clones containing the expected size insert were sequenced using the T7 Sequenase v2.0 Quick-Denature plasmid sequencing kit (Amersham Life Science, Cleveland, OH). GenBank accession numbers for the sequences are: AF079896, AF079897 and AF079898.

Restriction digests of PCR products

Half of each PCR reaction mixture (5 µl) was digested with 10 units of the restriction enzymes *Dde*I or *Dpn*II (New England Biolabs, Beverly, MA) in a final volume of 10 µl. The reactions were incubated at 37 °C for 2 h and separated on a 4% nuseive : agarose (3 : 1) gel (FMC Bioproducts, Rockland, ME). The DNA bands

Table 2. List of 10-mer primers used for RAPD analysis of *Phoma* species

Primer	Sequence	Primer	Sequence	Primer	Sequence
304	5′-AGTCCTCGCC-3′	317	5′-CTAGGGGCTG-3′	328	5′-ATGGCCTTAC-3′
305	5′-GCTGGTACCC-3′	318	5′-CGGAGAGCGA-3′	329	5′-GCGAACCTCC-3′
308	5′-AGCGGCTAGG-3′	319	5′-GTGGCCGCGC-3′	357	5′-AGGCCAAATG-3′
309	5′-ACATCCTGCG-3′	320	5′-CCGGCATAGA-3′	358	5′-GGTCAGGCCC-3′
310	5′-GAGCCAGAAG-3′	321	5′-ATCTAGGGAC-3′	359	5′-AGGCAGACCT-3′
311	5′-GGTAACCGTA-3′	322	5′-GCCGCTACTA-3′	360	5′-CTCTCCAGGC-3′
312	5′-ACGGCGTCAC-3′	323	5′-GACATCTCGC-3′	361	5′-GCGAGGTGCT-3′
313	5′-ACGGCAGTGG-3′	324	5′-ACAGGGAACG-3′	362	5′-CCGCCTTACA-3′
314	5′-ACTTCCTCCA-3′	325	5′-TCTAAGCTCG-3′	363	5′-ATGACGTTGA-3′
315	5′-GGTCTCCTAG-3′	326	5′-CGGATCTCTA-3′	364	5′-GGCTCTCGCG-3′
316	5′-CCTCACCTGT-3′	327	5′-ATACGGCGTC-3′	365	5′-TAGACAGAGG-3′

were visualized under ultraviolet light after staining the gel with ethidium bromide.

Results

Thin layer chromatography

All isolates of *P. foveata* and all varieties of *P. exigua* were assessed by TLC for the presence of anthraquinone pigment. None of the varieties of *P. exigua* showed the pigment (Table 3). However, pigment was present in 8 of the 10 *P. foveata* isolates. *P. foveata* isolates 90/480 and 28763 did not produce a pigment detectable either in culture or by TLC.

Screening for polymorphic RAPD patterns

Thirty-three random primers (Table 2) were analysed in RAPD reactions using *P. foveata* and *P. exigua* DNA to find a polymorphic DNA band between the two species. RAPD patterns were characteristic for each pathogen. All *P. foveata* isolates except one (28763) shared similar RAPD patterns and all isolates of *P. exigua* var. *exigua* shared similar patterns as well. Using primer #308 (Table 2), a 482 bp fragment was amplified strongly from *P. foveata* but only weakly or not at all from *P. exigua* var. *exigua* (Figure 1). This 482 bp fragment was chosen for its strong intensity and its size, which would facilitate its cloning.

Table 3. Biochemical and molecular characterization of *Phoma foveata* and *P. exigua* var. *exigua* isolates

Isolates	Pigment[1]	PCR-*Dde*I[2]	PCR-*Dpn*II[3]
Phoma foveata			
ATCC 24771	+	A	A
79/139	+	A	A
79/1040	+	A	A
90/480	–	A	A
90/481	+	A	A
94/1394	+	A	A
ATCC 28763	–	B	B
ATCC 32160	+	A	A
ATCC 48902	+	A	A
ATCC 64335	+	A	A
Phoma exigua			
94/1396	–	B	B
82/942[4]	–	B	B
90/482	–	B	B
90/731	–	B	B
90/835-3	–	B	B
ATCC 28964	–	B	B
ATCC 28967	–	B	B
ATCC 28980	–	B	B
ATCC 64336	–	B	B

[1]Presence or absence of pigment on TLC.
[2]RFLP pattern obtained after digestion of the PCR product with *Dde*I.
[3]RFLP pattern obtained after digestion of the PCR product with *Dpn*II.
[4]*Phoma exigua* var. *exigua* isolate 82/942 was recently re-identified as *P. exigua* var. *populi* (H. de Gruyter, pers. comm.). Significant genetic differences between the two varieties other than a lower PCR yield for the *populi* DNA were not detected.

Characterization of the 482 bp RAPD fragment

The 482 bp RAPD fragment amplified from *P. foveata* isolate 24771 was cloned for sequence analysis. From the sequence obtained, two specific primers (1L and Phoma-7) containing all or most of the RAPD primer #308 sequence were synthesized. Amplification of *P. foveata* (isolate 79/139) and *P. exigua* var. *exigua* DNA produced a 478 bp fragment. The 478 bp PCR fragments from *P. foveata* isolate 79/139 and *P. exigua* var. *exigua* isolate 94/1396 were cloned and sequenced.

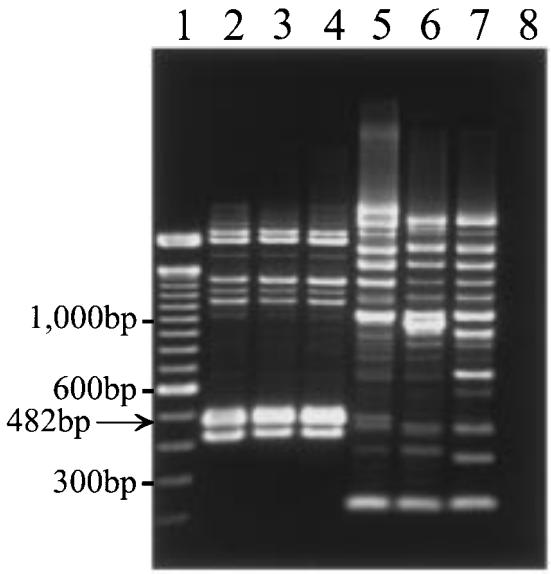

Figure 1. RAPD assay on selected isolates of *P. foveata* and *P. exigua* var. *exigua* using primer #308. Lane 1, 100 bp ladder (Gibco-BRL, Gaithersburg, MD); lane 2, *P. foveata* (24771); lane 3, *P. foveata* (90/480); lane 4, *P. foveata* (94/1394); lane 5, *P. exigua* var. *exigua* (94/1396); lane 6, *P. exigua* var. *exigua* (90/482); lane 7, *P. exigua* var. *exigua* (28980); lane 8, negative control. The sizes (bp) of selected fragments of the 100 bp ladder are indicated on the left.

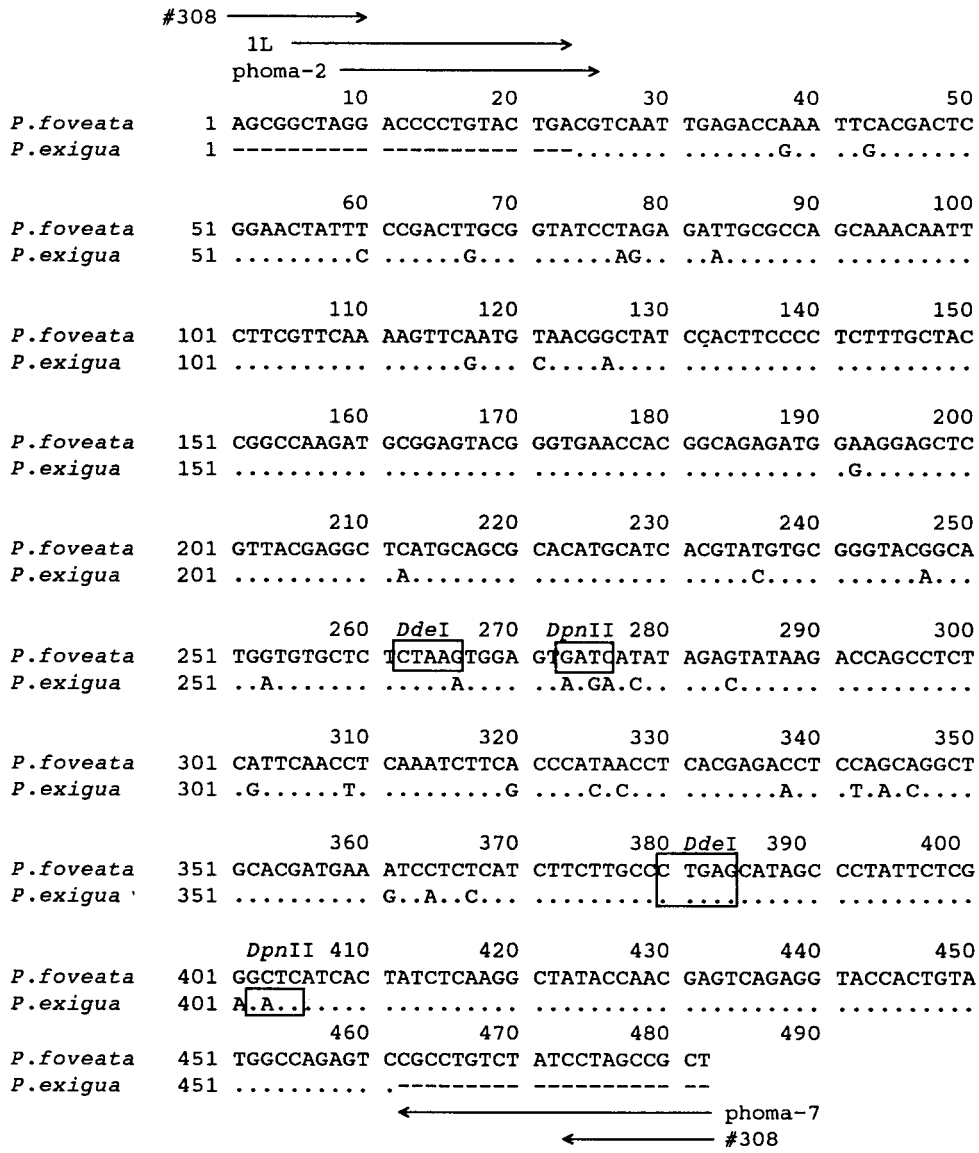

Figure 2. Alignment of *P. foveata* and *P. exigua* var. *exigua* marker sequences. *P. foveata* isolate 24771 was sequenced from a cloned, random amplified DNA band and *P. foveata* isolate 79/139 and *P. exigua* var. *exigua* isolate 94/1396 were sequenced from a cloned PCR fragment. The two *P. foveata* isolate sequences are identical and therefore are combined in this figure. Matches between the two fungi are indicated with dots and hyphens indicate unknown sequences. Primers used in this study are indicated by arrows. Boxes indicate restriction enzyme sites allowing the identification of *P. foveata* from *P. exigua*.

This allowed confirmation of the *P. foveata* RAPD marker sequence and detection of sequence differences between *P. foveata* and *P. exigua* var. *exigua* (see Figure 2). The sequence of the *P. foveata* isolate 24771 RAPD band is identical to the sequence of the *P. foveata* isolate 79/139 specific PCR band. The *P. exigua* var. *exigua* sequence is 92% identical to *P. foveata* (Figure 2: bases 26–436). There are 3 open reading frames of over 40 amino acids and 1 of the 3 continues past the 3′ end of the sequence. Using the BLAST search program (National Center for Biotechnology Information), there were no obvious similarities with any of the sequences available in the GenBank database.

PCR analysis of Phoma *species*

The first set of primers tested for PCR analysis of *Phoma* species, were 1L and Phoma-7 (Figure 2). Primer 1L contained 60% of the RAPD primer #308 sequence and Phoma-7 contains all of the #308 sequence. The stringency of the PCR conditions had to be reduced to reproducibly generate a PCR product for *P. exigua* var. *exigua*. Consequently, we developed another 5′ end primer, Phoma-2, that contains only 20% of the RAPD primer sequence. Amplification with Phoma-2 and Phoma-7 consistently produced a 474 bp PCR product. All isolates described in Table 1 were tested with Phoma-2 and Phoma-7. A DNA fragment of the expected size was amplified for all isolates of *P. foveata* and *P. exigua* vars. *exigua, diversispora, inoxydibilis* and *sambuci-nigrae* (Figure 3). There were no PCR products of the same intensity for *P. exigua* var. *linicola* or all the other *Phoma* species listed in Table 1 (data not shown). Occasionally, there was a weak PCR product for *P. exigua* var. *linicola* and *P. medicaginis* probably due to the presence of sequences resembling the PCR priming site. However, the DNA fragment(s) amplified for these two isolates were too weak to be visualised after restriction enzyme digestion. The *Phoma* DNAs that did not amplify the 474 bp fragment were tested with random primers to demonstrate that DNA quality and quantity were not the reason for the absence of the specific PCR band (data not shown).

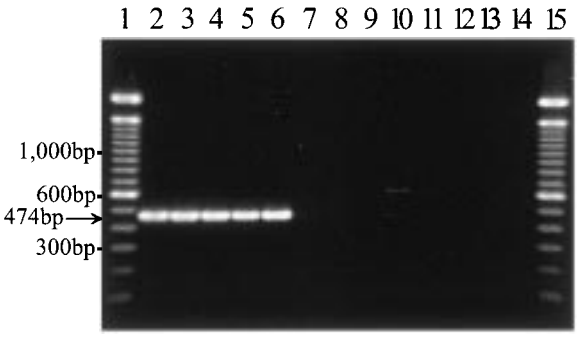

Figure 3. PCR assay on selected *Phoma* species using primers phoma-2 and phoma-7. Lanes 1 and 15, 100 bp ladder (Gibco-BRL, Gaithersburg, MD); lane 2, *P. foveata* (24771); lane 3, *P. exigua* var. *exigua* (94/1396); lane 4, *P. exigua* var. *diversispora* (58123); lane 5, *P. exigua* var. *inoxydibilis* (32161); lane 6, *P. exigua* var. *sambuci-nigrae* (32811); lane 7, *P. exigua* var. *linicola* (32332); lane 8, *P. eupyrena* (167330); lane 9, *P. glomerata* (214574); lane 10, *P. medicaginis* (174525); lane 11, *P. multirostrata* (216036); lane 12, *P. pomorum* (191265); lane 13, *P. sorghina* (194920); lane 14, negative control. The sizes (bp) of selected fragments of the 100 bp ladder are indicated on the left.

PCR-RFLP analysis of P. foveata *and* P. exigua *varieties*

The 474 bp PCR fragment amplified using primers Phoma-2 and Phoma-7 was digested using the restriction enzymes *Dde*I and *Dpn*II. As expected, the *Dde*I digestion generated DNA fragments of 254, 118 and 102 bp from the *P. foveata* PCR product and fragments of 372 and 102 bp from *P. exigua* var. *exigua* as well as from the varieties *diversispora, inoxydibilis* and *sambuci-nigrae* (Figure 4A). Using the restriction enzyme *Dpn*II, *P. foveata* was distinguished by its pattern of 264 and 210 bp fragments from the *P. exigua* varieties that generated a restriction pattern of 393 and 81 bp fragments (Figure 4B).

PCR-amplified bands for all isolates of *P. foveata* and *P. exigua* var. *exigua* were digested with *Dde*I and *Dpn*II (data not shown). All *P. foveata* isolates but one (28763) showed the pattern described in the previous paragraph for *P. foveata* and labelled as A in Table 3. *P. exigua* var. *exigua* isolates and *P. foveata* isolate 28763 showed the same pattern described previously for varieties of *P. exigua* and labelled as B in Table 3.

Discussion

A RAPD fragment obtained in large quantity from *P. foveata* was characterized to develop PCR primers. Primer pair Phoma-2 and Phoma-7 consistently amplified the *P. exigua* var. *exigua*, *diversispora*, *inoxydibilis* and *sambuci-nigrae* sequences as well as the *P. foveata* sequence. After digestion of the PCR product, *P. foveata* had a different PCR-RFLP pattern from those of *P. exigua* varieties (Figure 4). Isolates of *P. foveata* and *P. exigua* var. *exigua* including *P. foveata* isolates 90/480 and 28763, which did not show any pigment on TLC, were also assayed.

The RAPD and PCR-RFLP patterns of isolate 90/480 were similar to all *P. foveata* isolates that exhibited pigment. Isolate 90/480 is possibly a *P. foveata* that has lost its ability to produce pigment. If identification of isolate 90/480 relied only on pigment production than the failure of the isolate to produce pigment could result in misidentification of a quarantine pest which could have severe consequences. Isolate 28763 also did not produce any pigment but RAPD and PCR-RFLP

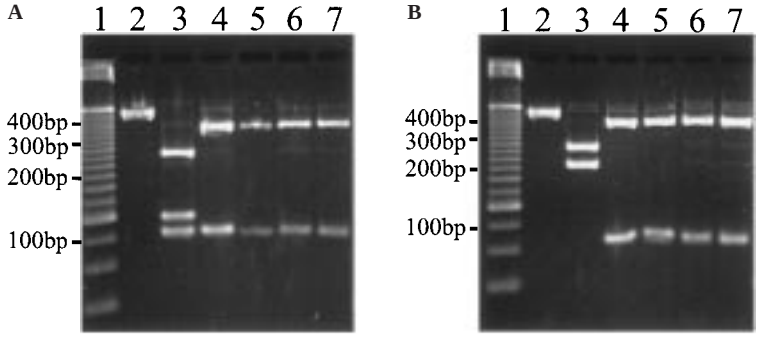

Figure 4. PCR assay and restriction digest of the amplified fragment of *Phoma* species. Panel A corresponds to the patterns obtained using the restriction enzyme *Dde*I and panel B corresponds to the patterns obtained using restriction enzyme *Dpn*II. Lane 1, 25 bp ladder (Gibco-BRL, Gaithersburg, MD); lane 2, undigested PCR product; lane 3, *P. foveata* (24771); lane 4, *P. exigua* var. *exigua* (94/1396); lane 5, *P. exigua* var. *diversispora* (58123); lane 6, *P. exigua* var. *inoxydibilis* (32161); lane 7, *P. exigua* var. *sambuci-nigrae* (32811). The sizes (bp) of selected fragments of the 25 bp ladder are indicated on the left of each panel.

patterns were similar to *P. exigua* var. *exigua*. According to supplier information, isolate 28763 is a strain *P. foveata*, however RAPD and PCR-RFLP patterns show that it is, instead, a form of *P. exigua*. Since pigment production cannot be the basis of this identification, the morphological similarity has most likely lead to the misidentification. Therefore, a nucleic acid-based identification test that specifically differentiates *P. foveata* from varieties of *P. exigua* is necessary to adequately regulate these pests. All other isolates consistently produced the expected *P. foveata* or *P. exigua* var. *exigua* PCR-RFLP patterns.

In summary, a PCR-based test has been developed which distinguishes *P. foveata* from varieties of *P. exigua*. PCR primers derived from a RAPD product generated a 474 bp fragment from both fungi which was then digested using restriction enzymes to identify *P. foveata* and *P. exigua* varieties. Although the assay has only been tested using pure mycelia, it could be used eventually to test DNA extracted from infected potato tissue. The availability of a rapid and reliable *P. foveata* identification test would reduce the risk of pest introduction and facilitate the application of proper measures for disease control.

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