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Single-strand-conformation polymorphism of ribosomal DNA for rapid species differentiation in genus *Phytophthora*

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Abstract

Single-strand-conformation polymorphism (SSCP) of ribosomal DNA of 29 species (282 isolates) of *Phytophthora* was characterized in this study. *Phytophthora boehmeriae*, *Phytophthora botryosa*, *Phytophthora cactorum*, *Phytophthora cambivora*, *Phytophthora capsici*, *Phytophthora cinnamomi*, *Phytophthora colocasiae*, *Phytophthora fragariae*, *Phytophthora heveae*, *Phytophthora hibernalis*, *Phytophthora ilicis*, *Phytophthora infestans*, *Phytophthora katsurae*, *Phytophthora lateralis*, *Phytophthora meadii*, *Phytophthora medicaginis*, *Phytophthora megakarya*, *Phytophthora nicotianae*, *Phytophthora palmivora*, *Phytophthora phaseoli*, *Phytophthora pseudotsugae*, *Phytophthora sojae*, *Phytophthora syringae*, and *Phytophthora tropicalis* each showed a unique SSCP pattern. *Phytophthora citricola*, *Phytophthora citrophthora*, *Phytophthora cryptogea*, *Phytophthora drechsleri*, and *Phytophthora megasperma* each had more than one distinct pattern. A single-stranded DNA ladder also was developed, which facilitates comparison of SSCP patterns within and between gels. With a single DNA fingerprint, 277 isolates of *Phytophthora* recovered from irrigation water and plant tissues in Virginia were all correctly identified into eight species at substantially reduced time, labor, and cost. The SSCP analysis presented in this work will aid in studies on taxonomy, genetics, and ecology of the genus *Phytophthora*.

Keywords: DNA fingerprinting; Genetics; Identification; ITS; *Phytophthora*; SSCP; Taxonomy

1. Introduction

Phytophthora de Bary is an economically important genus worldwide (Erwin and Ribeiro, 1996; Farr et al., 1989; Gregory, 1983). Species separation and identification are often required in the study of basic biology and epidemiology of *Phytophthora* diseases. Classic methods for distinguishing *Phytophthora* species are based upon morphological characters and growth characteristics (Gallegly's unpublished key; Ho, 1981; Stamps et al., 1990; Waterhouse, 1963). Use of these methods can be difficult and may lead to misidentification due to interspecific overlaps and intraspecific variations of morphological characteristics (Brasier, 1991; Erwin, 1983). These methods also are time-consuming.

An array of molecular approaches has been investigated in the search for alternative techniques for more effective species separation and for rapid identification within the genus *Phytophthora*. These approaches included protein electrophoresis, isozymes, DNA fingerprinting, and direct sequencing. The electrophoretic pattern of total protein was more sensitive than morphological features and growth behavior in distinguishing nine sub-groups among 93 isolates within *Phytophthora megasperma* (Hansen et al., 1986). It also effectively separated six *Phytophthora* species associated with deciduous tree crops (Bielenin et al., 1988). This approach, however, appears to be under-utilized (Brasier, 1991), partially due to the complexity of protein patterns.

Isozyme banding patterns are less complex than total protein patterns and are easier to differentiate and interpret (Nygaard et al., 1989). However, several isozymes have to be analyzed, and selection of isozymes is

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often important in separation of even a few *Phytophthora* species (Mills et al., 1991; Nygaard et al., 1989; Oudemans and Coffey, 1991a,b; Oudemans et al., 1994). These requirements may have undermined isozymic techniques as a taxonomic tool for the genus *Phytophthora*.

Analyses of ribosomal, nuclear, and mitochondrial DNAs show promise in establishing new systemics for the genus *Phytophthora*. DNA sequence analysis of ribosomal RNA genes are widely used in phylogenetics over a wide range of taxonomic levels with many organisms (Hibbett, 1992; White et al., 1990), including the genus *Phytophthora* (Cooke and Duncan, 1997; Crawford et al., 1996; Lee and Taylor, 1992). Specifically, internal transcribed spacer (ITS) sequences of *Phytophthora* are found variable among species but relatively conserved within individual species (Cooke and Duncan, 1997; Förster et al., 2000; Lee and Taylor, 1992). Based on the ITS sequences of genomic rDNA, Cooke et al. (2000) recently constructed a molecular phylogeny of *Phytophthora* and related Oomycetes.

Commercial kits, improved techniques and sequencing facilities have eased the application of ITS sequence analysis in taxonomy and phylogenetics. However, sequence analysis still can be expensive when many isolates need to be analyzed on a routine basis. In addition, sequence analysis requires specific software and expertise to use the software. Moreover, it may be impractical for separation of some species with little difference in DNA sequence.

Compared to sequence analysis, examination of DNA polymorphism or fingerprinting is less expensive and more direct. The fingerprints can be read either manually or automatically. Restriction fragment length polymorphism (RFLP) analysis is the most frequently used DNA fingerprinting technique for the genus *Phytophthora* (Förster and Coffey, 1991, 1993; Förster et al., 2000, 1989, 1990; Hong et al., 1999; Mills et al., 1991; Ristaino et al., 1998; Tooley et al., 1996). This technique separates DNA fragments by size. It offers finer resolution of species differences but is more time-consuming and expensive relative to isozyme electrophoresis (Nygaard et al., 1989). It also is subject to selection and use of multiple restriction enzymes or PCR primers.

The need for more effective DNA fingerprinting techniques to improve classification and identification of *Phytophthora* species is clearly evident (Gallegly, 1983). Single-strand-conformation polymorphism (SSCP) analysis is one of the most commonly used mutation detection methods (Sambrook and Russell, 2001). Single-stranded DNA molecules fold into complex three-dimensional structures as a result of intrastrand base pairing. Single strands of equal length but different sequence can therefore vary considerably in electrophoretic mobility as a result of the looping and compaction caused by intrastrand pairing. Alteration of the nucleotide sequence of the molecule by as little as a single base can reshape the secondary structure, with consequent changes in electrophoretic mobilities through native gels.

SSCP technique was initially developed for examining point mutations in human DNA (Orita et al., 1989a,b). It since has been extended for studying variability of plant pathogens including viruses (Kong et al., 2000; Magome et al., 1999; Rubio et al., 1996; Stavolone et al., 1998), nematodes (Clapp et al., 2000), and fungi (Kumeda and Asao, 1996; Moricca et al., 2000; Morrica and Ragazzi, 1998). It also was used as a complementary technique with species-specific PCR in the development of genetic markers for rapid identification of three *Phytophthora* species pathogenic on potato (Scott et al., 1998). These studies demonstrate that SSCP is a powerful tool for mutation detection and variation analysis. However, the potential of SSCP technique as a taxonomic tool in general and for genus *Phytophthora* in particular remains to be investigated.

In this study, we characterized the SSCP of amplified ribosomal DNA internal transcribed spacer 1 (ITS-1). The specific objectives were to (i) determine the SSCP pattern of 282 isolates, representing six groups and 29 species of *Phytophthora*, (ii) characterize the inter- and intraspecific variations of SSCP pattern, (iii) investigate how the SSCP patterns correspond to taxonomic entities, (iv) evaluate the reliability of PCR-SSCP procedure with 277 previously uncharacterized isolates, and (v) develop a single-stranded DNA (ssDNA) ladder for effective and efficient comparison of SSCP patterns within a gel and between gels.

2. Materials and methods

2.1. Isolates and culture

Two hundred and eighty two known isolates and 277 uncharacterized isolates, representing 29 species from six groups in the genus *Phytophthora*, were used in this study. These isolates originated from a wide range of host plants and substrates (plant tissue, soil, or irrigation water) in numerous geographic locations. The number of isolates and the origin of type isolates for individual SSCP groups characterized are listed in Tables 1 and 2. All isolates were cultured on V8 agar (20% clarified V8 juice, 0.4% $CaCO_3$, and 1.5% agar in distilled water) or 0.5× Lima Bean agar in 60-mm-diameter petri dishes at 23 °C in the dark for 1–2 weeks and then maintained at 15 °C.

2.2. DNA extraction

A one-step boiling method was used for DNA extraction. Briefly, about 2 cm^2 of mycelium was scraped

Table 1
SSCP analyses of *Phytophthora* species and origins and profiles of type isolates used in this study

Group[a]	Species	SSCP ID	No. isolates examined	SSCP profile and origin of type isolate					ITS-based clade[b]
				Lane[c]	Isolate	Host	Location	Original name (Supplier)[d]	
I	*P. cactorum*	Cac	10	13	22E8	*Malus* sp.	Rhodesia	P7 (MEG), ATCC 16694	1a
	P. pseudotsugae	Pse	1	12	23A5	NA[e]	NA	P92 (MEG)	1a
II	*P. boehmeriae*	Boe	1	14	22G7	NA	NA	S833 (AFS), P43 (MEG)	?[f]
	P. botryosa	Bot	1	24	22H8	*Heave rubber*	Thailand	62-2 (PHT), P44 (MEG)	2
	P. capsici	Cap	4	36	22F5	*Capsicum annum*	NC	P9 (MEG), ATCC 15427	2?
	P. citrophthora	Cip I	6	26	3E5	Nursery irrigation water	VA		2?
		Cip II	1	35	15D7	*Theobroma cacao*	Brazil	P.1210 (SNJ)	2?
	P. heveae	Hev	2	23	22J2	Soil	TN	P17 (MEG), ATCC 16701	5
	P. katsurae	Kat	1	8	22H6	*Castanea* sp.	Japan	P990 (PHT), P45 (MEG)	5
	P. meadii	Mea	2	27	22G4	*Citrus* sp.	India	P74 (MEG)	?
	P. megakarya	Mek	1	9	22H7	*Theobroma cacao*	Africa	203532 (PHT), P42 (MEG)	4
	P. nicotianae	Nic	58	7	22G1	*Nicotiana tabacum*	NC	P22 (MEG), ATCC 15409	1b
	P. palmivora	Pal	21	10	7A9	Nursery irrigation water	VA		4
	P. tropicalis	Tro	1	34	22H5	*Vanilla* sp.	Tahiti	FP59 (PHT), P27(MEG)	2?
III	*P. citricola*	Cil I	3	33	22F1	*Rhododendron* sp.	WV	P53 (MEG)	2?
		Cil II	2	15	22E9	*Kalmia latifolia*	WV	P101 (MEG)	2?
		Cil III	3	30	1E1	Nursery irrigation water	OK	SG-R-1 (SLV)	2?
		Cil VI	2	31	15C7	*Hedera helix*	SC	AF.018 (SNJ)	2?
	P. syringae	Syr	1	11	23A6	NA	NY	P35 (MEG)	8b
IV	*P. colocasiae*	Col	2	29	22F8	NA	NA	P113 (MDC), P47 (MEG)	2
	P. hibernalis	Hib	1	20	22H1	*Citrus sinensis*	Portugal	P115 (MEG), ATCC 60352	?
	P. ilicis	Ili	1	28	23A7	*Ilex* sp.	Canada	P113 (MEG), ATCC 56615	3
	P. infestans	Inf	4	21	22E4	*Lycopersicon esculentum*	NC	TLFL-1-1a (MEG)	1c
	P. phaseoli	Pha	2	18	23B4	*Phaseolus lunatus*	DE	P106 (MEG)	1c
V	*P. fragariae*	Fra	1	2	22G6	*Fragaria* sp.	MD	P114 (MEG), ATCC 11374	7a
	P. lateralis	Lat	1	22	22H9	*Chamaecyparis lawsoniana*	OR	P51 (MEG)	8a
	P. medicaginis	Med	1	25	23A4	*Medicago sativa*	OH	S797 (AFS), P37 (MEG)	8a
	P. megasperma	Meg I	6	6	23A3	*Actinidia chinensis*	CA	50 (EMH), P79 (MEG)	6?
		Meg II	2	19	22J8	*Pseudotsuga menziesii*	OR	20 (EMH), P78 (MEG)	6?
		Meg III	1	5	22J9	*Prunus* sp.	CA	62 (EMH), P82 (MEG)	6?
	P. sojae	Soj	2	3	22D8	*Glycine max*	Canada	P19 (MEG), ATCC 16705	7b

Table 1 (*continued*)

Group[a]	Species	SSCP ID	No. isolates examined	SSCP profile and origin of type isolate					ITS-based clade[b]
				Lane[c]	Isolate	Host	Location	Original name (Supplier)[d]	
VI	*P. cambivora*	Cam	3	4	22D7	*Prunus armeniana*	MD	P746 (PHT), P63 (MEG)	7a
	P. cinnamomi	Cin	13	1	23B2	*Persea americana*	Puerto Rico	P11 (MEG), ATCC 15401	7a
	P. cryptogea	Cry I	5	16	15E6	Soil	SC	D.200 (SNJ)	8a?
		Cry II	14	17	15E7	*Aster* sp.	CA	P12 (MEG), ATCC 15402	8a?
	P. drechsleri	Dre I	19	37	1D11	Nursery irrigation water	VA		8a?
		Dre II	83	32	1D12	Nursery irrigation water	VA		8a?

[a] Waterhouse's (1963) grouping.
[b] Cooke et al. (2000).
[c] SSCP profile of type isolate presented in Fig. 3.
[d] Original identifier of type isolates followed by the name of originator in parenthesis or ATCC catalog number. AFS, A Fritz Schmitthenner at Ohio State University, OH; EMH, Everett M. Hansen at Oregon State University in Corvallis, OR; MDC, Michael D. Coffey at University of California in Riverside, CA; MEG, Mannon E. Gallegly at West Virginia University in Morgantown, WV; PHT, Peter H. Tsao at University of California in Riverside, CA; RWG, Robert W. Goth at USDA in Beltsville, MD; SLV, Sharon L. von Broembsen at Oklahoma State University in Stillwater, OK; and SNJ, Steven N. Jeffers at Clemson University in Clemson, SC.
[e] Not available.
[f] The species was not assessed or it produced more than one SSCP pattern.

from 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 until further use.

2.3. Primers and PCR amplification

The major criterion for selection of primers was specificity for the genus *Phytophthora.* Additional considerations included DNA sequence, GC content, and size of fragment amplified for effective SSCP analysis (Hayashi, 1991; Sheffied et al., 1989). We targeted the ITS of ribosomal DNA. The two pairs of primers used in this study were (i) ITS6 and ITS7 (Cooke et al., 2000) and (ii) DC6 (Cooke et al., 2000) and PhytophR (Lee et al., 1993). The sequence of forward primer ITS6: 5′-GAA GGT GAA GTC GTA ACA AGG-3′, locates in 18S; the sequence of reverse primer ITS7: 5′-AGC GTT CTT CAT CGA TGT GC-3′, locates in the 5.8S. ITS6 and ITS7 favor oomycete amplification (Cooke et al., 2000). Alternatively, the sequence of forward primer DC6: 5′-GAG GGA CTT TTG GGT AAT CA-3′, locates in 18S and is specific for Peronosporales and Pythiales (Cooke et al., 2000). The sequence of reverse primer PhytophR, 5′-GCT ATT TAG TTA AAA GCA GA-3′, locates in ITS-1; this primer was originally an oligonucleotide probe specific for the *Phytophthora* genus (Lee et al., 1993).

PCR amplification was performed in a total volume of 25 μl that contained 2 μl of the boiled culture extract. Each reaction used 2.5 μl of the 10× PCR buffer, 2.5 μl each of 10 μM forward and reverse primers, 2 μl of 2 mM dNTPs, 0.1 μl (5 U/μl) of *Taq* polymerase (TaKaRa, Shuzo, Japan) and 13.4 μl of sterilized nanopure water (SDW). 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. Two microliters of PCR products were resolved by electrophoresis in 1% agarose gels and were stained with ethidium bromide. Images were captured using BioImaging & Chemi System (UVP Lab, Upland, CA).

2.4. SSCP analysis

The amount of PCR product was estimated by its band intensity on agarose gel in order to obtain an equal amount of DNA loading for SSCP electrophoresis. One to three microliters of PCR products were mixed with 7–9 μl of a denaturing buffer (95% formamide, 20 mM EDTA, and 0.05% bromophenol blue) to a total volume of 10 μl. After a brief spin, mixtures were heated at 96 °C for 10 min, and then chilled on ice. Five microliters of the denatured PCR products or a ssDNA ladder was loaded on an 8% acrylamide:bis (30:1) nondenaturing minigel (8.3 × 7.3 × 0.75 cm) cast using a Mini-PROTEAN 3 Cell (Bio-Rad Laboratories, Hercules, CA).

Additional steps of SSCP electrophoresis were performed as described previously (Kong et al., 2000; Rubio et al., 1996) with minor modifications depending on the fragment sizes of PCR products. The denatured PCR products amplified with ITS6 and ITS7 primers

Table 2
Origin of additional isolates of *Phytophthora* used in this study for illustrating identical SSCP pattern within a species or subspecies of species complex

Species	SSCP ID	Isolate	Host	Location	Original identifier (Supplier)[a]
P. cactorum	Cac	1B8	*Malus* sp.	VA	VA101 (MH)
		15C2	*Syringa meyeri*	WI	W.020 (SNJ)
		15C3	*Abies fraseri*	WI	W.177 (SNJ)
		15C4	*Malus domestica*	SC	HH-Apple (SNJ)
		15C5	*Malus domestica*	NY	NY.188 (SNJ)
		15C6	*Xfragaria ananassa*	WI	W.097 (SNJ)
		22E5	NA[b]		P59 (MEG)
		22E6	*Rhododendron* sp.	OH	P25 (MEG), S814 (AFS)
		22E7	NA		P6 (MEG)
P. cinnamomi	Cin	1A1	*Ginkgo biloba*	SC	SC-001 (SNJ)
		1A3	*Camellia japonica*	CA	D-001 (SNJ)
		1A10	*Persea americana*	CA	PC-40 (SNJ)
		1A11	Tester		(MH)
		1A12	Tester		(MH)
		1B7	*Heliamphora* sp.	VA	VA-86 (MH)
		1B12	*Rhododendron* sp.	SC	NC-2386
		1E7	Nursery irrigation water	OK	5N-L (SLV)
		23B1	*Camellia japonica*	NC	P10 (MEG)
P. nicotianae	Nic	1B11	*Catharanthus roseus*	NC	NC-2122 (DMB)
		1E2	Nursery irrigation water	OK	II-16 (SLV)
		1E3	Nursery irrigation water	OK	GLN9-3
		22F9	*Nicotiana tabacum*	NC	P23 (MEG)
		23B5	*Abies fraseri*	WV	P1 (MEG)
		23B8	*Citrus* sp.	India	P69 (MEG)
		23C3	*Solanum tuberosum*	NC	P77 (MEG)
		23C4	NA		P86 (MEG)
		23C7	Nursery irrigation water	VA	P107 (MEG)
P. citricola	Cil I	1C3	*Juglans* sp.	CA	GB-572 (GTB)
		22F3	*Rhododendron* sp.	OH	P33 (MEG), S813 (AFS)
	Cil II	15D2	*Rhododendron* sp.	WI	W.019 (SNJ)
		22F2	*Rhododendron* sp.	NY	P52 (MEG)
	Cil III	15C9	*Acer saccharum*	WI	W.001 (SNJ)
	Cil IV	15C8	Field soil	SC	AF.026 (SNJ)
P. drechsleri	Dre I	3J3	Nursery irrigation water	VA	
		3J4	Nursery irrigation water	VA	
		3J5	Nursery irrigation water	VA	
		4E4	Nursery irrigation water	VA	
	Dre II	3J6	Nursery irrigation water	VA	
		3D11	Nursery irrigation water	VA	
		5C11	Nursery irrigation water	VA	
		5D1	Nursery irrigation water	VA	
P. megasperma	Meg I	1C5	*Prunus* sp.	CA	GB-1374 (GTB)
		22J7	NA	OH	P49 (MEG), S502 (AFS)
		23A1	*Prunus* sp.	CA	P81 (MEG), 61 (EMH)
		23A2	*Malus sylvestirs*	NY	P80 (MEG), 60 (EMH)
		23C8	*Cornus florida*	WV	P60 (MEG)
	Meg II	22J6	*Trifolium* sp.	IL	P18 (MEG)

[a] Original identity of representative isolates followed by the name of originator in parenthesis. AFS, A Fritz Schmitthenner at Ohio State University, OH; DMB, D. Michael Benson at North Carolina State University, Raleigh, NC; EMH, Everett M. Hansen at Oregon State University in Corvallis, OR; GTB, Greg T. Browne at University of California in Davis, CA; MEG, Mannon E. Gallegly at West Virginia University in Morgantown, WV; MH, Mary Ann Hansen at Virginia Tech in Blacksburg, VA; SLV, Sharon L. von Broembsen at Oklahoma State University in Stillwater, OK; and SNJ, Steven N. Jeffers at Clemson University in Clemson.

[b] Host information not available.

were eletrophoresed in pre-chilled 1× TBE buffer (Tris–borate 89 mM, 2 mM EDTA, pH 8.0) at 200 V for 2 h at room temperature. The denatured PCR products amplified with DC6 and PhytophR primers were electrophoresed in pre-chilled 1× TBE buffer at a constant voltage of 200 volts for 6 h in an ice bath.

After electrophoresis, silver staining was carried out in a 12×12-cm plastic container using the procedure of Beidler et al. (1982) with some modifications. Specifically, polyacrylamide gels were peeled off carefully from the glass plate and soaked in 50 ml (for two gels) to 100 ml (for four gels) of 10% ethanol for 5 to 10 min until replaced by the same amount of 1% nitric acid for 3 min. After two brief washes with 100 ml dH_2O, the gels were soaked in 100 ml of 2 ppm silver nitrate (made from 100× stock stored at 4 °C) for 20 min and then washed three times with 300 ml dH_2O. The gels then were developed by briefly rinsing in 30 ml of 1 ppm formaldehyde in 3% sodium carbonate solution, and then changing the solution with 2× (twice) the volume when it became light brown/purple. The stain was fixed in 1% acetic acid when the desired intensity was reached.

SSCP banding patterns of individual isolates were determined with the aid of an ssDNA ladder. Intraspecific and interspecific variations of SSCP patterns were compared side by side when banding patterns were similar. The SSCP identity of individual species with a single pattern was named primarily by selecting the first three-characters of the specific epithet with the first letter capitalized. For subgroups within a species, a Roman numeral follows the three-character identifier representing the type of SSCP pattern. For example, "Cac" stands for *Phytophthora cactorum*, while "Dre I" and "Dre II" represent two SSCP patterns of *Phytophthora drechsleri* (Table 1). Exceptions included "Cil" for *Phytophthora citricola*, "Cip" for *Phytophthora citrophthora*, and "Mek" for *Phytophthora megakarya.*

2.5. ssDNA ladder

To facilitate comparison of SSCP patterns within a gel and between gels, three ssDNA ladders were developed and evaluated. ssDNA ladders were obtained by denaturing at 96 °C for 10 min in the denaturing buffer: (i) a 100-bp DNA ladder, (ii) a mixture of PCR products of several *Phytophthora* species that have distinct SSCP patterns, and (iii) a mixture of Φ X174 DNA/*Hae*III ladder (Promega, Madison, WI) with PCR products of *Phytophthora cinnamomi*. An aliquot of 25 ng of an ssDNA ladder was loaded per lane. For example, for ladder 3, 1 μl of 1:5 SDW-diluted Φ X174 DNA/*Hae*III ladder (1 μg/μl) was mixed with 2 μl of PCR products of *P. cinnamomi* and 37 μl of the denaturing buffer, which can be used for eight lanes. The primary criteria for selecting an ssDNA ladder were band number, location, separation, and reproducibility.

2.6. Morphological and physiological examination

Morphological and physiological characteristics of all isolates from Dr. Gallegly's personal collection were re-examined prior to SSCP analyses. All other reference isolates were also re-examined for their morphological identities. The majority of isolates from Virginia were examined morphologically prior to or after SSCP analysis. Some of these isolates, including all type isolates, were examined two or three times. Major structures examined included sporangia, sporangiophores, chlamydospores, mycelia, antheridia, oogonia, and oospores. Most of these structures were induced from the mycelium plugs of isolates. The plugs were taken from an actively growing area of pure culture, and submerged in 1% sterile soil water extract under fluorescent light overnight or for a few days depending on the species/isolates. Sexual structures of heterothallic species were produced following standard procedure. The identity of individual isolates was determined using the morphological keys to *Phytophthora* species (Gallegly's unpublished key; Ho, 1981; Stamps et al., 1990; Waterhouse, 1963).

2.7. Evaluation of reliability of PCR-SSCP procedure

The reliability of SSCP analysis of rDNA for species separation and identification was assessed by (i) repeating the entire procedure from DNA extraction to electrophoresis of denatured DNA of all type isolates (Table 1) and most of the unknown isolates at least three times, (ii) using an alternative gel (16.5×8.5×0.75 cm) cast with Wide Dual Cooled Mini V10-WCDC (Scie-Plas Ltd, England) for 140 min at the same electrophoretic conditions as described previously, and (iii) matching the SSCP identities with the morphological identities of 248 previously uncharacterized isolates.

3. Results

3.1. PCR-SSCP analysis

In a short PCR procedure of less than 2 h, DNA from boiled mycelia of all test isolates was amplified well with both pairs of primers. The PCR with primers ITS6 and ITS7 yielded a fragment of ca. 300 bp, whereas primers DC6 and PhytophR resulted in a fragment of ca. 600 bp (Fig. 1). The 600-bp fragment was resolved poorly in an 8% polyacrylamide gel after running for 6 h at 200 V. Therefore, only the SSCP analysis of PCR products amplified with primers ITS6 and ITS7 are presented here.

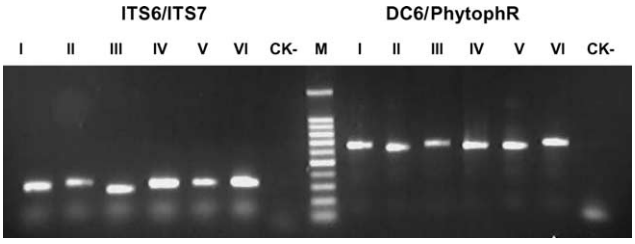

Fig. 1. Agarose gel electrophoresis of PCR products of *Phytophthora* species amplified with primers ITS6 and ITS7, and primers DC6 and PhytophR. Representatives of six morphological groups are listed on the top of lanes: I, *P. cactorum*; II, *P. nicotianae*; III, *P. citricola*; IV, *P. infestans*; V, *P. sojae*; and VI, *P. cinnamomi*. M, a 100-bp DNA ladder; CK, negative control with SDW.

Electrophoresis of the denatured 300-bp PCR products resulted in four stable major bands (Figs. 1–3). The locations of and distances between the upper and lower bands differed among the species. These variations constituted simple and distinct SSCP pattern(s) for individual species and subgroups. Additional fine bands sometimes appeared associated with some isolates within the same species or subgroup. However, these minor variations did not mask the major bands.

For twenty-four of the 29 species, each had an identical SSCP pattern among all isolates examined regardless of isolate origin (Tables 1 and 2) and mating type (Table 3). For example, among the 58 isolates of *Phytophthora nicotianae* examined were 14 A^1 and 42 A^2 mating types (Table 3); these isolates originated from diverse host ranges, substrates (plant tissues, soil, and irrigation water), and in numerous geographic locations (Tables 1 and 2); but they all had exactly the same SSCP pattern as illustrated in Fig. 2. These 24 species were *Phytophthora boehmeriae*, *Phytophthora botryosa*, *P. cactorum*, *Phytophthora cambivora*, *Phytophthora capsici*, *P. cinnamomi*, *Phytophthora colocasiae*, *Phytophthora fragariae*, *Phytophthora heveae*, *Phytophthora hibernalis*, *Phytophthora ilicis*, *Phytophthora infestans*, *Phytophthora katsurae*, *Phytophthora lateralis*, *Phytophthora meadii*, *Phytophthora medicaginis*, *P. megak-*

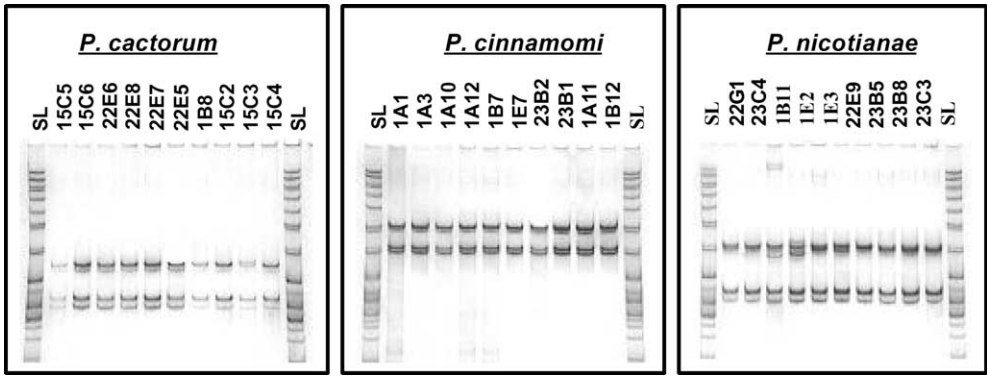

Fig. 2. Examples of single-patterned species include *P. cactorum*, *P. cinnamomi*, and *P. nicotianae*. The names of representative isolates of individual species are indicated on the top of lanes. SL represents the ssDNA ladder developed in this study.

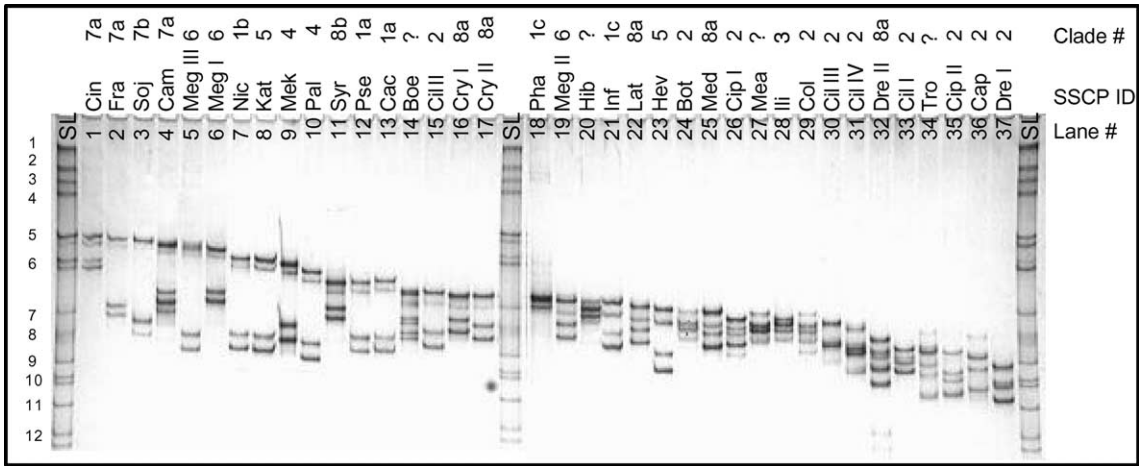

Fig. 3. Distinct SSCP patterns of type isolates of 29 species of *Phytophthora* present in a wide mimigel. Lane number and SSCP pattern identifier are listed on the top of each lane. Phylogenetic clade numbers of respective species as determined by Cooke et al. (2000) are also indicated for reference. SL is the ssDNA ladder. The numbers on the left of gel indicate the position of major fragments in the ladder.

Table 3
Summary of mating type isolates of heterothallic species of *Phytophthora* examined with single-strand-conformation polymorphism in this study

Species	SSCP ID	No. isolates examined	
		A^1	A^2
P. capsici	Cap	5	3
P. meadii	Med	1	1
P. nicotianae	Nic	14	42
P. palmivora	Pal	19	2
P. infestans	Inf	3	1
P. cambivora	Cam	3	2
P. cinnamomi	Cin	3	4
P. cryptogea	Cry I	1	1

arya, *P. nicotianae*, *Phytophthora palmivora*, *Phytophthora phaseoli*, *Phytophthora pseudotsugae*, *Phytophthora sojae*, *Phytophthora syringae*, and *Phytophthora tropicalis*. Among these 24 species, the SSCP pattern for each was unique (Fig. 3).

Each of the other five species produced more than one SSCP pattern. For instance, *P. citricola* had four SSCP patterns; *P. megasperma* had three SSCP patterns; *P. drechsleri* had two patterns (Fig. 4). *P. citrophthora* and *Phytophthora cryptogea* also had two patterns each. These patterns also were unique (Fig. 3); each pattern was associated with a subgroup of isolates within a species complex, but not with a specific mating type. For instance, both A^1 and A^2 isolates of *P. cryptogea* had Cry I pattern (Table 3).

3.2. ssDNA ladder

The mixture of Φ X174 DNA/*Hae*III ladder with PCR products of *P. cinnamomi* worked the best among the three ssDNA ladders tested. This ladder consistently exhibited 12 major bands (Fig. 3), which were evenly distributed and bordered all the SSCP banding patterns of *Phytophthora* spp. examined. In addition, migration of the ssDNA marker molecules was constant as displayed in Figs. 2–4. The other two ladders produced either too simple or too complex bands; they were excluded from further study.

3.3. Morphological and physiological examination

A total of 559 isolates were examined in this study. Interspecific overlaps of taxonomic characters were evident. As expected, isolates of *P. cryptogea* and *P. drechsleri* shared nearly every morphological trait and separation of these two species relied totally on the maxima of growth temperature. These two species also had numerous morphological overlaps with *P. megasperma*. *P. megasperma* is usually distinguished from *P. cryptogea* and *P. drechsleri* by producing oospores in single culture. However, many isolates of *P. megasperma* did not produce oospores in single culture in this study. These isolates would have been keyed into *P. cryptogea* without using SSCP profiles. Similarly, morphological overlaps were observed among *P. capsici*, *P. palmivora* and *P. tropicalis*, and among *P. citrophthora* and *P. syringae*.

Morphological variations also were common among isolates within some species such as *P. drechsleri*. Among the 102 isolates of *P. drechsleri* examined, all produced nonpapillate sporangia and grew at 35 °C. However, some isolates produced abundant lateral chlamydospores, while the other isolates had none. These two groups of isolates produced two distinct SSCP patterns: Dre II and Dre I, respectively.

3.4. Evaluation of reliability of the PCR-SSCP procedure

All SSCP patterns were reproducible among repeated analyses of the same species and isolates. Identical SSCP patterns also were observed for the same species and

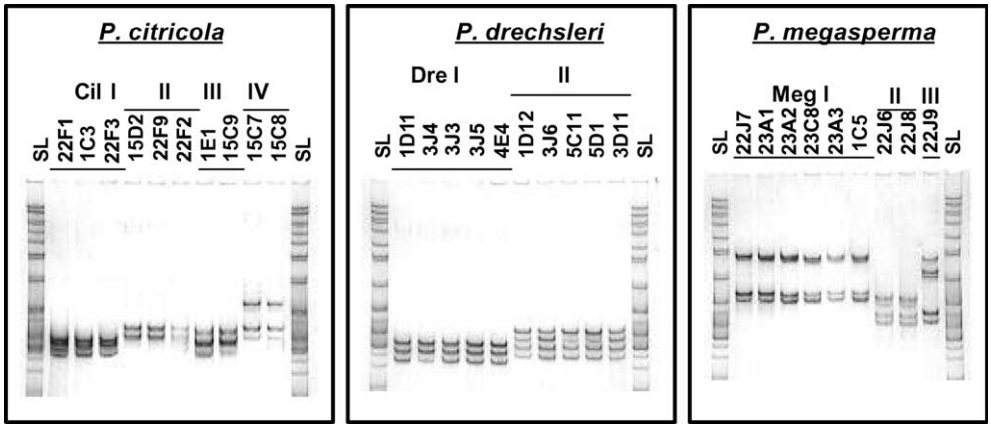

Fig. 4. Examples of multiple SSCP patterns produced by *P. citricola*, *P. drechsleri*, and *P. megasperma*. The names of representative isolates of individual species are listed on the top of lanes, and SL is the ssDNA ladder.

Table 4
Identification of *Phytophthora* isolates recovered from ornamental nurseries in Virginia using the SSCP analysis developed in this study followed by morphological examination to confirm their taxonomic identities

Species	SSCP ID	No. isolates examined		SSCP–morphology ID match	Isolate origin[a]
		SSCP	Morphology		
P. cactorum	Cac	3	3	3	W
P. cinnamomi	Cin	53	50	50	P
P. citricola	Cil I	27	14	14	P, W
	Cil II	6	6	6	W
P. citrophthora	Cip I	66	57	57	W
P. drechsleri	Dre I	9	9	9	W
	Dre II	60	56	56	W
P. megasperma	Meg I	24	24	24	W
P. nicotianae	Nic	26	26	26	P, W
P. palmivora	Pal	3	3	3	W

[a] P, plant tissues and W, irrigation water.

isolates regardless of gel electrophoresis unit [minigel (Figs. 2 and 4) versus wide minigel (Fig. 3)] used.

The 277 *Phytophthora* isolates from irrigation water and plant tissues produced 10 SSCP patterns (Table 4). These isolates were easily keyed into eight morphological species of five Waterhouse Groups with the SSCP profiles. Among the 248 isolates examined morphologically, each had the typical taxonomic characters of respective species.

4. Discussion

This study demonstrated that the SSCP of ITS-1 amplified with primers ITS6 and ITS7 can readily distinguish 29 species in the genus *Phytophthora*. It also can differentiate subgroups within some well-known species complexes. No other single existing taxonomic character or molecular fingerprint can distinguish so many species and subgroups of species complexes within the genus *Phytophthora* as the SSCP identified in this work.

As a new tool, SSCP analysis has a number of advantages over classical methods and other molecular fingerprinting. The entire procedure consists of a one-step boiling DNA extraction, a short PCR program, and an efficient SSCP protocol. The procedure involves no hazardous chemicals and takes no more than 6 h. It also uses less consumables and expensive equipment when compared to other molecular techniques (Ersek et al., 1994; Erselius and De Vallavieille, 1984; Förster et al., 1990; Lee et al., 1993; Old et al., 1984; White et al., 1990). The procedure is simple, efficient, and easy to use, requiring minimal training. The resultant SSCP profiles are uncomplicated and can be interpreted manually. In addition, the ssDNA ladder developed in this study facilitates comparison of SSCP patterns within a gel and between gels, which eliminates unnecessary runs for distinguishing profiles with similar patterns. The development of this procedure will facilitate studies of taxonomy, genetics, and ecology of the genus *Phytophthora*, as well as related plant disease diagnoses.

While recognizing the foundational importance of traditional taxonomic keys (Gallegly's unpublished key; Ho, 1981; Stamps et al., 1990; Waterhouse, 1963), we demonstrated that the SSCP is a powerful molecular fingerprint for studying taxonomy in the genus *Phytophthora* at both levels of species and subgroups within some species complexes. For example, SSCP analysis easily separated *P. cryptogea* and *P. drechsleri*, two morphologically identical taxa (Ho and Jong, 1991). This result supports the separation of these two groups as different taxa (Cooke et al., 2000; Mills et al., 1991). In the meanwhile, the representative isolates of *P. cryptogea* examined had two SSCP patterns, and those of *P. drechsleri* had two different patterns. SSCP groupings corresponded well with morphological entities such as Dre I and Dre II. These results indicate that the SSCP is a useful taxonomic tool for studies of species complexes.

Additional species complexes that were easily separated by the SSCP include *P. megasperma*, *P. citricola*, and *P. citrophthora*. *P. megasperma* is a broadly based species that includes a number of biotypes from different hosts (Erwin and Ribeiro, 1996). Three subgroups of isolates were removed from *P. megasperma* and placed in separate taxa: *P. sojae*, *P. medicaginis*, and *Phytophthora trifolii*, based on protein electrophoresis (Hansen et al., 1986), isozyme analyses (Nygaard et al., 1989), RFLP analyses of mitochondrial and nuclear DNAs (Förster and Coffey, 1993; Förster et al., 1989), and pathogenicity and growth characters (Hansen et al., 1986). The results of PCR-SSCP analyses support the establishment of new species associated with legume crops from this *P. megasperma* complex (Hansen and Maxwell, 1991). *P. palmivora/capsici* is another problematic species complex that includes many biotypes (Erwin and Ribeiro, 1996). The subgroup MF3 was

removed from *P. palmivora/capsici* complex and placed in a separate taxon: *P. megakarya* (Brasier and Griffin, 1979). More recently, isolates in subgroup MF4 also were placed in a new taxon: *P. tropicalis*, based on morphology and host specificity (Aragaki and Uchida, 2001). SSCP analysis genetically corroborated the justification for such separations.

Similar SSCP patterns were observed for *P. katsurae* (Kat) and *P. nicotianae* (Nic) and also for *P. pseudotsugae* (Pse) and *P. cactorum* (Cac). Separation of these species have to be accomplished by electrophoresing them side by side in a gel and comparing their SSCP profiles with those of suspected species. Since only one isolate each of *P. katsurae* and *P. pseudotsugae* was assessed in this study, additional isolates should be examined in future studies to improve application of the SSCP analysis for differentiating *P. katsurae* from *P. nicotianae* and *P. pseudotsugae* from *P. cactorum*.

This study also demonstrated that SSCP analysis is a reliable tool for taxonomy and identification within the genus *Phytophthora*. With this new tool, 277 previously uncharacterized isolates were easily and correctly identified into eight species in five of the six Waterhouse (1963, Groups). Application of this new technique also helped us to clarify the identities of several isolates that were misidentified previously (data not shown).

SSCP analysis can be used for studying genetic diversity in *Phytophthora* at the species level. Among the 24 species with a single, distinct SSCP pattern are known genetically uniform species such as *P. cactorum*, *P. nicotianae*, *P. palmivora*, and *P. cambivora* (Erwin and Ribeiro, 1996). The SSCP analyses of *P. cinnamomi* are in accord with the ITS sequence analyses (Cooke et al., 1996; Lee and Taylor, 1992), but differ from the analyses of isozymes and RFLP of mtDNA (Förster et al., 1990; Old et al., 1984; Oudemans and Coffey, 1991a). The genetic diversity of *P. cinnamomi* merits further investigation. The genetic diversity within species, where only one or a few isolates were analyzed in the present study, also is yet to be examined.

SSCP analysis also can be used for studying genetic diversity in *Phytophthora* at the subspecies level. Several species with multiple SSCP patterns including *P. megasperma*, *P.cryptogea*, and *P. drechsleri* are well known for their genetic diversity. The SSCP patterns conform to the protein profiles (Hansen et al., 1986) and RFLPs of mtDNA for the same isolates within a species (Förster and Coffey, 1993; Förster et al., 1989). For example, three SSCP groups of *P. megasperma* matched perfectly with three protein groups of Hansen et al. (1986): Meg I for "BHR" (isolates 50, 60, and 61), Meg II for "DF" (isolate 20), and Meg III for "AC" (isolate 62) (Tables 1 and 2). The genetic diversity within each of the other species with multiple SSCP patterns has not been thoroughly examined. This study indicates there are at least four genetically different subgroups of *P. citricola*, and two subgroups of *P. citrophthora*. The divergent SSCP patterns within *P. citrophthora* are in accord with respective sequence data (Cooke et al., 2000; Lee and Taylor, 1992).

SSCP analysis also can aid in *Phytophthora*-related disease diagnoses in several aspects. Use of SSCP analysis allows identification of multiple species of *Phytophthora*. This is particularly useful for diagnosing diseased plants such as citrus and strawberry that may be attacked by several species (Farr et al., 1989). Use of SSCP analysis also can lead to rapid and accurate identification of many isolates, which is important for growers to prevent a disease from spreading in commercial crop production. Finally, use of the SSCP analysis may eliminate false diagnoses due to cross-contamination (Yap et al., 1992), which is a serious problem commonly associated with enzyme-linked immunosorbent assay (ELISA) and species-specific detection (Schots et al., 1994). The minimal requirements for instrumentation, consumables, and training make this technique easily accessible for diagnosticians and more affordable for growers and consumers than other techniques.

Additional applications of SSCP analysis remain to be investigated. Cooke et al. (2000) grouped *Phytophthora* species into eight clades by DNA distance-based analysis of the combined ITS1, 5.8S subunit and ITS2 regions. Species of their clade 7 appeared to have the highest upper and lower bands, and those of clade 2 separated into the group with the lowest bands. Most species grouped in these two clades are located on the left (lanes 1–4) and right ends (lanes 24–37) of the gel, with a few exceptions, when the 29 species and 37 SSCP patterns are aligned by band position (Table 1 and Fig. 3). This implies that the SSCP analysis may aid in understanding the origin of *Phytophthora* species. Natural hybrids were recently found between *Phytophthora* species (Brasier et al., 1999). Test of this PCR-SSCP procedure for differentiating natural hybrids from their parental species of *Phytophthora* also is warranted.

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