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Phytophthora virginiana sp. nov., a high-temperature tolerant species from irrigation water in Virginia

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Abstract — Isolates belonging to a previously unknown species of *Phytophthora* were recovered from irrigation reservoirs at several ornamental plant nurseries in Virginia. Morphological features of this species include abundant lateral chlamydospores in clarified V8 juice agar and carrot agar and terminal, ovoid to obpyriform, nonpapillate and noncaducous sporangia in soil water extract. All tested isolates are silent A^1 mating type. They did not produce any sexual structures but induced A^2 mating type testers of *P. cinnamomi* and *P. cryptogea* to produce gametangia. Sequence analysis of the internal transcribed spacer (ITS) region confirmed that this species belongs to the high-temperature tolerant cluster within *Phytophthora* clade 9. Its ITS sequence differs from those of its two closest relatives, *P. hydropathica* and *P. parsiana*, by >26 bp. Based on these morphological, physiological, and molecular characteristics, we recognise it as a new species, described here as *Phytophthora virginiana*.

Key words —aquatic habitat, oomycetes, phylogeny, self-sterile, taxonomy

Introduction

The genus *Phytophthora* de Bary contains a number of destructive plant pathogens (Erwin & Ribeiro 1996). They are responsible for many historical and current epidemics of plant diseases including late blight of potatoes caused by *P. infestans*, ink disease of chestnut trees caused by *P. cambivora*, and sudden oak death caused by *P. ramorum* (Erwin & Ribeiro 1996, Goheen et al. 2002, Rizzo et al. 2002). One reason that *Phytophthora* species are widely spread plant pathogens is that they are well adapted to aquatic environments and dispersed by water (Blackwell 1944, Hong & Moorman 2005). If water is directly used for irrigation in crop production areas without proper decontamination, *Phytophthora* pathogens in the water can spread rapidly from field to field through irrigation systems, severely damaging ornamental and other crops.

Previous studies have revealed the presence of at least 20 *Phytophthora* species in aquatic environments (Hong & Moorman 2005), including many notorious plant pathogens such as *P. capsici* (Roberts et al. 2005), *P. nicotianae* (Thomson & Allen 1976), and *P. ramorum* (Werres et al. 2007). Other *Phytophthora* species regarded as opportunistic plant pathogens or lacking recorded pathogenicity also survive in the irrigation systems, such as *P. irrigata* (Hong et al. 2008), *P. hydrogena* (Yang et al. 2014), and *P. gonapodyides* (Pittis & Colhoun 1984). Many new *Phytophthora* taxa also exist in irrigation water and other aquatic environments. In Virginia alone, approximately 15 known *Phytophthora* species and hundreds of isolates representing unknown taxa have been recovered from irrigation water at ornamental plant nurseries (Hong et al. 2008). It still is not known whether these novel *Phytophthora* species pose a threat to nursery production (Hong et al. 2008, Yang et al. 2014). These species may also be confused with major plant pathogenic species. Hence, describing these new species is important for reducing the risk of misidentifying high-impact *Phytophthora* species and minimizing the threat that *Phytophthora* species pose to crops and natural plants (Gallegly & Hong 2008).

In this study we formally name a novel species, *Phytophthora virginiana*. Isolates were recovered during statewide surveys in Virginia, USA. We provide detailed morphological, physiological, and phylogenetic evidence to separate *P. virginiana* from all known *Phytophthora* species.

Materials & methods

Cultures

Isolates of *Phytophthora virginiana* were recovered from runoff containment basins and irrigation reservoirs at ornamental plant nurseries in several Virginia counties. The five representative isolates examined in this study were obtained during 2006–7 by baiting with rhododendron or camellia leaves (Bush et al. 2003). Agar blocks with actively growing cultures in 20% clarified V8 juice agar (CV8A) were transferred into microtubes with sterile distilled water for long-term storage at both 15 and –80°C. Sequences from all isolates were analyzed. Isolates 44G6 and 46A2 were also morphologically examined and tested for cardinal temperatures.

Sequencing and phylogeny

DNA extraction followed Hong et al. (2008). PCR amplifications of the internal transcribed spacer (ITS) region covering ITS1, 5.8S rRNA gene, and ITS2 were performed using primer pair ITS6F4R (Cooke et al. 2000). The reaction mix recipe and PCR program were applied according to Kong et al. (2003). Sequencing was done at the University of Kentucky Advanced Genetic Technologies Center (Lexington, KY). Sequences of both directions were visualized with Finch TV v. 1.4.0., aligned using Clustal W in MEGA5 (Tamura et al. 2011), and edited manually to correct obvious errors. Sequences of *P. virginiana* isolates were aligned with those of other clade 9 species and representative *Phytophthora* species from other clades using Clustal W in

MEGA5 (Tamura et al. 2011). *Pythium aphanidermatum* was used as an outgroup. The phylogeny was reconstructed in MEGA5 using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei 1993) with 1000 bootstrap replicates.

Cardinal temperatures

Isolates 44G6 and 46A2 were subcultured on CV8A and carrot agar (CA) and incubated at 5, 10, 15, 20, 25, 30, 35, and 40°C. Agar blocks (5 mm diam.) were taken from 7-day-old cultures using a flamed cork borer and placed upside down at the center of Petri dishes. For each isolate, triplicate dishes per medium were placed in an incubator at a designated temperature. Two colony growth measurements per colony were taken after 4 days when the fastest growing colony was c. 1 cm from the dish edge. This test for cardinal temperatures was repeated. Means (with standard errors) of daily radial growth were plotted against temperature using the gplots package v. 2.11.0 (Warnes et al. 2012) in R statistical software v. 2.15.0 (R Core Team 2012). Analysis of variance was conducted in R to determine whether the measurements were statistically different between two isolates and experiments.

Morphology

Colony morphology of *P. virginiana* was noted and photographed from 10-day-old cultures on CV8A, CA, malt extract agar (MEA), and potato dextrose agar (PDA) at 20°C in the dark.

Sporangia were produced by incubating agar blocks with actively growing cultures (≤ 2 weeks) in non-sterile 1.5% soil water extract solution (SWE, 15 g of non-*Phytophthora* containing nature soil/1 L distilled water) for 7–8 hours under cool-white fluorescent lamps at room temperature (c. 23°C). Mature chlamydospores were observed directly on 20-day-old cultures. Sporangia and chlamydospores were photographed using a Nikon Fujix Digital Camera HC-300Zi connected to a Nikon Labophot-2 microscope at 1000×. Both length and width of 50 randomly selected mature sporangia of *P. virginiana* and two perpendicular diameters of 30 chlamydospores were measured using Image-Pro® Plus *v*. 5.1.2.53.

Mating type of *P. virginiana* isolates was determined by placing each isolate with an A^1 or A^2 mating type tester of *P. cinnamomi* in dual cultures on hemp seed agar (HSA). The polycarbonate membrane method (Ko 1978, Gallegly & Hong 2008) was used to induce selfed gametangia of *P. virginiana* in the presence of its opposite mating type tester at room temperature. Several heterothallic species including *P. cinnamomi*, *P. cambivora*, *P. meadii*, *P. nicotianae*, and *P. cryptogea* were used as mating type testers. When no sexual organ was found after being paired for up to three months at room temperature in dark, additional polycarbonate membrane tests were conducted at 20 and 25°C.

Results

Sequencing and phylogeny

All *P. virginiana* isolates produced identical ITS sequences of 745 bp (ITS1 = 180 bp, 5.8s gene = 127 bp, ITS2 = 438 bp) that differed from those of all tested *Phytophthora* species. *Phytophthora virginiana* differed by 26 bp (11 bp in ITS1,

15 bp in ITS2) from its closest relative, the type isolate 5D1 of *P. hydropathica* (GenBank EU583793).

The ITS maximum likelihood phylogenetic trees (Fig. 1) placed *P. virginiana* in clade 9 of *Phytophthora* (Blair et al. 2008). *Phytophthora virginiana* isolates were supported as a taxon unique from all other known species by a strong bootstrap (Fig. 1). GenBank accession numbers generated in this study and used for phylogeny construction are included in Fig. 1.

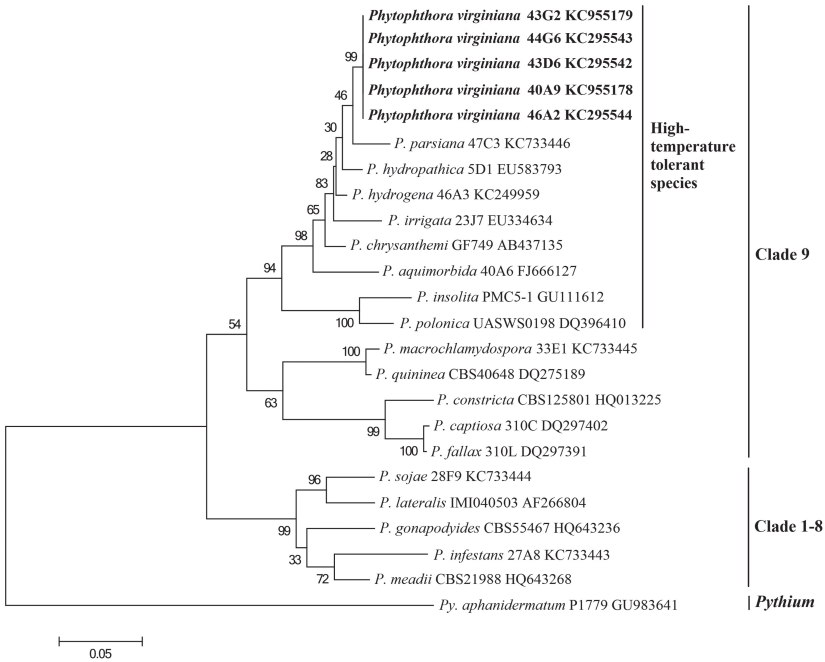

Figure 1. Maximum likelihood phylogenetic tree for species in *Phytophthora* clade 9 including *Phytophthora virginiana* and reference species in other clades based on ITS sequences. Alignment was conducted with Clustal W and the phylogenetic tree was generated in MEGA 5.

Cardinal temperatures

Measurements of radial growth were statistically identical between two *P. virginiana* isolates ($P = 0.21$) and two experiments ($P = 0.25$). Temperature-growth relation of *P. virginiana* isolates is shown in Fig. 2. Neither isolate grew on tested media at 5°C. The optimum temperature was 30°C on both CV8A and CA. Both isolates grew well at 35°C with 46A2 growing >5 mm daily on both media and 44G6 growing ~ 5 mm daily on CV8A. However, 44G6 grew relatively slowly on CA with an average growth rate of 1.6 $mm.d^{-1}$ in both experiments. No growth of 44G6 was observed on CA at 40°C, whereas 46A2 had notable growth on both media, as did 44G6 on CV8A at 40°C.

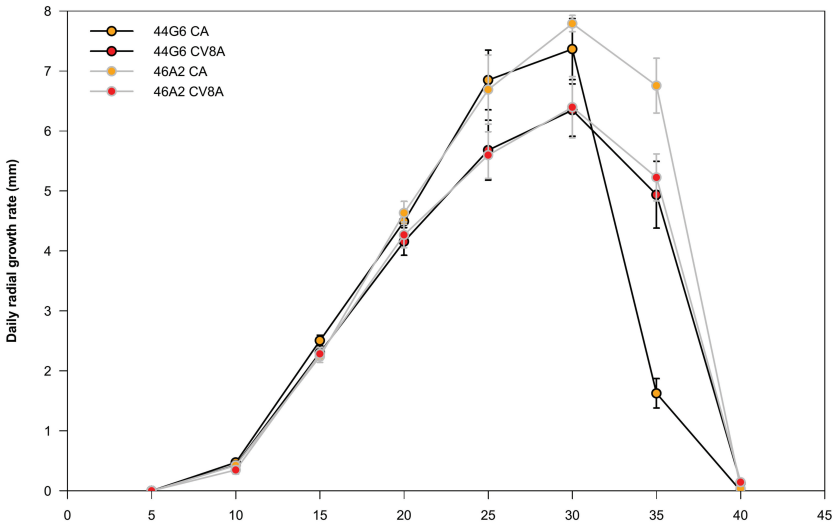

Figure 2. *Phytophthora virginiana.* Average daily radial growth of on carrot agar (CA) and clarified V8 juice agar (CV8A).

Colony morphology

After growing in the dark at 20°C for 10 days, the colonies of the two *P. virginiana* isolates were photographed (Fig. 3). The growth patterns of both isolates were similar on each tested medium. On CA, they grew fast and formed radiate colonies with relatively smooth edges, producing abundant aerial mycelia at the center and scattered at the colony intermediate and edge. On CV8A, they formed similar chrysanthemum colony patterns with cotton-like aerial mycelia at the colony center. On MEA, both formed tufted to chrysanthemum colonies with discontinuous edges. Colonies on PDA had dense aerial mycelia and expanded less than on CV8A.

Taxonomy

Phytophthora virginiana Xiao Yang & C.X. Hong, **sp. nov.** Fig. 4

MycoBank MB 804533

Differs from *Phytophthora hydropathica* by abundant, thin-walled, lateral chlamydospores.

Type: USA, Virginia, baited with camellia leaves from irrigation water of an irrigation runoff reservoir in a production perennial nursery, October 2007, collected by Chuanxue Hong. **Holotype,** ATCC MYA-4927; ex-type culture, 46A2 (GenBank KC295544).

Etymology: '*virginiana*' refers to the Commonwealth of Virginia, where the new species was initially recovered.

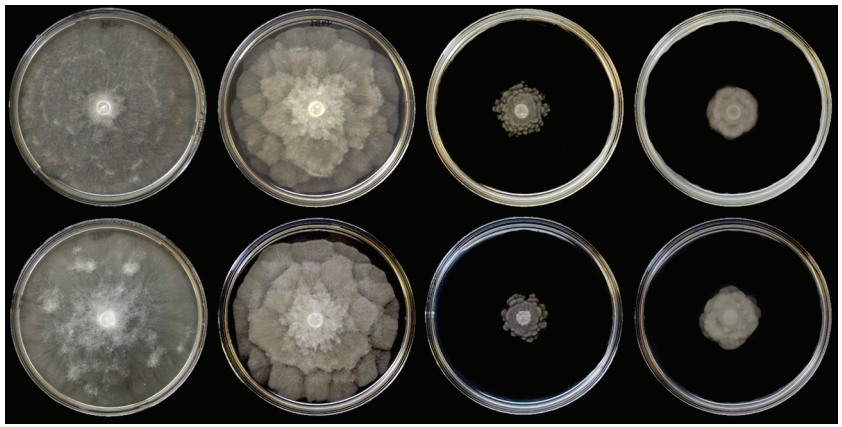

Figure 3. Colony morphology of *Phytophthora virginiana* isolates 44G6 (top) and 46A2 (bottom) after 10-days growth at 20°C on (left to right) carrot agar, clarified V8 juice agar, malt extract agar and potato dextrose agar.

Sporangia produced occasionally by aged cultures grown on carrot agar and 20% clarified V8 juice agar, and abundantly by culture plugs submerged in 1.5% soil water extract under light within 10 hours. Sporangial shape mostly ovoid to obpyriform and sometimes limoniform to ellipsoid with distorted-shaped sporangia such as peapod-shaped and peanut-shaped on culture plugs submerged in SWE after 20 hours. Sporangia terminal, nonpapillate and noncaducous; av. 51.7 × 32.5 µm. Internal proliferation of sporangiophore common, nested or extended. Sporangiophore erect, unbranched with occasional swelling. Hyphal swellings common, often peanut-shaped, angular, or variously distorted in shapes. Chlamydospores abundant, thin-walled, spherical and lateral produced by mature cultures av. 43.5 µm diam, sometimes on short stalks, with tapered base or clustered. Terminal chlamydospores rare.

Phytophthora virginiana is self-sterile, producing no sexual organ in single cultures. In the polycarbonate membrane tests, *P. virginiana* isolates belong to silent A^1 mating type, producing no sexual organ after up to 90-day-pairing, but stimulating A^2 mating type isolates of *P. cinnamomi* and *P. cryptogea* to produce gametangia after 30-day-pairing at 20, 25°C and room temperature (c. 23°C).

Additional isolates examined: **USA**, **Virginia**, baited with rhododendron or camellia leaves from irrigation runoff reservoirs in several perennial nurseries: June 2006, 40A9 (GenBank KC955178); November 2006, 43D6 (GenBank KC295542); May 2007, 43G2 (GenBank KC955179); May 2007, 44G6 (GenBank KC295543).

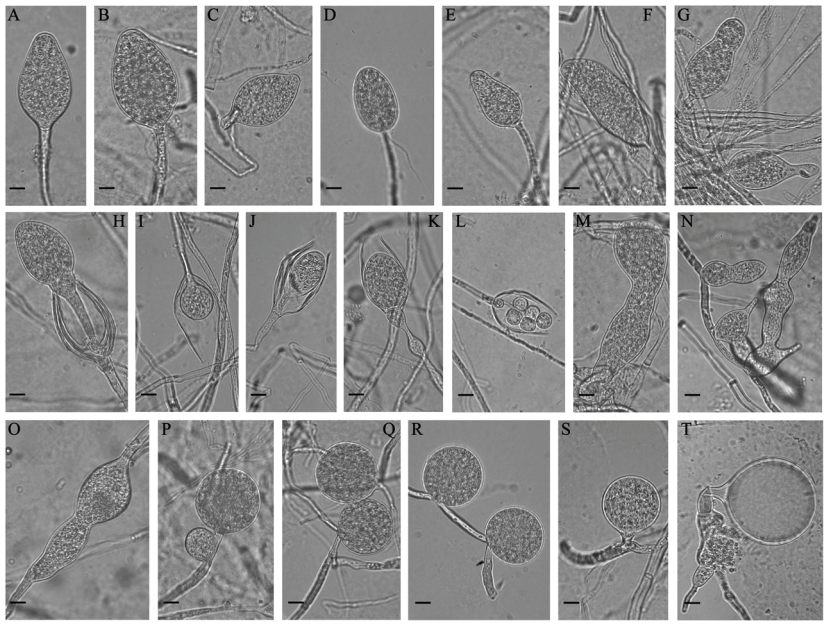

Figure 4. *Phytophthora virginiana*. Morphology of asexual structures. **A–G**: Sporangia in various shapes; A; Ovoid sporangium; B: Ovoid sporangium appearing semi-papillate just before releasing zoospores; C: Limoniform sporangium; D: Ellipsoid sporangium; E: Obpyriform sporangium; F: Distorted, peapod-shaped sporangium; G: Distorted, peanut-shaped sporangia. **H**: Extended proliferation. **I–K**: Nested proliferation with intercalary swelling on sporangiophore in K. **L**: Sporangium releasing zoospores with an incipient proliferation. **M–N**: Distorted hyphal swellings. **O**: Intercalary swelling. **P**: Lateral chlamydospore and hyphal swelling. **Q–S**: Lateral chlamydospores; S: Lateral chlamydospore with a tapered base on a short stalk. **T**: Terminal, aborted chlamydospore. Bars = 10 μm.

Discussion

Phytophthora virginiana was recovered from nursery irrigation water. This new species has unique morphological and physiological attributes as well as molecular signature, so that it can be readily distinguished from all known *Phytophthora* species. It is the only species that is heterothallic, grows well at 35°C, and produces abundant lateral chlamydospores. *Phytophthora insolita* (Ann & Ko 1980), which also grows well at 35°C and produces abundant thin-walled chlamydospores, is homothallic and produces terminal chlamydospores. *Phytophthora lateralis* (Tucker & Milbrath 1942) and *P. quininea* (Crandall 1947) also produce abundant lateral chlamydospores but are homothallic and did not sustain growth at 35°C (Tucker & Milbrath 1942, Crandall 1947). *Phytophthora quininea* also produces intercalary chlamydospores (Crandall 1947) whereas

P. virginiana does not. Among the known heterothallic, high-temperature tolerant species that grow well at 35°C, only *P. virginiana* produces abundant lateral chlamydospores. *Phytophthora melonis*, *P. hydrogena*, and *P. irrigata* do not produce chlamydospores in fresh agar media (Ho et al. 2007, Hong et al. 2008, Yang et al. 2014). Some isolates of *P. drechsleri* were reported to produce much smaller chlamydospores (diam. 7.9 µm) (Erwin & Ribeiro 1996) than those of *P. virginiana* (diam. 43.5 µm). *Phytophthora hydropathica* and some *P. parsiana* isolates frequently produce chlamydospores in fresh cultures (Hong et al. 2010, Mostowfizadeh-Ghalamfarsa et al. 2008). However, these two species produce mostly terminal chlamydospores, which are rare in *P. virginiana*. The average chlamydospore sizes of *P. hydropathica* (diam. 37 µm) and *P. parsiana* (diam. 27–37.5 µm) are also smaller than that of *P. virginiana*. Additionally, the ITS sequence of *P. virginiana* differs from its two closely related species by 26 bp (*P. hydropathica*) and 39 bp (*P. parsiana*). This sequence difference is another important criterion for separating *P. virginiana* from all other existing *Phytophthora* species.

The phylogenetic placement of *P. virginiana* provides further evidence to the argument that there is a high-temperature tolerant cluster (Fig. 1) in clade 9 of the genus *Phytophthora* (Yang et al. 2014). *Phytophthora virginiana* and all other species within this unique cluster grow well at 35°C and some survive at 40°C (Fig. 2). To date, species belonging to this cluster have been found on several continents including Europe, North America, South America, and Asia. *Phytophthora polonica* was first described in Poland (Belbahri et al. 2006). Irrigation reservoirs in Virginia contain diverse species belonging to this cluster including *P. aquimorbida* (Hong et al. 2012), *P. hydropathica* (Hong et al. 2010), *P. hydrogena* (Yang et al. 2014), *P. irrigata* (Hong et al. 2008), and this new species *P. virginiana*. *Phytophthora chrysanthemi*, *P. insolita* and *P. parsiana* were first reported from Asia (Ann & Ko 1980, Mostowfizadeh-Ghalamfarsa et al. 2008, Naher et al. 2011). Also, a provisional species belonging to this cluster, *Phytophthora* sp. "*lagoariana,*" recovered from the Amazonian rainforest, also grows well at 35°C (data not shown). The unique correlation between physiological and phylogenetic characteristics indicates that these high-temperature tolerant species may originate from tropical or subtropical environments, and increasing international trade may have contributed to current global distribution of these species.

The ecological and economic impacts caused by *P. virginiana* are not clear at this time. All isolates were recovered from irrigation water at several ornamental plant nurseries in Virginia by baiting. However, plants diseased by *P. virginiana* have not been observed at the same nurseries. This does not necessarily mean that *P. virginiana* will not cause severe damage when introduced to

new environments. Investigations into its host range and ecological roles in irrigation systems are warranted.

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