Occurrence and Identification of *Phytophthora* spp. Pathogenic to Pear Fruit in Irrigation Water in the Wenatchee River Valley of Washington State

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ABSTRACT

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Seven hundred forty-nine isolates of *Phytophthora* spp. were obtained from irrigation canals in eastern Washington State during the 1992 to 1995 and 1999 growing seasons. Isolates were retrieved using pear baiting techniques. All isolates were pathogenic to pear and were present in irrigation water beginning early in fruit development. Over the course of the 5 year study, 10 and 5% of isolates were identified as *P. cactorum* and *P. citricola*, respectively, using morphological criteria. The remaining isolates could not be identified using morphological criteria. Colony morphology of these isolates was characterized during all years of the study. In 1999, more detailed studies utilizing polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis of entire internal transcribed spacer (ITS) regions (ITS1, 5.8S, and ITS2) of ribosomal DNA for 180 isolates, and sequence analysis of ITS2 for 50 isolates, were used to investigate genetic variation and phylogenetic

relationships among isolates. Isolates were divided into 12 groups based on their growth type on corn meal agar. Restriction digestion of the entire ITS region with three enzymes revealed 11 restriction digestion patterns among 180 isolates. PCR-RFLP and sequence data were obtained for 12 reference *Phytophthora* spp. (two species in each of Waterhouse's six morphological groups). Phylogenetic analysis of ITS2 regions revealed nine clades, each with strong bootstrap support. Molecular analyses revealed 23 isolates that were in the *P. gonapodyides* clade, 9 in the *P. parasitica* clade, 1 in the *P. cactorum* clade, 7 in the *P. citricola/capsici* clade, and 4 in the *P. cambivora/pseudotsugae* clade. The three isolates comprising clade 5 were significantly distinct from all other *Phytophthora* spp. in the databases and may represent a new *Phytophthora* sp. Colony morphology was not consistently correlated to PCR-RFLP pattern or ITS2 phylogeny, suggesting that the former criterion is insufficient for species identification. The results of this study indicate that at least nine phylogenetically distinct taxa of *Phytophthora* pathogenic to pear are present in irrigation water in North Central Washington.

Additional keywords: systematics, water quality.

Phytophthora is a cosmopolitan genus comprised of 67 described species and varieties, many of which are economically important plant pathogens (14,38). Sprinkler rot of apple (*Malus domestica* Borkh.) and pear (*Pyrus communis* L.), which is reportedly caused by *Phytophthora cactorum* (Lebert & Cohn), was previously considered an occasional pathogen of apple and pear fruit, but has become widespread and common in the Wenatchee River Valley of Washington State (9,22). The disease is characterized by firm chocolate-brown lesions that may cover the fruit surface after several days. Pears are susceptible beginning early in fruit development, whereas the susceptibility of apple fruits increases as fruit matures (22). Irrigation water is also frequently contaminated with the pathogen and the disease is prevalent in orchards using over-the-canopy irrigation or when the sprinkler angles of under-the-canopy systems are sufficiently high to moisten fruit (9,22).

P. cactorum has been reported as the primary incitant of sprinkler rot of apple and pear (9,22) and was the predominant species isolated from infected fruit and irrigation water during previous studies in the Wenatchee River Valley (22,24). However, *P. citricola* Sawada, *P. cambivora* (Petri) Buisman, and unidentified nonpapillate species were also occasionally isolated from in-

fectured fruit and contaminated irrigation water during subsequent epidemics (G. G. Grove, *unpublished data*). The latter comprised the vast majority of isolates retrieved from irrigation water.

Copper is routinely added to irrigation water in order to manage sprinkler rot. Growers in Washington currently begin treatments when immature pears suspended in irrigation canals become infected. During some years, this approach fails to provide adequate warning of the presence of pathogenic species in irrigation water. Earlier studies showed that *Phytophthora* spp. retrieved from irrigation water vary in their sensitivity to copper (G. G. Grove, *unpublished data*), which complicates management decisions faced by the producer. If excessive copper is added, damage to developing fruit may occur, whereas insufficient copper may preclude effective disease management. Therefore, the accurate identification and initial occurrence of *Phytophthora* spp. present in irrigation water during the period of fruit susceptibility is required for determining the optimum concentration and timing of copper injections. Knowledge of the initial occurrence, frequency, and copper sensitivity of *Phytophthora* spp. in irrigation water could facilitate disease management by improving the timing and rates of copper applications.

The identification of *Phytophthora* spp. has been traditionally based on sporangial, oogonial, antheridial, and colony morphology (2,13,38). A tabular key has been devised to divide the genus into six groups based on morphological characters (13,41,44). However, these characters can be inconsistent and are sometimes difficult to assess. Morphological characters tend to overlap be-

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tween species and it is often difficult to induce sporulation in vitro (13,31,38). As a result, *Phytophthora* spp. have been frequently unidentified or misidentified. To overcome this problem, molecular analyses have been used for identification, genetic and epidemiological studies, and examination of evolutionary relationships within *Phytophthora* and closely related genera (1,4–8,10–12,15,16,19,20,21,23,25–27,32,33,39).

Intraspecific variation in *Phytophthora* and other fungi has been assessed using random amplified polymorphic DNA analysis (5, 28), random amplified microsatellites (23), and restriction fragment length polymorphism (RFLP) in nuclear and mitochondrial DNA (15–18). In most cases, isozyme analysis has been inadequate for studying intraspecific variation in the genus (16,19,31–33), although it has been used for analyzing interspecific hybrids (26). RFLP and sequence analysis of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) have been used to investigate relationships among Waterhouse's six morphological groups (6,7,9). The ITS region has a mutation rate that is appropriate for examination of evolutionary relationships at the species level in the genus (11). The objectives of this study were (i) to determine the seasonal occurrence and frequency of *Phytophthora* spp. that are pathogenic to pear and present in irrigation canals, and (ii) to use morphological characteristics, polymerase chain reaction (PCR)-RFLP, and sequence analyses of rDNA ITS to identify *Phytophthora* spp. pathogenic to pear and present in irrigation water.

MATERIALS AND METHODS

Sampling of irrigation water. Isolates were retrieved from irrigation water by regularly sampling canals within a 15-km radius of Wenatchee, WA, during April to September 1992 to 1995 and May to August 1999. Ten, five, and four canals were sampled in 1992 to 1994, 1995, and 1999, respectively. Immature pear fruits were either (i) suspended in jars containing irrigation water (in the laboratory) or (ii) suspended in pots that were placed into irrigation canals according to previously described methods (22, 24). When using the former technique during 1992 to 1995, irrigation water was collected weekly or biweekly in 1.1-liter glass jars and transported to the laboratory. One mature pear (cv. Bartlett) was suspended in the water for 3 days at 20 to 25°C in continuous light. To maximize the number of *Phytophthora* isolates collected in 1999, different incubation conditions were employed when using this technique. Samples were collected at 4- to 7-day intervals. On each sampling date, four water samples were collected from each canal with 1.1-liter canning jars. One mature pear (cv. Bartlett) was suspended in water for 3 days in either 24 h continuous light or 24 h continuous dark at 10°C or at room temperature (between 20 to 25°C). At the end of the incubation period, pears were rinsed with distilled water and incubated for 2 to 4 days at room temperature (20 to 25°C) in continuous light.

Isolation and culture preparation. Infected pears were rinsed with 95% EtOH, and isolations were performed by sampling tissue from lesion edges. Tissue was incubated on pentachloronitrobenzene-benomyl-neomycin sulfate-chloramphenicol-selective medium (40) at room temperature (20 to 25°C). When mycelia emerged from the edge of 3- to 5-day-old cultures, hyphal tips were transferred to V8 agar to obtain pure cultures (30). A total of 749 isolates were obtained. Pure cultures were stored in glass tubes (21 × 73 mm) containing 7 ml of lima bean agar (38). For long-term storage, isolates were covered with sterile mineral oil (47) (Sigma Chemical, St. Louis) after 3 to 5 days growth.

Pathogenicity and lesion type. Inoculations were performed to determine pathogenicity and investigate variability in lesion morphology. Isolates were grown on V8-juice agar medium at room temperature for 3 days. Immediately prior to inoculation, pear fruits were wounded with a flame-disinfested 4-mm-diameter cork borer. Each isolate was placed into two wounded pears ('D'Anjou'

or 'Bartlett', which are equally susceptible) (22). Mycelia from culture edges were placed in the wounds, covered with Parafilm, and incubated at 20 to 22°C for 3 to 4 days. Following incubation, the color, size, and margin characteristics of each lesion was recorded.

Morphological characterization. Isolates were transferred to various media to promote the production of oospores and sporangia (31,38,49) necessary for morphological characterization. Mycelial disks, 4 mm in diameter, were removed from cultures growing on V8 medium with a cork borer. Disks were transferred to lima bean agar, V8-juice agar, or soil extract (3) to stimulate the formation of sporangia. Soil extract was prepared by suspending 15 g of soil in 1 liter of distilled water for 12 h (3). Soil suspensions were then centrifuged in 40-ml plastic tubes in a centrifuge (Beckman J2-21; Beckman Inc., Redmond, WA) at 10,000 × g for 25 min and the aqueous phase was transferred to 250-ml wide-mouth bottles (Nalgene Inc., Rochester, NY). Morphological characterization of oospores and sporangia was based on the method of Stamps et al. (41).

To observe colony morphology, isolates grown on V8 agar were transferred to corn meal agar (CMA) (31). Four replicate plates were inoculated with each isolate, sealed with Parafilm, and inverted. Two of the replicate plates were incubated at 20 to 25°C, and two replicate plates were incubated at 20°C in a 12-h photoperiod. Colony morphology on CMA was recorded after 7 days (44–46).

Reference species. Two reference species were chosen from each of Waterhouse's six morphological groups (41). Reference isolates were obtained from cooperators for molecular comparison with the isolates (Table 1) from irrigation water. Because they have been reported as pathogenic to apple, pear, or both fruits (38), *P. cactorum*, *P. cinnamomi* Rands, *P. gonapodyides* (Petersen) Buisman, *P. palmivora* (E. J. Butler), *P. citricola*, and *P. parasitica* Dastur were included in this study (Table 1). In addition to live cultures of reference species, ITS2 sequence data was obtained from GenBank for *P. cambivora* and *P. citricola*.

Mycelium production. After sufficient colony growth on V8 agar, mycelia were scraped from the agar surface and transferred to 250-ml flasks containing 120 ml of lima bean broth amended with 10 µg/ml of chloramphenicol (Sigma Chemical). Twenty-five to thirty disks cut from the culture margins were used for inoculation. Cultures were incubated on an orbital shaker at 190 rpm for 7 to 10 days at 20 to 25°C. Mycelia were vacuum-filtered through Whatman No. 1 filter paper (Whatman, Maidstone, UK), placed in 2-ml microcentrifuge tubes, and frozen at –20°C for 2 h. Frozen mycelia were lyophilized, ground with a metal rod, and stored dry at –20°C for subsequent DNA extraction.

Genomic DNA extraction. Genomic DNA was extracted from 50 µg of lyophilized mycelia as described by Peever et al. (35) with modifications. Supernatant collected following potassium acetate precipitation was subjected to phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol) extraction followed by chloroform/isoamyl alcohol (24:1, vol/vol) extraction. DNA pellets were resuspended in sterile distilled water, and DNA concentrations were estimated visually in ethidium bromide-stained agarose gels (0.7%) by comparing band intensity with known quantities of *HindIII*-digested phage lambda DNA (Promega, Madison, WI). DNAs were routinely diluted to 50 ng/µl for PCR amplification.

PCR amplification and restriction digestion of rDNA ITS. Entire ITS regions encompassing ITS1, 5.8S, and ITS2 regions of nuclear rDNA were amplified and digested with restriction enzymes for PCR-RFLP analysis. ITS1 (5'-TCCGTAGGTGAAC-CTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC) universal primers were used to amplify the entire ITS region (48). PCR was performed in 50-µl reaction volumes with each reaction mixture containing 5 µl of 10× PCR buffer, 1 mM MgCl₂ (Promega), 20 µM deoxynucleoside triphosphates (Idaho Technologies, Idaho Falls, ID), 1 µM ITS1 and ITS4 primers, 2 units of *Taq* DNA

polymerase (Promega), and 50 ng of genomic DNA. This and all subsequent PCR amplifications were performed in a thermocycler (Hybaid Omne-E; Hybaid Ltd., Middlesex, UK). Cycling conditions included an initial 5 min denaturation at 96°C followed by 35 cycles of 96°C for 1 min (melt), 60°C for 1 min (anneal), and 72°C for 1 min (extension). The final extension cycle was 72°C for 10 min. PCR products were separated in 1.2% agarose gels (69 V for 5 h) with *HindIII*/*EcoRI*-digested lambda DNA (Promega) as a size standard, and visualized using a digital imaging system (UVP, Upland, CA). Amplicons obtained using ITS1 and ITS4 primers were digested with three different four-base-cutting restriction enzymes: *HaeIII* (GG/CC), *RsaI* (GT/AC), and *MspI* (C/CGG) (Life Technologies, Grand Island, NY). Restriction digestions were performed in 10-μl reaction volumes containing 3.7 μl of sterile distilled water, 1 μl of reaction buffer, 3 units of restriction enzyme, and 150 ng of PCR product. Digested ITS amplicons were separated on 2% agarose gels (92 V for 150 min) with *HindIII*/*EcoRI*-digested lambda DNA (Promega) as a size standard and visualized using the digital imaging system described previously.

Analysis of ITS PCR-RFLP data. The presence or absence of restriction fragments obtained with three different restriction enzymes was scored for each isolate. Each isolate was treated as the operational taxonomic unit, and only isolates with unique PCR-RFLP haplotypes were included in the analysis. A distance phenogram was constructed with the RESTDIST, NEIGHBOR, and CONSENSE programs in PHYLIP (Phylogeny Inference Package; J. Felsenstein, 1993, version 3.5c, Department of Genetics, University of Washington, Seattle). A distance matrix was estimated using the program RESTDIST, and distances were clustered using neighbor joining in the program NEIGHBOR. A majority-rule consensus tree was generated using CONSENSE, and statistical support for phenogram branches was estimated with 1,000 bootstrapped data sets generated by the program SEQBOOT. The program TREEVIEW (34) was used to visualize the phenogram.

Amplification and sequencing of ITS2. Isolates with different ITS PCR-RFLP patterns and one isolate of each reference species were further characterized by sequencing the ITS2 region and using distance analysis to estimate a phylogeny. At least one representative isolate with each PCR-RFLP pattern was chosen for sequencing. ITS2 regions of the sampled isolates were amplified using ITS3 (5'-GCATCGATGAAGAACGCAGC) and ITS4 universal primers (5'-TCCTCCGCTTATTGATATGC) (48). PCR reactions were conducted in 50-μl reaction volumes composed of 10× PCR buffer (Promega), 1 mM MgCl₂ (Promega), 20 μM deoxynucleoside triphosphate (Idaho Technologies), 0.25 μM ITS3 and ITS4 primers, 2 units of *Taq* DNA polymerase (Promega), and 50 ng of DNA template. An initial denaturation step was performed for 2 min at 95°C followed by 30 cycles of denaturation at

95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a 10 min final extension step at 72°C. ITS2 amplicons were purified with PCR purification spin columns (QiaQuick; Qiagen, Valencia, CA) following the manufacturer's directions. Purified PCR products were separated on ethidium bromide-stained 0.7% agarose gels, and DNA concentrations were estimated based on the intensity of the PCR product relative to *HindIII*-digested lambda DNA (Promega). Column-purified ITS2 amplicons were direct-sequenced on both strands using ITS3 and ITS4 primers. Each sequence reaction contained 40 to 90 ng of DNA, 320 nM primer, 4 μl of sequence reaction mix (BigDye Terminator Cycle Sequencing Ready Reaction Mix; Applied Biosystems, Foster City, CA), and sterile distilled water in 10-μl volumes. Cycling conditions consisted of 25 cycles of 15 s at 96°C, 15 s at 50°C, and 4 min at 60°C. Sequence products were purified with gel filtration cartridges (Centriflex; Edge BioSystems, Gaithersburg, MD), and sequenced on an automated DNA sequencer (PE Biosystems Model 377; Applied Biosystems, Norwalk, CT). Sequencing was performed in the Laboratory for Biotechnology and Bioanalysis, School of Molecular Biosciences, Washington State University, Pullman.

Sequence alignment and phylogenetic analysis. To determine the phylogenetic relationships of sampled isolates and their relationships to the reference species, ITS2 regions were aligned and phylogenetic analysis was performed. Some isolates shared identical ITS2 sequences and duplicate sequences were removed from the data set prior to phylogenetic analysis. Isolates from irrigation water and reference species were sequenced, and ITS2 sequences were obtained from GenBank. One isolate of each reference species was sequenced and included in the phylogenetic analysis, except two *P. cactorum* and *P. infestans* isolates were sequenced. The two *P. cactorum* isolates retrieved from irrigation water were included in the phylogenetic analysis because they had different colony morphologies. A *P. cambivora* isolate retrieved from a naturally infected pear and a *P. citricola* isolate obtained from irrigation water were also included. Two *P. cambivora* sequences (GenBank Accession Nos. AF266763 and AF139369) and a *P. citricola* isolate (GenBank Accession No. AF266788) were obtained and included in the phylogenetic analysis. Sequences were aligned using Clustal-X (42), and the unrooted phylogeny was estimated by distance analysis in PHYLIP. One thousand bootstrapped data sets were generated using SEQBOOT and fed into DNADIST to compute a distance matrix with the Kimura 2-parameter nucleotide substitution model with a transition/transversion ratio of 2.2 estimated from the data set. Distances were clustered using neighbor joining in the program NEIGHBOR with random input order of taxa, and the consensus phylogram was constructed using CONSENSE. Branch lengths were estimated by maximum likelihood in the program DNAML

TABLE 1. Reference *Phytophthora* spp.

Species	Source	Waterhouse group
<i>P. cactorum</i> 1 (Lebert & Cohn) Schröter	G. G. Grove, Washington State University	I
<i>P. cactorum</i> 2 (Lebert & Cohn) Schröter	G. G. Grove, Washington State University	I
<i>P. pseudotsugae</i> Hamm & Hansen	E. M. Hansen, Oregon State University	I
<i>P. palmivora</i> (E. J. Butler) (A1 mating type)	J. E. Adaskaveg, University of California, Riverside	II
<i>P. capsici</i> Leonian	J. B. Ristaino, North Carolina State University	II
<i>P. parasitica</i> 1 Dastur	J. B. Ristaino, North Carolina State University	III
<i>P. parasitica</i> 2 Dastur	J. B. Ristaino, North Carolina State University	III
<i>P. citricola</i> Sawada	G. G. Grove, Washington State University	III
<i>P. infestans</i> (Mont.) de Bary (US1)	D. A. Johnson, Washington State University	IV
<i>P. infestans</i> (US11)	D. A. Johnson, Washington State University	IV
<i>P. mirabilis</i> Galindo & Hohl (A1)	J. E. Adaskaveg, University of California, Riverside	IV
<i>P. megasperma</i> 1 (Drechs.)	G. G. Grove, Washington State University	V
<i>P. megasperma</i> 2 (Drechs.)	G. G. Grove, Washington State University	V
<i>P. lateralis</i> Tucker & Milbrath	E. M. Hansen, Oregon State University	V
<i>P. cinnamomi</i> 1 Rands	G. G. Grove, Washington State University	VI
<i>P. cinnamomi</i> 2 Rands	G. G. Grove, Washington State University	VI
<i>P. gonapodyides</i> (Petersen) Buisman	E. M. Hansen, Oregon State University	VI

with the distance tree as the user tree. Statistical support for phylogram branches was estimated by bootstrapping with 1,000 bootstrapped data sets. The program TREEVIEW (34) was used to visualize the phylogram.

RESULTS

Seasonal occurrence, pathogenicity, and lesion types. Seven hundred forty-nine isolates were retrieved from irrigation water using direct and indirect baiting methods. All isolates were pathogenic to pear in subsequent inoculations. The initial detection of *P. cactorum* occurred on 29, 28, 28, and 9 May 1992, 1993, 1994, and 1995, respectively. *P. citricola* was first detected on 1 July, 4 June, 24 June, 9 July, and 16 July 1992, 1993, 1994, 1995, and 1999, respectively. Nonpapillate isolates were first detected on 22 May, 7 May, 22 April, 17 April, and 8 June 1992, 1993, 1994, 1995, and 1999, respectively. The proportion of canals infested and relative proportions of isolates classified as *P. cactorum*, *P. citricola*, or unidentified nonpapillate isolates are presented in Figure 1. These latter isolates were detected in all canals sampled during all years of the study. Baiting in jars was somewhat more efficient for detection of the nonpapillate isolates. In one canal, 64.3, 63.6, and 72.2% of the nonpapillate, unidentified isolates were detected by this technique in 1992, 1993, and 1994, respectively. Based on color, homogeneity of color, size, and distinction of lesion margin as criteria, eight different lesion types were observed (data not shown). Lesion types that developed on artificially inoculated pears were frequently different from lesion types observed on the original bait pears. Approximately 5% of isolates gave the same lesion types for three independent observations (one on original pear bait, two on inoculated pears).

Morphological characterization. Only 15% of the isolates retrieved during the course of the study produced sporangia and oospores in numbers sufficient for positive identification. Seventy-nine isolates were identified as *P. cactorum*. Thirty-six isolates (including 1999 isolates 910, 928, 948, 954, and 964) were identified as *P. citricola*. Only two of the isolates produced papillate sporangia and, due to lack of oospore production, could not be identified to species. The remaining 634 isolates could not be identified by morphological criteria. These isolates had nonpapillate sporangia and were placed in Waterhouse groups V or VI. Two of the isolates with unique growth morphologies produced some paragynous antheridia and a few internally proliferous, nonpapillate sporangia. Although these two isolates had features characteristic of Waterhouse group V, the observed morphological characteristics were insufficient for species identification. In 1999, 12 different growth types were observed among 180 isolates (Tables 2 and 3). The majority (85%) of the isolates could be classified into growth types 1, 2, 3, and 9.

PCR-RFLP analysis of nuclear rDNA ITS. A PCR amplicon of 900 to 950 bp was obtained for all 180 isolates during 1999 using the ITS1 and ITS4 universal primers (48). Variation was investigated using restriction enzyme digestion with three enzymes: *MspI*, *RsaI*, and *HaeIII*. A phenogram based on similarity of RFLP fragments revealed several clusters of isolates with low (50 to 65%) bootstrap support. Two large clusters of isolates (labeled A and B) had bootstrap support greater than 80% (Fig. 2). All except one of the 180 isolates sampled from irrigation water in 1999 fell into cluster A with high bootstrap support (Fig. 2). Cluster A also included reference species *P. lateralis*, *P. capsici*, *P. citricola*, *P. pseudotsugae*, *P. cinnamomi*, *P. gonapodyides*, *P. parasitica* (isolate 1), *P. megasperma* (isolate 1), and 179 isolates from irrigation water. Cluster B included *P. palmivora*, *P. mirabilis*, *P. infestans*, *P. parasitica* (isolate 2), *P. megasperma* (isolate 2), *P. cactorum*, and one sampled isolate (isolate 894). Multiple isolates of each reference species had the same PCR-RFLP haplotype and clustered together, except *P. megasperma* isolates 1 and 2 and *P. parasitica* isolates 1 and 2 (Fig. 2).

Phylogenetic analysis of nuclear rDNA ITS2. Fifty isolates from the 1999 sampling were selected for phylogenetic analysis based on growth type on CMA and PCR-RFLP analysis. Selected isolates included at least one representative of each RFLP pattern and each growth type. ITS2 regions of selected isolates and 14 isolates representing 12 reference *Phytophthora* spp. were amplified and amplicons of the expected size (590 to 620 bp) were obtained using ITS3 and ITS4 universal primers. ITS2 sequences examined in the phylogenetic analysis ranged from 561 to 583 bp for sampled isolates and 558 to 583 bp for reference *Phytophthora* spp. Phylogenetic analysis revealed nine clades among the *Phytophthora* spp. retrieved from irrigation water, each with bootstrap support greater than 80% (Fig. 3). Clade 1 contained one isolate (871) and no reference species. Clade 2 included two isolates (857 and 969) and two reference species, *P. cambivora* (GeneBank Accession No. AF266763) and *P. pseudotsugae*. The third clade contained isolates 910, 881 (representing five isolates with identical sequence), and 928 and reference species *P. citricola* and *P. capsici*. Clade 4 contained one isolate with papillate sporangia (isolate 894) and *P. cactorum*. Clade 5 contained isolates 776, 777, and 829 and no reference species. BLAST searches revealed that the most similar sequences to these isolates were represented by *P. gonapodyides*, which formed another well-separated clade in our phylogram (clade 9). The sixth clade contained isolates 783 and 793, and clade 7 contained isolates 772 (representing five isolates with identical sequence), 812, 955, and 810 plus reference species *P. parasitica*. Clade 8 contained isolate 951 and no reference species, and clade 9 contained isolates 861, 808, 775 (repre-

Fig. 1. A, Proportions of canals infested and **B,** *Phytophthora* spp. isolates retrieved and classified as *P. cactorum*, *P. citricola*, or unidentified nonpapillate species 1992 to 1995 and 1999. Isolates were obtained from irrigation canals in eastern Washington using immature pears as baits.

senting 19 isolates with identical sequence), 923, and 784 and reference species *P. megasperma* and *P. gonapodyides*. All isolates producing sporangia in this clade had nonpapillate sporangia. *P. gonapodyides* had an ITS2 haplotype identical to the 19 isolates represented by isolate 775.

The phenogram based on PCR-RFLP (Fig. 2) and the phylogram based on ITS2 sequences (Fig. 3) were not strictly correlated although they had similar topology. The level of resolution obtained with the ITS2 sequence data was much higher than that obtained with ITS-RFLP.

Comparison of morphological and molecular data. Colony morphology of all isolates was compared with PCR-RFLP and ITS2 sequence variation to detect possible correlations. Consistent correlation between colony morphology and ITS haplotype was observed in only three cases (Table 2). Colony morphologies 6, 14, and 20 were consistently associated with ITS2 clades 2, 4, and 3, respectively. The remainders of the colony morphologies were not consistently associated with ITS haplotype (Table 2). Isolates from four of seven PCR-RFLP clusters exhibited more than one growth type per haplotype. Conversely, the same growth types were observed for several different PCR-RFLP haplotypes.

DISCUSSION

During all years of the study, *Phytophthora* spp. pathogenic to pear were present in irrigation water beginning during the early stages of fruit development and susceptibility. There was temporal and spatial variability in initial occurrence and species composition of irrigation water, indicating that producers should assume that inoculum is present throughout the fruit development period and treat irrigation water accordingly. *P. cactorum* is considered the primary incitant of sprinkler rot of apple and pear, and was the predominant species in irrigation water during earlier studies (22). This species comprised only 10% of the isolates in this long-term study using conventional and modified pear baiting techniques. The nonpapillate isolates were spatially and temporally more prevalent in water and more frequently isolated when baits were suspended in jars. These isolates were present in all 10 of the irrigation canals sampled during 1992 to 1995 and were occasionally isolated from naturally infected fruit during sprinkler rot epidemics that occurred during the study period. However, infection of pears using our baiting technique does not necessarily indicate that all retrieved isolates could infect pears under orchard conditions. The role of the nonpapillate isolates in the etiology and epidemiology of sprinkler rot needs further study. Our results also indicate that the conventional method of suspending pears in irrigation canals for several days may be not be sensitive enough for the results to be used to time the initial copper treatment of irrigation water.

Irrigation water has been reported as a source of several *Phytophthora* spp. including *P. capsici*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, and *P. parasitica* (14). *P. cactorum*, which has been reported as the causal agent of sprinkler rot of pear and apple, was

isolated from irrigation water in several earlier studies (3,22,24), and was isolated 79 times during the course of this study. Over the 5 year study, 36 isolates from irrigation water were identified as *P. citricola*. *P. citricola* was previously found in irrigation water and recorded as pathogenic to apple roots (29). However, *P. citricola* has not been reported as an incitant of fruit rot of pear and apple, but has been occasionally isolated from naturally infected pear fruit in Washington State. This species therefore should be considered a hazard to developing fruit, particularly in orchards irrigated with water from contaminated canals or ponds.

Several morphologically distinct isolates were retrieved in 1999, but they could not be identified to species using conventional methods. Based on molecular analyses, the predominant species retrieved from irrigation water during the course of this study was *P. gonapodyides*. Nineteen of the 1999 isolates had identical ITS2 sequences to the reference species. Some of these isolates produced nonpapillate sporangia but no oospores and could not be positively identified. *P. gonapodyides* is a heterothallic species and requires both mating types for production of sexual propagules, which could account for the absence of oospores in our cultures (14,38,41). Although *P. gonapodyides* has been reported as the causal organism of root rot diseases of several hosts, the first isolation of *P. gonapodyides* was performed from decaying apple fruit in 1910 (36). Phylogenetic analysis showed that there were several isolates from irrigation water that are not closely related to any described *Phytophthora* sp. It is possible that these isolates might represent new *Phytophthora* spp.

Phylogenetic analysis indicated that there were at least nine different phylogenetic taxa of *Phytophthora* in irrigation water. *P. cactorum* was infrequently isolated during the course of this study, but was isolated throughout the growing season in previous studies (21,23). None of the 1999 isolates sampled from irrigation water were identified as *P. cactorum* based on morphological characteristics. Only one isolate (isolate 894) had a similar RFLP pattern and resided in the same clade with *P. cactorum* in the phylogram, strongly suggesting that it was *P. cactorum*. These results are in contrast with previous studies in which *P. cactorum* was the common *Phytophthora* sp. recovered from irrigation water in this geographical area (21,23). All isolates obtained in the early studies were obtained by suspending pears in irrigation canals, whereas the majority of isolates obtained during the current study were isolated from pears suspended in irrigation water contained in jars in the laboratory. The use of the latter technique may add precision to investigations of the *Phytophthora* spp. composition of irrigation water.

In several studies, phylogenetic analysis of ITS sequences has not supported the differentiation of *Phytophthora* spp. based on papillal, antheridial, or thallic characteristics (5,6,10). The grouping of semipapillate and papillate or semipapillate and nonpapillate species were frequently observed by several workers (5–

TABLE 2. Relationships among clusters defined with internal transcribed spacer (ITS) polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) data, clades defined with ITS2 sequence data, and colony morphologies/growth types

PCR-RFLP cluster ^a	ITS2 clade ^b	Colony morphology ^c
A	4	1, 3, 4, 5, 18, 19
B	5	2, 3, 5, 19
C	5	1, 2, 9
D	6	6
D	7	6, 14, 16
E	2	14
F	1	20

^a Clusters defined based on bootstrap values greater than 75% (Fig. 1).
^b Clades defined based on bootstrap values greater than 75% (Fig. 2).
^c Colony morphology determined on corn meal agar.

TABLE 3. Growth type of *Phytophthora* isolates on corn meal agar^a

Growth type	Characteristics of growth types	Number of isolates
1	Segmented	42
2	Radiating, shiny, irregular margin	59
3	Radiating, smooth margin	12
6	Lanceolate, petiolate	2
9	Radiating, fine mycelium	45
14	Fairly fluffy, not radiating, curly	5
15	Weak growth, no pattern	1
16	Fluffy, not radiating, curly	1
17	Radiating, not shiny, not smooth	4
18	Fairly fluffy, lanceolate	1
19	Slightly fluffy, slightly curly	6
20	Fast growth, no pattern	5

^a Isolates were retrieved from irrigation water using immature pear fruits as baits. Colonies were determined according to the classification system of Ribeiro (40).

8,16). In this study, grouping of *P. mirabilis* and *P. infestans* with semipapillate sporangia (group IV) and *P. cactorum* with papillate sporangia (Waterhouse group I) supported the results of previous studies. A similar situation was observed in clade 7 including some nonpapillate and papillate isolates and group II species *P. parasitica*. In addition, nonpapillate Waterhouse group VI species *P. cambivora*, five isolates from irrigation water (two of which produced nonpapillate sporangia), and Waterhouse group I species *P. pseudotsugae* were placed in the same clade. Although *P. cambivora* was recorded as pathogenic to apple and pear in previous studies (29), morphological characteristics were not sufficient to describe these five isolates as *P. cambivora*. Therefore, our results also suggest that Waterhouse groups do not represent a natural assemblage. However, this classification scheme has been a valuable tool for the identification of *Phytophthora* spp. prior to the advent of molecular techniques. Additional molecular studies evaluating different genomic regions are required for better resolution of the genus.

We observed different RFLP patterns among multiple isolates of *P. parasitica* and *P. megasperma*. RFLP patterns for ITS regions of *P. megasperma* (17,39) and *P. citrophthora* (26) isolates were distinct (within species) in previous studies. Nuclear DNA analysis also showed intraspecific variation within *P. megasperma*, *P. palmivora*, *P. parasitica*, *P. capsici*, and *P. megakarya* isolates (18).

Colony morphology has been characterized by various growth media. Corn meal, potato dextrose, and carrot agars have been suggested as the most useful media to detect distinct cultural patterns (14,38). In this study, isolates were divided into 12 groups

Fig. 2. Phenogram of genetic similarity among samples of *Phytophthora* spp. from irrigation water based on polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) of internal transcribed spacer region. Representative isolate numbers and reference species are indicated. Numbers in parentheses indicate the total number of isolates with identical PCR-RFLP haplotype. Numbers at the major branches indicate the percentage occurrence of the cluster to the right of the branch in 1,000 bootstrapped phenograms. Clusters containing sampled isolates with bootstrap values greater than 80% are labeled A and B. Scale represents 10% differentiation.

based on colony morphology on CMA. Our results indicated that isolates with different genotypes could be of similar growth type and vice-versa. Similar colony morphologies (clade 5 containing *P. citricola* and clade 3 with unique isolates) were observed in some clades, suggesting this criterion may be used as a distinguishing characteristic for some species. Although variation in colony morphology has been considered by a number of researchers to have limited utility for identification (14), the criterion has in some instances been used as a differentiating characteristic (41,44–46). However, it is not a consistent criterion for identification and should be used in conjunction with other characters.

The molecular data reported here suggest that revision in the taxonomy of *Phytophthora* is required. Although entire ITS

Fig. 3. Internal transcribed spacer 2 (ITS2) phylogeny estimated among *Phytophthora* spp. sampled from irrigation water using distance analysis and neighbor joining. Representative isolate numbers and reference species are indicated. Numbers in parentheses indicate the total number of isolates with identical ITS2 haplotype. Numbers at the major branches indicate the percentage occurrence of the cluster to the right of the branch in 1,000 bootstrapped phylograms. Clades with sampled isolates and bootstrap values greater than 80% are labeled 1 through 9. Branch separating clade 7 from clade 6 (91% bootstrap support) is too short to be visible. Scale represents 0.1 nucleotide substitutions per site.

regions of rDNA have been useful for reevaluation of the genus and other oomycete genera, this region is not always useful for species differentiation (8). Additional studies that evaluate different regions of the genome are needed (5,8,10,17). Because morphological characters are required for accurate identification, studies on methods that promote sporulation in vitro would be useful.

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