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Rapid identification of *Phytophthora ramorum* using PCR-SSCP analysis of ribosomal DNA ITS-1

P. Kong[1], C.X. Hong[1], P.W. Tooley[2], K. Ivors[3], M. Garbelotto[3] and P.A. Richardson[1]

[1]Department of Plant Pathology, Physiology, and Weed Science, Hampton Roads Agricultural Research and Extension Center, Virginia Polytechnic Institute and State University, Virginia Beach, VA, USA, [2]United States Department of Agriculture-Agricultural Research Service, Fort Detrick, MD, USA, and [3]Department of ESPM-ES, University of California, Berkeley, CA, USA

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ABSTRACT

P. KONG, C.X. HONG, P.W. TOOLEY, K. IVORS, M. GARBELOTTO AND P.A. RICHARDSON. 2004.

Aims: The primary objectives of this study were to determine if a single-strand conformation polymorphism (SSCP) analysis can be used for rapid identification of *Phytophthora ramorum*, an important quarantine plant pathogen worldwide, and to further assess the potential of the SSCP technique as a taxonomic tool for the genus *Phytophthora*.

Methods and Results: SSCP of ribosomal DNA internal transcribed spacer 1 was characterized for 12 isolates of *P. ramorum*, using a recently reported protocol. The SSCP patterns of this species then were compared with those of 18 closely related *Phytophthora* species. *Phytophthora ramorum* had a unique pattern and was easily distinguished from genetically, morphologically and ecologically close relatives.

Conclusion: An immediate benefit of this study is provision of a highly effective and efficient identification tool for *P. ramorum* in the quarantine process.

Significance and Impact of the Study: This study also provides additional evidence demonstrating that the SSCP is an ideal DNA marker for species differentiation within the genus *Phytophthora*.

Keywords: *Phytophthora ramorum*, *Rhododendron*, sudden oak death, taxonomy, *Viburnum*.

INTRODUCTION

Phytophthora ramorum was first described on *Rhododendron* spp. and *Viburnum* sp. in Germany and the Netherlands in 2001 (Werres *et al.* 2001). Recently, this pathogen also was found responsible for a Phytophthora canker disease in central coastal CA and in Curry Co., southwestern OR, USA (Goheen *et al.* 2002a; McWilliams *et al.* 2002; Rizzo *et al.* 2002). *Phytophthora ramorum* has a wide range of hosts including many important forest trees such as oak, Douglas fir and redwood (Davidson *et al.* 2002; Maloney *et al.* 2002; McWilliams *et al.* 2002; Parke *et al.* 2002; Rizzo *et al.* 2002; Tooley and Englander 2002). Spread of this pathogen may have devastating consequences on natural forests and landscape, and plant nurseries in the USA and other countries.

To prevent pathogen spread, both United States Department of Agriculture (USDA)/Animal and Plant Health Inspection Service and local regulatory institutions have enforced strict measures, regulating shipment of host plant materials from the quarantine areas in CA and OR. An eradication effort also was undertaken in the infested regions of OR, followed by intensive monitoring of treated and perimeter areas (Goheen *et al.* 2002b). These quarantine efforts, however, may not be as effective as expected. This disease occurs in popular recreational parks and urban and wild forest interfaces (Rizzo *et al.* 2002), where the pathogen has been reported to survive in soil on hiking trails. In addition, the host list of *P. ramorum* is growing rapidly

Correspondence to: C.X. Hong, Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University, Hampton Roads Agricultural Research and Extension Center, Virginia Beach, VA 23455, USA (e-mail: chhong2@vt.edu).

(Davidson *et al.* 2002; Maloney *et al.* 2002; McWilliams *et al.* 2002; Parke *et al.* 2002; Tooley and Englander 2002). Thus, spread of this pathogen is still a potential threat. Many states are taking an aggressive approach regarding inspection of plant materials. Specifically, five south-eastern states in the USA (GA, NC, SC, TN and VA) are considered high-risk regions for this disease because of the similarity of ecological environments and annual receipts of large quantities of host or potential host plant stocks from CA and OR. Parallel surveys for this pathogen at nurseries and the surrounding forestry areas in these regions are underway.

Rapid and accurate microbial identification is essential for any pathogen inspection and survey programme. Morphological identification of *P. ramorum* requires substantial experience in species differentiation within the genus *Phytophthora* and considerable time. It can be difficult and may lead to misidentification because of interspecific overlaps and intraspecific plasticity of diagnostic characters (Waterhouse 1963; Gallegly 1983; Brasier 1991). *Phytophthora ramorum* generally is characterized with semi-papillate, deciduous sporangia and numerous large chlamydospores, by its slow growth rate and low cardinal temperature (Werres *et al.* 2001). However, *P. ramorum* may be confused with other species that have similar morphology, such as *P. palmivora* (Werres *et al.* 2001).

Phytophthora ramorum also is a genetically close relative to *P. lateralis*; they differ in ITS-1 and ITS-2 regions by only three and eight nucleotides, respectively (Werres *et al.* 2001). This implies the difficulty of using fragment length-based DNA fingerprinting techniques for distinguishing *P. ramorum* from *P. lateralis* and other genetically similar relatives. DNA sequencing of ITS regions is an effective means for identification of *P. ramorum*, however, use of this method can be time consuming and expensive when numerous isolates need to be identified on a routine basis.

Single-strand conformation polymorphism (SSCP) analysis is a powerful tool, which can detect single base mutations or variations (Orita *et al.* 1989; Rubio *et al.* 1996; Kong *et al.* 2000; Sambrook and Russell 2001). A protocol for SSCP analysis of ribosomal DNA for species separation within the genus *Phytophthora* was reported recently (Kong *et al.* 2003). SSCP analysis worked well for all 29 species tested, but *P. ramorum* was not characterized in that study (Kong *et al.* 2003). Thus, the primary objective of this study was to determine if the SSCP analysis can be used for rapid identification of *P. ramorum*. This was accomplished by characterizing the SSCP pattern of *P. ramorum* and comparing it with those of 18 other closely related species. Another objective was to further assess the potential of SSCP analysis as a taxonomic tool for identifying *Phytophthora* species.

MATERIALS AND METHODS

Isolates, culture and DNA extraction

Twelve isolates of *P. ramorum* and 24 type isolates of other species that are closely related to *P. ramorum* were included in this study (Table 1). All species except *P. ramorum* were cultured and culture DNA was extracted at Virginia Tech in Virginia Beach, VA. Isolates were subcultured on V8 agar (20% clarified V8 juice, 0·4% $CaCO_3$ and 1·5% agar in distilled water) in 60-mm diameter Petri dishes at 23°C in the dark for 1–2 weeks and maintained at 15°C. DNA extraction of these cultures was performed using a one-step boiling method, unless stated otherwise. Mycelium was scraped from a 2-cm^2 surface area of a 1–2 week-old culture plate and transferred to a 1·5-ml microtube containing 500 μl of 10 mM Tris–Cl (pH 7·5). DNA was released by boiling mycelia in a heat block for 20 min then vortexing for 3 min. The supernatant was used immediately or stored at −20°C prior to further use.

Isolates of *P. ramorum* were cultured and DNA extracted using different procedures at USDA-Agricultural Research Service facility in Ft Detrick, MD and University of California in Berkeley, CA. Eight isolates of *P. ramorum* originating from CA, Germany and the Netherlands were grown on a synthetic liquid medium (Xu *et al.* 1982) at 20°C for 14 days in darkness. Genomic DNA was isolated from 60 mg of lyophilized mycelium using the method of Goodwin *et al.* (1992). The other four isolates of *P. ramorum* originating from OR were grown in potato dextrose broth on a rotary shaker (50 rev min^{-1}) at room temperature for 10 days. Genomic DNA was isolated from 75 mg of lyophilized mycelium using the following modified cetyltrimethylammoniumbromide (CTAB) extraction procedure. Lyophilized tissue was pulverized in a FastPrep® instrument (Bio101, Carlsbad, CA, USA) for 10 s at 5000 rev min^{-1}. Pulverized tissue was incubated in 500-ml CTAB on dry ice for 2 min, then thawed at 75°C for 2 min. This freeze-thaw step was repeated twice, with the final thaw for 30 min. DNA was purified in phenol : chloroform : isoamyl alcohol (25 : 24 : 1), further cleaned by using the GeneClean® Turbo Nucleic Acid Purification kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's instructions, and eluted in 30 μl ultra-pure water.

PCR-SSCP analysis

DNA amplification and SSCP analysis of the PCR products were performed at Virginia Tech, as described previously (Kong *et al.* 2003). Amplification utilized a pair of primers that favour oomycetes (Cooke *et al.* 2000) – forward primer ITS6: 5′-GAA GGT GAA GTC GTA ACA AGG-3′, located in the 18S rDNA and reverse primer ITS7: 5′-AGC GTT CTT CAT CGA TGT GC-3′, located in the 5·8S

Table 1 Origins of *Phytophthora* species and isolates analysed in present study

Species	SSCP ID	Isolate*	Host	Location	Alternative source†
P. ramorum	Ram	0-13	*Lithocarpus densiflorus*	CA	0-13 (PWT), Pr-5 (DMR)
	Ram	0–16	*Quercus agrifolia*	CA	0-16 (PWT), Pr-6 (DMR)
	Ram	0-217	*Rhododendron* cv. 'Gomer Waterer'	CA	0-217 (PWT), Pr-52 (DMR)
	Ram	73101	*Lithocarpus densiflorus*	CA	73101 (PWT)‡
	Ram	PRG-1	*Rhododendron* cv. 'Schneewolke'	Germany	PRG-1 (PWT), BBA 69082 (SW), CBS101548
	Ram	PRG-2	*Rhododendron* cv. 'Catawbiense'	Germany	PRG-2 (PWT), BBA 9/95 (SW), CBS 101553 (ex-type)
	Ram	PRN-1	*Rhododendron* sp.	the Netherlands	PRN-1 (PWT), BBA 9/95 (SW), CBS 101553 (ex-type)
	Ram	PRN-2	*Rhododendron* sp.	the Netherlands	PRN-2 (PWT), PD 94/844 (SW), CBS 101332
	Ram	0-661	*Lithocarpus densiflorus*	ON	0–661 (MG)
	Ram	0-662	*Lithocarpus densiflorus*	ON	0–662 (MG)
	Ram	0–663	*Lithocarpus densiflorus*	ON	0–663 (MG)
	Ram	0–664	*Lithocarpus densiflorus*	ON	0-664 (MG)
P. cactorum	Cac	22E8	*Malus* sp.	Rhodesia	P7 (MEG), ATCC 16694
P. cambivora	Cam	22D7	*Prunus armeniana*	MD	P746 (PHT), P63 (MEG)
P. cinnamomi	Cin	23B2	*Persea americana*	Puerto Rico	P11 (MEG), ATCC 15401
P. citricola	Cil I	22F1	*Rhododendron* sp.	West VA	P53 (MEG)
	Cil III	1E1	Nursery irrigation water	OK	SG-R-1 (SLV)
	Cil IV	22G2	*Hedera helix*	SC	AF.018 (SNJ)
	Cil II	22E9	*Kalmia latifolia*	West VA	P101 (MEG)
P. citrophthora	Cip I	3E5	Nursery irrigation water	VA	
	Cip II	15D7	*Theobroma cacao*	Brazil	P.1210 (SNJ)
P. colocasiae	Col	22F8	NA	NA	P113 (MDC), P47 (MEG)
P. cryptogea	Cry I	15E6	Soil	SC	D.200 (SNJ)
	Cry II	22G2	*Aster* sp.	CA	P12 (MEG), ATCC 15402
P. drechsleri	Dre I	1D11	Nursery irrigation water	VA	
	Dre II	1D12	Nursery irrigation water	VA	
P. gonapodyides	Gon	21J5	Vegetable debris in water	UK	ATCC 46726
P. heveae	Hev	22J2	Soil	TN	P17 (MEG), ATCC 16701
P. hibernalis	Hib	22H1	*Citrus sinensis*	Portugal	P115 (MEG), ATCC 60352
P. ilicis	Ili	23A7	*Ilex* sp.	Canada	P113 (MEG), ATCC 56615
P. infestans	Inf	22E4	*Lycopersicon esculentum*	NC	TLFL-1-1a (MEG)
P. lateralis	Lat	22H9	*Chamaecyparis lawsoniana*	ON	P51 (MEG)
P. medicaginis	Med	23A4	*Medicago sativa*	OH	S797 (AFS), P37 (MEG)
P. nicotianae	Nic	22G1	*Nicotiana tabacum*	NC	P22 (MEG), ATCC 15409
P. phaseoli	Pha	23B4	*Phaseolus lunatus*	DE	P106 (MEG)
P. syringae	Syr	23A6	NA	NY	P35 (MEG)

*Identifier of isolates or DNA samples at Virginia Tech in Virginia Beach, VA.
†Original identity of test isolate followed by name of originator in parenthesis. ATCC, American type Culture Collection; CBS, Central bureau voor Schimmelcultures, Utrecht, the Netherlands; DMR, David M. Rizzo at University of CA in Davis, CA; MDC, Michael D. Coffey at University of California in Riverside, CA; MEG, Mannon E. Gallegly at West VA University in Morgantown, West Virginia; MG, Matteo Garbelotto at University of California in Berkeley, CA; PHT, Peter H. Tsao at University of California in Riverside, CA; PWT = Paul W. Tooley at USDA/ARS, Ft Detrick, MD; SLV, Sharon L. von Broembsen at Oklahoma State University, Stillwater, OK and SNJ, Steven N. Jeffers at Clemson University, Clemson, SC; SW, Sabine Werres, Institute for Plant Protection in Horticulture, Braunschweig, Germany.
‡Isolate obtained from Tim Tidwell, California Department of Food and Agriculture.
NA, not available.

rDNA. PCR was performed in a total volume of 25 μl containing 2 μl of boiled culture extract or a 100–1000 $\times$ dilution of DNA extracts. Each reaction used 2·5 μl of the 10 $\times$ PCR buffer, 2·5 μl of 10 μM forward and reverse primers, 2 μl of 2 mM dNTPs, 0·1 μl (5 U μl^{-1}) of Taq^{TM} polymerase (TaKaRa, Shuzo Co. Ltd, Kyoto, Japan) and 13·4 μl of sterilized nanopure water. PCR was programmed with an initial denaturing at 96°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min, with a final extension at 72°C for 10 min.

One microlitre of individual PCR products was mixed with 9 ml of the denaturing buffer (95% formamide, 20 mM

EDTA and 0·05% bromophenol blue). After a brief spin, mixtures were heated at 96°C for 10 min then chilled on ice. Five microlitres of each mixture was loaded on an 8% acrylamide : Bis (29 : 1) nondenaturing minigel (8·3 × 7·3 × 0·75 cm) cast using a Mini-PROTEAN 3 Cell (Bio-Rad Laboratories, Hercules, CA, USA). An aliquot of 25 ng of a single-stranded DNA (ssDNA) ladder also was included in both left and right lanes of a gel to facilitate comparison of SSCP patterns (Kong *et al.* 2003). Denatured PCR products were eletrophoresed in prechilled 1 × TBE buffer (Tris–borate 89 mM, 2 mM EDTA, pH 8·0) at 200 V for 2 h at room temperature.

After electrophoresis, polyacrylamide gels were peeled from the glass plate and soaked in 50 ml (for two gels) of 10% ethanol for 10 min, and placed in the same amount of 1% nitric acid for 3 min. After two brief washes with 100 ml dH_2O, gels were stained in 50 ml of 2 ppm silver nitrate (made from 100 × stock stored at 4°C) for 20 min then rinsed three times in 200 ml dH_2O. Gels were developed by briefly rinsing in 30 ml of 1 ppm formaldehyde in 3% sodium carbonate until desired band intensity was reached. The stain was fixed in 1% acetic acid once the SSCP patterns were visible. Images were captured using BioImaging and Chemi System (UVP Lab Inc., Upland, CA, USA) for documentation and comparison analysis between species. SSCP banding patterns of individual isolates were analysed with the aid of the ssDNA ladder.

RESULTS AND DISCUSSION

All isolates of *P. ramorum* tested had an identical SSCP banding pattern (Ram) regardless of their origin (Fig. 1). Four bands were evenly distributed with the top one higher than rung 8, and the bottom one above rung 9 of the ssDNA ladder.

Ram is a unique SSCP pattern for *P. ramorum* when compared with its genetically close relatives (Fig. 2). For example, *P. lateralis* is the closest known relative to *P. ramorum* and also produced four evenly distributed bands (Lat), each of these bands was lower than respective bands of Ram (Fig. 2). Therefore, *P. ramorum* and *P. lateralis* can be differentiated by their SSCP patterns. This indicates that SSCP analysis is a useful technique for distinguishing *P. ramorum* from genetically close relatives. Initially, SSCP analysis was developed for monitoring mutations in human DNA (Orita *et al.* 1989) and can detect single base mutations (Sambrook and Russell 2001). Therefore, it was not unexpected that *P. ramorum* had a distinct SSCP pattern from *P. lateralis*, although both species differ by only three base pairs in the ITS-1 region (Werres *et al.* 2001). This study provides additional evidence that SSCP analysis is a powerful tool for detection of nucleotide variations.

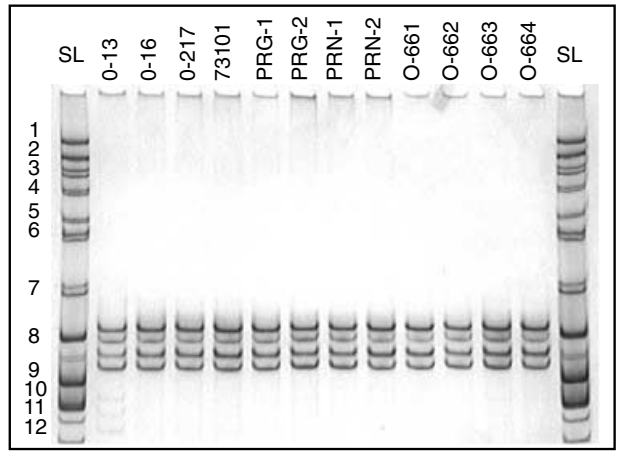

Fig. 1 Uniformity of single-strand conformation polymorphism profiles on polyacrylamide gel of ITS-1, amplified using primers ITS6/ITS7 of 12 isolates of *Phytophthora ramorum* from different geographical origins. Isolates 0-13 and 0-16 originated in Marin Co., CA; 0–217 in Santa Cruz Co., CA; 73101 in Sonoma Co., CA; isolates PRG-1 and PRG-2 originated in Germany, and PRN-1 and PRN-2 in the Netherlands; isolates O-661 to O-664 originated in Curry Co., Oregon. SL is a single strand DNA ladder with rung numbers listed on the left side of the left ladder

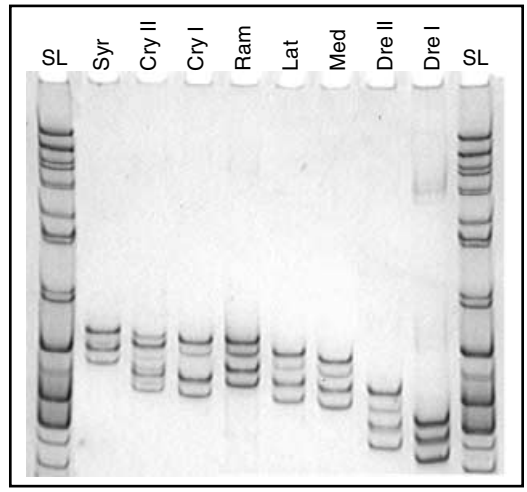

Fig. 2 Single-strand-conformation polymorphism profiles of ITS-1 for *Phytophthora ramorum* (Ram) and its genetically close relatives. Cry I and Cry II, *P. cryptogea* subgroups I and II; Dre I and Dre II, *P. drechsleri* subgroups I and II; Lat, *P. lateralis*; Med, *P. medicaginis* and Syr = *P. syringae*. SL represents a single strand DNA ladder

Ram also is different from the SSCP patterns of other *Phytophthora* species that are morphologically similar to *P. ramorum* (Fig. 3). *Phytophthora palmivora* is considered morphologically similar to *P. ramorum* (Werres *et al.* 2001), but the two species can be easily distinguished by SSCP

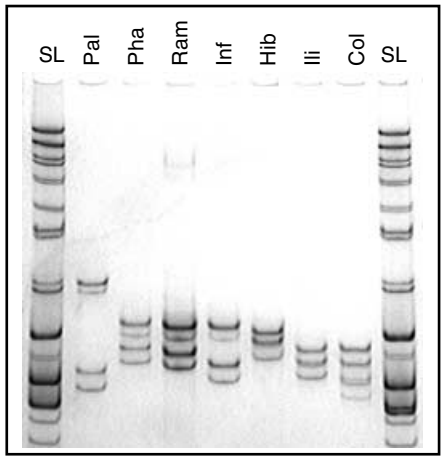

Fig. 3 Single-strand-conformation polymorphism profiles of ITS-1 for *Phytophthora ramorum* (Ram) and morphologically similar species. Col, *P. colocasiae*; Hib, *P. hibernalis*; Ili, *P. ilicis*; Inf, *P. infestans*; Pal, *P. palmivora* and Pha, *P. phaseoli*. SL represents a single strand DNA ladder

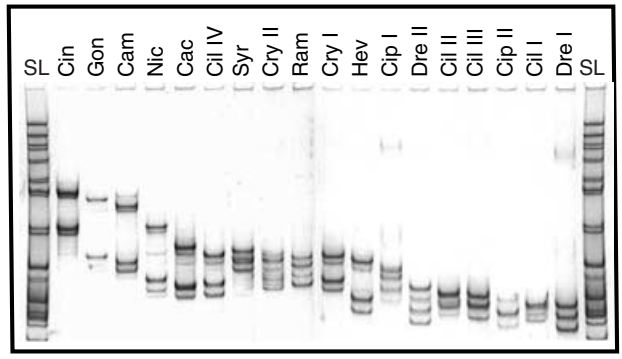

Fig. 4 Single-strand conformation polymorphism profiles of ITS-1 for *Phytophthora ramorum* (Ram) and its ecologically close associates. Cac, *P. cactorum*; Cam, *P. cambivora*; Cil I–IV, *P. citricola* subgroups I–IV; Cin, *P. cinnamomi*; Cip I and Cip II, *P. citrophthora* subgroups I and II; Cry I and Cry II, *P. cryptogea* subgroups I and II; Dre I and Dre II, *P. drechsleri* subgroups I and II; Gon, *P. gonapodyides*; Hev, *P. heveae*; Nic, *P. nicotianae* and Syr, *P. syringae*. SL represents a single strand DNA ladder

patterns (Fig. 3). *Phytophthora ramorum* belongs to Waterhouse's group IV within the genus *Phytophthora* based on morphology and heterothallism (Waterhouse 1963). This species can be separated from other members within the same group using morphological characters and host ranges; this study adds another effective character for species separation within this morphological group.

Phytophthora cambivora, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. gonapodyides*, *P. heveae*, *P. hibernalis*, *P. lateralis*, *P. nicotianae* and *P. syringae* have overlaps of host range with *P. ramorum* (Hoitink *et al.* 1986; Erwin and Ribeiro 1996; Werres *et al.* 2001; Linderman *et al.* 2002; Maloney *et al.* 2002; Parke *et al.* 2002; Rizzo *et al.* 2002; Tooley and Englander 2002). Of particular concern are *P. cactorum*, *P. citricola*, *P. heveae*, *P. nicotianae*, *P. citrophthora* and *P. cinnamomi*, which may cause leaf spots and/or twig dieback similar to those caused by *P. ramorum* (Werres *et al.* 2001; Linderman *et al.* 2002; Rizzo *et al.* 2002). This study indicates that none of these species has an identical SSCP pattern to Ram (Figs 2–4). *Phytophthora ramorum* can be easily distinguished from all other species examined, especially those that may cause similar foliage symptoms on the same plants.

Compared with classical methods and existing molecular fingerprinting techniques, SSCP analysis is an effective alternative tool for differentiating *P. ramorum* from other *Phytophthora* species. Identification of *Phytophthora* isolates by classical methods involves several steps: (i) examining the sexual type (homothallic vs heterothallic), antheridial configuration (amphigynous vs paragynous), and sporangium papillation (papillate vs non- or semi-papillate) to narrow down to one of Waterhouse's six groups; (ii) determining the persistence of sporangia and the length of pedicel and (iii) assessing the presence and number of chlamydospores and hyphal swelling produced, etc. to further key into species (Waterhouse 1963; Erwin and Ribeiro 1996). Although *P. ramorum* has rather distinct morphological characters (Werres *et al.* 2001), identification of an unknown isolate with classic methods must involve these steps. Examining morphological characters requires substantial experience and time. In contrast, the SSCP analysis assessed in this work is rapid and efficient; SSCP profiles easily distinguish *P. ramorum* from other described *Phytophthora* species. Further determination of other species as causal pathogens is possible by comparing SSCP profiles with the *Phytophthora* species examined in this study and reported previously (Kong *et al.* 2003). These features make SSCP analysis a superior technique to other existing molecular fingerprinting methods such as restriction fragment length polymorphism (RFLP) (Förster *et al.* 1989; Ristaino *et al.* 1998) and isozyme analysis (Nygaard *et al.* 1989; Oudemans and Coffey 1991); both methods usually require examining several molecular profiles to key an isolate to species.

Use of the SSCP technique has several other advantages. The entire procedure takes <6 h, and SSCP profiles of 26–90 isolates can be examined each time, depending on the capacity of the thermocycler and the electrophoresis unit employed. SSCP patterns in silver-stained gels can be differentiated visually without specialized equipment normally required by other DNA fingerprinting methods for differentiation of *Phytophthora* spp. (Förster *et al.* 1989; Tooley *et al.* 1997; Ristaino *et al.* 1998). In addition, SSCP analysis is a DNA sequence-based technique, yet does not

require a DNA sequencer or expensive sequence analysis software. This technique is not plagued by cross contamination or false positives, a serious problem associated with ELISA and species-specific PCR-based identification (Yap *et al.* 1992; Schots *et al.* 1994).

In summary, this study provides essential data supporting SSCP analysis as an effective alternative for distinguishing *P. ramorum* from its close relatives. This research provides a simple, rapid and reliable tool for confirming positive detections in ongoing surveys for *P. ramorum* at nurseries and surrounding forest areas in the south-eastern USA and in inspections of plant material outside quarantine areas. Additional investigations are warranted to assess the potential of this technique for direct detection of the pathogen from plant materials, soil and water samples. This study also provides additional evidence that SSCP of ITS-1 is an ideal DNA marker for species differentiation and identification within the genus *Phytophthora*.

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