Limited Genetic Diversity in North American Isolates of *Phytophthora erythroseptica* Pathogenic to Potato Based on RAPD Analysis

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ABSTRACT

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Pink rot of potato (*Solanum tuberosum*), caused by *Phytophthora erythroseptica*, is found wherever potatoes are grown, and in the last decade, it has reemerged as an economically important disease in Canada and the United States. A selection of isolates of *P. erythroseptica* from major potato-growing regions in North America, namely Prince Edward Island and New Brunswick, Canada, and Maine and Idaho, U.S.A., was assessed for genetic diversity with randomly chosen decanucleotide primers which were used to amplify regions of DNA to reveal polymorphisms among templates (random amplified polymorphic DNA [RAPD]). The isolates varied in their geographic origin as well as in their sensitivity to mefenoxam, as determined by an in vitro assay. In three separate RAPD screens (I, II, and III) with 23 isolates of *P. erythroseptica* chosen from a larger collection, 1,410, 369, and 316 robust, scorable bands were amplified, respectively. However, among the bands amplified in screens I, II, and III, only 3, 1, and 3 bands, respectively, were polymorphic. When three primers yielding polymorphisms were used to screen 106 isolates from Prince Edward Island and New Brunswick, or a representative collection of 32 isolates from Prince Edward Island, New Brunswick, Maine, and Idaho, no major variation was discovered. RAPD markers were not correlated with geographic origin or mefenoxam sensitivity of the isolates. From an evolutionary standpoint, the absence of genetic diversity among the isolates of *P. erythroseptica* we examined may be attributable to the relatively recent introduction of a small founding population of the pathogen in North America.

Phytophthora erythroseptica Pethyb. is an oomycete and therefore belongs to a group of organisms closely related to chromophyte algae, such as diatoms (9). Isolates of *P. erythroseptica* are diploid, homothallic (self-fertile; 17,42), and produce oospores that can survive for many years in soil (49).

Diversity within or among populations of *P. erythroseptica* has not been well documented. Strains of the pathogen specific to a particular plant species have been reported, including isolates pathogenic to potato (*Solanum tuberosum* L.; 34,35), raspberry (*Rubus* spp.; 3), tomato (*Lycopersicon esculentum* L.; 12), clover (*Tri-*

folium spp.; 36), lupine (*Lupinus* spp.; 46), white calla (*Zantedeschia aethiopica* Spreng.; 44), and pea (*Pisum sativum* L.; 5). In addition, isolates of *P. erythroseptica* that are resistant to mefenoxam, a systemic fungicide generally used for suppression of diseases caused by oomycetes, have been recovered from various locations in the United States (14,20,43).

Pink rot of potato is found in most major potato-growing regions of the world (48), and propagules of *P. erythroseptica* are endemic in almost all cultivated soils where potatoes are grown (21). Poorly drained land and warm, wet conditions at harvest tend to exacerbate disease incidence and severity (2,37). All underground potato tissues can become infected by *P. erythroseptica*. Root and stem infection can result in plant wilting and death, while tuber infection leads to pink rot development in the field and in storage.

The re-emergence of potato pink rot as an economically important disease has been partly fueled by the increase in occurrence of mefenoxam-resistant strains of the pathogen and the lack of disease-resistant potato cultivars. Mefenoxam (or its parent isomer metalaxyl) is a systemic fungicide

commonly applied for control of potato diseases caused by oomycetes. Prior to the 1990s, metalaxyl was routinely used prophylactically for the control of late blight caused by *Phytophthora infestans* (Mont.) de Bary. However, in the mid-1990s, metalaxyl-sensitive strains of *P. infestans* were displaced by migrant, resistant genotypes such as US-8 in the United States and Canada (15,16,29,30), resulting in loss of fungicide utility. Even so, mefenoxam is still applied to the potato crop for the suppression of pathogens causing tuber rot diseases such as leak (causal agent: *Pythium ultimum* var. *ultimum* Trow) and pink rot. In recent years, the recovery of mefenoxam-resistant strains of *P. erythroseptica* in the United States and Canada (14,20,43) has created concern that successful control of pink rot also is in jeopardy.

Pathogen diversity can play a critical role in disease dynamics and, consequently, in the success of disease-management strategies, including the development of cultivars resistant to pink rot. Random amplified polymorphic DNA (RAPD; 53) analysis has been used successfully to assess diversity among isolates of other *Phytophthora* species (6,23–25,27,50). Therefore, our aim was to assess the genetic diversity in collections of isolates of *P. erythroseptica* from North America pathogenic to potato using the RAPD method.

MATERIALS AND METHODS

Source of isolates. Tubers with symptoms of pink rot were collected from harvested fields and warehouses in Prince Edward Island and New Brunswick, Canada, during 1999 to 2001. Individual tubers were cut longitudinally, and small tissue samples (10 × 5 × 3 mm) were taken from the margins of necrotic regions with a sterile scalpel, surface-sterilized in 0.6% sodium hypochlorite for 30 s, rinsed twice in sterile distilled water, and blotted dry on sterile filter paper (Whatman no. 4). Tissue pieces were then plated onto 1.5% water agar in small petri dishes (60 × 15 mm). Petri dishes were incubated in the dark at 22°C for 3 to 4 days. Following incubation, hyphal tips from the margins of ac-

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tively growing cultures were removed with a sterile probe and plated onto clarified V8 medium (33) to generate pure cultures. In addition, pure cultures growing in agar medium were obtained from Maine and Idaho. The entire collection of isolates was comprised of 104 from Prince Edward Island, 2 from New Brunswick, 32 from Maine, and 30 from Idaho. All isolates were identified to the species level using the keys of Newhook et al. (26) and Gerretson-Cornell (11). Isolates in pure culture were used to generate single-zoospore isolates using a protocol described previously (33).

Mefenoxam sensitivity of isolates. Single-zoospore isolates were characterized for mefenoxam sensitivity using clarified V8 medium amended with 0, 0.5, 1, 5, 10, 50, 100, or 500 µg of mefenoxam per ml as described in Peters et al. (33). Calculated EC₅₀ values (the mefenoxam concentration inhibiting the growth of the pathogen by 50%) were used to recognize three categories of sensitivity: sensitive (S; EC₅₀ < 1 µg/ml), moderately resistant (MR; EC₅₀ = 1 to 100 µg/ml), and highly resistant (HR; EC₅₀ > 100 µg/ml).

Genomic DNA extraction. Agar plugs (5 mm diameter) taken from the margins of 3-day-old single-zoospore cultures of *P. erythroseptica* were placed in 15 ml of clarified V8 broth (33) in 50-ml plastic disposable sterile centrifuge tubes to generate mycelium for DNA extraction. Tubes were placed in the dark at 18°C for 3 days to allow growth of the pathogen. Following incubation, the tubes were centrifuged at 5,000 rpm (4,137 × g) for 5 min to collect the mycelium. Excess V8 broth was decanted, and each mycelial pellet was then washed twice with sterile distilled water and centrifuged for a further 5 min at 5,000 rpm (4,137 × g). After decanting wash water, mycelial pellets were drawn up the side of the tube with a sterile spatula and pressed to remove excess water. Pellets were transferred to labeled 1.5-ml centrifuge tubes and stored at -80°C prior to DNA extraction.

DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen Inc. - Canada, Mississauga, Ontario) according to the manufacturer's instructions. Sterile sea sand (autoclaved Fisher S25-500) was added to the mortar during grinding in liquid nitrogen to enhance tissue disruption. DNA absorbance was measured at 260 and 280 nm using a SmartSpec 3000 (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario) to determine extraction yields.

RAPD polymerase chain reaction (PCR). RAPD analysis of selected isolates of *P. erythroseptica* was performed in three separate screens. In screen I, 360 RAPD primers from Kits A, B, C, D, E, F, H, and Y (Operon Technologies, Inc., Alameda, CA) and UBC Sets 1 and 8 (University of British Columbia, Nucleic Acid-Protein

Service Unit, Vancouver, BC Canada) were used in reactions with DNA templates from four selected isolates of *P. erythroseptica* from Prince Edward Island and Maine (Table 1). These isolates originated from four fields in different regions within Prince Edward Island and Maine and also showed diversity for mefenoxam sensitivity (Table 1). Primers that revealed polymorphisms were then used to assess a larger collection of 106 single-zoospore isolates of *P. erythroseptica* from Prince Edward Island and New Brunswick, Canada. In screen II, 80 RAPD primers, chosen for their ability to produce reproducible banding patterns in screen I, were used in reactions with DNA templates from 10 selected isolates of *P. erythroseptica* from Prince Edward Island and New Brunswick, Canada (Table 1). Finally, in screen III, 60 RAPD primers, chosen for their ability to produce reproducible banding patterns in screens I and II, were used in reactions with DNA templates from 10 selected isolates of *P. erythroseptica* from Prince Edward Island, New Brunswick, Maine, and Idaho (Table 1). Again, these isolates originated from 10 fields representing different potato production areas of North America and also showed diversity for mefenoxam sensitivity (Table 1). Following screens I to III, the three primers (OPF 01, OPH 12, UBC 730) that consistently revealed polymorphisms were used to assess a representative collection of 32

single-zoospore isolates of *P. erythroseptica* from Prince Edward Island, New Brunswick, Maine, and Idaho.

Reactions were performed in 25-µl volumes containing 1× buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCL, 2 mM MgCl₂), 0.2 mM dNTPs, 0.45 µM primer, 1 U of AmpliTaq DNA polymerase (PerkinElmer Life and Analytical Sciences, Woodbridge, Ontario), and 50 ng of genomic DNA template. DNA amplification was performed in a DNA Engine (MJ Research, Inc., Waltham, MA) with an initial cycle at 94°C for 5 min, 33°C for 2 min, and 72°C for 2 min, followed by 35 cycles at 94°C for 1 min, 33°C for 1 min, and 72°C for 2 min, and a final incubation at 72°C for 10 min. Reaction conditions were optimized for each primer individually to enhance the detection of polymorphic bands.

RAPD PCR products (10 µl) were loaded into 1% agarose gels and run for 1 h at 90 V. The gels were visualized with a UV light box after staining with Syber-Green nucleic acid gel stain (Molecular Probes, Inc., Eugene, OR), and the resulting images were then captured with a Kodak DC290 digital camera. Gel lanes were arranged so that templates with the same primer were run side by side to allow a quick visual assessment of RAPD patterns among isolates. All reactions were run twice to resolve questionable results or confirm polymorphisms. For each primer, nonreproducible fragments between the

Table 1. Number, source, and mefenoxam sensitivity of single-zoospore isolates of *Phytophthora erythroseptica* used in each of three random amplified polymorphic DNA (RAPD) screens

Screen	Primers ^a	Isolates ^b	Isolate source ^c	Mefenoxam sensitivity ^d
I	OPA 1-20, OPB 1-20, OPC 1-20,	PE9909-1BSZ1	PE	S
	OPD 1-20, OPE 1-20, OPF 1-20,	PE9913-2ASZ1	PE	S
	OPH 1-20, OPY 1-20,	PE99C3SZ3	ME	S
	UBC 1-100, UBC 701-800	PE99C5SZ3	ME	HR
II	OPA 03,04,11,18,19; OPB 07,10,17;	PE9906-1BSZ2	PE	S
	OPC 02; OPD 01,02,03,11,18;	PE9910-1ASZ1	PE	S
	OPE 08,09,15,16,19,20;	PE9911-1ASZ2	PE	S
	OPF 04,06,13,14,16;	PE9915-1ASZ1	PE	S
	OPH 02,03,04,12,13,19,20;	PE9916-2ASZ1	PE	S
	OPY 08,14,15,16,17,20;	PE9924-1DSZ1	PE	S
	UBC 29,38,51,60,71,72,75,77,79,82,	PE9927-2CSZ1	PE	S
	83,84,88,90,91,96,97,98,703,704,	PE0104-10SZ2	PE	S
	710,712,714,718,726,727,728,729,	PE0105-SZ1	NB	S
	730,731,732,733,734,735,751,	PE0106-SZ1	NB	S
	754,755,770,778,790,791,798			
	OPA 03,04,11,18,19; OPB 07,10,17;	PE9910-1ASZ1	PE	S
	OPC 02; OPD 01,02,03,11,18;	PE0106-SZ2	NB	S
	OPE 08,09,15,19,20; OPF 13,14,16;	PE0201	ME	S
III	OPH 02,03,04,13,19,20;	PE0210	ME	S
	OPY 08,14,15, 16,20;	PE0221	ME	HR
	UBC 60,72,75,79,82,83,84,90,	PE0227	ME	HR
	91,703,712,714,718,727,728,730,	PE0237	ID	S
	731,732,733,734,751,755,770,778,	PE0242	ID	S
	790, 791,798	PE0248	ID	HR
		PE0260	ID	HR

^a OP = Operon Technologies, Inc., Alameda, CA; UBC = University of British Columbia, Nucleic Acid-Protein Service Unit, Vancouver, BC Canada.

^b Isolate codes in the culture collection housed in Agriculture and Agri-Food Canada, Crops and Livestock Research Centre, Charlottetown, PEI, Canada.

^c PE = Prince Edward Island, Canada; NB = New Brunswick, Canada; ME = Maine, U.S.A.; ID = Idaho, U.S.A.

^d S = sensitive; HR = highly resistant.

repeated tests were disregarded. Comparison of profiles for each primer was done on the basis of the presence or absence of RAPD fragments presumed to be the same length and scored as identical.

RESULTS AND DISCUSSION

All isolates of *P. erythrosetpica* from Prince Edward Island and New Brunswick were sensitive to mefenoxam, whereas isolates from Maine and Idaho were either sensitive or highly resistant.

Visualization of randomly amplified PCR products revealed clear banding patterns that were reproducible (Fig. 1). In screen I, using DNA templates of isolates from Prince Edward Island and Maine, 293 of 360 random primers yielded a total of 1,410 scorable bands (Table 2). Somewhat surprisingly, only three bands were polymorphic. This minor variation was insufficient for further meaningful population analysis and did not correlate with isolate source or mefenoxam sensitivity. Nevertheless, the three RAPD markers were used to assess a larger collection of single-zoospore isolates from Prince Edward Island (104 isolates) and New Brunswick (2 isolates). Again, only minor variation was found (two haplotypes) with primer OPF 01 (Fig. 1) that distinguished several isolates from one tuber from one field (a polymorphic fragment of ~1,500 bp was absent in 8 isolates from this field and present in 7 isolates from this field).

To examine a greater range of DNA templates with a large primer set (screen II), 10 isolates of *P. erythrosetpica* recovered from individual fields from across Prince Edward Island (8 isolates) and New Brunswick (2 isolates) were screened with 80 primers chosen from screen I for their ability to produce clear, reproducible banding patterns (Table 1). A total of 369 scorable bands was amplified, but only one was polymorphic (Table 2).

A final screen (screen III) was conducted with DNA templates from isolates of *P. erythrosetpica* from different geographic locations including Prince Edward

Island, New Brunswick, Maine, and Idaho, and that showed diversity in sensitivity to mefenoxam (Table 1). Sixty primers chosen from screens I and II for their ability to produce clear, reproducible banding patterns (Table 1) amplified a total of 316 scorable bands, of which only 3 were polymorphic RAPD markers (Table 2). Again, this minor variation was insufficient for further meaningful population analysis and did not correlate with isolate source or mefenoxam sensitivity. Even so, the three RAPD markers were used to assess a representative collection of 32 single-zoospore isolates from Prince Edward Island (16 isolates), New Brunswick (2 isolates), Maine (6 isolates), and Idaho (8 isolates). Again, only minor variation was found (Table 3).

At the start of this study, our intent was to identify a series of primers and RAPD markers that could then be used to assess a large collection of single-zoospore isolates from geographically diverse potato grow-

ing regions in North America. As these collections included isolates differing in mefenoxam sensitivity, it was hoped that an RAPD marker to detect this trait would be found. However, only very minor genetic variation was revealed by RAPD screening of representative isolates, and this variation was not correlated with isolate source or mefenoxam sensitivity. This is somewhat surprising given the ease with which RAPD markers are generally found in screens with DNA templates of other *Phytophthora* species. For example, in heterothallic species, 9 random decanucleotide primers amplified 217 polymorphic bands in a collection of isolates of *P. cinnamomi* Rands from Taiwan (6), 16 RAPD markers were generated from 4 random decanucleotide primers used to amplify regions of DNA from collections of *P. cinnamomi* from South Africa and Australia (23), 9 Operon primers chosen from a screen of 40 primers for reliability and polymorphism revealed 33 polymor-

Table 2. Summary of the results of three random amplified polymorphic DNA (RAPD) primer screens using isolates of *Phytophthora erythrosetpica* obtained from Prince Edward Island and New Brunswick, Canada, and Maine and Idaho, U.S.A.

	Screen I	Screen II	Screen III
Number of isolates	4	10	10
Number of primers assessed	360	80	60
Number of primers yielding amplified polymerase chain reaction products	293	80	60
Total number of amplified bands	1,410	369	316
Number of polymorphic bands (RAPD markers)	3	1	3

Table 3. Polymorphic loci and number of random amplified polymorphic DNA (RAPD) haplotypes detected by each primer among 32 isolates of *Phytophthora erythrosetpica* from Prince Edward Island, New Brunswick, Maine, and Idaho

Primers ^a	Primer sequences	Total number of bands amplified	Number of polymorphic markers	RAPD haplotypes
OPF 01	5'-ACGGATCCTG	3	1	2
OPH 12	5'-ACGCGCATGT	8	1	2
UBC 730	5'-CCACACCCAC	5	1	2
Total		16	3	

^a OP = Operon Technologies, Inc., Alameda, CA; UBC = University of British Columbia, Nucleic Acid-Protein Service Unit, Vancouver, BC Canada.

Fig. 1. *Phytophthora erythrosetpica* isolates from Prince Edward Island assayed with Operon Primer F01 revealing polymorphic patterns. A total of 10 µl of each polymerase chain reaction (PCR) product was loaded per lane on a 1% agarose gel. They were loaded in sequential order with the exception of lanes 1, 10, 19, 28, and 37, which contained marker DNA. Lanes 5, 6, and 12 are missing the band of interest at ~1,500 bp. Reactions from lanes 7 and 16 were rerun and assessed on another gel.

phic amplified products in a collection of isolates of *P. megakarya* Brasier & M.J. Griffin (27), and 6 Operon primers chosen from a screen of 40 primers for reliability and polymorphism revealed 77 polymorphic markers in a collection of isolates of *P. infestans* (Mont.) de Bary (24). In *P. sojae* Kaufm. & Gerd., a homothallic species similar to *P. erythroseptica* (18), a screen of 220 decanucleotide primers revealed 16 that detected polymorphisms and yielded 23 polymorphic fragments in a collection of isolates from the United States (25). In a similar study of *P. sojae* isolates collected from China, 13 decanucleotide primers revealed 68 polymorphic RAPD markers (50).

The genetics of mefenoxam resistance in *P. erythroseptica* are still unclear. Studies with *P. infestans* have suggested that resistance to mefenoxam (metalaxyl) is governed by a single nuclear locus exhibiting incomplete dominance (40), and a similar hypothesis was put forward by Goodwin and McGrath (14) to explain metalaxyl resistance in *P. erythroseptica* and the origin of a class of isolates that were intermediate in resistance. However, more recent work with *P. infestans* has provided contrasting evidence, including reports that metalaxyl resistance is conferred by a single dominant gene with minor genes causing variation in isolate response (22), or that resistance is governed by at least two major loci with other minor loci contributing additive effects (8,19). The wide range of metalaxyl sensitivity found in a large collection of Canadian isolates of the US-8 genotype of *P. infestans* classed as moderately resistant implied that more than one gene was involved in phenotypic expression (polygenic control; 29). This may also be the case for *P. erythroseptica*, but the occurrence of this intermediate form seems to be relatively rare, as isolates in this class comprised only 5% of 805 isolates collected from 1997 to 2000 in the United States (43). RAPD markers have been linked to metalaxyl insensitivity loci in *P. infestans* (8). Unfortunately, we were unable to find a RAPD marker that correlated with mefenoxam sensitivity in our collection of isolates. Such a marker would be a useful tool in the on-going effort to monitor the spread of mefenoxam-resistant strains of *P. erythroseptica* in North America.

Host specificity has generally been used to separate strains of *P. erythroseptica*. However, isolates of *P. erythroseptica* pathogenic to potato have also caused disease in tomato, spinach, and tulip (21). In addition, isolates pathogenic to potato have been recovered from the asymptomatic roots of several nonsolanaceous hosts, including wheat and rye (51). Although commercial potato cultivars vary in susceptibility to pink rot (32,38), none is immune to disease, and the type of race-

specific resistance that has been well documented for other oomycete-host interactions, including *P. sojae*-soybean (4) and *P. infestans*-potato (1), has not been reported for *P. erythroseptica*-potato interactions. Indeed, studies examining potato host response to pathogen attack have uncovered no pathogenic diversity among isolates used for inoculation (31,32,38,41). These reports of the lack of pathogenic diversity among potato-infecting strains of *P. erythroseptica* are consistent with our general findings of low diversity in the pathogen genome as a whole.

In the field, *P. erythroseptica* has an active sexual cycle, and so individual isolates are most likely to be extensively homozygous as a result of repeated selfing, as has been found for isolates of the homothallic *P. sojae* (10,47). Variation, such as at loci conferring resistance to mefenoxam, would be generated mainly by mutation. In addition, migration of strains in infected seed tubers (7) may introduce new genotypes into a growing region. Outcrossing has been found to play a significant role in the generation of genetic variation in other homothallic oomycetes including *Aphanomyces euteiches* Drechs. (39) and *P. sojae* (10,47,52). Outcrossing has, as yet, never been demonstrated experimentally between isolates of *P. erythroseptica*, and so its impact on pathogen populations is unknown.

From an evolutionary standpoint, the absence of genetic diversity among the isolates of *P. erythroseptica* we examined may be attributable to the relatively recent introduction of a small founding population of the pathogen in North America. Pink rot of potato was first described in Ireland by Pethybridge in 1913 (34) and was initially reported in the United States in Maine in 1938 (2). The heterothallic *P. infestans* apparently evolved in the highlands of central Mexico, and it is here that sexual reproduction and the most genetic diversity among isolates is found (45). Genetic variation in clonal A1 (US-1) North American populations of *P. infestans* was most likely generated only by mutation and asexual processes such as mitotic recombination in the absence of sexual reproduction with the opposite A2 mating type (13), prior to the migration of A2 strains into Canada and the United States in the 1990s (15). Even so, these processes were sufficient to generate a number of physiologic races of the pathogen (28). In contrast, the evolutionary origin of *P. erythroseptica* is unknown, but migration via infected plant tissues, particularly seed tubers (7), has likely contributed to the pan-global distribution of strains pathogenic to potato.

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