New Species and Phylogeny of the Genus *Phytophthora*

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ABSTRACT

The genus *Phytophthora* includes many agriculturally and ecologically important plant pathogens. Characterization of new *Phytophthora* species is the first and a most critical step to understanding their biology, ecology and economic importance. Six novel *Phytophthora* species recovered from irrigation systems at ornamental plant nurseries in Mississippi and Virginia were described based on morphological, physiological and molecular characters:

1. *Phytophthora mississippiae* sp. nov. produces a mix of non-papillate and semi-papillate sporangia, and catenulate hyphal swellings. It is a heterothallic species. All examined isolates of *P. mississippiae* are A^1. When paired with A^2 mating type testers, *P. mississippiae* produces ornamented oogonia and amphigynous antheridia. It is phylogenetically grouped in *Phytophthora* subclade 6b based on sequences of the rRNA internal transcribed spacer (ITS) region and the mitochondrially encoded cytochrome *c* oxidase 1 (*cox* 1) gene.
2. *Phytophthora hydrogena* sp. nov. is heterothallic. It produces non-caducous and non-papillate sporangia. It is characterized by frequently producing widening at the pedicel tip of sporangiophores or tapered sporangial based toward the point of attachment. This species is phylogenetically placed in a high-temperature tolerant cluster in *Phytophthora* clade 9. All members in this cluster grow well at 35℃.
3. *Phytophthora virginiana* sp. nov. is a self-sterile species. All examined isolates are silent A^1. It produces non-caducous and non-papillate sporangia and is also

placed in the high-temperature tolerant cluster in clade 9. Morphologically, it is characterized by producing abundant thin-walled, lateral chlamydospores in carrot agar and clarified V8 juice agar.

4. *Phytophthora macilentosa* sp. nov. is a heterothallic species. Only A^1 isolates have been found. It produces characteristic elongated, non-papillate sporangia. It is also a member of the high-temperature cluster in clade 9.
5. *Phytophthora stricta* sp. nov. is a heterothallic species. It produces unique non-papillate and slightly caducous sporangia with one to three constrictions on its sporangiophore. Phylogenetically, *P. stricta* represents a new ITS clade within the genus.
6. *Phytophthora* ×*stagnum* nothosp. nov. is a novel hybrid species with *P.* taxon PgChlamydo as its paternal parent and a *P. mississippiae*-like species as its maternal parent. This new hybrid produces intercalary chlamydospores and catenulate hyphal swellings, which are morphological characters of *P.* taxon. PgChlamydo and *P. mississippiae,* respectively. It also produces both smooth-walled and ornamented oogonia, which may be indicative of oogonial characters of its paternal and maternal parents, respectively.

By incorporating new *Phytophthora* species, clusters and clades, phylogenies including approximately 128 *Phytophthora* taxa were constructed based on sequences of five genetic markers. Among the selected genetic markers, the beta-tubulin (B-tub) gene provided the highest phylogenetic resolution. General phylogenetic structure of the B-tub phylogeny was similar to that in previous multi-locus phylogenies, except that *P.*

cinnamomi, *P. parvispora*, *P. quercina*, *P. stricta*, and a provisional species, *P.* sp. e1, were not clustered in any of the 10 known *Phytophthora* clades and represented new clades. The B-tub phylogeny was also used to study the correlations between phylogeny and morphological characteristics including sporangial papillation, caducity, homothallism, and antheridial configuration, as well as maximum growth temperature. The results indicated that the character of sporangial papillation was mostly consistent among species within individual subclades. Maximum growth temperature was also generally correlated with phylogenetic positions. Consistency in caducity, homothallism or antheridial configuration was not found. A new multi-locus phylogeny based on sequences of 11 genetic markers of more than 146 *Phytophthora* species was proposed to validate new clades and clusters, as well as investigate detailed phylogenetic relations among species in this quickly expanding, taxonomically complex group of plant pathogens.

DEDICATION

To my grandfather, Mr. Yuan.

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Chapter 1 Introduction

The Genus 'Plant Destroyer'

The genus *Phytophthora* was first described by Heinrich Anton de Bary in 1876 (Erwin & Ribeiro, 1996). "*Phytophthora*" was from the Greek word "Φυτόφθορα", which means "plant destroyer". The name evidences the destructiveness of *Phytophthora* species as plant pathogens.

Many notorious *Phytophthora* species are responsible for historical and current plant disease epidemics of agricultural, forestry and horticultural plants. *P. infestans* is the first described and type species of the genus. It causes late blight on potato, which was the major staple food in Ireland during the 1840s. When *P. infestans* attacked potatoes and caused the Great Famine in Ireland from 1845 to 1849, 1.1 to 1.5 million people died because of lacking food (Kennedy, 1999) and even more people emigrated from Ireland (Ghabhann, 1997). During World War I, another late blight-caused famine took place in Germany due to lack of copper to make copper sulfate fungicide, which was the most effective fungicide to control *P. infestans* at that time. The famine led to 70 thousand deaths (Carefoot & Sprott, 1969). Although it has been 160 years after its damage was first noticed, late blight is still the most important disease of potato. It causes yield losses of 16% of the world potato production, which are estimated at more than $5 billion per year (Haverkort *et al.*, 2009). *Phytophthora ramorum* causes sudden oak death (SOD), which has killed millions of trees in the forests of California and Oregon since the early

1990s. It also causes ramorum blight on hundreds of under-story shrubs and ornamental plant species (Werres *et al.*, 2001; Goheen *et al.*, 2002; Rizzo *et al.*, 2002; Rizzo *et al.*, 2005). The SOD disaster prompted the quarantine of *P. ramorum* to prevent its spread (USDA-APHIS-PPQ, 2002). Another example of notorious *Phytophthora* pathogens is *P. cinnamomi*. It causes Phytophthora root rot on chestnut trees, which was responsible in concert with the chestnut blight for the near-extinction of wild American chestnut trees in the eastern United States (Zentmyer, 1980; Freinkel, 2007). Additionally, it affects a wide range of woody ornamentals such as azalea, camellia, and boxwood and has been named as one of the 100 world worst invasive species (Lowe *et al.*, 2000).

Phytophthora Biology

Phytophthora species produce various asexual and sexual structures for different biological and ecological functions. *Phytophthora* species grow as filamentous hyphal cells which are similar to most fungi. However, *Phytophthora* species produces coenocytic, aseptate hyphae which usually contain diploid nuclei except a transient haploid phase prior to fertilization (Dick, 1990), while fungi are haploid or dikaryotic. The cell walls of *Phytophthora* species are composed of β-glucans and cellulose, while those of fungi contain chitin (Erwin & Ribeiro, 1996; Rossman, 2006; Fry & Grünwald, 2010). Specialized hyphae including appressoria and haustoria are important for penetrating plant cells, infecting and colonizing plant hosts.

Sexual organs called gametangia are the key structures of *Phytophthora* species in evolutionary processes such as speciation and adaptation to novel ecosystems, by producing hybrids and polyploids (Brasier & Hansen, 1992; Bertier *et al.*, 2013). Although they have never been observed for some *Phytophthora* species such as *P. fluvialis*, *P. gemini*, and *P. macrochlamydospora*, most species produce gametangia in their sexual stage. A gametangium is either the female structure oogonium or the male structure antheridium. *Phytophthora* species are usually classified into homothallic and heterothallic species. Homothallic species can produce gametangia in single culture when conditions are suitable, while heterothallic species require both mating type strains, designated as A^1 and A^2, to produce gametangia (Erwin & Ribeiro, 1996). Morphological characters of these sexual organs are important traits for species identification and taxonomy (Waterhouse, 1963; Gallegly & Hong, 2008).

The major infective structures of *Phytophthora* species are zoospores. They are wall-less, motile, asexual spores released by sporangia. Each zoospore has two heterokont flagella for swimming: a long whiplash flagellum producing major motive force and a short tinsel flagellum assisting direction (Blackwell, 1944; Erwin & Ribeiro, 1996). Zoospores can remain motile for hours, then stop swimming and encyst through discarding flagella and developing cell walls (Bimpong & Clerk, 1970; Erwin & Ribeiro, 1996). Encysted zoospores can survive for hours to two weeks depending on environmental conditions (Kong *et al.*, 2012). Sporangia are important asexual reproductive structures of *Phytophthora* species. In addition to producing and releasing zoospores, they can germinate directly to produce germ tubes and then infect plant hosts. Sporangia are of

diverse morphologies. They differ in papillation, caducity, proliferation structure, as well as size and shape. These sporangial characteristics are important morphological traits for characterizing and identifying *Phytophthora* species (Waterhouse, 1963; Gallegly & Hong, 2008). Also of importance for characterizing *Phytophthora* species is the presence or absence, and morphology of chlamydospores. They are various in size and shape as well as wall-thickness. Chlamydospores are resting structures which can survive for long periods in soil (Erwin & Ribeiro, 1996; Fry & Grünwald, 2010). They are important survival structures of many plant pathogens, such as *P. cinnamomi* and *P. ramorum*, which threaten container-grown plants.

Description of Novel *Phytophthora* Species

The number of formal *Phytophthora* species has reached approximately 140 since *P. infestans* was described in 1876. Before 2000, description of new *Phytophthora* species was mainly based on morphological characters and sometimes on additional physiological and host specificity features. Approximately 60 species were described from the 1870s to the 1990s (Erwin & Ribeiro, 1996). After 2000, molecular technologies, such as sequencing barcoding genetic markers: the internal transcribed spacer (ITS) region and mitochondrially encoded cytochrome *c* oxidase 1 (*cox* 1) gene have changed the species concept of *Phytophthora* from being morphology- to phylogeny-based. Also, the SOD disaster has reminded us of the importance of early detection and invigorated surveys of *Phytophthora* species in previously unexplored ecosystems. Since then, 38

novel *Phytophthora* species have been described during the 2000s and additional 42 have been described between 2010 to present.

Aquatic *Phytophthora* Species in Irrigation Systems

It is interesting that most species of the genus *Phytophthora*, which have been grouped into water molds for 70 years (Blackwell, 1944), were first noted from diseased plants or terrestrial substrates instead of aquatic environments. Before 2000, only a couple of *Phytophthora* species were first detected from aquatic ecosystems (Hong *et al.*, 2008). *Phytophthora gonapodyides* is now recognized as a resident aquatic species often recovered from water. It was described by examining isolates recovered from a decaying apple in a reservoir (Petersen, 1910). Also, the description of *P. insolita* included a few isolates recovered from ditch water and soil slurries (Ann & Ko, 1980). Since extensive surveys have been exploring *Phytophthora* diversity in aquatic ecosystems, more new species have been recovered and described from aquatic habitats. For example, *P. amnicola*, *P. borealis*, *P. fluvialis*, *P. riparia*, and *P. siskiyouensis* were all described based on aquatic isolates recovered from rivers and creeks (Reeser *et al.*, 2007; Crous *et al.*, 2011; Crous *et al.*, 2012; Hansen *et al.*, 2012).

Recent studies have detected a wide diversity of *Phytophthora* species in irrigation systems (Themann *et al.*, 2002; Bush *et al.*, 2003; Hong & Moorman, 2005; Werres *et al.*, 2007; Ghimire *et al.*, 2011; Stewart-Wade, 2011; Yang *et al.*, 2012; Loyd *et al.*, 2014; Parke *et al.*, 2014; Zappia *et al.*, 2014). Many of them are plant pathogens, such as *P.*

ramorum (Werres *et al.*, 2007), *P. nicotianae* (Thomson & Allen, 1976), and *P. capsici* (Roberts *et al.*, 2005). In Virginia alone, at least 25 well-known *Phytophthora* species have been recovered from irrigation systems (unpublished data). If *Phytophthora*-contaminated water is used for irrigation, it is very likely that these plant pathogens cause plant health issues and threaten crop production. Besides the well-known species, a number of *Phytophthora* isolates representing provisional and previously unknown species have been recovered from irrigation systems. For example, three novel species and one new taxon of *Phytophthora*, namely *P. irrigata* (Hong *et al.*, 2008), *P. hydropathica* (Hong *et al.*, 2010), *P. aquimorbida* and *P.* taxon aquatilis (Hong *et al.*, 2012) have been recovered from irrigation systems and described recently. In contrast to well-known plant pathogens, new species lack characterizations in biology, physiology, phylogeny, and pathogenicity. The knowledge gap also makes it impossible to develop sound management measures for growers. Obviously, characterization of new *Phytophthora* species is the first and a most critical step to understanding their biology, ecology and economic importance. It is also critical to first responders in accurately diagnosing the diseases caused by these new species and deliver correct management recommendations.

Phylogeny of the Genus *Phytophthora*

Before molecular technologies were adapted for classifying *Phytophthora* species, traditional taxonomy of *Phytophthora* was mainly based on morphological characters. The most recognized and widely used morphology key was developed by Waterhouse

(1963). She divided *Phytophthora* species into six groups based on variations in morphological traits including sporangial papillation, caducity, homothallism and antheridial configuration.

When DNA sequencing data started becoming available, the genus *Phytophthora* became phylogenetically classified into 10 clades based on a series of sequence analyses (Cooke *et al.*, 2000; Kroon *et al.*, 2004; Blair *et al.*, 2008; Martin *et al.*, 2014). In 2000, Cooke *et al.* developed a phylogeny based on ITS sequences (Cooke *et al.*, 2000). Kroon *et al.* constructed a phylogeny in 2004 based on sequences of four nuclear and mitochondrial genes (Kroon *et al.*, 2004). In 2008, Blair *et al.* constructed a phylogeny based on sequences of seven nuclear genetic markers. This multi-locus phylogeny divided 82 *Phytophthora* species into 10 phylogenetic clades (Blair *et al.*, 2008). In 2014, Martin *et al.* constructed another multi-locus phylogeny by combining sequences of the seven nuclear genes with those of four additional mitochondrial genes (Martin *et al.*, 2014). Phylogenetic analyses have greatly advanced the taxonomy of *Phytophthora* species. However, they also have a few problems. First, there is no phylogeny which included vast majority of *Phytophthora* species. Although the most recent phylogeny included 90 formal and 17 provisional species (Martin *et al.*, 2014), at least 30 formal and provisional species were not included. And this problem becomes more important when increasingly more novel species have been described every year and continuously challenge the current system. Second, although the general phylogenetic structures of different analyses were similar, a variety of individual genes or combinations of genetic markers were used by different studies and variations have been found among the results. Third, although the

correlation between morphology and phylogeny for the genus *Phytophthora* has been studied (Ersek & Ribeiro, 2010; Martin *et al.*, 2012), the exact relationship between traditional and modern phylogeny-based taxonomies is not yet clear.

Research Objectives

Isolates of three previously unknown *Phytophthora* species were recovered in 2006 or 2007 from irrigation systems in ornamental plant nurseries in the eastern Virginia. Three other novel species were recovered in 2012 from irrigation reservoirs in a nursery in Mississippi. The objectives of this research were 1) to characterize these novel *Phytophthora* species by describing their morphological, physiological and molecular features; 2) to compare five genetic markers and select the most phylogenetically informative genetic markers; 3) to construct a phylogeny of the most informative genetic marker and use the phylogeny to study the correlation between phylogeny and morphology of *Phytophthora* species.

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Chapter 2 *Phytophthora mississippiae* sp. nov., a new species recovered from irrigation reservoirs at a plant nursery in Mississippi

Yang X, Copes WE & Hong CX (2013) *Phytophthora mississippiae* sp. nov., a new species recovered from irrigation reservoirs at a plant nursery in Mississippi. *Journal of Plant Pathology Microbiology* **4**: 180. http://dx.doi.org/10.4172/2157-7471.1000180

Abstract

A previously unknown *Phytophthora* species was recovered from irrigation water in Mississippi. This novel species produced both nonpapillate and semipapillate sporangia, and catenulate hyphal swellings. All examined isolates were compatibility type A^1. Ornamented oogonia with amphigynous antheridia and plerotic oospores were produced when this novel species was paired with A^2 mating type testers of *P. cryptogea* and *P. nicotianae* in polycarbonate membrane tests. Sequence analyses of the rDNA internal transcribed spacer (ITS) region and the mitochondrially encoded cytochrome *c* oxidase 1 (*cox* 1) gene placed this species in clade 6 of the genus *Phytophthora*. Based on the morphological, physiological and molecular features, this new species is named as *Phytophthora mississippiae* sp. nov. The implications of these results are discussed.

Introduction

The genus of *Phytophthora* includes a group of destructive plant pathogens (Erwin & Ribeiro, 1996). This genus was divided into 10 clades following phylogenetic analyses (Cooke *et al.*, 2000; Kroon *et al.*, 2004; Blair *et al.*, 2008; Martin *et al.*, 2014). Among the 10 clades designated by previous phylogenetic analyses, clade 6 has a strong association with forest and riparian environments (Jung *et al.*, 2011; Kroon *et al.*, 2012). It currently consists of 18 formal species. All were described after the year 2000, except for *P. gonapodyides* (Buisman, 1927), *P. humicola* (Ko & Ann, 1985) and *P. megasperma* (Drechsler, 1931). Clade 6 also includes a number of provisional species such as *P.* taxon Forestsoil, *P.* taxon Oaksoil, and *P.* taxon Pgchlamydo (Brasier *et al.*, 2003), as well as many other undescribed taxa.

Several factors have contributed to the recent increase in the number of species in clade 6. First, advancements in molecular biology and sequence analysis provide viable alternatives to morphospecies concepts used in traditional taxonomic systems such as the taxonomy key of *Phytophthora* species developed by Waterhouse (Waterhouse, 1963). Accompanying these advancements is identification of definitive characters and phylogenetic analysis tools that have greatly facilitated re-examination of *Phytophthora* collections and description of new species. For example, *P. rosacearum* and *P. sansomeana* were recently separated from the '*P. megasperma* complex' after sequence analyses (Hansen *et al.*, 2009). *Phytophthora* sp. O-group isolated in the 1990s was formally named as *P. inundata* (Brasier *et al.*, 2003). *Phytophthora* taxon Salixsoil first

isolated in the 1970s was assigned as *P. lacustris* (Nechwatal *et al.*, 2013). Many newly isolated species in clade 6 such as *P. bilorbang* (Aghighi *et al.*, 2012) and *P. gemini* (Man in't Veld *et al.*, 2011) were also described by taking advantage of phylogenetic analysis. Second, recent occurrence of sudden oak death (SOD) caused by *P. ramorum* in the United States (Goheen *et al.*, 2002; Rizzo *et al.*, 2002) and forest declines caused by several *Phytophthora* species in other countries (Shearer *et al.*, 2004; Hansen, 2008) has motivated global surveys of natural habitats and waterways for these pathogens. These surveys done in natural environments recovered a number of new species such as *P. borealis* and *P. riparia* (Hansen *et al.*, 2012), plus other taxa that belong to clade 6. Third, parallel surveys of irrigation systems have been greatly intensified to address growing concerns over the increasing *Phytophthora* disease risk as agricultural industries increasingly use recycled water in the light of global water scarcity (Bouwer, 2002; Hong & Moorman, 2005). The surveys in irrigation systems also recovered a number of novel *Phytophthora* species (Hong *et al.*, 2008; Hong *et al.*, 2010; Hong *et al.*, 2012) and many new taxa in clade 6.

The objective of this study was to characterize and describe a group of isolates belonging to a previously unknown *Phytophthora* species recovered from irrigation reservoirs in Mississippi. We describe the morphological, physiological and molecular characters of this new taxon and formally name it as *Phytophthora mississippiae* sp. nov.

Materials and Methods

Isolation and isolate maintenance

The origin of four *Phytophthora mississippiae* isolates examined in this study is shown in table 2.1. They were recovered from the surface, middle, or bottom of water columns in irrigation reservoirs at an ornamental plant nursery of Mississippi in 2012 by baiting with rhododendron leaves (Bush *et al.*, 2003; Ghimire *et al.*, 2011). These baits were deployed in the surveyed reservoirs for seven days then transferred to a laboratory. They were then cut into approximately 1×1 cm^2 sections and plated onto PARP selective media (contains pimarcin, ampicillin, rifampicin, and pentachloronitrobenzene). *Phytophthora* colonies emerging from the edge of baits were hyphal-tipped onto 20% clarified V8 juice agar (CV8A) to obtain pure cultures (Erwin & Ribeiro, 1996). Cultures were maintained on CV8A and blocks of fresh agar cultures were transferred into microtubes with sterile distilled water for long-term storage at 15℃. The holotype isolate MYA-4946 was deposited at the American Type Culture Collection in Manassas, Virginia, USA.

Colony morphology and cardinal temperatures

Ten-day-old colony morphology of the four isolates of *P. mississippiae* on carrot agar (CA), CV8A, malt extract agar (MEA), and potato dextrose agar (PDA) grown at 20℃ in the dark was noted and photographed.

Cardinal temperatures of the four isolates were assessed on CV8A and CA. Agar blocks (5 mm in diameter) taken from actively growing areas of 7-day-old cultures were placed on fresh media at the center of 10-cm Petri dishes. Triplicate Petri dishes per isolate per temperature were placed in the dark at 5, 10, 15, 20, 25, 30, 35, and 40℃. Two perpendicular measurements of each colony were taken after eight days. This test was repeated. Following the analysis of variance using R statistical software *v*. 2.15.0 (R Core Team, 2012), data from repeating tests were pooled together. Radial growths along with their standard errors were plotted against temperature using the gplots package *v*. 2.11.0 (Warnes *et al.*, 2012) in R.

Morphology of sporangia and gametangia

Sporangia were produced by transferring agar plugs (10×10 mm^2) from actively growing area of 10-day-old cultures on CV8A to Petri dishes containing non-sterile, 1.5% soil water extract solution (SWE, 15 g of sandy loam soil/1 L distilled water) or filtered, non-sterile pond water. Mature sporangia developed after incubating at room temperature (*c*. 23℃) under cool-white fluorescent light.

Mating type of these isolates was determined by placing each with an A^1 or A^2 mating type tester of *P. cinnamomi* in dual cultures on hemp seed agar (HSA). Selfed sexual structures were produced at room temperature using the polycarbonate membrane method to physically separate *P. mississippiae* isolates from their reverse mating type testers (Ko,

1978; Gallegly & Hong, 2008). Several heterothallic species including *P. cinnamomi*, *P. cambivora*, *P. meadii*, *P. nicotianae*, and *P. cryptogea* were used as mating type testers.

Sporangia and gametangia were photographed with a Nikon Fujix Digital Camera HC-300Zi connected to a Nikon Labophot-2 microscope. Fifty randomly selected mature sporangia were measured for length and width while 30 gametangia were measured for the size of oogonia, oospores, and antheridia with Image-Pro® Plus *v.* 5.1.2.53.

DNA extraction, amplification and sequencing

Isolates were grown in 20% V8 juice broth at room temperature for one week. Mycelial masses were harvested and lysed using a FastPrep®-24 system (MP Biomedicals, Santa Ana, CA). DNA was extracted as instructed using the DNeasy® Plant Mini kit (Qiagen, Valencia, CA). Amplifications were performed with forward primer ITS6 and reverse primer ITS4 (Cooke *et al.*, 2000) for the internal transcribed spacer (ITS) region covering ITS1, 5.8S rRNA gene, and ITS2, following previously described reaction mix recipe and PCR program (Kong *et al.*, 2003). Primers COXF4N and COXR4N were used to amplify the mitochondrial cytochrome *c* oxidase 1 (*cox* 1) gene (Kroon *et al.*, 2004). Sequencing was performed in both directions at the University of Kentucky Advanced Genetic Technologies Center (Lexington, KY) using the same primers. Sequences of both directions were visualized with Finch TV *v.* 1.4.0. and aligned using Clustal W.

Sequence analyses

Sequences generated in this study were compared with those of all other species in the same clade and species representing other clades (Table 2.1). Sequences were aligned using Clustal W. Phylogeny reconstruction was conducted in MEGA 5.1 (Tamura *et al.*, 2011) using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993) with 1,000 replications of bootstrap.

Results

Colony morphology

The four isolates of *P. mississippiae* had a similar growth pattern at 20℃ on each medium (Figure 2.1). Colonies had none to sparse aerial mycelia and grew at the greatest rates on CA. The colony pattern on CA was radiate with a smooth edge. Isolates 57J1, 57J3 and 57J4 had a moderate growth rate on CV8A while isolate 57J2 had a slow growth rate. All isolates produced hispid aerial mycelia. Colony pattern on CV8A was radiate to slightly petaloid with a relatively smooth edge. On MEA, all isolates had limited but discernible growth with irregular colony patterns. Isolates had a moderate growth rate on PDA and produced tomentose aerial mycelia. Colony pattern on PDA was petaloid (isolates 57J1, 57J2 and 57J3) to slight cottony (isolate 57J4).

Cardinal temperatures for vegetative growth

Radial growth rates were different among isolates ($P < 0.01$) but not between repeating experiments ($P = 0.17$). Isolates 57J1, 57J3, and 57J4 had similar growth rates (Figure 2.2A). Isolate 57J2 grew more slowly than the other isolates at 10 to 30℃ on both CV8A and CA (Figure 2.2B). The optimum temperature for the growth of *P. mississippiae* was 25℃ on CA and 30℃ on CV8A. Limited but notable growth was observed at 5 and 35℃. No growth occurred at 40℃. After the experiments were completed, cultures from all temperatures were relocated to room temperature. Additional growth occurred on plates previously maintained at 5℃ but not those maintained at 40℃.

Sequence analyses and phylogenetic position

GenBank accession numbers of sequences generated in this study and used in the sequence analyses are shown in table 2.1. All isolates of *P. mississippiae* have 818-bp ITS sequences. Isolates 57J1 and 57J2 have an identical ITS sequence, while 57J3 and 57J4 have an identical ITS sequence (Table 2.2). These two subgroups differ by 3 bp. These ITS sequences of *P. mississippiae* were distinct from those of all known *Phytophthora* species. Two species with most similar ITS sequences are *P. borealis* and *P.* gonapodyides. *P. mississippiae* differs from *P. borealis* (GenBank accession no. HM004232) and *P. gonapodyides* (GenBank accession no. KF112854) in the ITS sequence by 7 and 8 bp, respectively (Table 2.2).

The four *P. mississippiae* isolates have an identical 867-bp *cox* 1 sequence, which also is distinct from those of all known species. The *cox* 1 sequence of *P. mississippiae* differs from two proximal sequences, those of *P. thermophila* (GenBank accession no. HQ012872) and *P. borealis* (GenBank accession no. JQ626625) by 30 and 31 bp, respectively.

Sequence analyses of both ITS and *cox* 1 sequences placed *P. mississippiae* in clade 6 of the genus *Phytophthora* (Blair *et al.*, 2008; Jung *et al.*, 2011). The four *P. mississippiae* isolates form a distinct taxon in the phylogenetic trees based on ITS (Figure 2.3) and *cox* 1 (Figure 2.4) sequences.

Taxonomy

Phytophthora mississippiae X. Yang, W. E. Copes, and C. X. Hong., sp. nov. (Figures 2.1, 2.5, 2.6)

MycoBank MB804659

Phytophthora mississippiae produced abundant sporangia in 1.5% SWE after 15 hours under light. Sporangia were mostly ovoid to obpyriform (Figure 2.5A-D, F-G, H, J). It occasionally produced slight ellipsoid sporangia (Figure 2.5E, I). Sporangia were non-caducous, mostly non-papillate (Figure 2.5A-G) and sometimes semi-papillate (Figure 2.5H-J). Primary sporangia were terminal and averaged 60.5 µm in length and 31.7 µm in

width. Secondary lateral sporangia were observed on the mycelial plug after submersion in SWE for more than 40 hours. Nested and extended internal proliferation was common (Figure 2.5L, M). Sporangial characteristics among four *P. mississippiae* isolates are summarized in table 2.3. Mycelia were flat (Figure 2.5N), coiled (Figure 2.5O), or swollen (Figure 2.5P). Hyphal swellings were commonly elongated with irregular shapes, especially in aged cultures (> 30-day-old). Catenulate, globose hyphal swellings were frequently observed in both fresh and aged cultures (Figure 2.5Q). Chlamydospores were not observed.

Phytophthora mississippiae is self-sterile. Gametangia were produced in dual cultures where *P. mississippiae* isolates were paired with an A^2 mating type tester of *P. cinnamomi* suggesting that all four isolates examined in this study are A^1. In the polycarbonate membrane test, gametangia were produced by isolates 57J3 and 57J4 after 50-day-breeding when paired with A^2 mating type testers of *P. cryptogea* (Figure 2.6A, B) and *P. nicotianae* (Figure 2.6C, D). Oogonia had characteristic ornamented protuberances on the surface and oogonial wall was pigmented to golden-brown with maturation (Figure 2.6A-D). Many oogonia had a tapered base (Figure 2.6A, B, D). Oogonial diameter averaged 38.2 µm. Plerotic oospores averaged 34 µm in diameter (Figure 2.6A-D). All antheridia were amphigynous (Figure 2.6A-D) and averaged 19.5 µm depth and 14.3 µm width. Sometimes bi-cellular antheridia were produced (Figure 2.6A).

Holotype

ATCC MYA-4946 (exo-type: 57J3) from irrigation water of a nursery reservoir, Mississippi, USA, February, 2012

Etymology

'*mississippiae*' refers to the state of Mississippi where this new species was isolated.

Habitat

Irrigation water of an ornamental plant nursery, Mississippi, USA.

Discussion

This study characterized a novel species of *Phytophthora* morphologically, physiologically and phylogenetically then named it as *P. mississippiae*. This is the first and critical step to understanding the biology, ecology and economic significance of any novel species. The description of *P. mississippiae* will help the first responders in diagnosing the disease caused by this new species. It also will reduce misidentification of high-impact pathogens like *P. ramorum* (Rizzo *et al.*, 2002) and *P. kernoviae* (Brasier *et al.*, 2005).

Phytophthora mississippiae can be readily distinguished from all known *Phytophthora* species by its morphological and molecular characters. Within the genus *Phytophthora*, only five species, *P. alni* (Brasier *et al.*, 2004), *P. cambivora* (Buisman, 1927), *P. gibbosa* (Jung *et al.*, 2011), *P. katsurae* (Ko & Chang, 1979), and this new species, *P. mississippiae* produce ornamented oogonia with bullate protuberances. *P. mississippiae* is easily separated from three homothallic species, *P. alni*, *P. gibbosa* and *P. katsurae* (Ko & Chang, 1979; Brasier *et al.*, 2004; Gallegly & Hong, 2008; Jung *et al.*, 2011) by its heterothallism. Both *P. cambivora* and *P. mississippiae* are heterothallic, but they can be separated by the papillation of sporangia and presence of hyphal swellings. *Phytophthora mississippiae* produces both nonpapillate and semipapillate sporangia, while *P. cambivora* produces only nonpapillate sporangia (Gallegly & Hong, 2008). *Phytophthora mississippiae* also frequently produces catenulate hyphal swellings, while *P. cambivora* typically does not. Similarly, *P. mississippiae* can be easily differentiated from other clade 6 species including *P. borealis*, *P. thermophila*, and *P. gonapodyides* in *cox* 1 sequences (> 30 bp difference).

Like many other species in clade 6, the economic importance of *P. mississippiae* is not known at this point. Many clade-6 species are abundant in natural habitats and frequently recovered from natural water and soil environments, but usually do not cause apparent disease symptoms on plants (Kroon *et al.*, 2012). Only a few clade-6 species have been found to cause diseases on agricultural and horticultural plants (Jung *et al.*, 2011). Examples include *P. asparagi* which causes root rot of asparagus (Saude *et al.*, 2008) and *P. megasperma* which causes crown rot of hollyhock (Drechsler, 1931). A saprophytic

lifestyle for many species in this clade may play an important role in decomposing plant debris (Kroon *et al.*, 2012). Unlike many other clade-6 species initially detected in natural environments, *P. mississippiae* was first recovered from irrigation water in a plant production facility. It is possible that *P. mississippiae* was carried into agricultural irrigation water systems from surrounding habitats through flash flood runoff that occurred in this area during heavy rains. This hypothesis is supported by the fact that this new species was found only in Mississippi but not in any of nursery irrigation systems surveyed in Virginia during the past 14 years and in Alabama during the past two years. Nevertheless, investigations into its origin, pathogenicity and host range are warranted.

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Table 2.1 Origin and GenBank accession numbers of *Phytophthora mississippiae* isolates and reference species

Species	ITS clade	Isolate	Location	Substrate	Date	GenBank accession no.	
						ITS	cox 1
Phytophthora mississippiae	6	57J1	Mississippi, USA	Irrigation water	2012	KF112850	KF112858
		57J2	Mississippi, USA	Irrigation water	2012	KF112851	KF112859
		57J3 [T]	Mississippi, USA	Irrigation water	2012	KF112852	KF112860
		57J4	Mississippi, USA	Irrigation water	2012	KF112853	KF112861
P. amnicola	6	DH228	Australia	Still water	2009	JQ029956	JQ029948
P. asparagi	6	SP326	Michigan, USA	*Asparagus officinalis*	2008	EF185089	n/a [*]
		CBS121536	The Netherlands	*Asparagus officinalis*	n/a	n/a	JX524163
P. bilorbang	6	CBS161653	Australia	Rhizosphere soil of dying *Rubus* sp.	2012	JQ256377	JQ256375
P. borealis	6	AKWA58.1-0708	Alaska, USA	Creek water	2012	HM004232	JQ626625
P. fluvialis	6	MURU 468	Australia	River water	2009	JF701436	JF701442
P. gemini	6	CBS123381	The Netherlands	*Zostera marina*	1998	FJ217680	JX262931
P. gibbosa	6	CBS127951	Australia	Root soil of dying *Acacia pycnantha*	2009	HQ012933	HQ012846
P. gonapodyides	6	34A8, CBS55467	United Kingdom	Fruit bait	1967	KF112854	KC733448
P. gregata	6	CBS127952	Australia	Root soil of dying *Patersonia* sp.	2009	HQ012942	HQ012858
P. humicola	6	32F8, P3826	Taiwan	Soil slurries	1977	KF112855	KF112862
P. inundata	6	30J3, P894	Spain	*Olea* roots	1966	KF112856	KF112863
P. lacustris	6	P245	United Kingdom	*Salix matsudana*	1972	AF266793	JF896561
P. litoralis	6	CBS127953	Australia	Root soil of dying *Banksia* sp.	2008	HQ012948	HQ012866
P. megasperma	6	CBS40272	Washington, D.C. , USA	*Althaea rosea*	1931	HQ643275	n/a
		IMI133317	Australia	n/a	1968	n/a	AY564194
P. pinifolia	6	CMW26668	Chile	*Pinus radiata*	2007	EU725806	JN935961
P. riparia	6	3-100B9F	Oregon, USA	Creek water	2006	HM004225	n/a
P. rosacearum	6	22J9, OSU 62	California, USA	Cherry	n/a	KF112857	KF112864
P. thermophila	6	CBS127954	Australia	Root soil of dying *Eucalyptus* sp.	2004	EU301155	HQ012872
P. infestans	1	27A8, KDT-2C	Mexico	*Solanum tuberosum*	1992	KC733443	KC733447
P. meadii	2	CBS21988	India	*Hevea brasiliensis*	1987	HQ643268	AY564192
P. sojae	7	28F9, P6497	Mississippi, USA	*Glycine max*	1974	KC733444	AY564162
P. lateralis	8	IMI040503, CBS16842	Oregon, USA	*Chamaecyparis lawsoniana*	1942	AF266804	AY564191
P. aquimorbida	9	40A6	Virginia, USA	irrigation reservoir	2006	FJ666127	GQ294536
P. macrochlamydospora	9	33E1, P10264	Australia	*Glycine max*	2003	KC733445	KC733454
Pythium aphanidermatum	*Pythium*	P1779	n/a	n/a	n/a	GU983641	n/a
		P2	n/a	n/a	n/a	n/a	AY564163

[T] exo-type

[*] n/a = not available

Table 2.2 Polymorphic nucleotides in the sequences of internal transcribed spacer region (ITS) among *Phytophthora mississippiae* isolates and the type isolates of *P. borealis* and *P. gonapodyides*. Compared to the sequence of *P. mississippiae* type isolate 57J3, the differential nucleotides of isolates 57J1 and 57J2, *P. borealis*, and *P. gonapodyides* are shaded.

Isolate	ITS1						ITS2									
	15	43	44	98	106	173	469	517	553	582	588	608	652	740	744	787
Phytophthora mississippiae																
57J1, 57J2	A	T	A	C	A	C	C	T	C	G	G	C	T	T	G	-
57J3, 57J4	A	T	A	C	G	T	T	T	C	G	G	C	T	T	G	-
Phytophthora borealis																
AKWA58.1-0708	-	T	A	Y	G	C	T	T	C	S	G	Y	G	G	G	-
Phytophthora gonapodyides																
34A8	A	A	T	C	G	T	T	G	T	G	A	C	G	T	A	T

Table 2.3 Morphological variations of sporangial characters among isolates of *Phytophthora mississippiae* in this study

Character	Isolate				Species average
	57J1	57J2	57J3	57J4	
Shape (%)					
ovoid to obpyriform	86	93	96	90	92
slightly ellipsoid	12	7	4	2	6
distorted	1	-	-	8	2
Papilla (%)					
nonpapillate	66	70	53	69	64
semipapillate	33	30	47	31	36
Size range (µm)	47.3-70.0 x 21.1-43.3	48.8-64.3 x 26.8-38.6	55.4-71.9 x 28.6-41.9	47.3-77.3 x 20.4-37.1	47.3-77.3 x 20.4-43.3
Average size (µm)	61.5±7.2 x 34.0±3.5	57.4±3.9 x 31.1±2.5	62.6±3.8 x 34.9±3.2	60.3±7.2 x 26.8±3.4	60.4±6.0 x 31.3±4.5
L:W ratio	1.82	1.85	1.80	2.27	1.96

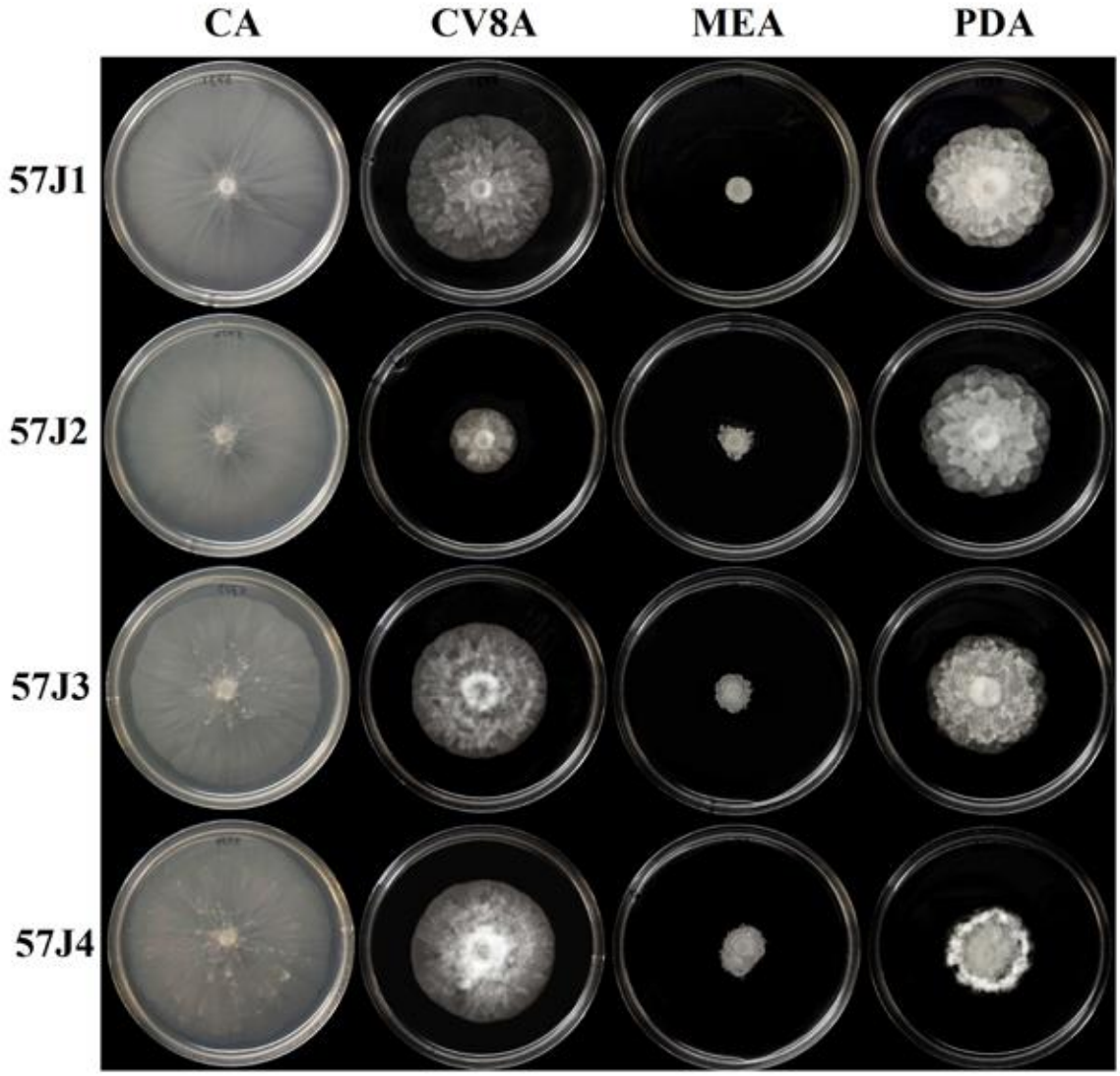

Figure 2.1 Colony morphology of *Phytophthora mississippiae* isolates on various media incubated at 20℃ for 10 days in the dark: CA = carrot agar; CV8A = 20% clarified V8 juice agar; MEA = malt extract agar; PDA = potato dextrose agar

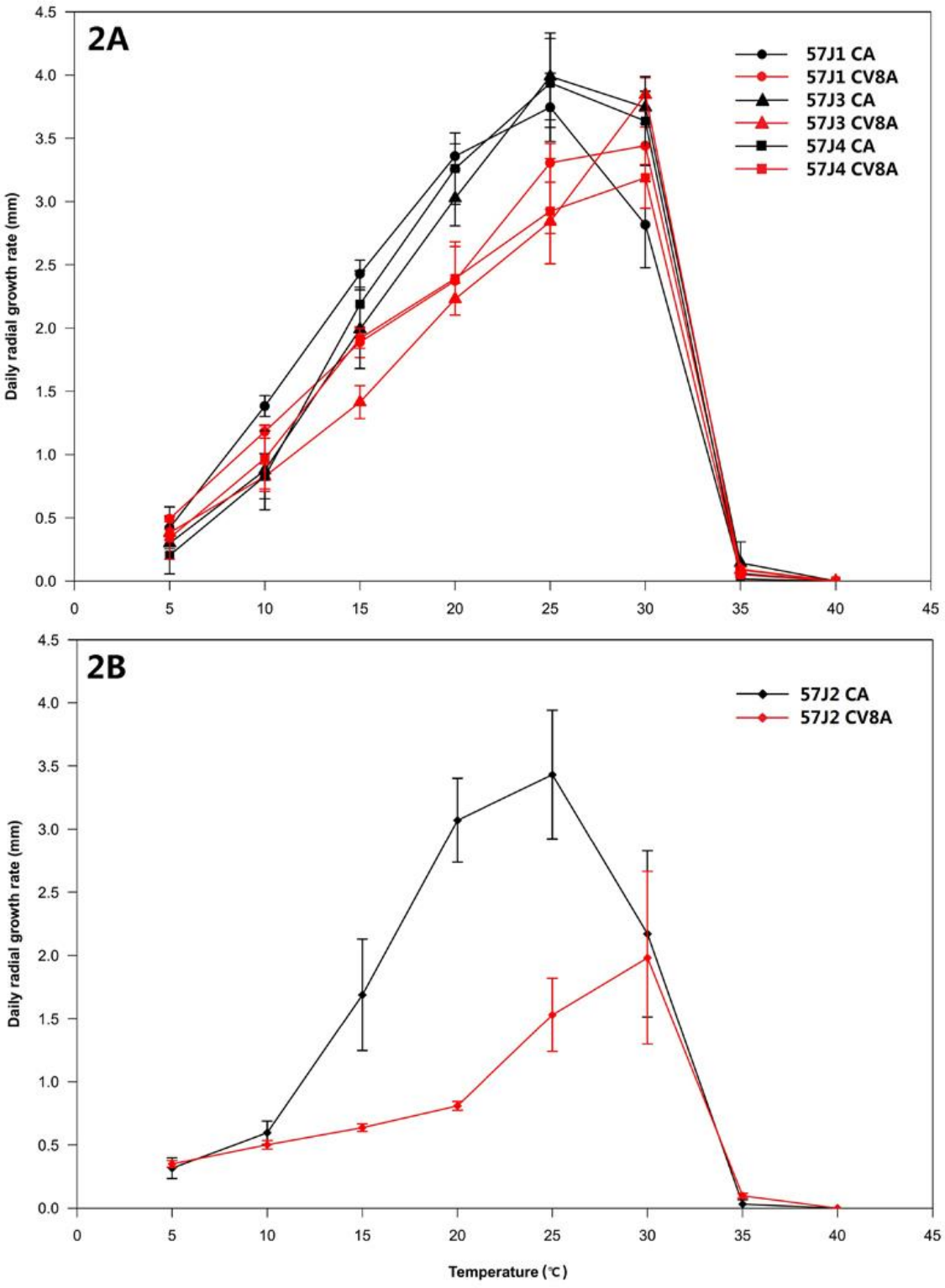

Figure 2.2 Differential radial growth of *Phytophthora mississippiae* isolates on carrot agar (CA) and 20% clarified V8 juice agar (CV8A): A. Daily radial growth rates of isolates 57J1, 57J3, and 57J4; B. 57J2

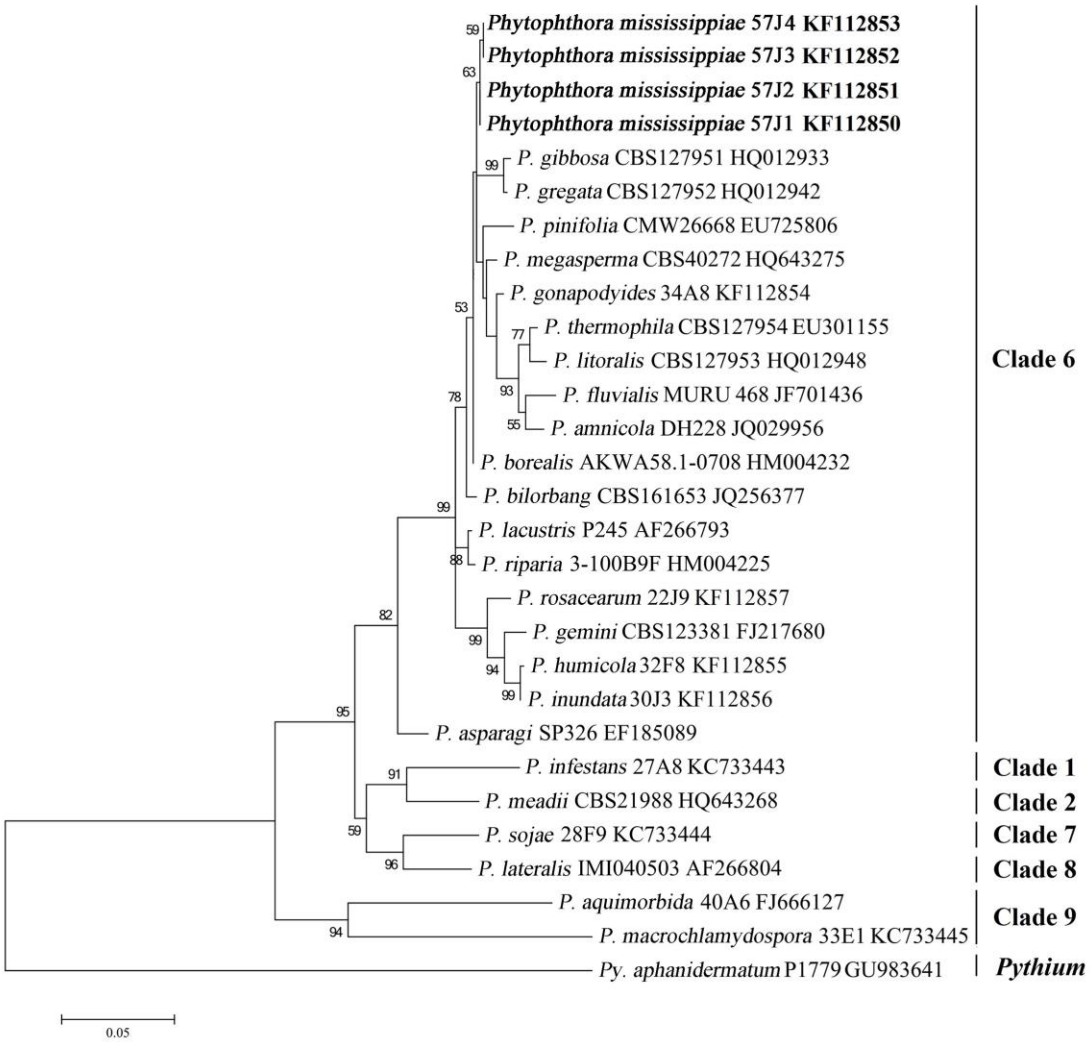

Figure 2.3 A maximum likelihood phylogenetic tree generated in MEGA 5.1, based on the alignment of ITS sequences with Clustal W. The numbers on branches are bootstrap values (1,000 replicates; values less than 50% are not shown).

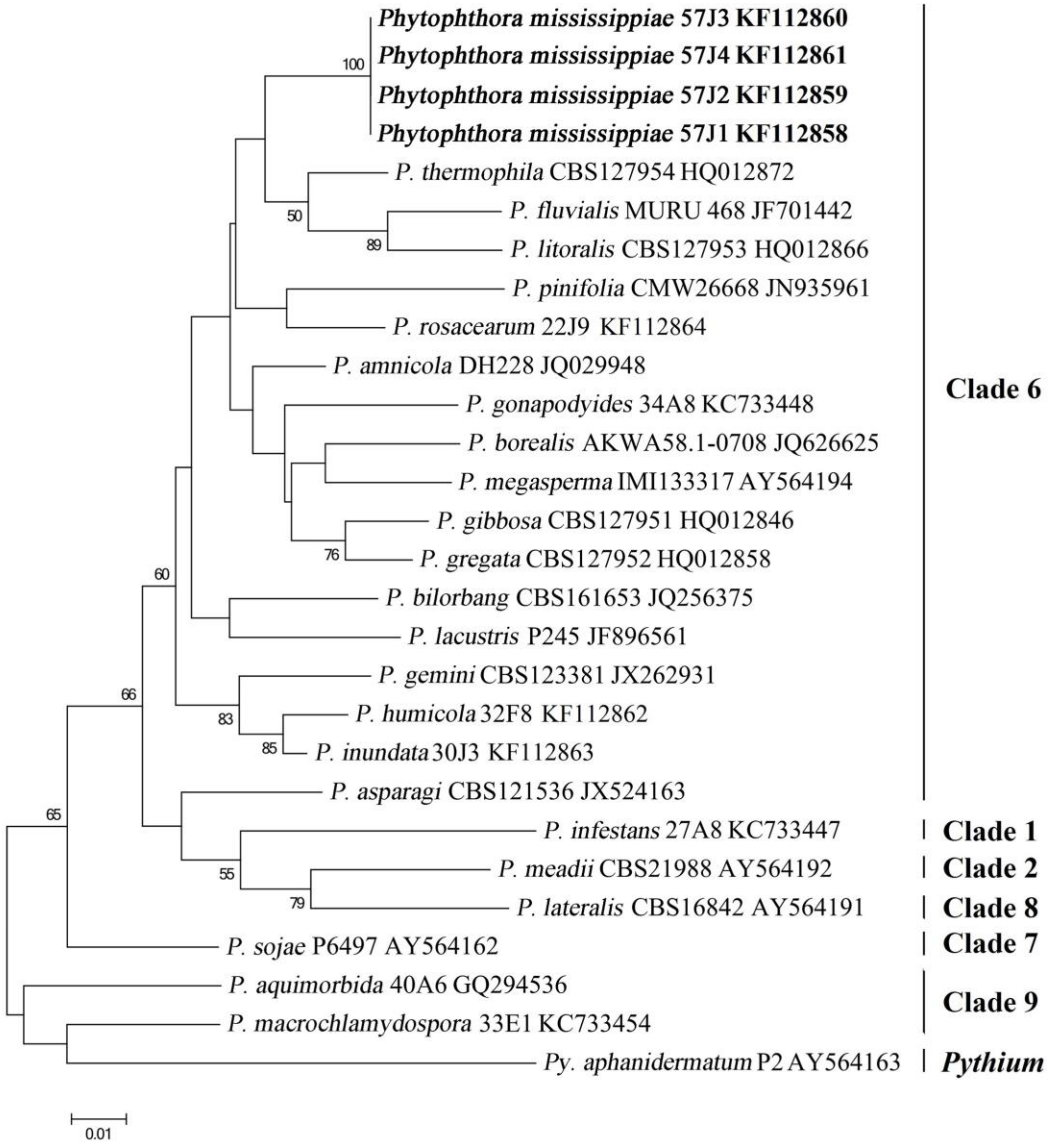

Figure 2.4 A maximum likelihood phylogenetic tree generated in MEGA 5.1, based on the alignment of partial *cox* 1 sequences with Clustal W. The numbers on branches are bootstrap values (1,000 replicates; values less than 50% are not shown).

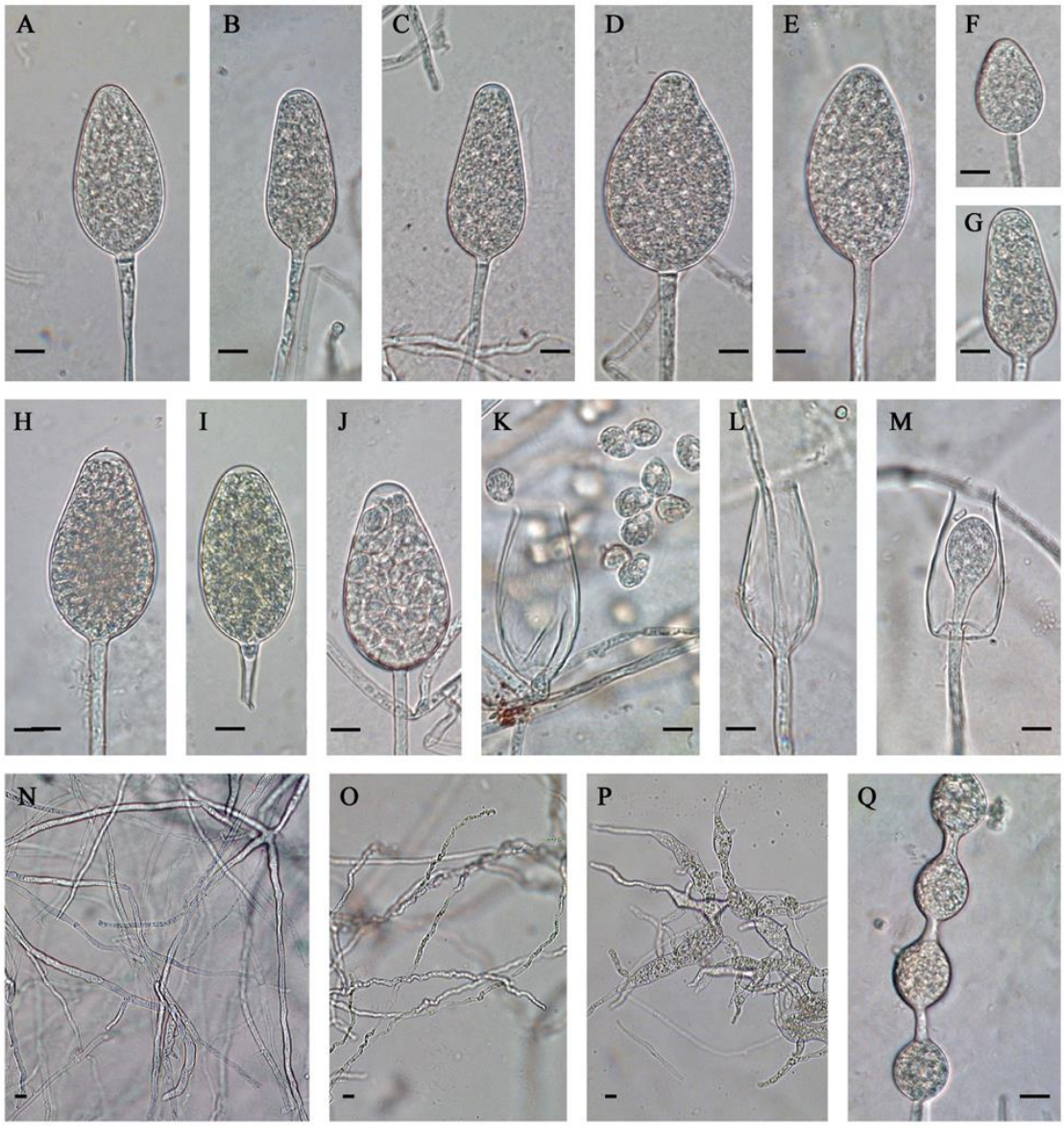

Figure 2.5 Morphology of asexual structures of *Phytophthora mississippiae*: A-G. Nonpapillate, noncaducous sporangia in various shapes; A. An obpyriform to ovoid sporangium; B, C. Obpyriform sporangia; D. An ovoid sporangium; E. An slightly excentric, ellipsoid sporangium; F. A secondary, ovoid sporangium; G. A secondary, obpyriform sporangium; H. A semipapillate, ovoid sporangium; I. A semipapillate, ellipsoid sporangium; J. A semipapillate sporangium right before releasing zoospores; K. A sporangium releasing zoospores; L. Internal extended proliferation; M. Nesting proliferation; N. Smooth, flat mycelia; O. Coiled mycelia; P. Swollen mycelia; Q. Catenulate hyphal swellings. Bars = 10 µm.

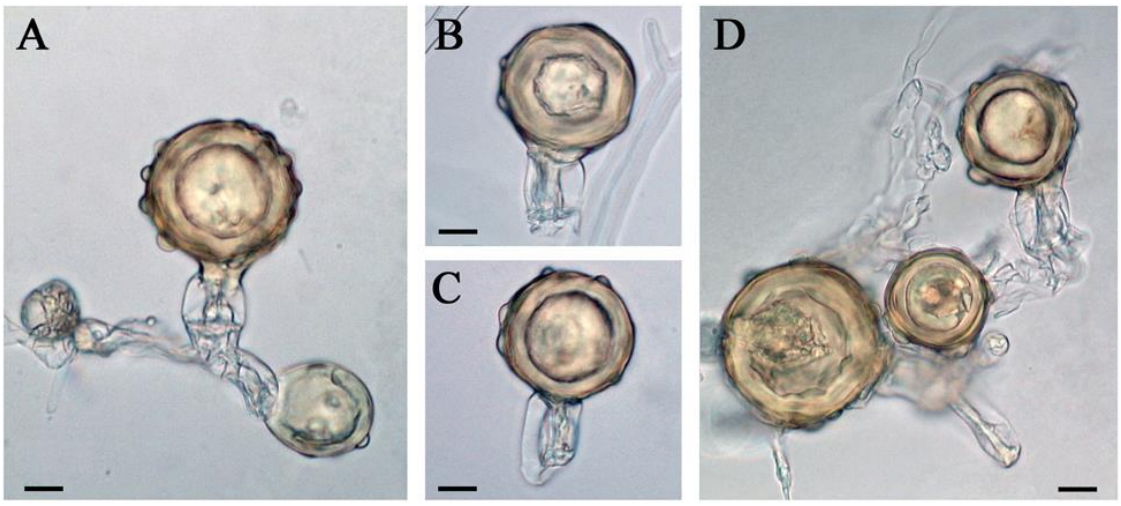

Figure 2.6 Morphology of gametangia of *Phytophthora mississippiae*: A, B. Selfing gametangia induced by *P. cryptogea*; A. An oogonium with an ornamented surface and a tapered base, a bi-celled, amphigynous antheridium, and two immature gametangia; B. An oogonium with ornamented surface and tapered base; C, D. Selfing gametangia induced by *P. nicotianae*; C. A plerotic oospore with a cylindroid, amphigynous antheridium; D. Three different sized oogonia. Bars = 10 µm.

Chapter 3 A high-temperature tolerant species in clade 9 of the genus *Phytophthora*: *P. hydrogena* sp. nov.

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Abstract

A previously unknown *Phytophthora* species was isolated from irrigation water in Virginia, USA. This novel species produces abundant noncaducous and nonpapillate sporangia in soil water extract solution. It sometimes produces chlamydospores and hyphal swellings in aged cultures and in Petri's solution. This species has optimum vegetative growth at 30℃ and grows well at 35℃. The lowest and highest temperatures for growth are 5 and 40℃. All isolates examined in this study are compatibility type A^1 and produce mostly plerotic oospores when paired with an A^2 mating-type tester of *P. cinnamomi*. Sequence analyses of the rDNA internal transcribed spacer (ITS) regions and the mitochondrially encoded cytochrome *c* oxidase 1 (*cox* 1) gene placed this species in clade 9 of the genus *Phytophthora*. These characteristics support the description of this taxon as a new species for which we propose the name *P. hydrogena* sp. nov. Further phylogenetic and physiological investigations of clade 9 species revealed a high-temperature tolerant cluster including *P. hydrogena*, *P. aquimorbida*, *P. hydropathica*, *P.*

irrigata, *P. chrysanthemi*, *P. insolita*, *P. polonica* and *P. parsiana*. These species all grow well at 35℃. The monophyly of the species in this heat-tolerant cluster except *P. insolita* and *P. polonica* is highly supported by the maximum-likelihood analyses of the ITS and *cox* 1 sequences.

Introduction

Many species in the genus *Phytophthora* have been considered destructive plant pathogens. Two notorious examples are that *P. infestans* caused Irish potato famine 1845-1849 (Erwin & Ribeiro, 1996) and *P. ramorum* caused sudden oak death (SOD), which has killed millions of trees in the forests of Oregon and California (Goheen *et al.*, 2002; Rizzo *et al.*, 2002). The SOD disaster reminded the science community and the public of the constant plant biosecurity threat posed by *Phytophthora* species and invigorated research in these pathogens (Grünwald *et al.*, 2012). To date the total number of formally described *Phytophthora* species has reached approximately 130, which is double the number 17 years ago (Erwin & Ribeiro, 1996).

Traditional taxonomy of *Phytophthora* species was based on morphological characteristics, as exemplified by the classic morphological key by Waterhouse (1963), which separated the genus into six groups and is still widely used today (Waterhouse, 1963). However, due to the plasticity and overlapping of morphological characteristics among species, taxonomy based on morphology cannot be used to reliably distinguish some species within the genus (Erwin & Ribeiro, 1996; Naher *et al.*, 2011).

Phylogenetic analyses of various DNA regions have greatly advanced the taxonomy of this important genus. Ten clades were proposed for genus *Phytophthora* after phylogenetic analyses of mitochondrial and nuclear DNA sequences (Cooke *et al.*, 2000; Martin & Tooley, 2003; Kroon *et al.*, 2004; Villa *et al.*, 2006; Blair *et al.*, 2008; Robideau *et al.*, 2011). By taking advantage of the phylogenetic analyses, many new species have been separated from some well-known species complexes. For instance, *P. brassicae* (Man in't Veld *et al.*, 2002) was separated from *P. porri*. *Phytophthora obscura* (Grünwald *et al.*, 2012) was separated from *P. syringae*. Similarly *P. hydropathica* (Hong *et al.*, 2010) and *P. irrigata* (Hong *et al.*, 2008) were separated from *P. drechsleri*. Likewise *P. plurivora* (Jung & Burgess, 2009) and *P. pini* (Hong *et al.*, 2011) were separated from *P. citricola*. In addition, many new species, such as *P. aquimorbida* (Hong *et al.*, 2012), *P. bilorbang* (Aghighi *et al.*, 2012), *P. borealis* and *P. riparia* (Hansen *et al.*, 2012), were discovered.

Among the 10 *Phytophthora* clades, clade 9 contains many relatively new members. The only exceptions are *P. insolita* (Ann & Ko, 1980), *P. macrochlamydospora* (Irwin, 1991) and *P. quininea* (Crandall, 1947). All other species in this clade were described after 2005. Many members in this clade, such as three recently named species *P. irrigata* (Hong *et al.*, 2008), *P. hydropathica* (Hong *et al.*, 2010) and *P. aquimorbida* (Hong *et al.*, 2012), are well adapted to the irrigation reservoir environment. The objective of this study was to examine another group of water isolates with distinct features and formally describe it as *Phytophthora hydrogena* sp. nov.

Materials and Methods

Isolation and isolate maintenance

Three isolates examined in this study (Table 3.1) were recovered by baiting with fresh rhododendron or camellia leaves (Hong *et al.*, 2002; Bush *et al.*, 2003; Ghimire *et al.*, 2011) from irrigation reservoirs in an ornamental plant nursery in Virginia, USA, in May and Oct 2007. Cultures were grown on 20% clarified V8 juice agar (CV8A) (Erwin & Ribeiro, 1996), and blocks of fresh agar cultures were transferred into microtubes with sterile distilled water for long-term storage at 15℃. The ex-type was deposited at the American Type Culture Collection (MYA-4919) in Manassas, Virginia. The dry culture as holotype was deposited at the Massey Herbarium of Virginia Polytechnic Institute and State University (VTMH 14882) in Blacksburg, Virginia.

Colony morphology

To examine colony morphology, cultures were grown on CV8A, carrot agar (CA), malt extract agar (MEA) and potato dextrose agar (PDA). Each 10 cm Petri dish with 12 mL medium was seeded upside down with an agar block (5 mm diam) taken from 10-day cultures with the aid of a flamed cork borer. The dishes were placed in the dark at 20℃. Colony morphology was noted after 10 days.

DNA extraction, amplification and sequencing

A 5×5 mm^2 agar plug from an actively growing culture was placed in 20% V8 juice broth (Erwin & Ribeiro, 1996) at room temperature (ca. 23℃) for seven days to produce mycelial mass. The mycelial samples were harvested, dried and lysed with a FastPrep®-24 system (MP Biomedicals, Santa Ana, California). Purified DNA was extracted as instructed with the DNeasy® Plant Mini kit (Qiagen, Valencia, California). Amplifications were performed with forward primer ITS6 and reverse primer ITS4 (Cooke *et al.*, 2000) for the internal transcribed spacer (ITS) region covering ITS1, 5.8S rRNA gene and ITS2, following previously described reaction mix recipe and PCR program (Kong *et al.*, 2003). Amplifications also were performed with primer pair COXF4N and COXR4N (Kroon *et al.*, 2004) for sequencing the mitochondrially encoded cytochrome *c* oxidase 1 gene (*cox* 1) following the PCR protocol with a 45℃ annealing temperature. Excess primer and dNTPs were removed from PCR products with shrimp alkaline phosphatase and exonuclease I (USB 70092Y and 70073Z). One U each enzyme was added to 15 mL PCR product, incubated at 37℃ for 30 min, followed by heat inactivation at 65℃ for 15 min. Sequencing was performed in both directions for all regions with the same primers as for PCR by the University of Kentucky Advanced Genetic Technologies Center (Lexington, Kentucky). Sequences of both directions were viewed with Finch TV 1.4.0. and aligned with Clustal W.

Phylogenetic analysis

Sequences generated in this study were compared with those of all other species in the same clade and selected species in other clades (Table 3.1). Sequences were aligned with Clustal W. Phylogeny reconstruction was conducted in MEGA 5 (Tamura *et al.*, 2011) with the maximum-likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993) with 1000 bootstrap replications. GenBank accession numbers of the sequences derived in this study are provided (Table 3.1). The alignments and trees are available in TreeBASE (S14412).

Growth temperature studies

Three isolates were assessed for cardinal temperatures on CV8A and CA. Agar blocks (5 mm diam) taken from actively growing areas of 10-day cultures were placed at the center of Petri dishes with fresh media. These dishes were placed in the dark at 5, 10, 15, 20, 25, 30, 35, 36, 37, 38, 39 or 40℃, triplicate dishes per isolate per temperature. Two perpendicular diameter measurements of each colony were taken when the margin of the fastest growing colony was about 1 cm from the dish edge. The test was repeated. Means of radial growth along with standard errors were plotted against temperature with the gplots package 2.11.0 (Warnes *et al.*, 2012) in R statistical software 2.15.0 (R Core Team, 2012).

Sporangial morphology

Sporangia were produced by transferring 10 ×10 mm square agar plugs of 1 to 2-week old colonies on 10% CV8A to Petri dishes containing non-sterile 1.5% soil water extract solution (SWE, 15 g non-Phytophthora containing nature soil/1 L distilled water) and incubating at room temperature approximately 10 hours until mature sporangia formed. Sporangia were photographed with a Nikon Fujix Digital Camera HC-300Zi connected to a Nikon Labophot-2 microscope. At least 50 randomly selected mature sporangia were measured for length and width with Image-Pro® Plus 5.1.2.53.

Breeding system of gametangia

Because gametangia were not observed in single cultures on any media, several methods were employed to determine the sexuality and mating type of three isolates of *P. hydrogena*. They were tested in dual culture with either A^1 or A^2 mating type tester of *P. cinnamomi* on hemp seed agar (HSA) or CV8A. Also, the polycarbonate technique (Ko, 1978; Gallegly & Hong, 2008) was used to induce selfing and prevent mixing the gametangia of target isolates and testers. Specifically, each isolate was paired with either mating type of *P. cinnamomi* on CA or HSA amended with 30 mg β-sitosterol L^{-1} then incubated at 20 and 25℃ as well as room temperature. For isolates that did not produce oospores, additional tests were conducted with the mating type testers of *P. meadii* and *P. cambivora* at the same temperatures.

Statistical analysis

Analyses of variances were carried out with R (R Core Team, 2012) to determine the differences in the measurements of morphological and physiological characteristics among repeating experiments and isolates.

Results

Colony morphology

After growing at 20℃ in dark for 10 days, three isolates of *P. hydrogena* showed a similar growth pattern (Figure 3.1). Overall, colonies expanded the fastest on CA among four tested media. Colonies on CA had a relatively smooth edge and produced radiate to slightly chrysanthemum-like patterns. On CV8A and PDA, all isolates formed typical chrysanthemum-like colonies with irregular edges, although colonies expanded more slowly and were denser on PDA than on CV8A. Only isolate 46A4 had limited but discernible growth with irregular colony and radiate mycelia on MEA (Figure 3.1).

Sequence analysis and phylogenetic position

Three isolates of *P. hydrogena* have an identical 744-bp ITS sequence and an identical 867-bp partial *cox* 1 sequence. *Phytophthora hydrogena* is placed within *Phytophthora* clade 9, according to the maximum-likelihood phylogenetic trees based on ITS and *cox* 1

sequences. It forms a distinct new group with its closest relatives *P. hydropathica*, *P. irrigata* and *P. parsiana*, with strong bootstrap support (Figure 3.2). *Phytophthora hydrogena* differs in ITS sequence from *P. hydropathica*, *P. irrigata* and *P. parsiana* by 31 bp, 53 bp and 43 bp respectively. In *cox* 1 sequence, *P. hydrogena* is different from *P. hydropathica*, *P. irrigata* and *P. parsiana* by 25 bp, 28 bp and 26 bp respectively.

Cardinal temperatures for vegetative growth

Vegetative growth rates were statistically identical among three isolates ($P = 0.98$) and between two experiments ($P = 0.26$). The average daily radial growth of three isolates were plotted against temperature (Figure 3.3). Generally, *P. hydrogena* grew discernibly faster on CA than on CV8A. The optimum temperature for growth was 30℃ and it grew well at 35℃ (Figure 3.3). Only notable growth was observed at 38 and 39℃ and no growth at 5 and 40℃ after five days (Figure 3.3).

Taxonomy

Phytophthora hydrogena X. Yang and C. X. Hong., sp. nov. (Figure 3.4)

MycoBank MB803274

Phytophthora hydrogena produced few sporangia in aged CA and CV8A. When mycelial plugs were submerged in 1.5% SWE under light, they produced abundant sporangia

within 10 hours. Sporangia varied from ovoid to obpyriform, limoniform and ellipsoid (Figure 3.4A-G). Sporangia were terminal, nonpapillate and noncaducous; they averaged 44.58 mm long and 29.27 mm wide. Most sporangia had a conspicuous cell wall at the joint point between sporangial base and pedicel tip. Approximately half of the sporangia observed in this study had characteristic widening at the pedicel tip of sporangiophores or tapered sporangial base toward the point of attachment (Figure 3.4K-M). Sporangia also commonly had a conspicuous basal plug (Figure 3.4H, K). Nested and extended internal proliferation (Figure 3.4I, J) was common. Knobby and angular mycelia were observed especially in aged cultures (Figure 3.4S, T). Hyphal swelling of sporangiophores (Figure 3.4P, R) occasionally was observed when submerged in modified Petri's solution. Chlamydospores were not observed in fresh culture on any agar media or in SWE. However, a limited number of chlamydospores, average 34.5 mm diam, were observed in aged cultures (> 50 days) in CA and CV8A or cultures incubated in modified Petri's solution for more than 12 hours under light (Figure 3.4N, O).

Phytophthora hydrogena is heterothallic and all examined isolates are A^1. None of these isolates produced any sexual structure in single culture. Polycarbonate membrane tests on CA and HSA at 20, 25℃ and room temperature induced the formation of gametangia in A^2 tester isolates of *P. cambivora* and *P. cinnamomi*. Gametangia were not observed in any isolates of *P. hydrogena* or testers of *P. meadii*. Gametangia also were observed when each *P. hydrogena* isolate was paired with an A^2 tester of *P. cambivora* or *P. cinnamomi* in the dual culture without separation by a polycarbonate membrane. Most of the oogonia produced in the dual cultures were typical of *P. cinnamomi*, which had a

funnel-shaped base or broad, tapering stalk through an antheridium (Figure 3.5I). A small number of oogonia were distinct from those typical oogonia produced by *P. cinnamomi*. They most likely were produced by *P. hydrogena* (Figure 3.5A-H). These oogonia were globose with golden to brown pigment, averaged 41.1 mm diam. Oospores, average 37.6 mm, were mostly plerotic (Figure 3.5A-C, E, F), sometimes aplerotic (Figure 3.5D, G) and eccentric (Figure 3.5G). These oogonia were attached by antheridia that were mostly amphigynous and averaged about 15 mm wide and deep (Figure 3.5A-H). Multiple paragynous antheridia occasionally were associated with an oogonium (Figure 3.5H).

Holotype

VTMH 14882 (Virginia Tech Massey Herbarium, Blacksburg, Virginia, USA), dried culture of an isolate from irrigation water of a nursery reservoir, Virginia, USA, Oct 2007.

Ex-holotype: 46A3 (ATCC MYA-4919).

Etymology

'*hydrogena*' refers to the aquatic habitat where it was isolated.

Habitat

Irrigation water of a perennial plant nursery, Virginia, USA.

Discussion

This study described a novel species, *Phytophthora hydrogena*, based on its distinct phylogenetic position, morphology and physiology, and revealed a cluster of high-temperature tolerant species within *Phytophthora* clade 9. These findings have several ramifications.

Description of *P. hydrogena* is the first and critical step to assess the potential plant biosecurity threat posed by this species and also will help reduce the risk in misidentification of high-impact pathogens within this genus. *Phytophthora hydrogena* is morphologically, physiologically and phylogenetically distinct from any other known Phytophthora species. As a heterothallic species, *P. hydrogena* can be easily distinguished from homothallic species. Among heterothallic species, *P. drechsleri*, *P. hydropathica*, *P. inundata*, *P. irrigata*, *P. melonis* and *P. parsiana* as well as this new species, *P. hydrogena*, produce nonpapillate sporangia and grow well at 35℃ (Gallegly & Hong, 2008; Hong *et al.*, 2008; Hong *et al.*, 2010). *Phytophthora hydrogena* differs from *P. drechsleri*, *P. inundata*, *P. melonis*, and *P. parsiana* by producing much smaller sporangia. It also differs from *P. drechsleri*, *P. hydropathica*, *P. inundata*, *P. melonis* and *P. parsiana* by not producing chlamydospores in CV8A, CA, MEA or PDA. Morphologically *P. hydrogena* is very similar to *P. irrigate*, but the former can be distinguished by its characteristic widening of sporangiophore tips and tapered sporangial bases.

Many species in *Phytophthora* clade 9 share a common physiological character of high-temperature tolerance. Analyses of ITS and *cox* 1 sequences grouped *P. hydrogena* with *P. aquimorbida* (Hong *et al.*, 2012), *P. hydropathica* (Hong *et al.*, 2010), *P. irrigata* (Hong *et al.*, 2008), *P. chrysanthemi* (Naher *et al.*, 2011), P. insolita (Ann & Ko, 1980), *P. polonica* (Belbahri *et al.*, 2006) and *P. parsiana* (Mostowfizadeh-Ghalamfarsa *et al.*, 2008) in a distinct cluster (Figure 3.2). Species in this cluster have an optimum temperature around 30℃, and they all grow well at 35℃. This unique characteristic of high-temperature tolerance has not been found from other species in clade 9 or in members of any other *Phytophthora* clade. The monophyly of species in this high temperature-tolerant cluster, except *P. insolita* and *P. polonica*, is highly supported by the maximum-likelihood analyses of the ITS (99%) and *cox* 1 (98%) sequences (Figure 3.2). Many species in this cluster including *P. aquimorbida*, *P. hydropathica*, *P. irrigata* and *P. hydrogena* initially were isolated from irrigation reservoirs. The host ranges of these aquatic species are unknown with the only exception of *P. hydropathica*, which is known to attack *Kalmia latifolia* and other horticultural crops (Hong *et al.*, 2010). These findings might imply that the ancestors of these high-temperature tolerant species may originate from tropical or subtropical environments.

It is intriguing that all isolates of this novel species are A^1. Similar observations were made in two other heterothallic species, *P. hydropathica* (Hong *et al.*, 2010) and *P. irrigata* (Hong *et al.*, 2008), that also were recovered initially from irrigation reservoirs in Virginia. The implication of this phenomenon remains unknown. One possibility is that

two mating types of these heterothallic species were separated according to geographic distributions after their global migrations. This geographical separation of mating-type isolates has been reported in other *Phytophthora* species. For example, the A^2 mating type of *P. infestans* was not found outside Mexico until 1984 (Hohl & Iselin, 1984). Another example is all European isolates of *P. ramorum* before 2003 were A^1 mating type while all North American isolates were A^2 (Hansen *et al.*, 2003). Similarly, it is possible that only A^1 mating-type isolates of *P. hydrogena*, *P. hydropathica* and *P. irrigata* migrated from their origins to Virginia.

The economic and ecological significance of this new species is not clear at this point. Symptomatic plant samples infected by this species have never been recorded from the Disease Clinic at Hampton Road AREC, Virginia Tech, in Virginia Beach. *Phytophthora hydrogena* caused asymptomatic infection on roots of *Rhododendron catawbiense*, but it was a poor survivor in potting mix in a preliminary pathogenicity test (data not shown). Further studies are warranted on its host range, ecological role in aquatic and terrestrial ecosystems, as well as its origin.

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Table 3.1 Origins and sequence accession numbers of *Phytophthora hydrogena* and reference species

Species	Clade	Isolate	Substrate/host	Origin	Year	Sequence accession no. [#]	
						ITS	*cox* 1
P. hydrogena	9	44G8	Irrigation water	Virginia	2007	**KC249958**	**KC249961**
		46A3 [T]	Irrigation water	Virginia	2007	**KC249959**	**KC249962**
		46A4	Irrigation water	Virginia	2007	**KC249960**	**KC249963**
P. andina	1	EC3421	*Solanum muricatum*	Ecuador	n/a [*]	AY770738	AY564160
P. hedraiandra	1	CBS111725	*Viburnum* sp.	the Netherlands	2004	AY707987	AY769115
P. infestans	1	2C	*Solanum tuberosum*	Mexico	1992	**KC733443**	**KC733447**
P. meadii	2	CBS21988	*Hevea brasiliensis*	India	1987	HQ643268	AY564192
P. ilicis	3	CBS114348	*Ilex aquifolium*	the Netherlands	1957	JX524158	JX524159
P. heveae	5	CBS29629	*Hevea brasiliensis*	Malaysia	1929	AF266770	AY564182
P. gonapodyides	6	CBS55467	Fruit bait from water	UK	1967	HQ643236	**KC733448**
P. humicola	6	CBS20081	Soil slurries	Taiwan	1977	HQ643243	AY564184
P. pinifolia	6	CMW26668	*Pinus radiata*	Chile	2007	EU725806	JN935961
P. cinnamomi	7	CBS14422	*Cinnamomum burmannii*	Indonesia	1922	HQ643189	n/a
		10A6	*Persea americana*	n/a	n/a	n/a	AY564169
P. rubi	7	CBS96795	Base of raspberry cane	Scotland	1985	HQ643340	DQ674736
P. foliorum	8	LT192	*Rhododendron* sp.	Tennessee	2004	HQ261560	EU124918
P. aquimorbida	9	40A6	Irrigation water	Virginia	2006	FJ666127	GQ294536
P. captiosa	9	310C	*Eucalyptus saligna*	New Zealand	1992	DQ297402	**KC733449**
P. chrysanthemi	9	GF749	*Chrysanthemum* sp.	Japan	2000	AB437135	n/a
P. constricta	9	CBS125801	Soil	Australia	2006	HQ013225	**KC733450**
P. fallax	9	310L	*Eucalypti delegatensis*	New Zealand	1997	DQ297391	**KC733451**
P. hydropathica	9	5D1	Irrigation water	Virginia	2000	EU583793	**KC733452**
P. insolita	9	PMC5-1	Soil slurries	Taiwan	1980	GU111612	AY564188
P. irrigata	9	23J7	Irrigation water	Virginia	2000	EU334634	**KC733453**
P. macrochlamydospora	9	UQ778	*Glycines max*	Australia	1974	**KC733445**	**KC733454**
P. parsiana	9	SUC25	*Ficus carica*	Iran	1991	**KC733446**	**KC733455**
P. polonica	9	UASWS0198	*Alni glutinosae*	Poland	2004	DQ396410	n/a
		P15005	n/a	US	n/a	n/a	**KC733456**
P. quininea	9	CBS40648	*Cinchona officinalis*	Peru	1947	DQ275189	n/a
		CBS40748	*Cinchona officinalis*	Peru	1947	n/a	AY564200
Pythium aphanidermatum	*Pythium*	P1779	n/a	n/a	n/a	GU983641	n/a
		P2	n/a	n/a	n/a	n/a	AY564163

[#] Sequence accession numbers in bold are those generated in this study. [T] exo-type isolate [*] n/a = not available

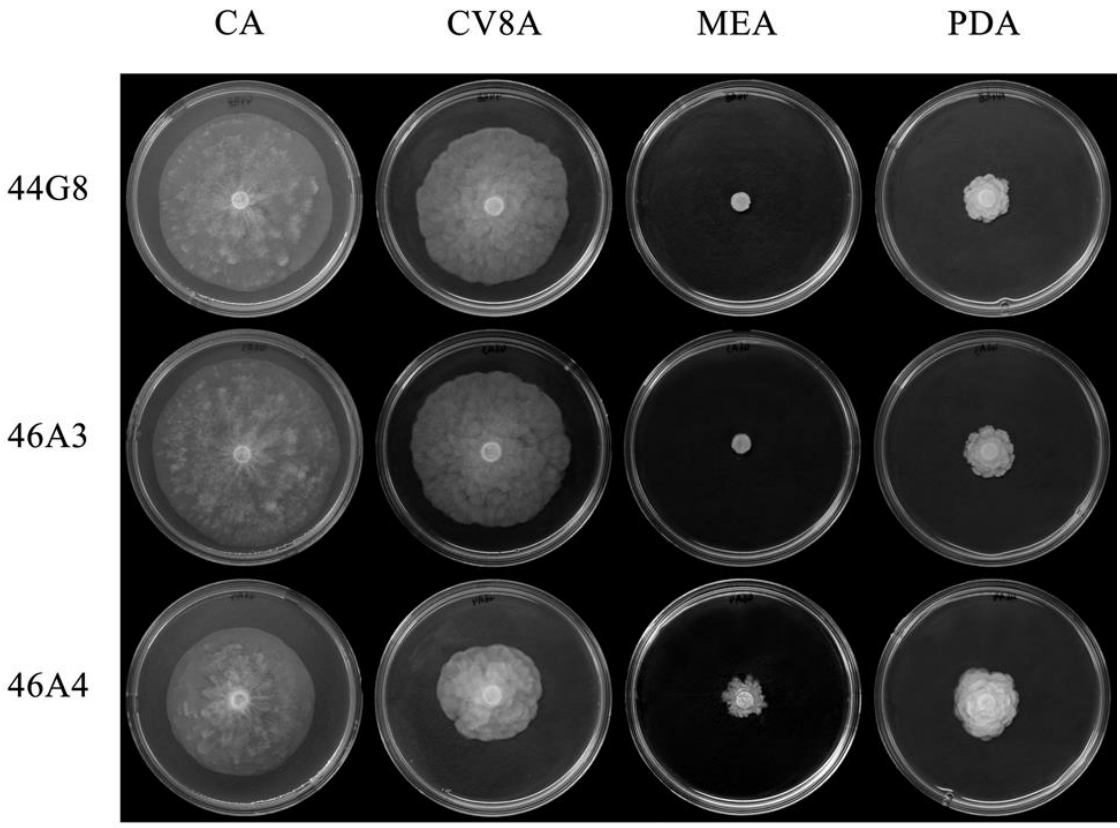

Figure 3.1 Colony morphology of *Phytophthora hydrogena* isolates on various media incubated at 20℃ for 10 days in the dark. CA = carrot agar; CV8A = 20% clarified V8 juice agar; MEA = malt extract agar; PDA = potato dextrose agar.

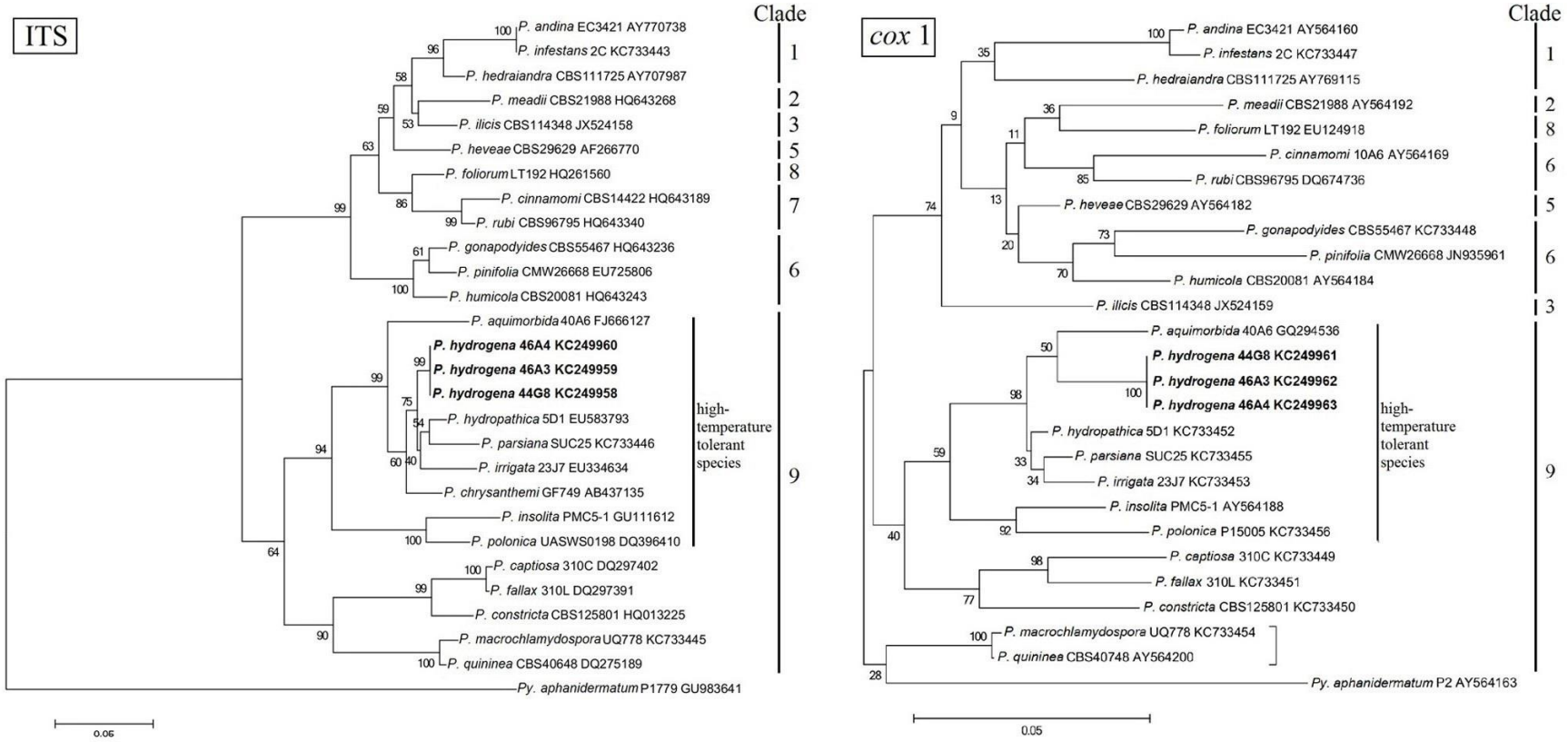

Figure 3.2 Maximum likelihood phylogenetic trees based on ITS and *cox* 1 sequences. Alignment was conducted with Clustal W and the phylogenetic tree was generated in MEGA5.

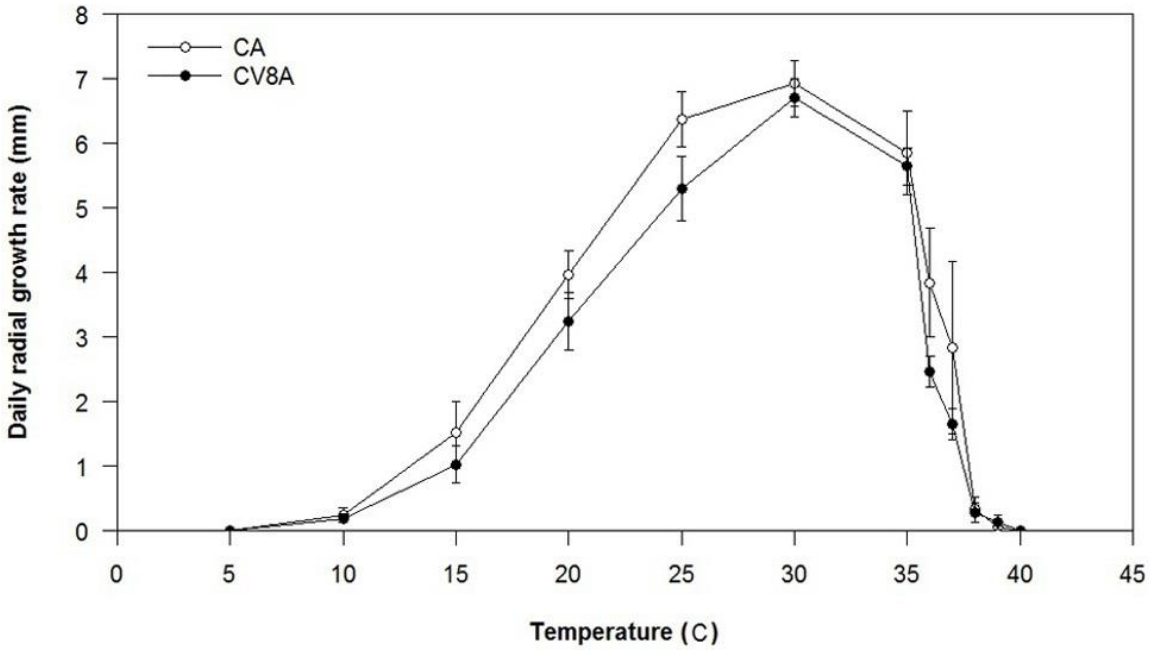

Figure 3.3 Radial growth of *Phytophthora hydrogena* isolates in carrot agar (CA) and 20% clarified V8 juice agar (CV8A) over a 5-day period.

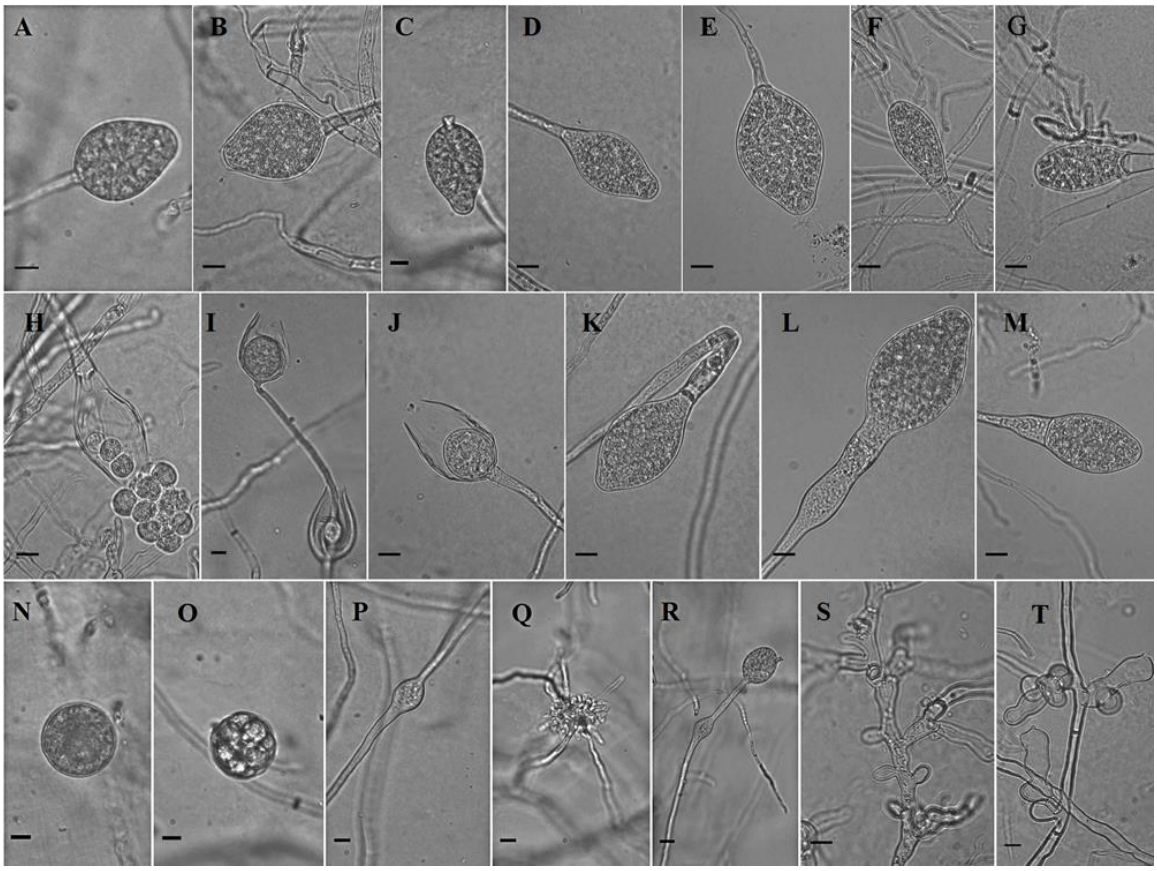

Figure 3.4 Morphology of asexual structures of *Phytophthora hydrogena*. A-G. Nonpapillate sporangia in various shapes. A, B. Ovoid sporangia. C. An obpyriform to ovoid sporangium. D, E. Limoniform sporangia with a tapered base. F, G. Ellipsoid sporangia. H. A sporangium with a conspicuous basal plug releasing zoospores. I, J. Nested proliferation. K. A sporangium with a conspicuous basal plug, a tapered base and a widening sporangiophore tip L, M. Sporangia with a tapered base and a widening sporangiophore tip. N, O. Chlamydospores infrequently produced in modified Petri's solution. P, R. Intercalary swelling on sporangiophore occasionally produced in modified Petri's solution. Q. Hyphal aggregation rarely formed in modified Petri's solution. S, T. Knobby and angular coralloid-like mycelia. Bars = 10 µm.

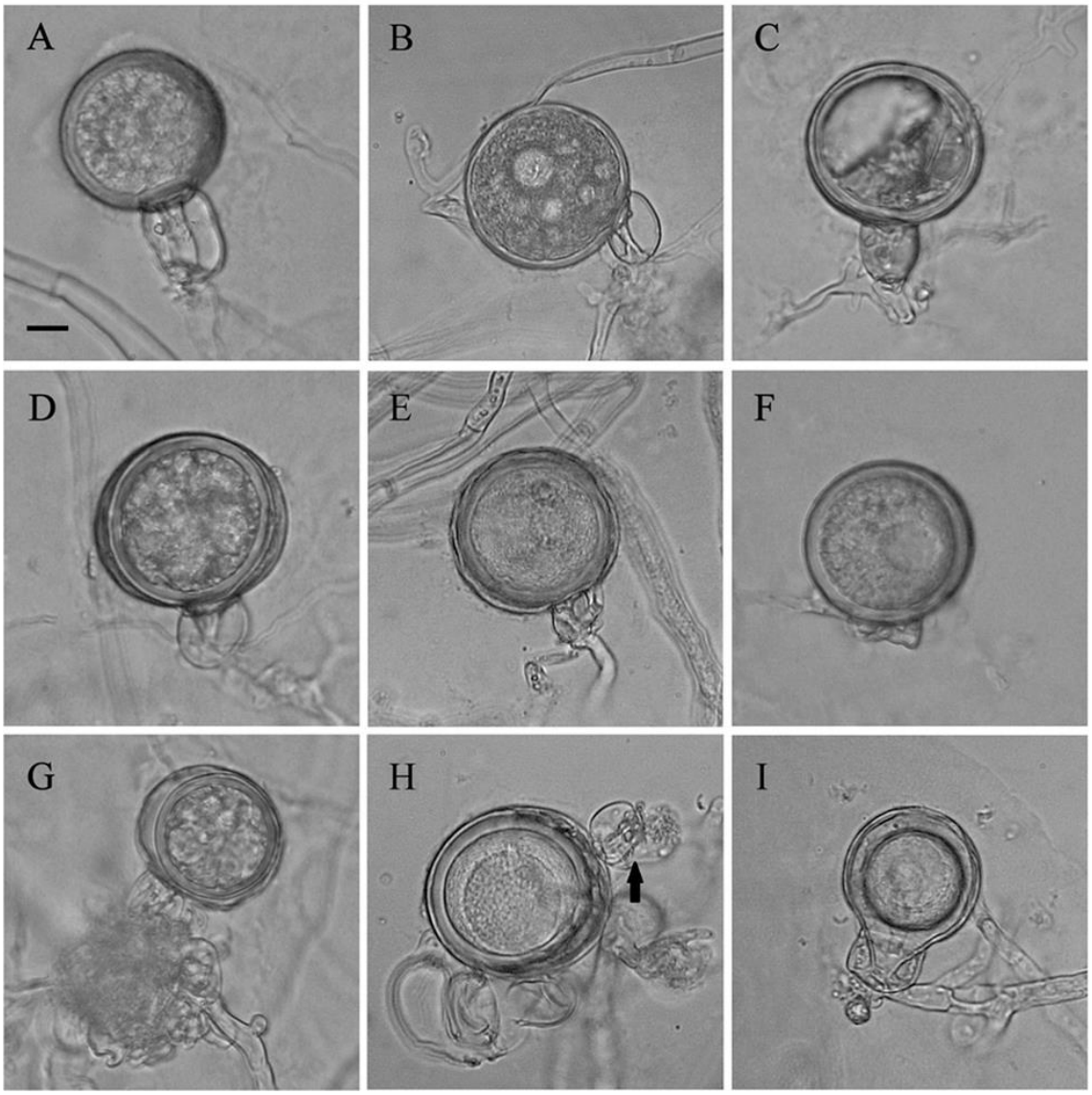

Figure 3.5 Morphology of sexual organs of *Phytophthora hydrogena*. A. A plerotic oospore with a long, cylindroid, amphigynous antheridium. B. A plerotic oospore with a globose antheridium. C. A plerotic oospore with a golden coniform antheridium. D. A slightly aplerotic oospore with a globose antheridium. E, F. Plerotic oospores with a short antheridium. G. Excentric aplerotic oospore. H. A slight aplerotic oospore with an amphigynous antheridium (arrow) and multiple paragynous antheridia. I. A typical sexual organ produced by *Phytophthora cinnamomi* with a characteristic broad tapering stalk in the antheridium and a funnel-shaped base of the oogonium within the antheridium. Bar = 10 µm.

Chapter 4 *Phytophthora virginiana* sp. nov., a high-temperature tolerant species from irrigation water in Virginia

Yang X & Hong CX (2013) *Phytophthora virginiana* sp. nov., a high-temperature tolerant species from irrigation water in Virginia. *Mycotaxon* **126**: 167-176.

http://dx.doi.org/10.5248/126.167

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*.

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. cinnamomi* (Freinkel, 2007), 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 25 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.

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 and 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. 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℃. 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 ITS6&4 (Cooke *et al.*, 2000). 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℃. Agar blocks (5 mm diam.) were taken from 7-day-old cultures using a famed 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 four 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℃ 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 florescent lamps at room temperature (*c.* 23℃). 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 ImagePro® 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℃.

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 (Figure 4.1) placed *P. virginiana* in clade 9 of *Phytophthora*. *Phytophthora virginiana* isolates were supported as a taxon unique from all other known species by a strong bootstrap (Figure 4.1). GenBank accession numbers generated in this study and used for phylogeny construction are included in figure 4.1.

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 figure 4.2. Neither isolate grew on tested media at 5℃. The optimum temperature was 30℃ on both CV8A and CA. Both isolates grew well at 35℃ 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℃, whereas 46A2 had notable growth on both media, as did 44G6 on CV8A at 40℃.

Colony morphology

After growing in the dark at 20℃ for 10 days, the colonies of the two *P. virginiana* isolates were photographed (Figure 4.3). 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 X. Yang and C. X. Hong, sp. nov. (Figure 4.4)

MycoBank MB 804533

Isolate examined

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).

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).

Etymology

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

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℃ and room temperature (*c.* 23℃).

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℃, and produces abundant lateral chlamydospores. *Phytophthora insolita* (Ann & Ko, 1980), which also grows well at 35℃ 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℃ (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℃, 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 (Mostowfizadeh-Ghalamfarsa *et al.*, 2008; Hong *et al.*, 2010). 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 (Figure 4.1) 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℃ and some survive at 40℃ (Figure 4.2). 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, *P.* sp. lagoariana recovered from the Amazonian rainforest, also grows well at 35℃ (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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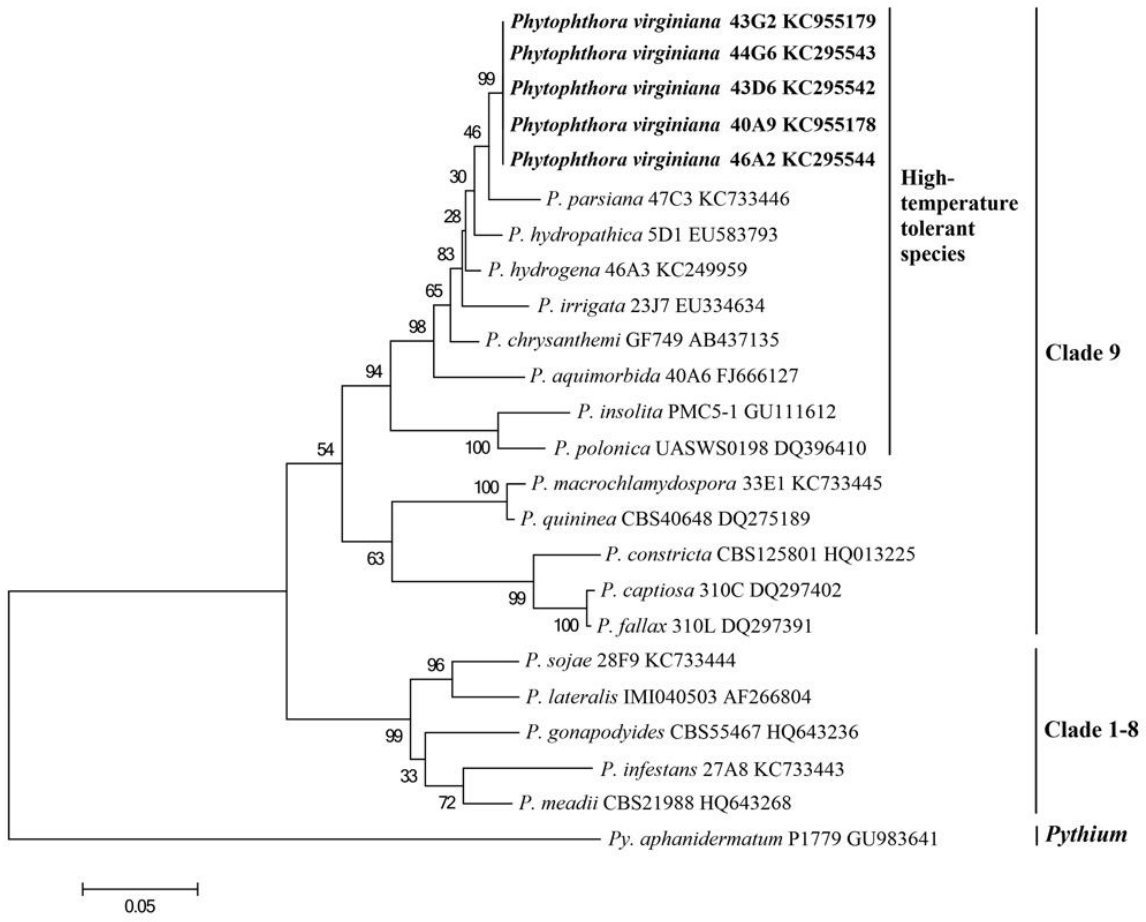

Figure 4.1 Maximum likelihood phylogenetic tree for species in *Phytophthora* clade 9 including *Phytophthora virginiana* and reference species in other clades based on ITS sequences. Alignment was conducted with Clustal W and the phylogenetic tree was generated in MEGA 5.

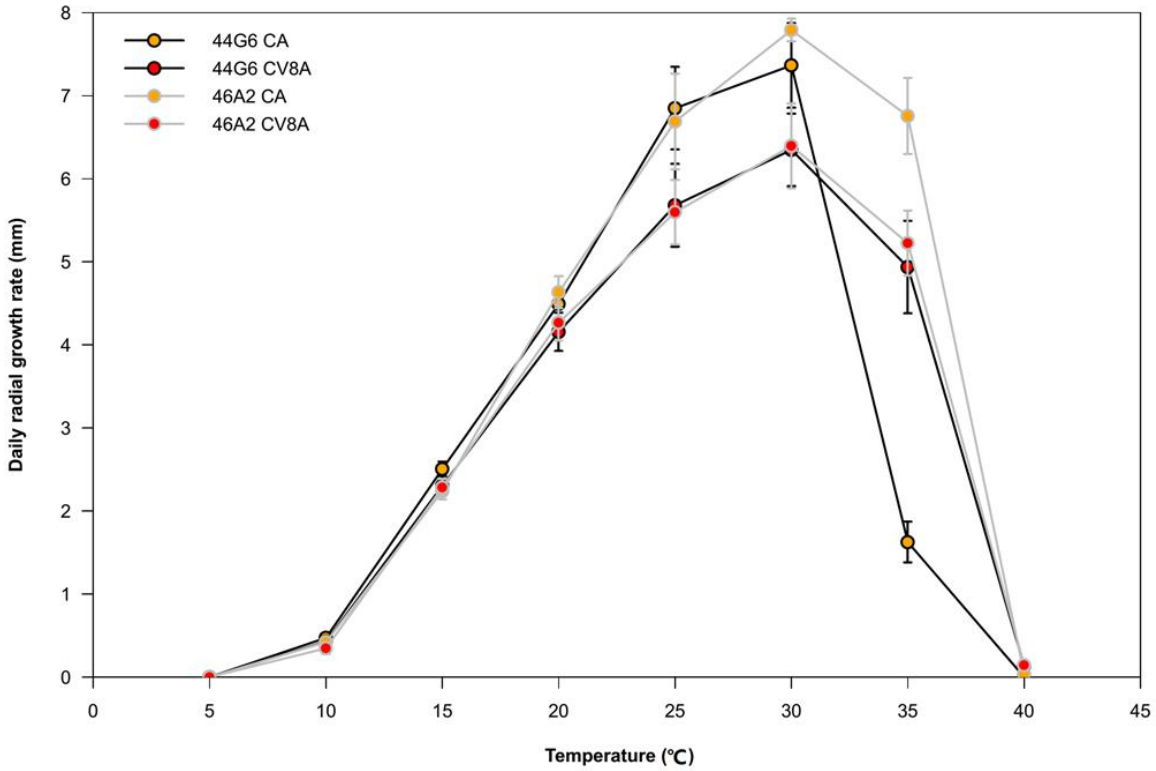

Figure 4.2 *Phytophthora virginiana.* Average daily radial growth of on carrot agar (CA) and clarified V8 juice agar (CV8A).

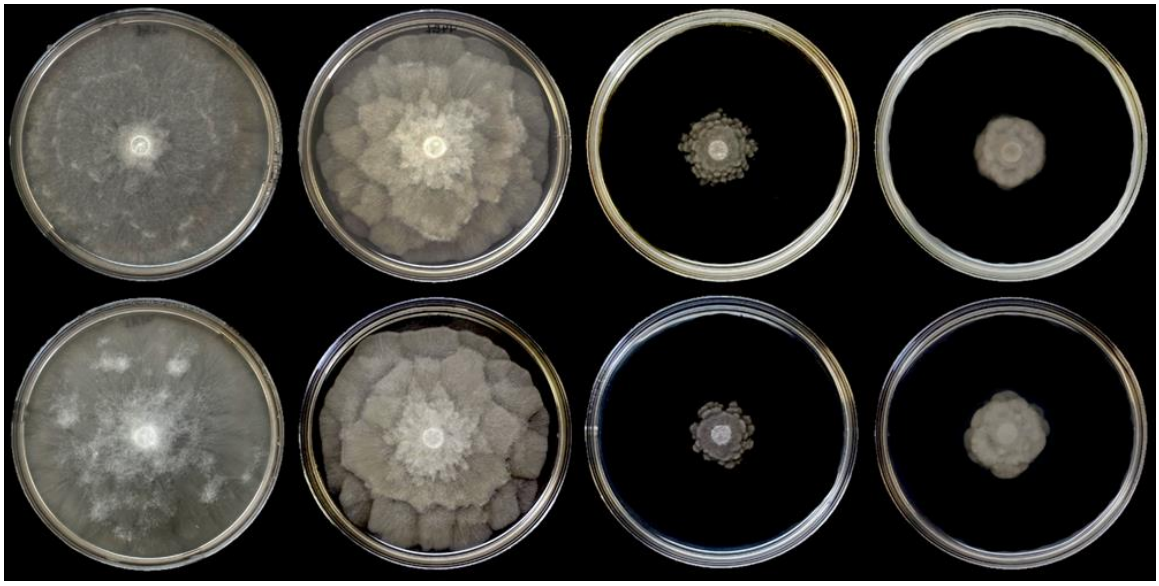

Figure 4.3 Colony morphology of *Phytophthora virginiana* isolates 44G6 (top) and 46A2 (bottom) after 10-days growth at 20℃ on (left to right) carrot agar, clarified V8 juice agar, malt extract agar and potato dextrose agar.

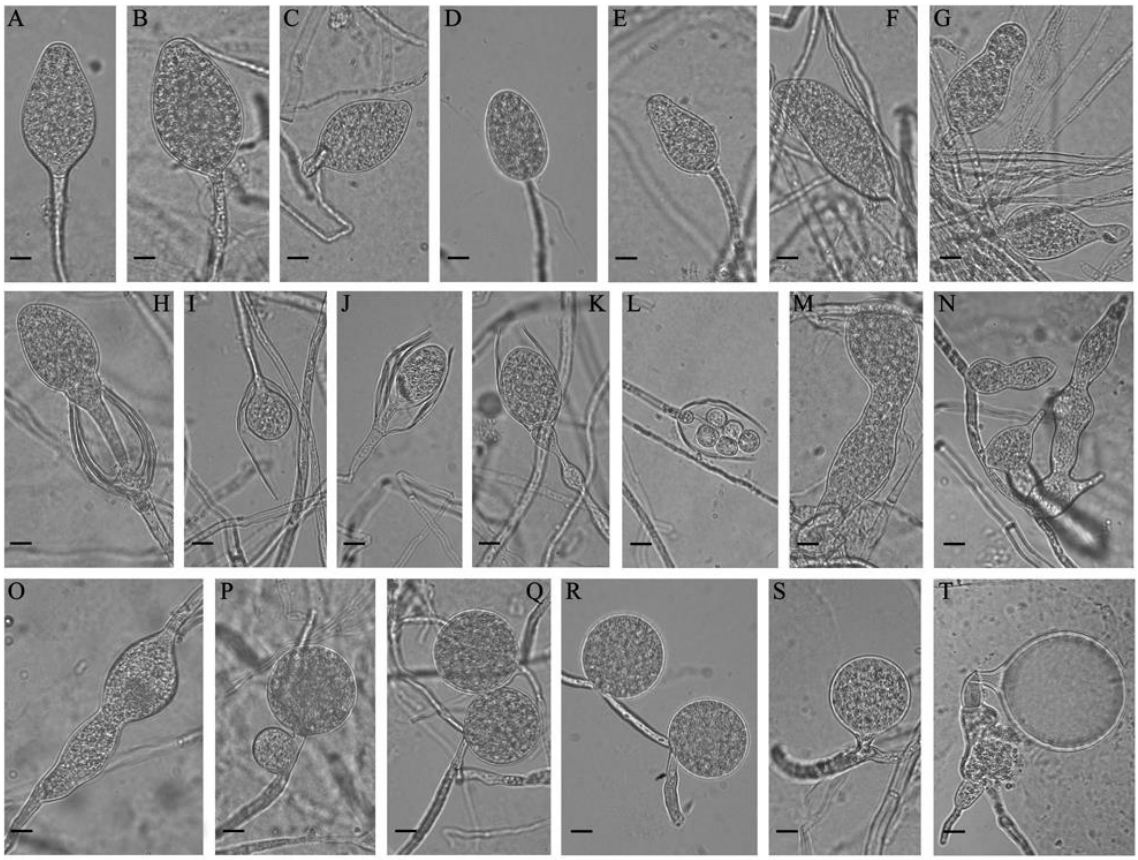

Figure 4.4 *Phytophthora virginiana*. Morphology of asexual structures. A-G: Sporangia in various shapes; A; Ovoid sporangium; B: Ovoid sporangium appearing semi-papillate just before releasing zoospores; C: Limoniform sporangium; D: Ellipsoid sporangium; E: Obpyriform sporangium; F: Distorted, peapod-shaped 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.

Chapter 5 Two novel species *Phytophthora stricta* and *P. macilentosa* representing a new clade and cluster of *Phytophthora*

Yang X, Copes WE & Hong CX (2014) Two novel species representing a new clade and cluster of *Phytophthora*. *Fungal Biology* **118**: 72-82

http:/dx.doi.org/10.1016/j.funbio.2013.11.003

Abstract

Phytophthora stricta sp. nov. and *Phytophthora macilentosa* sp. nov. are described based on morphological, physiological and molecular characters in this study. *Phytophthora stricta* represents a previously unknown clade in the rRNA internal transcribed spacer (ITS)-based phylogeny. *Phytophthora macilentosa*, along with nine other species, consistently forms a high temperature-tolerant cluster within ITS clade 9. These observations are supported by the sequence analysis of the mitochondrial cytochrome *c* oxidase 1 gene. Both species are heterothallic and all examined isolates are A^1 mating type. *Phytophthora stricta* produces nonpapillate and slightly caducous sporangia. This species is named after its characteristic constrictions on sporangiophores. *Phytophthora macilentosa* produces nonpapillate and noncaducous sporangia, which are mostly elongated obpyriform with a high length to breadth ratio. Both species were recovered from irrigation water of an ornamental plant nursery in Mississippi, USA and *P. stricta* was also recovered from stream water in Virginia, USA.

Introduction

Genus *Phytophthora* includes a diverse group of ecologically and economically important plant pathogens. This diversity has been rapidly unfolding over the past decade, partly due to extensive survey of many previously unexplored habitats. The genus currently has more than 130 known species (Lévesque, 2011; Kroon *et al.*, 2012; Martin *et al.*, 2012), which is almost twice as many as that in 1996 (Erwin & Ribeiro, 1996). These diverse members of the genus *Phytophthora* currently are classified morphologically or phylogenetically. The most comprehensive framework of morphology-based classification was established by Waterhouse (1963). She placed *Phytophthora* species into six groups based primarily on the papillation of sporangium, sexual type and antheridium configuration. This six-group system has since been the cornerstone in the classification of this important genus and individual groups are often referred to as the "Waterhouse Group" (Waterhouse, 1963). The first comprehensive phylogeny of *Phytophthora* species was constructed by Cooke *et al.* (2000) based on the rRNA internal transcribed spacer (ITS) sequences. This phylogeny consists of ten clades with a vast majority of the 48 *Phytophthora* species examined residing in clades 1 to 8. This 10-clade phylogeny is generally supported by subsequent analyses of many additional DNA regions (Martin & Tooley, 2003; Kroon *et al.*, 2004; Villa *et al.*, 2006; Blair *et al.*, 2008; Robideau *et al.*, 2011; Runge *et al.*, 2011). The main radiation of the genus (clades 1 to 8) is considered as a mononphyletic group and each clade is also monophyletic with the exception of clades 4 and 9 (Blair *et al.*, 2008). However, the monophyly of *Phytophthora* genus has been challenged by the rapid increasing number of new species

and more recent phylogenetic analyses. Specifically, a multi-locus phylogenetic analysis shows that the genus *Phytophthora* is much more paraphyletic than previously thought (Runge *et al.*, 2011). This analysis supports the hypothesis that *Phytophthora* species and downy mildews are closely related (Brasier & Hansen, 1992).

The relationship between traditional morphology-based classification and modern phylogenetic taxonomy has been a central focus of several recent studies. Generally, species in the clades 1-5 and 10 as well as subclades 8b-c produce papillate to semipapillate and caducous sporangia, while those in clades 6, 7, and 9 plus subclade 8a produce nonpapillate and noncaducous sporangia (Blair *et al.*, 2008; Kroon *et al.*, 2012). This generalization, however, is challenged by recent discovery of new species. For examples, both *P. gibbosa* (Jung *et al.*, 2011) and *P. mississippiae* (Yang *et al.*, 2013) phylogenetically belong to clade 6, while *P. constricta* (Rea *et al.*, 2011) belongs to clade 9. These species produce semipapillate sporangia, in addition to nonpapillate sporangia typical of species in clades 6 and 9.

Among the most rapidly expanding phylogenetic groups of *Phytophthora* species is clade 9. Eleven of the fourteen species in this clade were described recently. These new species include *P. aquimorbida* (Hong *et al.*, 2012), *P. captiosa* and *P. fallax* (Dick *et al.*, 2006), *P. chrysanthemi* (Naher *et al.*, 2011), *P. constricta* (Rea *et al.*, 2011), *P. hydrogena* (Yang *et al.*, 2014), *P. hydropathica* (Hong *et al.*, 2010), *P. irrigata* (Hong *et al.*, 2008), *P. polonica* (Belbahri *et al.*, 2006), *P. parsiana* (Mostowfizadeh-Ghalamfarsa *et al.*, 2008), and *P. virginiana* (Yang & Hong, 2013). This paraphyletic clade (Blair *et al.*,

2008; Runge *et al.*, 2011) includes a cluster of species that appear tolerant of high temperature (Yang & Hong, 2013; Yang *et al.*, 2014). The origin and their interrelationship of these high temperature-tolerant species are unknown at this point (Yang *et al.*, 2014), but it is interesting that many of these species were first recovered from aquatic environments such as irrigation water (Hong *et al.*, 2008; Hong *et al.*, 2010; Hong *et al.*, 2012; Yang & Hong, 2013; Yang *et al.*, 2014).

In this study we describe two new species, *Phytophthora stricta* sp. nov. and *Phytophthora macilentosa* sp. nov., based on their unique morphological, physiological and molecular features. Isolates of the two species were recovered from aquatic environments in Mississippi and Virginia, USA. We also demonstrate that *P. stricta* represents a new clade in this important genus and *P. macilentosa* along with nine other species consistently forms a high temperature-tolerant cluster within clade 9.

Materials and Methods

Isolation and isolate maintenance

Isolates of *Phytophthora stricta* and *P. macilentosa* were recovered from irrigation reservoirs at an ornamental plant nursery of Mississippi in 2012 by baiting with rhododendron leaves. *Phytophthora stricta* was also recovered from stream water in Virginia. Rhododendron whole leaf baits were deployed in the surveyed reservoir and streams for 7 or 14 days, respectively, and then transferred to a laboratory. They were cut

into approximately 1 ×1 cm^2 sections and plated onto clarified V8 juice agar (CV8A)-P_{10}ARP containing pimaricin, ampicillin, rifampicin, and pentachloronitrobenzene (Jeffers & Martin, 1986; Ferguson & Jeffers, 1999). *Phytophthora* colonies emerging from the edge of bait leaf pieces were hyphal-tipped onto 20% CV8A to obtain pure cultures. Cultures were maintained on CV8A and blocks of fresh agar cultures were transferred into microtubes with sterile distilled water for long-term storage at 15℃. Four isolates of each novel species were used in morphological, physiological and sequence analyses. The holotype isolates were deposited at the American Type Culture Collection in Manassas, Virginia, USA.

DNA sequencing and sequence analyses

Isolates were grown in 20% V8 juice broth at room temperature (*c*. 23℃) for one week. Mycelial masses were harvested and lysed using a FastPrep®-24 system (MP Biomedicals, Santa Ana, CA). DNA was extracted as instructed using the DNeasy® Plant Mini kit (Qiagen, Valencia, CA). Amplifications were performed with forward primer ITS6 and reverse primer ITS4 (Cooke *et al.*, 2000) for the internal transcribed spacer (ITS) region covering ITS1, 5.8S rRNA gene, and ITS2, following previously described reaction mix recipe and PCR program (Kong *et al.*, 2003). Primers COXF4N and COXR4N were used to amplify the mitochondrial cytochrome *c* oxidase 1 (*cox* 1) gene (Kroon *et al.*, 2004). Sequencing was performed in both directions at the University of Kentucky Advanced Genetic Technologies Center (Lexington, KY) using the same primers. Sequences of both directions were visualized with Finch TV *v.* 1.4.0. and aligned using Clustal W.

Sequences generated in this study were compared with other selected sequences in GenBank (www.ncbi.nlm.nih.gov/genbank/) to determine the phylogenetic positions. Sequences were aligned using Clustal W. Phylogeny reconstruction was conducted in MEGA 5.1 (Tamura *et al.*, 2011) using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993) with 1,000 replications of bootstrap.

Morphology

Sporangia were produced by transferring agar plugs (10×10 mm^2) from actively growing cultures (< 30-day-old) on CV8A to Petri dishes containing 1.5% non-sterile soil water extract solution (SWE, 15 g of sandy loam soil in 1 L of distilled water). Mature sporangia developed overnight after incubating at room temperature under cool-white fluorescent light.

Mating type of eight isolates was determined by dual culture of each with an A^1 or A^2 mating type tester of *P. cinnamomi* on hemp seed agar (HSA). Selfed gametangia were produced on HSA using the polycarbonate membrane method (Ko, 1978; Gallegly & Hong, 2008) with *P. cinnamomi*, *P. cambivora*, *P. meadii*, *P. nicotianae*, and *P. cryptogea* isolates as mating type testers.

Fifty randomly selected mature sporangia were photographed with a Nikon Fujix Digital Camera HC-300Zi connected to a Nikon Labophot-2 microscope and measured for length

and width with Image-Pro® Plus *v*. 5.1.2.53. Gametangia produced in polycarbonate membrane tests were measured for the size of oogonia, oospores and antheridia.

Cardinal temperatures

Cardinal temperatures of four isolates for each species were assessed on CV8A and carrot agar (CA). Agar blocks (5 mm in diameter) taken from actively growing areas of 7-day-old cultures were placed on fresh media at the center of 10-cm Petri dishes. Triplicate dishes per isolate per temperature were placed in the dark at 5, 10, 15, 20, 25, 30, 35, and 40°C. Two perpendicular measurements of each colony were taken for *P. stricta* and *P. macilentosa* after 5- and 6-day incubation when the margins of the fastest growing colonies were about 1 cm away from the dish edge. The cardinal temperature tests were repeated once. Analysis of variance was performed using R statistical software *v*. 2.11.0 (R Core Team, 2012) to determine the difference in growth rates among temperatures and isolates within the same species as well as between media and repeating assays. Radial growths along with their standard errors were plotted against temperature using the gplots package *v*. 2.11.0 (Warnes *et al.*, 2012) in R.

Results

Sequence analyses and phylogenetic positions

All isolates of *P. stricta* produced 813-bp ITS sequences. Isolates 58A1, 58A2 and 58A4 have an identical ITS sequence, which differs from that of isolate 58A3 by 1 bp. The *cox* 1 sequences of all *P. stricta* isolates are identical. Among four *P. macilentosa* isolates, 58A5 and 58A6 have an identical 772-bp ITS sequence, which differs from that of isolates 58A7 and 58A8 by 1 bp. Isolates 58A5 and 58A6 also have an identical 867-bp *cox* 1 sequence, which differs from that of 58A7 and 58A8 by a single base mutation. The ITS and *cox* 1 sequences of *P. stricta* and *P. macilentosa* were distinct from those of all known *Phytophthora* species.

By the sequences of both ITS region (Figure 5.1) and *cox* 1 gene (Figure 5.2), *P. macilentosa* is placed in clade 9 of the *Phytophthora* genus. *Phytophthora stricta* is not clustered into any known clade of *Phytophthora* based on the ITS sequences (Figure 5.1). In the phylogenetic tree of 120 *Phytophthora* species based on ITS sequences, four *P. stricta* isolates form a distinct taxon which is basal to ITS clades 1-8 (Figure 5.1). In the phylogenetic tree of 80 *Phytophthora* species based on *cox* 1 sequences, *P. stricta* is not grouped in, but close to species within *Phytophthora* subclade 8a, including *P. sansomeana*, *P. medicaginis*, *P. drechsleri*, *P. cryptogea*, *P. erythroseptica* and *P. richardiae* (Figure 5.2). GenBank accession numbers of sequences used in the sequence analyses and alignments as well as uncompressed phylogenetic trees are available in TreeBASE (S14477).

Cardinal temperatures

Radial growth rates were essentially the same between two repeating temperature tests for *Phytophthora stricta* ($P = 1.00$) and *P. macilentosa* ($P = 0.92$). Also, there was no difference in growth rates among the four isolates of *P. stricta* ($P = 0.99$) and among those of *P. macilentosa* ($P = 0.49$). Thus, data from repeating tests were pooled and averages of four isolates for each species were used for subsequent analysis. Temperature growth relationships for *P. stricta* and *P. macilentosa* are shown in Figures 5.3A and 5.3B, respectively. *Phytophthora stricta* had a much higher growth rate on CA than on CV8A. The optimum temperature for its growth was 25℃ on CA and 30℃ on CV8A. Very limited growth of *P. stricta* was observed at 5℃ and no growth at 35 and 40℃. The optimum temperature for growth of *P. macilentosa* was 30℃ and it also grew well at 35℃ on both media. No growth of *P. macilentosa* occurred at 5℃ and little growth occurred at 40℃.

Taxonomy

Phytophthora stricta X. Yang, W. E. Copes and C. X. Hong, sp. nov. (Figures 5.4, 5.6A, B, C)

MycoBank MB 804933

Phytophthora stricta produced sporangia after submerging mycelial plugs in 1.5% soil water extract solution for approximately 18 hours. Sporangia were mostly ovoid (Figures 5.4A, B) to various shapes including limoniform (Figure 5.4C), obpyriform (Figure 5.4D), ellipsoid (Figure 5.4E), pyriform (Figure 5.4F), and obovoid (Figure 5.4G). They were nonpapillate and slightly caducous. Caducous sporangia usually had a long pedicel (Figure 5.4P) which averages 129.8 µm in length but sometimes had a short pedicel (Figure 5.4Q). All undisturbed sporangiophores had one to three constrictions (Figures 5.4B, C, H, J, O). These constriction sites located from just beneath the base of the sporangium (Figure 5.4C) to 350 µm away from the sporangial base (Figure 5.4O). Primary sporangia were terminal, averaged 61.8 µm in length and 35.3 µm in width. Secondary lateral sporangia were observed and mostly grew from the constricted section of sporangiophores (Figures 5.4G, H). Sporangia often had a conspicuous basal plug (Figures 5.4B, C, I, J, K, L). Nesting (Figure 5.4J) and internal proliferation (Figure 5.4K) were common. External proliferation was also observed (Figure 5.4L). Terminal chlamydospores were occasionally produced in aged cultures (> 60 days). They were thin-walled, average 34.7 µm in diameter, mostly spherical (Figure 5.4M) or peanut-shaped (Figure 5.4N).

Phytophthora stricta is heterothallic and all examined isolates are A^1. Gametangia were produced in dual cultures when *P. stricta* was paired with an A^2 mating type tester of *P. cinnamomi*. In polycarbonate membrane tests, selfed gametangia were observed after 20-day pairing with an A^2 mating type tester isolate of *P. meadii*. The oogonia of *P. stricta* were globose (Figures 5.6A, B, C) and averaged 29.3 µm in diameter, yet commonly

aborted (Figure 5.6C). Oogonial wall was pigmented to golden with maturation. Oospores were plerotic (Figures 5.6A, B, C) and averaged 24.5 µm in diameter and sometimes with two ooplasts (Figure 5.6B). Antheridia were amphigynous, often in a long cylindrical shape (Figures 5.6A, C), and averaged 18.6 µm in depth and 15.4 µm in width.

Holotype

ATCC MYA-4944 (exo-type: 58A1), recovered from the surface water in a nursery reservoir, Mississippi, USA, February, 2012. Other isolates examined: 58A2, 58A3, and 58A4, recovered from the same reservoir and depth at the same time; 45D6, from water of St. Mary River, Virginia, USA, May, 2007; 49D4, from water of Kennedy Creek, Virginia, USA, February, 2009.

Etymology

'*stricta*' refers to the constrictions of sporangiophores.

Phytophthora macilentosa X. Yang, W. E. Copes and C. X. Hong, sp. nov. (Figures 5.5, 5.6D, E, F)

MycoBank MB 804934

Phytophthora macilentosa produced abundant sporangia after submerging mycelial plugs in 1.5% soil water extract solution for approximately 15 hours. Sporangia were mostly elongated obpyriform (Figures 5.5A, B, C, D). Fifty-two percent of sporangia had a length to width (L:W) ratio greater than 2.0; 17% with a L:W ratio >2.5; and 5% with a L:W ratio >3.0. Sporangium shapes ranged from obpyriform (Figure 5.5E) to ovoid (Figure 5.5F), limoniform (Figure 5.5G), ellipsoid (Figure 5.5H), pyriform (Figures 5.5K, L) and distorted shapes such as peanut-shaped (Figure 5.5J) and peapod-shaped (Figure 5.5I). They were nonpapillate and noncaducous. Primary sporangia were mostly terminal, average 72.5 µm in length and 34.8 µm in width. Lateral sporangia were observed (Figure 5.5M). Direct germination of sporangia was observed (Figure 5.5N). Nesting (Figure 5.5P) and internal proliferation (Figure 5.5Q) were common. Hyphal swellings (Figures 5.5R, S, T) were common; some were catenulate (Figure 5.5S) or elongated with irregular shapes (Figure 5.5T), especially in aged cultures (> 60 days). Knobby and angular mycelia were often observed (Figure 5.5U). Hyphal aggregation was common. No chlamydospore was observed in this study.

Phytophthora macilentosa is self-sterile. Sexual bodies were produced in dual culture tests when paired with an A^2 tester of *P. cinnamomi*, suggesting that four isolates of *P. macilentosa* are A^1. Selfed gametangia of *P. macilentosa* were induced by an A^2 mating type isolate of *P. nicotianae* in polycarbonate membrane tests after a 20-day pairing. Oogonia were spherical, thick-walled, became golden to dark brown at maturity (Figures 5.6D, E, F), averaged 35.4 µm in diameter, commonly aborted (Figure 5.6F). Oospores

were mostly aplerotic (Figures 5.6D, E, F) and averaged 26.3 µm in diameter. Antheridia were amphigynous, mostly globose (Figures 5.6E, F), and averaged 11.5 µm in diameter.

Holotype

ATCC MYA-4945 (exo-type: 58A7), recovered from the middle of water column in a nursery reservoir, Mississippi, USA, February, 2012. Other isolates were recovered from the bottom (58A5 and 58A8) and middle (58A6) of water column in the same reservoir at the same time.

Etymology

'*macilentosa*' refers to the elongated sporangia with great length to width ratio frequently produced by this species.

Discussion

Two new species, *Phytophthora stricta* and *P. macilentosa*, were described based on morphological, physiological and sequence analyses in this study. *Phytophthora stricta* represents a new clade in the genus *Phytophthora*. *Phytophthora macilentosa*, along with nine other species, consistently forms a high temperature-tolerant cluster within clade 9. These findings constitute important stepping stones towards understanding the diversity and biology of *Phytophthora* species. Specifically, they will aid first responders in

correctly diagnosing diseases caused by either novel species, while reducing the risk in misidentification of high-impact pathogens such as *P. ramorum* (Rizzo et al. 2002)(Rizzo *et al.*, 2002) and *P. kernoviae* (Brasier *et al.*, 2005).

Morphological and physiological distinctions of *P. stricta* and *P. macilentosa*

Both *P. stricta* and *P. macilentosa* can be readily distinguished from all other *Phytophthora* species by their unique morphological characteristics. Besides *P. stricta,* only *P. pinifolia* (Duran *et al.*, 2008) and *P. constricta* (Rea *et al.*, 2011) produce slightly caducous sporangia and constricted sporangiophores. *Phytophthora stricta* can be differentiated from *P. constricta* by its heterothallism and nonpapillate sporangia. Also, the constrictions on sporangiophores are near the base of sporangium for *P. pinifolia* (Duran *et al.*, 2008) and *P. constricta* (Rea *et al.*, 2011), whereas the majority of those are >100 µm away from the sporangial base for *P. stricta*. In addition, 2 to 3 constrictions in single sporangiophores are common for *P. stricta* but not for the other species. Furthermore, internal and external proliferations in sporangium production are common for *P. stricta*, but not for *P. pinifolia* (Duran *et al.*, 2008).

Phytophthora macilentosa also has distinct morphological and physiological attributes. *Phytophthora elongata* (Rea *et al.*, 2010) is the only other species producing abundant elongated sporangia than *P. macilentosa*. These two species are distinct in sexual behavior, sporangial papillation and temperature response. *Phytophthora macilentosa* is heterothallic and produces nonpapillate sporangia, whereas *P. elongata* is homothallic

and produces semipapillate sporangia. Also, the optimum growth temperature is 30℃ for *P. macilentosa*, whereas it is 25℃ for *P. elongata* (Rea *et al.*, 2010).

A new clade represented by *P. stricta*

Several lines of evidence support *P. stricta* representing a previously unknown clade in the genus *Phytophthora*. First, the phylogenetic analysis of 120 species based on ITS sequences placed *P. stricta* in a distinct position that is basal to clades 1 to 8 but apart from clades 9 and 10 (Figure 5.1). *Phytophthora stricta* differs from its closest relatives: *P. sansomeana* (GenBank accession no. GU258782), *P. medicaginis* (DQ821174), and *P. cryptogea* (HQ643216) of subclade 8a in the ITS sequence by 98 bp, 99 bp, and 116 bp, respectively. Second, the analysis of *cox* 1 sequences also showed that *P. stricta* is close to but not clustered in subclade 8a (Figure 5.2). Third, *P. stricta* produces slightly caducous sporangia, representing an intermediate between subclade 8a with species producing noncaducous sporangia and those clades with species producing caducous sporangia. Fourth, constricted sporangiophores of *P. stricta* also evidence its transitional morphological status, evolving to be caducous or degenerating to be noncaducous. The discovery of *P. stricta* in this study presents a challenge to the current 10-clade phylogeny of *Phytophthora* species (Cooke *et al.*, 2000; Kroon *et al.*, 2004; Blair *et al.*, 2008; Kroon *et al.*, 2012; Martin *et al.*, 2012). A new phylogeny incorporating this new clade is warranted. Further phylogenetic analyses are underway to reconstruct the phylogeny for the genus *Phytophthora*.

A high temperature-tolerant cluster represented by *P. macilentosa*

Phytophthora macilentosa, along with nine other species, consistently forms a high temperature-tolerant cluster within clade 9 (Figures 5.1 and 5.2). This cluster currently includes *P. aquimorbida* (Hong *et al.*, 2012), *P. chrysanthemi* (Naher *et al.*, 2011), *P. hydrogena* (Yang *et al.*, 2014), *P. hydropathica* (Hong *et al.*, 2010), *P. insolita* (Ann & Ko, 1980), *P. irrigata* (Hong *et al.*, 2008), *P. parsiana* (Mostowfizadeh-Ghalamfarsa *et al.*, 2008), *P. polonica* (Belbahri *et al.*, 2006), and *P. virginiana* (Yang & Hong, 2013). *Phytophthora macilentosa* is the latest addition to this cluster. These species have the optimum growth temperature at ~ 30℃ and all grow well at 35℃. This strong affinity between the physiological feature and phylogenetic position has not been found in any other clade of *Phytophthora.* This monophyly is supported by strong bootstrap (99%) in the sequence analysis of the ITS region (Figure 5.1), but to a lesser degree by the *cox* 1 sequences (Figure 5.2). Further analyses are warranted to pin down the phylogenetic relationships among these high temperature-tolerant species.

Origins and potential impacts of the two novel species

Four isolates each of *P. stricta* and *P. macilentosa* examined in this study were recovered from an irrigation reservoir in Mississippi, USA. It was interesting that all four isolates of *P. stricta* were from the surface water (10 cm) while those of *P. macilentosa* were from either middle (100 cm) or bottom (175 cm) depths of the water column in the same

reservoir. *Phytophthora stricta* also was isolated from two streams during a statewide survey in Virginia, USA. Neither species has been recovered from diseased plants in the same nursery and the stream surrounding areas. These observations, however, do not necessarily mean that both new species described in this study present low risk to ornamental crops. This is particularly true in cases when these species might be accidentally introduced into new areas. Investigations into their pathogenicity and host range are needed before the potential threats posed by *P. stricta* and *P. macilentosa* to horticultural crops locally and plant biosecurity globally can be assessed.

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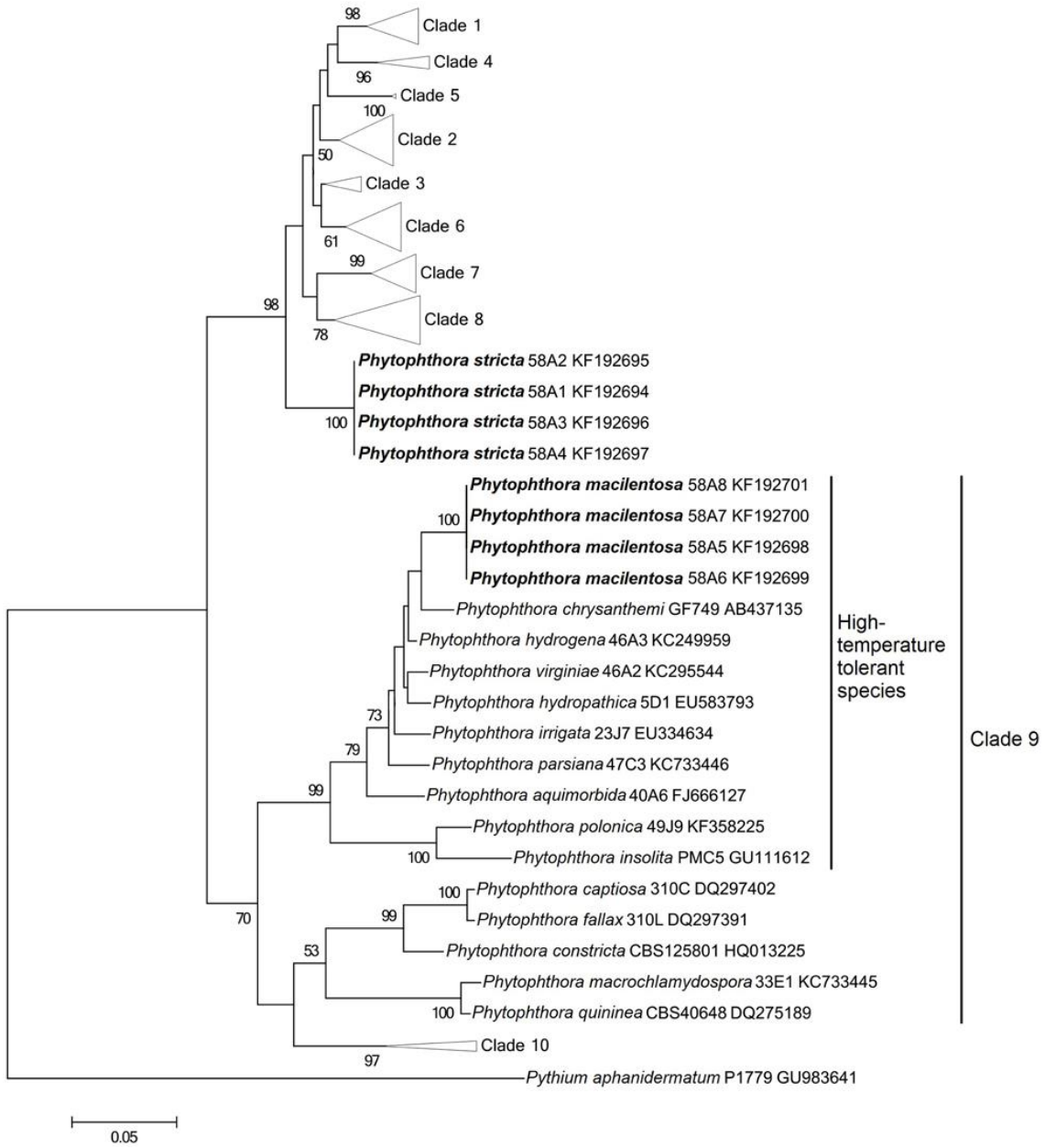

Figure 5.1 Maximum likelihood phylogeny based on ITS sequences. Alignment was conducted with Clustal W and the phylogenetic tree was generated and compressed in MEGA5. Bootstrap values less than 50% are not shown. GenBank accession numbers of sequences are given following the species names and isolate codes. An uncompressed tree is available in TreeBASE (S14477).

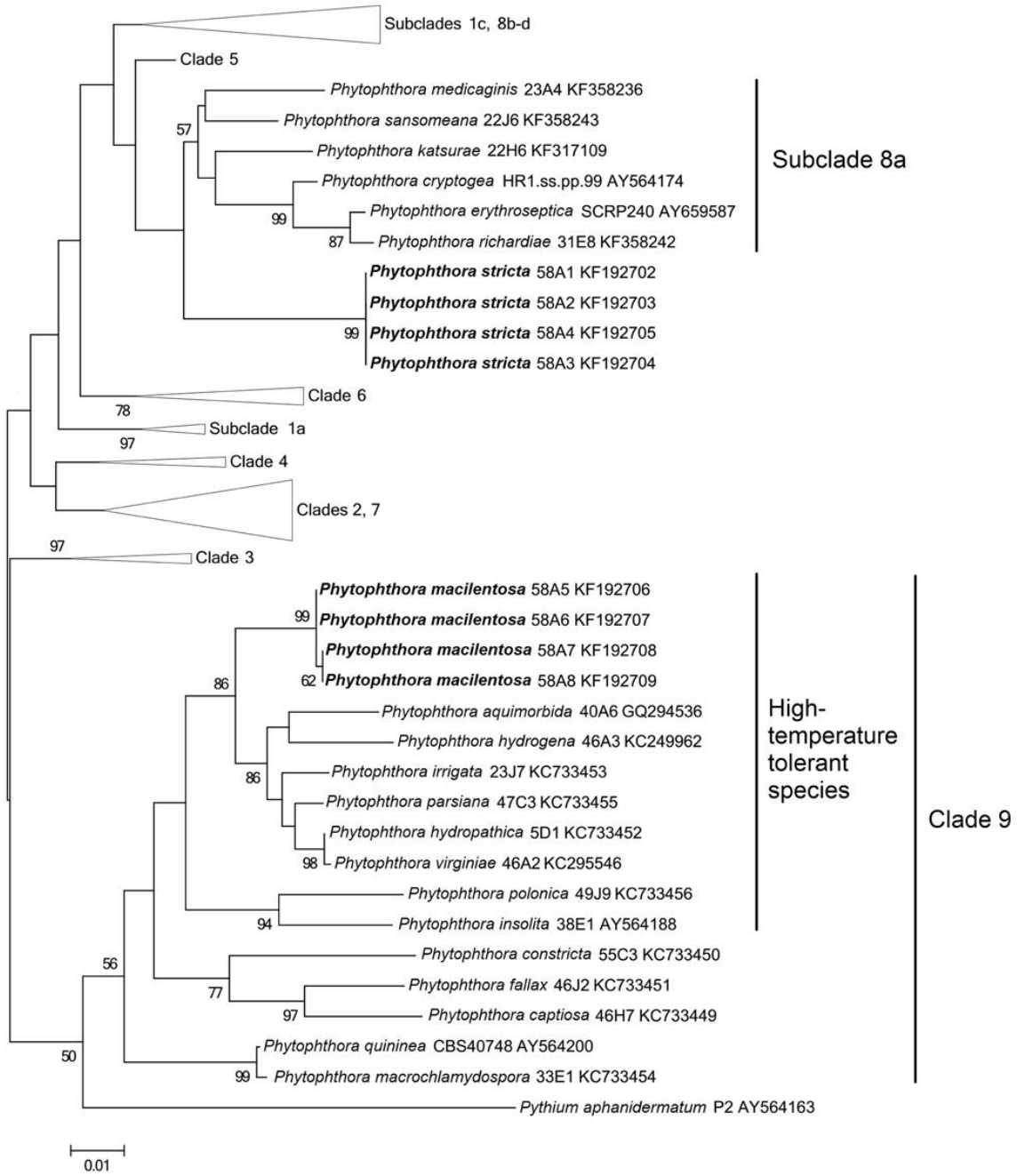

Figure 5.2 Maximum likelihood phylogeny based on *cox* 1 sequences. Alignment was conducted with Clustal W and the phylogenetic tree was generated and compressed in MEGA5. Bootstrap values less than 50% are not shown. GenBank accession numbers of sequences are given following the species names and isolate codes. An uncompressed tree is available in TreeBASE (S14477).

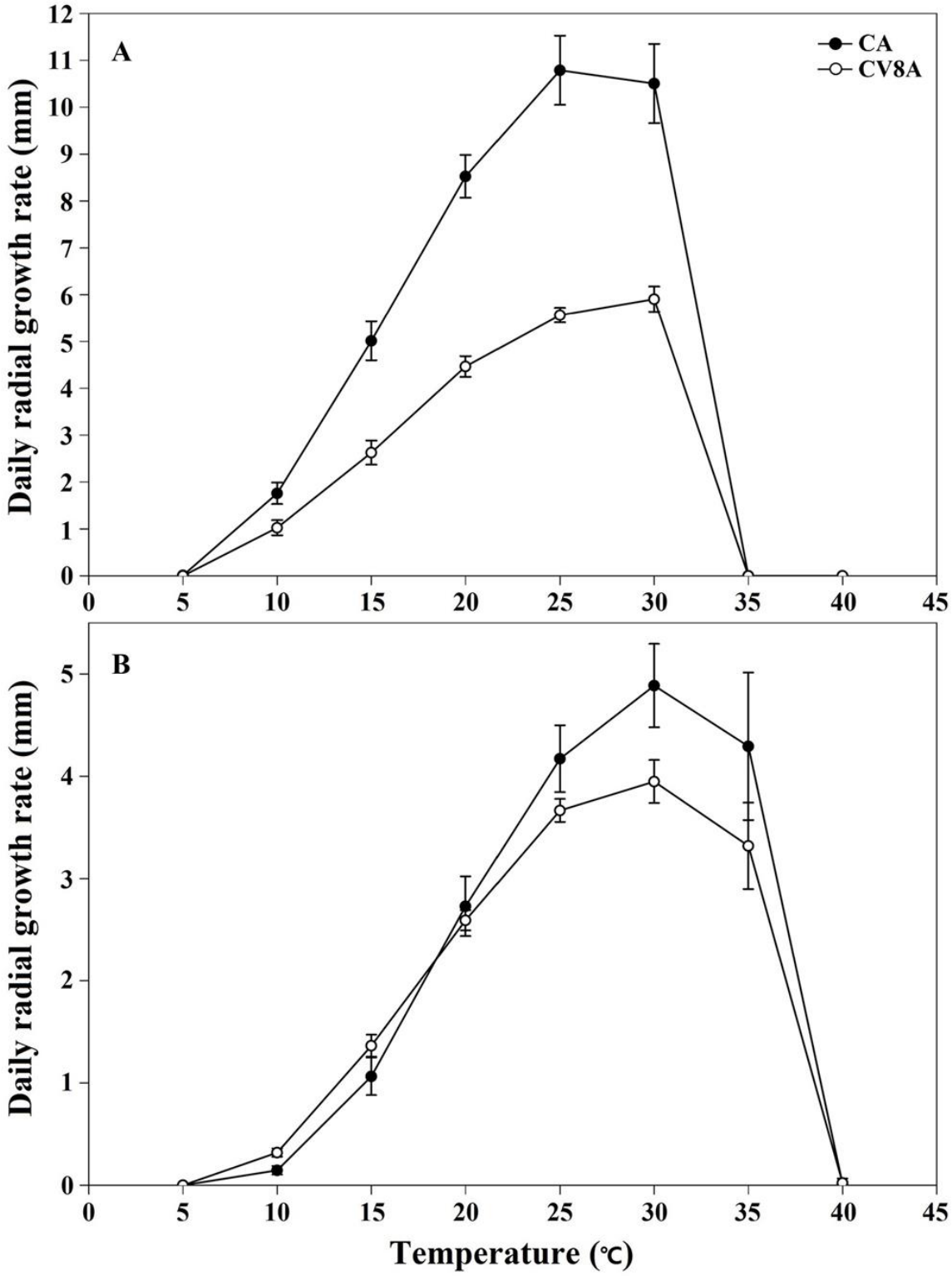

Figure 5.3 Daily radial growth of (A) *Phytophthora stricta* and (B) *P. macilentosa* on carrot agar (CA) and 20% clarified V8 juice agar (CV8A).

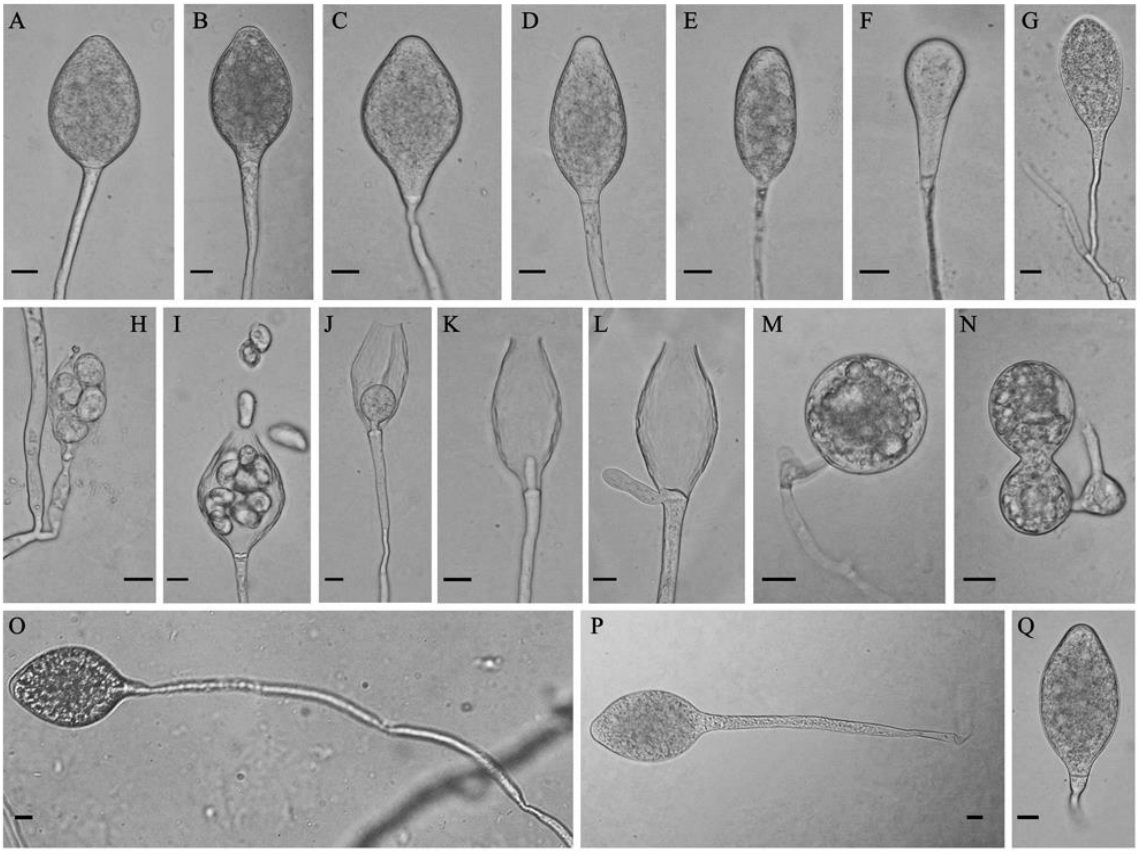

Figure 5.4 Morphology of asexual structures of *Phytophthora stricta*. A-G: Nonpapillate sporangia in various shapes. A: An ovoid sporangium. B: An ovoid sporangium with a conspicuous basal plug and a constriction of the sporangiophore. C: A limoniform sporangium with a conspicuous basal plug and a constriction near the base. D: An obpyriform sporangium. E: An ellipsoid sporangium. F: A pyriform sporangium. G: An obovoid, lateral, secondary sporangium grown from the constriction of a sporangiophore. H: A lateral, secondary sporangium containing four zoospores, grown from the constriction of a sporangiophore. I: A sporangium releasing zoospores. J: A sporangium with nested proliferation resembling a basal plug and a constriction of the sporangiophore. K: Internal proliferation resembling a basal plug. L: External proliferation with a basal plug. M: A globose, terminal chlamydospore. N: A peanut-shaped, terminal chlamydospore. O: A sporangiophore with three constrictions. P: A caducous sporangium with a long pedicel. Q: A caducous sporangium with a short pedicel. Bars = 10 μm.

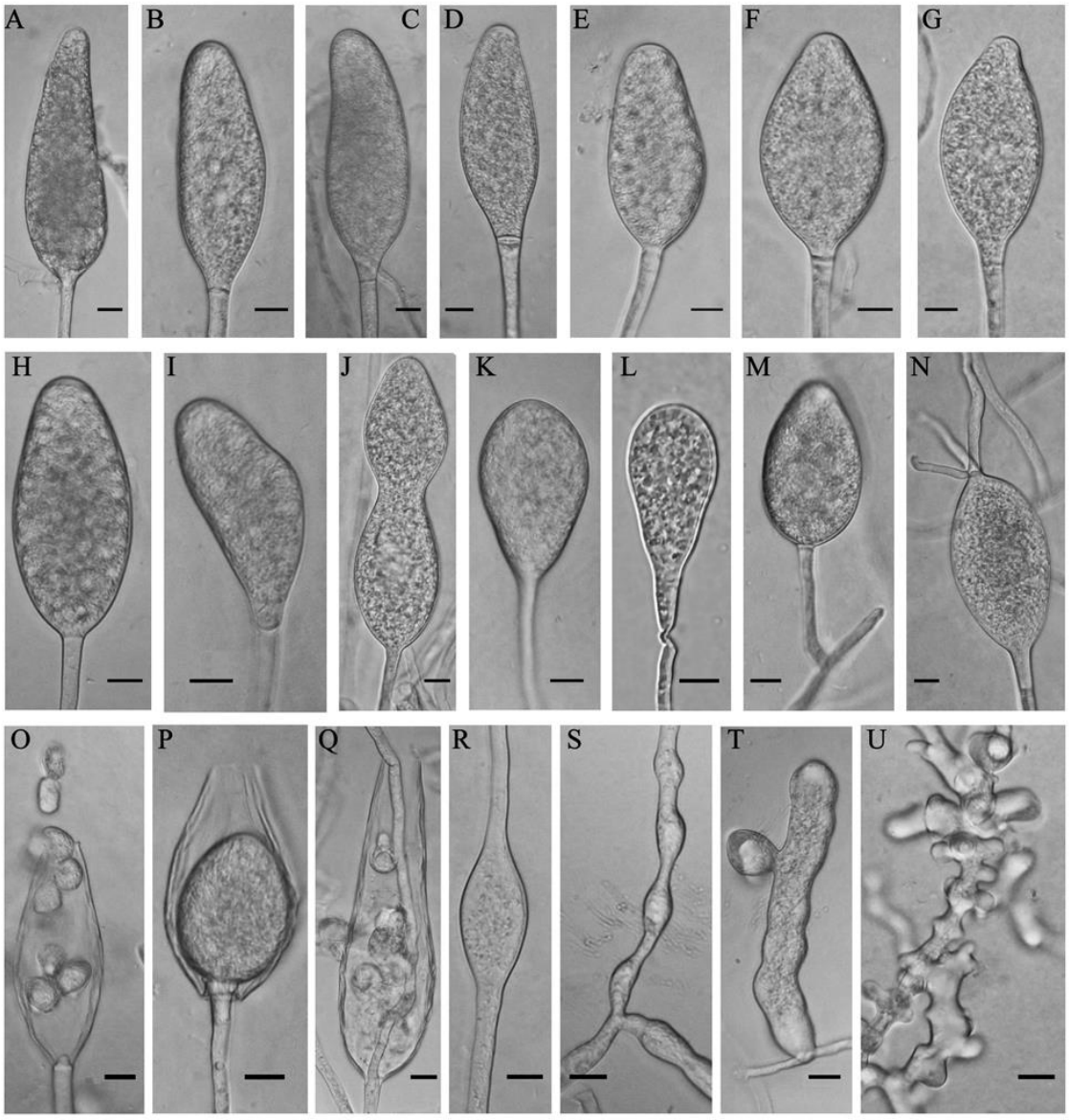

Figure 5.5 Morphology of asexual structures of *Phytophthora macilentosa*. A-D: Elongated obpyriform sporangia produced by four isolates. E: An obpyriform sporangium. F: An ovoid sporangium. G: A limoniform sporangium. H: An ellipsoid sporangium. I: A peapod-shaped sporangium. J: An elongated, peanut-shaped sporangium. K, L: Pyriform sporangia. M: A lateral ovoid sporangium. N: Direct germination of a sporangium. O: A sporangium with a basal plug, releasing zoospores. P: Nested proliferation. Q: Extended, internal proliferation. R: Intercalary swelling. S: Catenulate hyphal swellings. T: Elongated, irregular swellings. U: Knobby and angular mycelia. Bars = 10 µm.

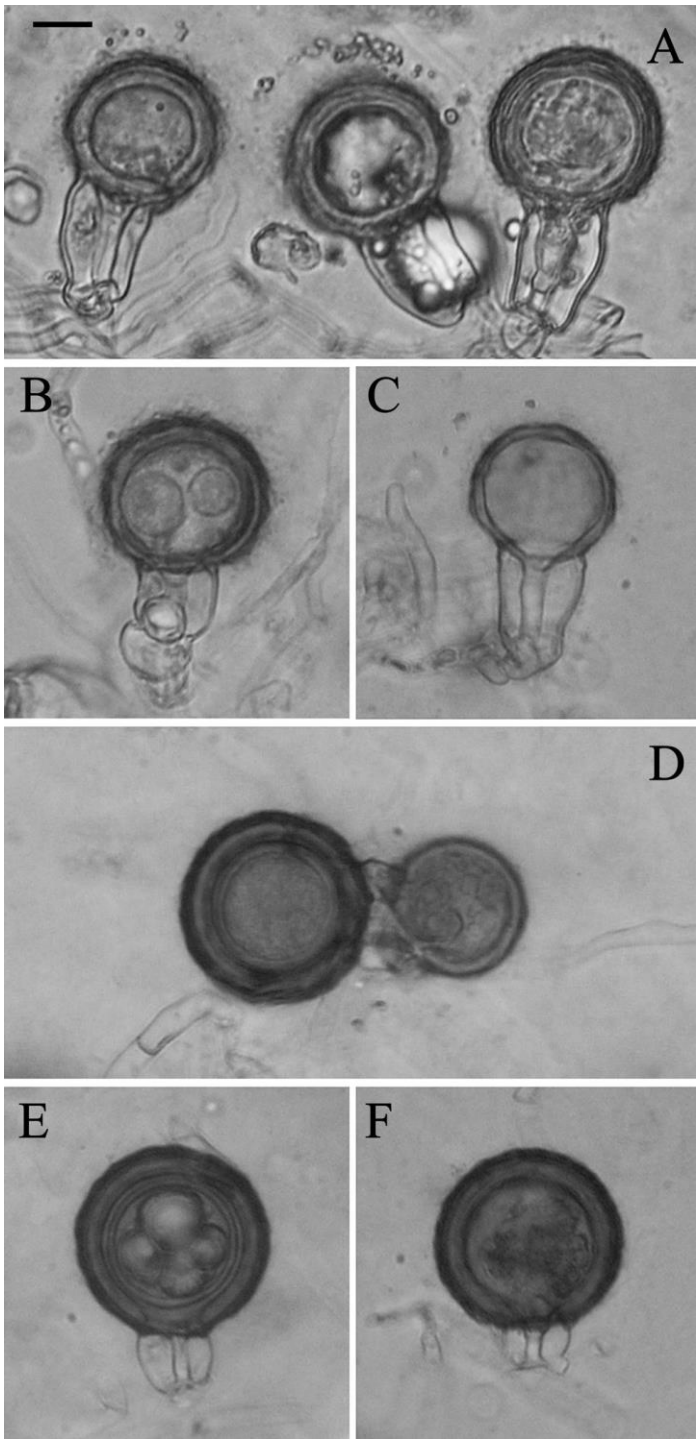

Figure 5.6 Gametangia of (A-C) *Phytophthora stricta* and (D-F) *P. macilentosa*. A: Three plerotic oogonia with amphigynous, cylindrical-shaped antheridia. B: An plerotic oogonium containing two ooplasts. C: An aborted oogonium and a long antheridium. D: A dark-brown pigmented, aplerotic oogonium and an immature gametangium. E: An aplerotic oogonium with an amphigynous, globose antheridium. F: An aborted oogonium with a globose antheridium. Bar = 10 µm.

Chapter 6 *Phytophthora* ×*stagnum* nothosp. nov., a new hybrid from irrigation reservoirs at ornamental plant nurseries in Virginia

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Abstract

A novel *Phytophthora* species was frequently recovered from irrigation reservoirs at several ornamental plant production facilities in eastern Virginia. Initial sequencing of the internal transcribed spacer (ITS) region of this species generated unreadable sequences due to continual polymorphic positions. Cloning and sequencing the ITS region as well as sequencing the mitochondrially encoded cytochrome *c* oxidase 1 and beta-tubulin genes revealed that it is a hybrid between *P.* taxon PgChlamydo as its paternal parent and an unknown species genetically close to *P. mississippiae* as its maternal parent. This hybrid has some diagnostic morphological features of *P.* taxon PgChlamydo and *P. mississippiae*. It produces catenulate hyphal swellings, characteristic of *P. mississippiae*, and chlamydospores, typical of *P.* taxon PgChlamydo. It also produces both ornamented and relatively smooth-walled oogonia. Ornamented oogonia are another important diagnostic character of *P. mississippiae*. The relatively smooth-walled oogonia may be

indicative of oogonial character of *P.* taxon PgChlamydo. The new hybrid is described here as *Phytophthora* ×*stagnum*.

Introduction

The genus *Phytophthora* includes many agriculturally and ecologically important plant pathogens. It currently contains approximately 120 species (Martin *et al.*, 2012). These species were traditionally divided into six groups by morphological features (Waterhouse, 1963). They have been classified into 10 clades according to phylogenetic analyses of nuclear and mitochondrial sequences (Cooke *et al.*, 2000; Martin & Tooley, 2003; Kroon *et al.*, 2004; Villa *et al.*, 2006; Blair *et al.*, 2008; Robideau *et al.*, 2011). Members of this genus are capable of surviving in a variety of terrestrial and aquatic habitats (Erwin & Ribeiro, 1996). However, species in certain clades or subclades are better adapted to specific ecosystems. For example, most clade 1 species such as *P. infestans* (Erwin & Ribeiro, 1996) and *P. hedraiandra* (de Cock & Lévesque, 2004) appear as terrestrial pathogens which attack above-ground plant tissues, while many species in subclade 6b and clade 9 are often associated with aquatic environments such as irrigation reservoirs (Hong *et al.*, 2008; Hong *et al.*, 2010; Hong *et al.*, 2012; Yang *et al.*, 2013; Yang & Hong, 2013; Yang *et al.*, 2014; Yang *et al.*, 2014), rivers and riparian ecosystems (Brasier *et al.*, 2003; Jung *et al.*, 2011).

Even though *Phytophthora* species were among the earliest described plant pathogens, investigations into *Phytophthora* interspecific hybridization were initiated only recently.

One of the first studies describing this phenomenon was conducted in 1991 which revealed that some isolates initially assigned as *P. meadii* were actually polyploid and might be hybrids based on cytological evidence (Sansome *et al.*, 1991). Thereafter, several artificial hybrids: *P. infestans*×*P. mirabilis*, *P. nicotianae*×*P. capsici*, *P. sojae* ×*P. vignae*, and *P. capsici*×*P. tropicalis* have been produced by pairing in dual culture (Goodwin & Fry, 1994; May *et al.*, 2003; Donahoo & Lamour, 2008), zoospore fusion (Ersek *et al.*, 1995; English *et al.*, 1999), and nuclear transplantation (Gu & Ko, 2001). In the meanwhile, 10 natural *Phytophthora* hybrids have been reported. These include *P.* × *pelgrandis* (*P. nicotianae*×*P. cactorum*) (Man in't Veld *et al.*, 1998; Bonants *et al.*, 2000; Nirenberg *et al.*, 2009; Man in't Veld *et al.*, 2012), *P. alni* including three subspecies: *P. alni* subsp. *alni*, *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis* (Brasier *et al.*, 1999; Brasier *et al.*, 2004; Ioos *et al.*, 2006), *P. andina* with *P. infestans* as one parent (Gomez-Alpizar *et al.*, 2008; Oliva *et al.*, 2010; Goss *et al.*, 2011), *P.* × *serendipita* (*P. cactorum*×*P. hedraiandra*) (Man in't Veld *et al.*, 2007; Man in't Veld *et al.*, 2012), four hybrids in subclade 6b: *P. amnicola*×*P.* taxon PgChlamydo (A-PG), *P.* taxon PgChlamydo×*P. amnicola* (PG-A), *P. thermophila*×*P. amnicola* (T-A), and *P. thermophila*×*P.* taxon PgChlamydo (T-PG) (Nagel *et al.*, 2013), as well as three hybrids in subclades 8b: *P. porri*×*P.* taxon parsley, *P. porri*×a *P. primulae*-like species, and a third hybrid with two unknown species as parents (Bertier *et al.*, 2013). It is interesting to note that parents of most individual hybrids belong to the same *Phytophthora* clade. The only inter-clade hybrid is *P. nicotianae* (clade 1)×*P. capsici* (clade 2), which was

produced by zoospore fusion (Ersek *et al.*, 1995; English *et al.*, 1999) and nuclear transplantation (Gu & Ko, 2001).

A number of *Phytophthora* hybrids are emerging plant pathogens. By inheriting and recombining alleles or genes from both parents followed by rapid evolution (Brasier, 2000; Brasier, 2001; Bertier *et al.*, 2013), these hybrids have broader host ranges (Bertier *et al.*, 2013) and produce new virulence factors with higher aggressiveness, while overcoming weaknesses of their parental species. For example, *P. alni* and its variants are destructive pathogens that have killed more than 10,000 riparian *Alnus* trees in Europe in 1996 alone (Brasier *et al.*, 1999). *Phytophthora ×pelgrandis* was found infecting plants in the genera of *Cyclamen, Eriobotrya, Lavandula, Lewisia, Pelargonium, Primula,* and *Spathiphyllum* in the Netherlands, Germany, Italy, Peru and Taiwan (Man in't Veld *et al.*, 1998; Bonants *et al.*, 2000; Hurtado-Gonzales *et al.*, 2009; Nirenberg *et al.*, 2009; Man in't Veld *et al.*, 2012; Faedda *et al.*, 2013). *Phytophthora ×serendipita* has been isolated from hosts in the genera of *Idesia*, *Penstemon*, *Allium*, *Rhododendron*, *Kalmia*, and *Dicentra* in Europe and the United States, while its parent *P. hedraiandra* only infects *Rhododendron* and *Viburnum* species, indicating this emerging hybrid pathogen has successfully utilized new habitats and adapted to novel hosts (Man in't Veld *et al.*, 2007; Man in't Veld *et al.*, 2012). *Phytophthora porri×P.* taxon parsley in subclade 8b has shown a similar expansion of host range including *Allium victorialis, Allium grayi*, *Pastinaca sativa, Chrysanthemum* species, and *Parthenium argentatum*, while its parents only infect leek and parsley (Bertier *et al.*, 2013). Although their host ranges are unknown, the four subclade 6b hybrids A-PG, PG-A, T-A, and T-PG, which originated in

Australia, have exploited new habitats in South Africa (Nagel *et al.*, 2013). It must be noted that sexual reproduction of most *Phytophthora* hybrids is compromised due to their nature of allopolyploidy and resulting genetic incompatibility. Most *Phytophthora* hybrids are sterile, nonfunctional in meiosis, or produce numerous abortive oospores (Brasier *et al.*, 2004; Man in't Veld *et al.*, 2012; Bertier *et al.*, 2013; Nagel *et al.*, 2013).

Since 2005 we have obtained more than twenty isolates of a previously unknown *Phytophthora* species from irrigation systems. It has distinct morphology from all known species. Also, continual polymorphic sequences in the internal transcribed spacers (ITS) region of all isolates suggest that this is a *Phytophthora* hybrid. Here, we examine and describe its morphological, physiological and molecular characters and name this new hybrid as *Phytophthora* ×*stagnum* nothosp. nov.

Materials and Methods

Isolate collection and maintenance

Phytophthora ×*stagnum* isolates were recovered from irrigation runoff containment basins of several private ornamental plant nurseries in eastern Virginia, USA, by baiting with rhododendron leaves. Pure cultures were obtained by subculturing hyphal tips of colonies emerging from the edge of leaf baits followed by single-spore isolation (Erwin & Ribeiro, 1996). They were maintained and routinely subcultured onto 20% clarified V8 juice agar (CV8A) in the present study. Agar blocks with actively growing cultures in

CV8A were transferred into microtubes with sterile distilled water for long-term storage at 15℃. The holotype was deposited at the American Type Culture Collection (MYA-4926) in Manassas, Virginia.

DNA extraction

Four representative isolates, 36H8, 36J7, 43F3, and 44F9, were grown in 20% clarified V8 broth at room temperature (*c*. 23℃) for 7 days to produce mycelial masses which were then dried and lysed using a FastPrep®-24 system (MP Biomedicals, Santa Ana, CA, USA). DNA was extracted using the DNeasy® Plant Mini kit (Qiagen, Valencia, CA, USA).

Sequence analysis of the maternally-inherited *cox* 1 genes

To elucidate the maternal parent of *P.* ×*stagnum*, primers COXF4N and COXR4N (Kroon *et al.*, 2004) were used to amplify the maternal-inherited mitochondrial cytochrome *c* oxidase 1 (*cox* 1) gene. Sequences in both directions were visualized with Finch TV *v*. 1.4.0 (Geospiza Inc., Seattle, WA, USA), aligned using Clustal W and edited manually to correct obvious errors. The *cox* 1 sequences were aligned using MAFFT online version 7 (Katoh & Standley, 2013) and the G-INS-I algorithm (Katoh *et al.*, 2005). Maximum likelihood (ML) inference was carried out with MEGA5.1 (Tamura *et al.*, 2011) using the Tamura-Nei model (Tamura & Nei, 1993) with 1,000 bootstrap replicates. *Pythium aphanidermatum* was used as an outgroup.

Sequence analyses of ITS and beta-tubulin genes

To investigate the parentage of *P.* ×*stagnum*, cloned ITS region and the single-copy beta-tubulin genes were sequenced and analyzed.

PCR amplifications were performed using the forward primer ITS6 and reverse primer ITS4 (Cooke *et al.*, 2000) for the ITS region. Amplification products were cloned into a pGEM-T Easy Vector System, which was then transformed into *Escherichia coli* competent JM109 cells (Promega, Madison, WI, USA). The cells were plated on Luria-Bertani (LB) agar (Becton, Dickinson and Company, Sparks, MD, USA) amended with ampicillin and ChromoMax IPTG/X-Gal Solution (Fisher Scientific, USA) and incubated at 37℃. Transformed cells with recombinant plasmids were identified by blue-white screening, subcultured into 2-mL centrifuge tubes containing 1.5 mL LB broth using toothpicks, and incubated overnight at 37℃ with moderate shaking. Plasmid DNA was extracted from the liquid cultures using the Alkaline Lysis with SDS: Minipreparation method (Sambrook & Russell, 2006). The ITS primer pair 6F/4R was used to amplify the plasmid DNA. A total of 94 amplification products including 23, 23, 25, and 23 from isolates 36H8, 36J7, 43F3, and 44F9, respectively, were purified and sequenced at the University of Kentucky Advanced Genetic Technologies Center (Lexington, KY, USA) in both directions using the same ITS primer pair.

Primers Btub_F1 and Btub_R1 (Blair *et al.*, 2008) were used to amplify the single-copy beta-tubulin gene. To analyze hybrid characteristic of *P.* ×*stagnum*, edited sequences were compared to those of putative parent species. Alignments were done with Clustal W.

Colony morphology

To examine colony morphology, cultures of four representative isolates were grown on carrot agar (CA), CV8A, malt extract agar (MEA), and potato dextrose agar (PDA). Colony patterns were photographed after incubation for 10 days in the dark at 20℃.

Cardinal temperatures

Representative isolates were examined for their cardinal temperatures on CA and CV8A. Agar blocks (5 mm in diameter) taken from actively-growing areas of 10-day old cultures were placed at the center of 10-cm Petri dishes with freshly made media. Triplicate dishes per isolate per temperature were placed in the dark at 5, 10, 15, 20, 25, 30, 35, and 40℃. Two perpendicular measurements of each colony were taken after 8 days. The cardinal temperature test was repeated once. Means of radial growth along with standard errors were plotted against temperature with the gplot package 2.11.0 (Warnes *et al.*, 2012) in R statistical software 2.15.0 (R Core Team, 2012). Analysis of variance was also conducted with R to determine the differences in radial growth measurements between repeated experiments and among representative isolates.

Morphology

Sporangia of *Phytophthora* ×*stagnum* were produced by transferring agar plugs (10×10 mm) from actively growing cultures on CV8A to Petri dishes containing non-sterile, soil water extract (SWE, 15 g of sandy loam soil/1 L water). Mature sporangia developed after incubating at room temperature under cool-white fluorescent light. Chlamydospores were produced in aged cultures in CV8A (after >30 days).

The mating type of representative isolates was determined in dual culture with an A^1 or A^2 tester of *P. cinnamomi* on CV8A. Selfed gametangia of *P.* ×*stagnum* were induced in polycarbonate membrane tests with an opposite mating type tester of *P. nicotianae* using hemp seed agar (HSA) (Ko, 1978; Gallegly & Hong, 2008).

Asexual and sexual bodies were photographed with a Nikon Fujix Digital Camera HC-300Zi connected to a Nikon Labophot-2 microscope. More than 50 randomly selected mature sporangia per isolate, more than 30 chlamydospores and all observed gametangia were measured using Image-Pro Plus *v*. 5.1.2.53.

Results

Sequence analysis of *cox* 1 gene

All four representative isolates of *P.* ×*stagnum* produced an identical 867-bp *cox* 1 sequence, which is distinct from those of all known *Phytophthora* species. This new species and *P. mississippiae* isolate 57J3 (GenBank Accession No. KF112860) differ by 18 bp in the alignment of *cox* 1 sequences. In the ML phylogenetic tree based on *cox* 1 sequences of *P.* ×*stagnum* and other selected species, *P.* ×*stagnum* isolates clustered in a distinct taxon which is closely related to *P. mississippiae* (Figure 6.1), indicating the maternal parent of *P.* ×*stagnum* is genetically close to *P. mississippiae*.

Sequence analysis of ITS clones

Among the 94 clones of the ITS region, 61 resulted in high-quality sequences. These included 16, 16, 16, and 13 from isolates 36H8, 36J7, 43F3, and 44F9, respectively. In the alignment of these 61 ITS sequences, 35 rare single-nucleotide polymorphism (SNP) sites occurred at low frequencies (~1/61). These rare SNPs were mostly intraspecific polymorphisms of parent species of *P.* ×*stagnum*. We also observed six frequent SNPs (four in ITS1 region and two in ITS2 region) and three indels in the ITS1 region (Table 6.1) at high frequencies (10–31/61).

These 61 clones can be generally grouped into three types by the six frequent SNPs and three indels in the ITS sequence. Thirty-one clones produced two types of 816-bp sequences while the other 30 clones produced an identical 819-bp sequence. According to the sequence alignment (Table 6.1), the two 816-bp sequences are 99% identical to that of *P. mississippiae*, while the 819-bp sequence is ~ 100% identical to those of *P.* taxon

PgChlamydo in GenBank (www.ncbi.nlm.gov/genbank/). Clones of individual representative isolates produced all ITS sequence types.

Sequence analysis of beta-tubulin gene

Isolates 36H8, 36J7, and 44F9 resulted in an identical 1124-bp beta-tubulin sequence with 26 polymorphic positions (Table 6.2). Isolate 43F3 also produced a 1124-bp sequence with 24 of the same 26 polymorphic positions. In spite of the polymorphic positions, beta-tubulin sequences of *P.* ×*stagnum* are identical to that of *P.* taxon PgChlamydo and 11 bp different from that of *P. mississippiae* (Table 6.2). Sequences of *P.* taxon PgChlamydo and *P. mississippiae* are distinct and both occur at 18 of the 26 polymorphic positions of *P.* ×*stagnum* containing ambiguous sequences such as positions 99, 102, and 261 (Table 6.2). At the other eight polymorphic positions such as positions 93, 306, and 450, both species share the same sequences which also occur as one of the ambiguous polymorphic sequences of *P.* ×*stagnum* (Table 6.2). Putative sequences of the maternal parent of *P.* ×*stagnum* are shown in table 6.2. The maternal parent is approximately 21 bp different from *P. mississippiae* in beta-tubulin sequence.

Colony morphology

The four representative isolates had a similar growth pattern after 10-days incubation in the dark at 20℃ (Figure 6.2). Colony pattern on CA and CV8A was stellate to radiate

with a relatively smooth edge and abundant aerial mycelia at the center. Colony pattern on MEA and PDA was rosaceous except isolate 43F3, which produced a slightly cottony colony on PDA. Colony growth of all isolates was slowest on MEA among tested media.

Cardinal temperatures for vegetative growth

Radial growth rates were similar among four representative isolates ($P = 0.71$) and between two cardinal temperature tests ($P = 0.74$). Thus, data from the repeated tests were pooled and averages were plotted against temperature (Figure 6.3). The optimum temperature for the vegetative growth of *P.* ×*stagnum* in both media was 25℃. It also grew well at 30℃ on both media. Limited growth occurred at 5 and 35℃. No growth was observed at 40℃.

Taxonomy

Phytophthora ×*stagnum*. X. Yang and C. X. Hong nothosp. nov. (Figure 6.4)

MycoBank MB807978

Sporangia were occasionally produced by aged cultures (> 30 days) grown in CA and CV8A. Abundant sporangia were produced from fresh mycelial plugs submerged in 1.5% SWE within 10 hours. Sporangial shape varied from ovoid (Figures 6.4a, b) to ellipsoid (Figures 6.4c, d), obpyriform (Figures 6.4e, f) and distorted shapes (Figure 6.4g).

Sporangia were terminal, nonpapillate and noncaducous. They ranged from 30.5 to 89.7 µm in length (average 54.3 ± 11.0 µm) and 17.5 to 40.4 µm in width (average 30.3 ± 3.9 µm). Direct germination of sporangia was frequently observed (Figures 6.4g, h). Nested and extended internal proliferations were common (Figures 6.4j, k). Hyphal swellings in irregular shapes were abundantly produced in both young and aged cultures (Figures 6.4l, m). Catenulate, globose hyphal swellings were frequently observed in aged cultures (Figure 6.4m). Intercalary chlamydospores were observed in aged cultures of all examined isolates (Figures 6.4n, o, p). They were mostly thin-walled (Figures 6.4n, o), rarely thick-walled (Figure 6.4p), and averaged 33.5 ± 4.9 µm in diameter.

Phytophthora ×*stagnum* is heterothallic and all isolates examined are A^1. They produced no sexual structure in single culture. Oogonia were produced in dual culture when each *P.* ×*stagnum* isolate was paired with an A^2 tester of *P. cinnamomi*. In the polycarbonate test, a limited number of gametangia (~40) were produced by the four isolates after being paired with an A^2 mating type tester of *P. nicotianae* for more than 50 days. Two distinct groups of gametangia were observed. Isolates 36H8 and 36J7 mostly produced ornamented oogonia with characteristic protuberances (Figures 6.4q, r, s, t). These oogonia averaged 33.6 ± 8.1 µm in diameter. Oogonial wall was pigmented golden at maturity. All observed ornamented oogonia aborted (Figures 6.4q, r, s, t). Antheridia were amphigynous, commonly distorted (Figures 6.4s, t). They averaged 19.4 µm in depth and 14.2 µm in width. Isolates 43F3 and 44F9 mostly produced oogonia with a relatively smooth surface (Figures 6.4u, v). These oogonia averaged 28.0 ± 5.6 µm in diameter. The oogonial wall was darkly golden-brown. Plerotic oospores (Figure 6.4u)

were also mostly aborted (Figure 6.4v). Antheridia were amphigynous, globose or distorted, and averaged 10.0 µm in depth and 12.3 µm in width (Figures 6.4u, v).

Holotype

ATCC MYA-4926 (exo-type: 43F3), recovered from an irrigation runoff reservoir, Virginia, USA, January, 2007. Other representative isolates were recovered from the same location: isolates 36H8 and 36J7, recovered in March, 2007; 44F9, recovered in May, 2007.

Etymology

'*stagnum*' refers to the irrigation reservoirs where this novel hybrid species was recovered.

Discussion

Sequence analyses of the *cox* 1, ITS, and beta-tubulin genes have demonstrated that *Phytophthora* ×*stagnum* is a hybrid species with a species genetically close to *P. mississippiae* as its maternal and *P.* taxon PgChlamydo as its paternal parent. First, the mitochondrial *cox* 1 sequence of *P.* ×*stagnum* is mostly analogous to that of *P. mississippiae* (Figure 6.1), suggesting that its maternal parent is genetically close to *P. mississippiae*. Second, cloning of the ITS region of *P.* ×*stagnum* isolates consistently

resulted in two types of 816-bp sequences and one type of 819-bp sequence. The 819-bp sequence is identical or only 1-bp different from those of authentic *P.* taxon PgChlamydo isolates (Brasier *et al.*, 2003). The two types of 816-bp sequences only differ from that of the *P. mississippiae* type isolate (Yang *et al.*, 2013) by 3 or 6 bp (Table 6.1). Third, *P.* × *stagnum* contains the beta-tubulin sequences of *P.* taxon PgChlamydo and *P. mississippiae* at 26 polymorphic positions (Table 6.2). Its sequences at non-polymorphic positions are identical to that of *P.* taxon PgChlamydo and only ~10 bp different from that of *P. mississippiae*. These results of ITS and beta-tubulin sequence analyses indicate that *P.* ×*stagnum* is a hybrid between *P.* taxon PgChlamydo and a species genetically close to *P. mississippiae*.

This hybrid species has diagnostic morphological and physiological characters of *P.* taxon PgChlamydo and *P. mississippiae*. For instance, *P.* ×*stagnum* is similar to *P.* taxon PgChlamydo in producing chlamydospores, which are not produced by *P. mississippiae* (Yang *et al.*, 2013). However, both *P.* ×*stagnum* and *P. mississippiae* produce abundant catenulate hyphal swellings (Figure 6.4m) in aged cultures, as well as nested or extended internal proliferations (Figures 6.4j, k). Also, both *P.* ×*stagnum* and *P. mississippiae* produce ornamented oogonia (Figures 6.4q-t). The relatively smooth-walled oogonia produced by *P.* ×*stagnum* (Figures 6.4u, v) may implicate the oogonial morphology of *P.* taxon PgChlamydo although it has not been reported. In addition, *P.* × *stagnum* is similar to *P. mississippiae* in colony morphology and growth rate on CV8A (Yang *et al.*, 2013). Both species produce radiate to slightly petaloid colonies with a relatively smooth edge (Figure 6.2) and the fastest growth on CV8A occurs at 25℃

(Figure 6.3). *Phytophthora* ×*stagnum* can be separated from both parents by its optimal growth temperature on CA at 25℃ (Figure 6.3), while it occurs at 30℃ for *P. mississippiae* (Yang *et al.*, 2013) and about 28℃ for *P.* taxon PgChlamydo (Brasier *et al.*, 2003).

Although we have identified the two parent species of *P.* ×*stagnum* by molecular and morphological evidences, the mechanism by which this subclade 6b hybrid was produced remains unknown. It seems likely that this new hybrid formed asexually. One major reason is that species in subclade 6b tend to be homothallic as exemplified by *P. gibbosa, P. gregata*, and *P. megasperma* (Drechsler, 1931; Jung *et al.*, 2011), or "sterile" with unknown sexual structures such as *P. amnicola, P. thermophila* and *P.* taxon PgChlamydo (Brasier *et al.*, 2003; Jung *et al.*, 2011; Crous *et al.*, 2012). This tendency may be a result of their adaptation to aquatic habitats (Brasier *et al.*, 2003; Jung *et al.*, 2011; Nagel *et al.*, 2013). The four subclade 6b hybrid species reported in 2013, PG-A, A-PG, T-A, T-PG also produced no gametangia (Nagel *et al.*, 2013). In this study, we only observed a limited number of sexual bodies of *P.* ×*stagnum* (~40) in five polycarbonate-membrane tests. These results along with previous findings indicate that hybrids in subclade 6b were more than likely formed asexually via hyphal anastomosis or zoospore fusion. However, Nagel *et al.* suggested that the conditions used in laboratory mating tests may be not conducive to the formation of sexual bodies of subclade 6b species, while suitable conditions may exist in natural environments (Nagel *et al.*, 2013). The formation mechanism of sexual structures of these subclade 6b hybrid species warrants further investigations.

Aquatic environments are ideal for the development and survival of natural *Phytophthora* hybrids. Many known *Phytophthora* hybrids have close association with aquatic environments. Examples include the four subclade 6b hybrids recovered from river and riparian ecosystems (Nagel *et al.*, 2013); *Phytophthora alni* and its variants associated with riparian *Alnus* trees (Brasier *et al.*, 1999; Brasier *et al.*, 2004); and *Phytophthora* × *pelgrandis* initially recovered from horticultural plants grown in hydroponic systems (Man in't Veld *et al.*, 1998). The fact that most natural *Phytophthora* hybrid species were initially identified from aquatic environments is interesting. First, natural aquatic ecosystems such as rivers, streams, and riparian habitats provide ideal environments for many plant species to grow. Consequently, *Phytophthora* species from various plant hosts have greater chances to aggregate and subsequently form hybrids under suitable conditions, such as *P. alni* and subclade 6b hybrids described in 2013. Similarly, hundreds of ornamental plants are grown in nurseries using hydroponic or recycling irrigation systems which greatly increase the chance of close contact between species. *Phytophthora* ×*pelgrandis*, *P.* ×*serendipita*, and *P.* ×*stagnum* may have formed in these systems by mating or anastomosis (Man in't Veld *et al.*, 1998). Second, newly formed *Phytophthora* hybrids may have a better opportunity to survive and adapt to aquatic ecosystems that contain a diverse variety of plant species. Third, aquatic environments favor asexual reproduction via motile zoospores or chlamydospores. This may be important for species that are sterile or nonfunctional in sexual reproduction as are all known *Phytophthora* hybrids. Fourth, for the saprophytic *Phytophthora* species in subclade 6b including PG-A, A-PG, T-A, T-PG (Brasier *et al.*, 2003; Jung *et al.*, 2011;

Nagel *et al.*, 2013) as well as *P.* ×*stagnum* in this study, the abundant plant debris in aquatic environments provides ideal microhabitats and nutrient sources. Fifth, water also offers hybrids vehicles for mobility compared to terrestrial environments, which may allow them to migrate into new habitats. In summary, aquatic environments may provide favorable conditions for *Phytophthora* hybrids to form, survive and disseminate.

All four representative isolates of *Phytophthora* ×*stagnum* are genetically stable. They were routinely subcultured on artificial media during the experimental period (~ 2 years) and did not revert to either parent type. Also, sequencing of the ITS region of representative isolates was conducted several times in three years (2008, 2012, and 2013), and all ITS sequences obtained displayed similar polymorphisms. In addition, isolates of *P.* ×*stagnum* have been continually recovered from the same irrigation reservoirs since 2005. These observations suggest that this new hybrid species is relatively stable in the laboratory and in nature, and may have adapted to the irrigation systems of the surveyed nurseries in eastern Virginia.

Similar to the other four *Phytophthora* hybrids in subclade 6b (Nagel *et al.*, 2013), the pathogenicity of *P.* ×*stagnum* is yet to be determined. No diseased plant samples associated with this novel hybrid species has been received in the Disease Clinic at Hampton Roads Agricultural Research and Extension Center in Virginia Beach, Virginia. Also, in a preliminary pathogenicity test, *P.* ×*stagnum* caused little if any dieback on rhododendron plants (data not shown). The low aggressiveness of *P.* ×*stagnum* may be inherited from its parent species. *Phytophthora* taxon PgChlamydo is considered as an

opportunistic plant pathogen (Brasier *et al.*, 2003), although it has been found to cause leaf spot on nursery stocks in California (Blomquist *et al.*, 2012). The maternal parent species of *P.* ×*stagnum* is close to *P. mississippiae*, which has an unknown host range (Yang *et al.*, 2013).

Origin of this novel hybrid is not known at this time. Although *P.* taxon PgChlamydo has been frequently recovered from the same irrigation reservoirs, the maternal parent of *P.*× *stagnum* has never been isolated. This observation may suggest that the new hybrid had been introduced to these nurseries via incoming ornamental plant materials. Crop health risk posed by this new hybrid species has yet to be assessed.

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Table 6.1 Internal transcribed spacer (ITS) sequence alignment of *Phytophthora* ×*stagnum*, *P. mississippiae* and *P.* taxon PgChlamydo. Position numbers are given based on the alignment. Yellow indicates sequences belong to *P.* taxon PgChlamydo authentic isolates. Blue indicates sequences belong to *P. mississippiae* type isolate 57J3.

Species	Isolate/Sequence type	GenBank Accession	ITS1							ITS2		
			15 [a]	59	110	148	175	179	182	653	741	798
P. t. PgChlamydo	P236	AF541900	-	C	A	A	T	T	A	G	G	C
	P1056	AF541901	A	C	A	A	T	T	A	G	G	C
P. ×*stagnum*	819-bp type	KJ705086	A	C	A	A	T	T	A	G	G	C
	816-bp type 2 [b]	KJ705085	-	T	G	G	-	-	T	T	G	T
	816-bp type 1	KJ705084	-	C	G	G	-	-	A	T	G	C
P. mississippiae	57J3	KF112852	A	C	G	G	T	-	A	T	T	C

[a] Position 15 is in the poly(A) region of ITS 1, which may contain sequencing errors. Thus, it is excluded from the analysis of hybridization. However, the indel of position 15 among three types of *P.* ×*stagnum* clones explains continual polymorphism and unreadable sequences of the ITS 1 regions amplified with the forward primer ITS6F in the initial sequencing before cloning.

[b] Type 1 and 2 occurred 21 and 10 times among 31 clones producing 816-bp sequences.

Table 6.2 Beta-tubulin sequence alignment of *Phytophthora* ×*stagnum*, *P. mississippiae* and *P.* taxon PgChlamydo. Position numbers are given based on the alignment. Shading color: green indicates ambiguous sequences of polymorphic positions; yellow indicates sequences of the paternal parent *P.* taxon PgChlamydo; blue indicates sequences of the putative maternal parent.

Species	Isolate	GenBank Accession	Position in aligned beta-tubulin sequences [a]																																				
			75	93	99	102	186	240	261	276	306	342	354	429	450	457	462	517	519	561	571	594	609	621	634	646	667	681	684	702	726	747	759	765	771	783	993	1098	1104
P. t. PgChlamydo	P236	KF750602	C	C	C	C	C	C	C	T	G	A	C	T	C	C	A	C	G	G	T	C	C	T	T	C	C	G	T	T	C	C	C	G	G	T	T	T	T
P. ×*stagnum*	43F3	KJ883155	C	Y	Y	Y	C	C	Y	Y	S	M	Y	Y	Y	C	R	C	R	S	Y	M	C	Y	Y	C	Y	G	T	Y	Y	C	M	G	G	Y	Y	Y	T
	36H8	KJ883153	C	Y	Y	Y	C	C	Y	Y	S	M	Y	Y	Y	C	R	C	R	S	Y	M	C	Y	Y	Y	Y	G	Y	Y	Y	C	M	G	G	Y	Y	Y	T
Putative maternal parent	n/a	n/a	C	T	T	T	C	C	T	C	C	C	T	C	T	C	G	C	A	C	C	A	C	C	C	T	T	G	C	C	T	C	A	G	G	C	C	C	T
P. mississippiae	57J3	KJ883157	T	C	T	T	A	T	T	C	G	C	T	C	C	T	G	T	R	C	C	C	T	C	C	C	C	A	T	Y	T	T	C	A	A	C	C	C	Y

[a] Y = T and C; S = G and C; M = A and C; R = G and A

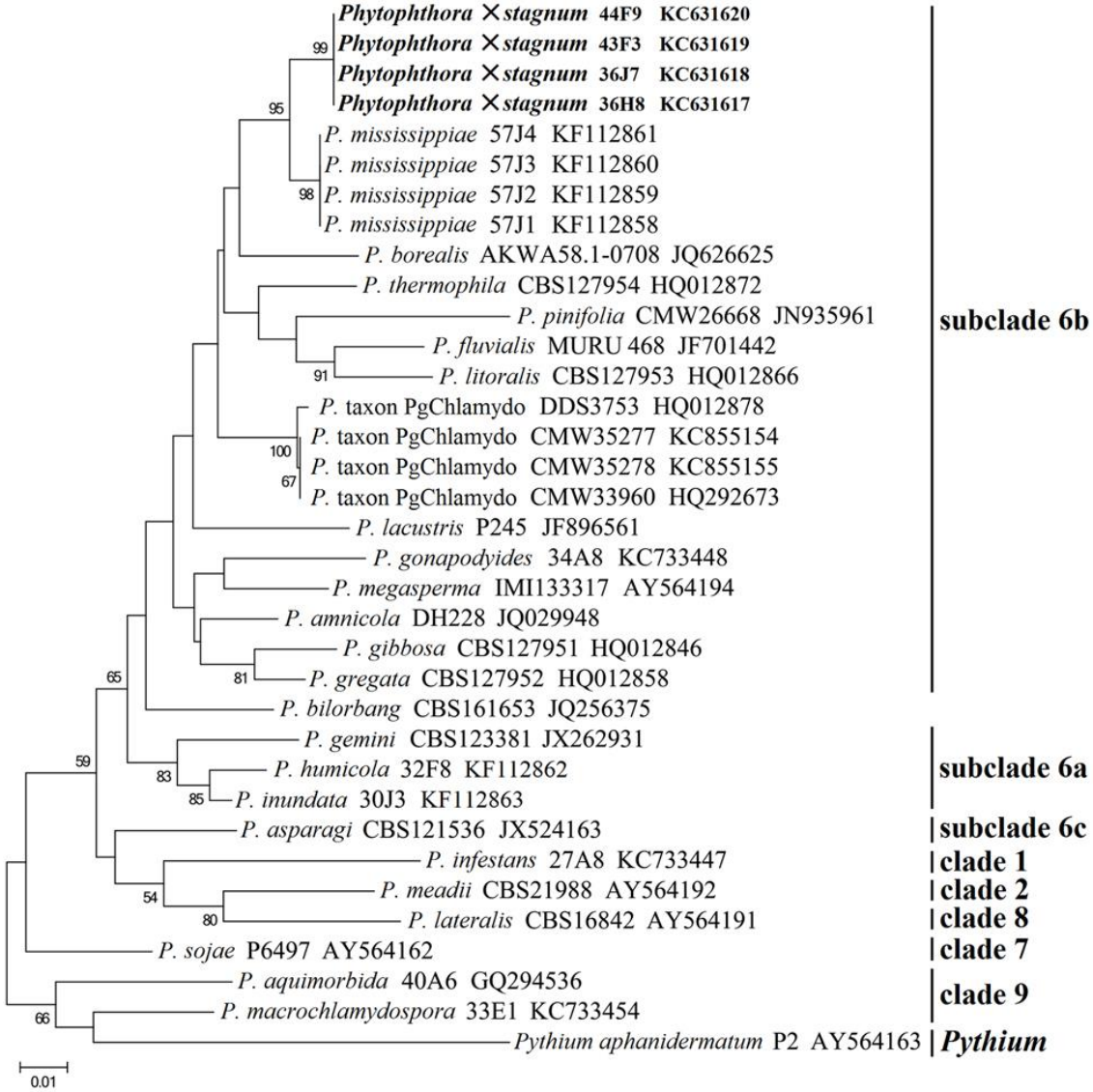

Figure 6.1 Maximum Likelihood phylogenetic tree based on mitochondrial *cox* 1 sequences of *Phytophthora* ×*stagnum* and representative species. Alignment was conducted with MAFFT version 7. Phylogenetic tree was generated in MEGA5. GenBank accession numbers of sequences are given following the species names and isolate codes. Bootstrap values are shown on branches (1,000 replicates; values < 50% are not shown).

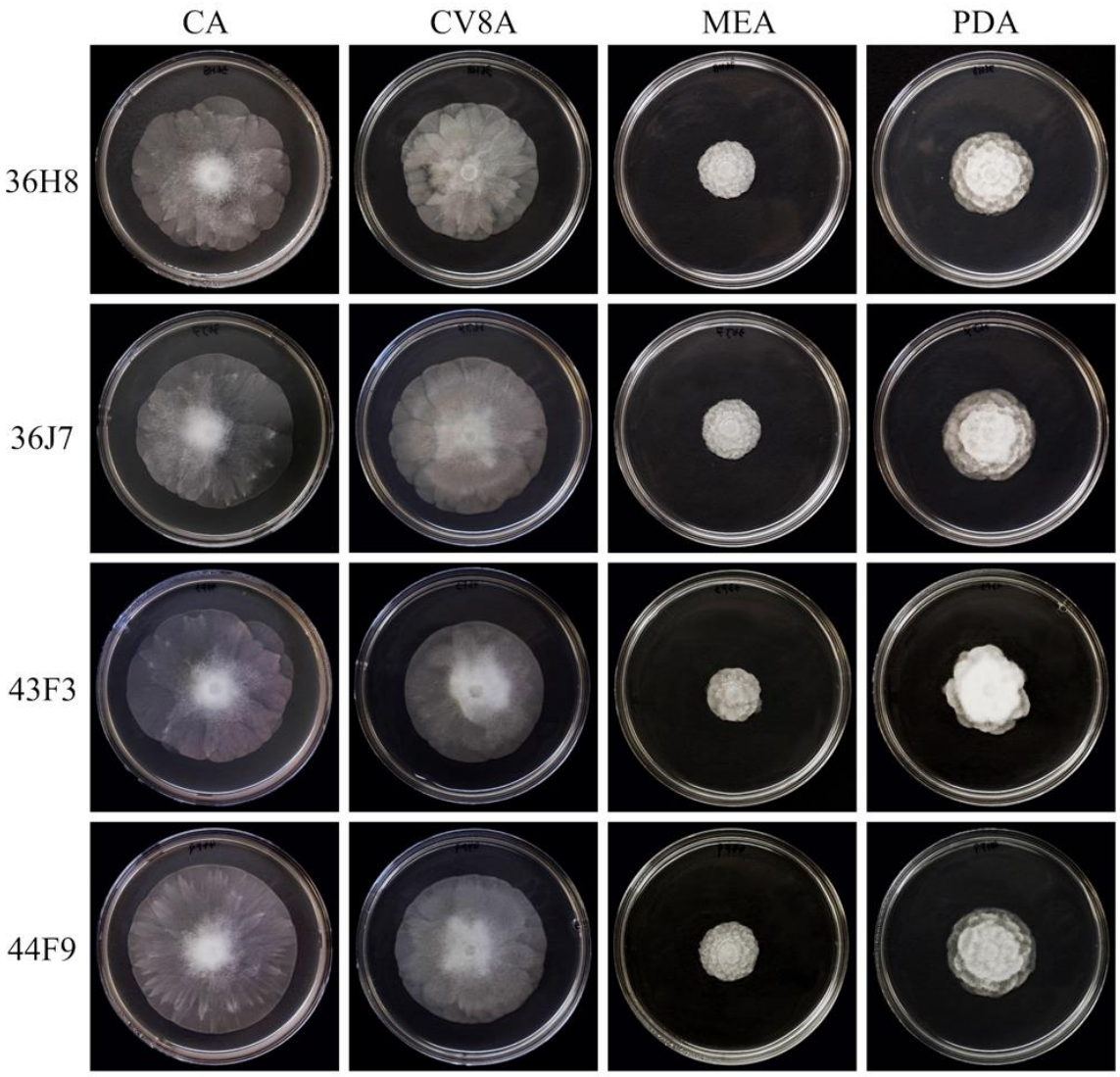

Figure 6.2 Colony morphology of *Phytophthora* ×*stagnum* representative isolates on various media incubated at 20℃ for 10 days in the dark. CA = carrot agar, CV8A = 20% clarified V8 juice agar, MEA = malt extract agar, PDA = potato dextrose agar.

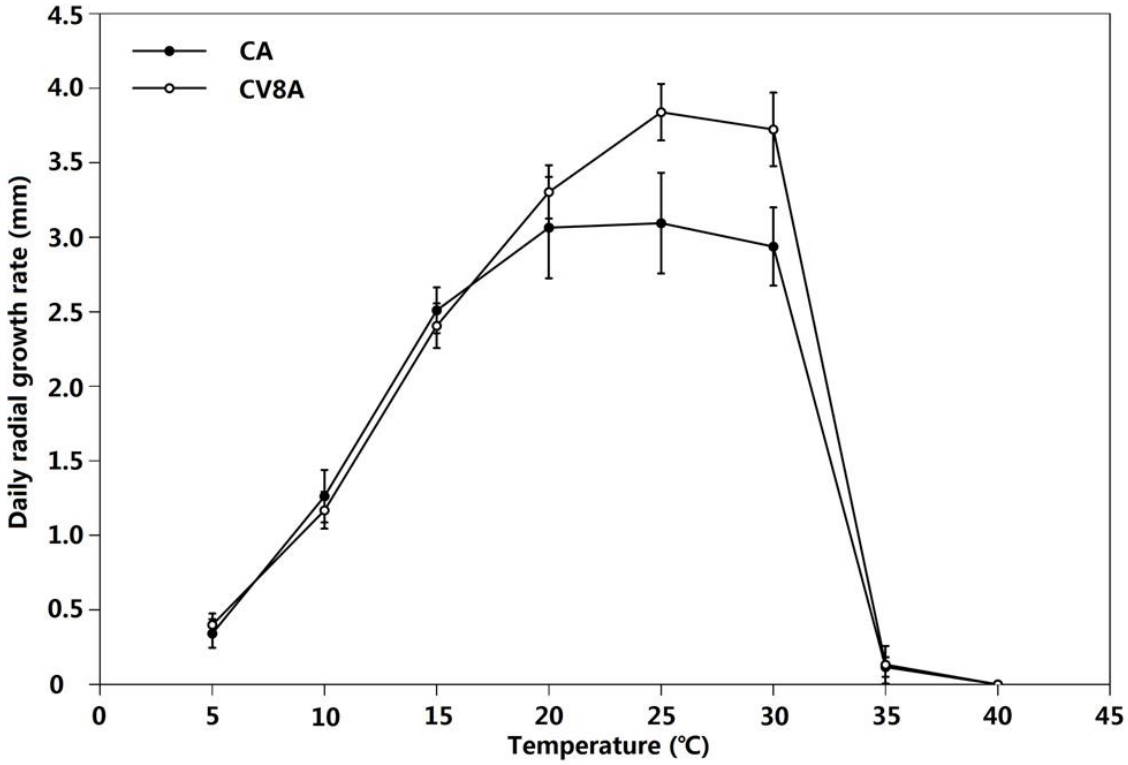

Figure 6.3 Average daily radial growth of *Phytophthora* ×*stagnum* representative isolates in carrot agar (CA) and 20% clarified V8 juice agar (CV8A) over an 8-days period.

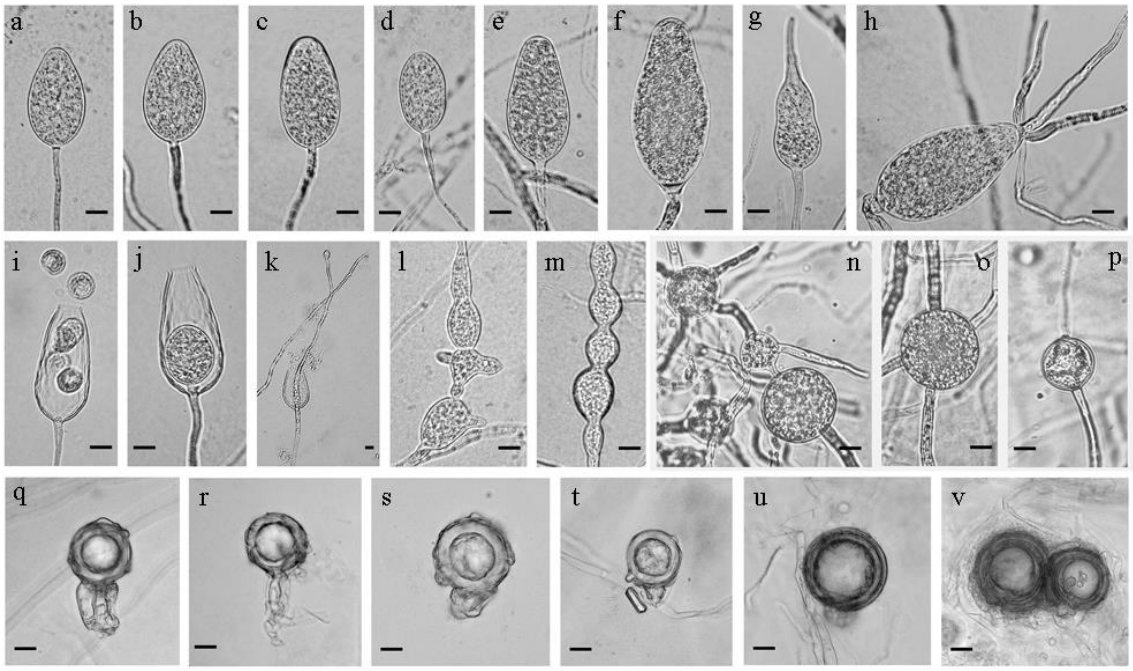

Figure 6.4 Morphology of *Phytophthora* ×*stagnum*. (a-g) Nonpapillate and noncaducous sporangia in various shapes; (a, b) Ovoid sporangia; (c, d) Ovoid to ellipsoid sporangia; (e, f) Obpyriform sporangia; (g) A germinated sporangium in distorted shape; (h) Direct germination of an obpyriform sporangium; (i) A sporangium releasing zoospores; (j) Nested internal proliferation; (k) Extended internal proliferation; (l) Hyphal swellings; (m) Catenulate hyphal swellings; (n) Thin-walled intercalary chlamydospores and hyphal swellings; (o) A thin-walled intercalary chlamydospore; (p) A thick-walled chlamydospore; (q-t) Ornamented, aborted oogonia produced by isolates 36H8 and 36J7; (q) An oogonium with an amphigynous antheridium; (r-t) Ornamented oogonia with distorted antheridia; (u, v) Relatively smooth-walled, darkly pigmented oogonia produced by isolates 43F3 and 44F9; (u) An oogonium containing a plerotic oospore with a globose antheridium; (v) Oogonia with abortive oospores. Bars = 10 μm.

Chapter 7 Constructing a new phylogeny for the genus *Phytophthora*

Yang X, Tyler B & Hong CX (2014) Constructing a new phylogeny for the genus *Phytophthora*. *Phytopathology* **104** (S3): 133.

Abstract

Traditional taxonomy of the genus *Phytophthora* was based on morphological traits such as sporangial papillation and caducity, homothallism, and antheridial configuration. Phylogeny-based classification has emerged by taking advantage of modern molecular technologies, especially when the number of formal species in the genus has doubled during the past decade to approximately 140 and it is increasingly difficult to classify *Phytophthora* species by morphology alone. The current 10-clade phylogeny was established based on sequences of seven nuclear genetic markers in 2007-2008 and it included approximately 80 *Phytophthora* species. The most recent multi-locus phylogeny in 2014 included 90 formal and 17 provisional species. By incorporating new species, clusters and clades, sequences of five genetic markers of approximately 130 *Phytophthora* taxa were analyzed in this study. Phylogeny based on sequences of beta-tubulin (B-tub) gene provided the highest phylogenetic resolution among selected genetic markers. General phylogenetic grouping of individual species in the B-tub phylogeny was similar to that of previous phylogenetic analyses, except that *P. cinnamomi*, *P. parvispora*, *P. quercina*, *P. stricta*, and *P.* sp. e1 were not clustered in any of the 10

known clades. The B-tub phylogeny was also used to map morphological and physiological characters of individual species including sporangial papillation, caducity, homothallism, antheridial configuration and maximum growth temperature, and to review the relationships between phylogeny and these morphological and physiological characters. The results indicated that the character of sporangial papillation was consistent among species within most subclades of the B-tub phylogeny. Maximum growth temperature was also generally correlated with phylogenetic positions in the B-tub phylogeny. No correlation was found between phylogeny and caducity, homothallism or antheridial configuration. A new multi-locus phylogeny based on sequences of 11 genetic markers of more than 146 *Phytophthora* species was proposed to validate new clades and clusters, examine the exact relations between phylogeny and morphology, as well as investigate detailed phylogenetic relations among species in this quickly expanding, taxonomically complex group of plant pathogens.

Introduction

Traditional taxonomy of the genus *Phytophthora* was mainly based on morphological characters. The most successful morphology-based classification of *Phytophthora* species was established by Waterhouse (1963). She placed *Phytophthora* species into six morphological groups based on morphology of sporangia, homothallism and configuration of antheridia (Waterhouse, 1963). Generally, homothallic species producing papillate sporangia and paragynous antheridia were classified in Group I. Group-II species were similar to those of Group I in sporangial morphology, while their

oospores were not formed in single-strain culture and they produced amphigynous antheridia. Semi-papillate species were classified in either Groups III or IV. Homothallic species with paragynous antheridia were classified in Group III, while species in Group IV produced amphigynous antheridia and oospores were rarely produced in single-strain culture. Species producing non-papillate and non-caducous sporangia were classified in Groups V or VI. Similarly, species that were homothallic and produced paragynous antheridia were classified in Group V, while species producing amphigynous antheridia and rare oospores in single-strain culture were classified in Group VI (Waterhouse, 1963). Other morphological keys of *Phytophthora* species were also based on morphology of sporangia and gametangia, plus morphological traits of chlamydospores, growth patterns and cardinal temperatures (Tucker, 1967; Newhook *et al.*, 1978; Ho, 1981; Stamps *et al.*, 1990; Ho, 1992; Gallegly & Hong, 2008; Ristaino, 2012). These morphological keys have been widely used for identifying *Phytophthora* species. Nevertheless, as acknowledged by Waterhouse herself and many other colleagues, plasticity in morphological characters of individual species is significant, so is the overlapping in morphological characters among species. For example, *P. constricta*, *P. gibbosa*, *P. mississippiae,* and *P. multivesiculata* produce a mixture of semi-papillate and non-papillate sporangia, which is ambiguous under traditional taxonomy (Kroon *et al.*, 2012; Yang *et al.*, 2013). Two recently described species, *P. hydropathica* and *P. irrigata*, are similar to *P. drechsleri* in morphology while recent sequence analyses indicated they are distinct species (Erwin & Ribeiro, 1996; Hong *et al.*, 2008; Hong *et al.*, 2010). Also, production and observation of many morphological structures as well as physiological features require substantial training and expertise, and they are very tedious

and time-consuming. It is not uncommon that some isolates do not produce all structures required for species identification under laboratory conditions. Additionally, species number of the genus *Phytophthora* has doubled during the past decade due to extensive surveys in previously unexplored ecosystems (Martin *et al.*, 2012) such as natural forests, streams, riparian ecosystems and irrigation systems. The total number of formal species in this genus was about 58 in 1996, but now in 2014 it is approximately 140, plus a number of provisionally named species. The rapid increase in the species number has increasingly challenged morphology-based taxonomy and made it impossible for many species.

Modern molecular technologies have greatly advanced the species identification and systematics for the genus *Phytophthora* and the taxonomic concepts have evolved from morphology- to phylogeny-based (Martin & Tooley, 2003; Kroon *et al.*, 2004; Villa *et al.*, 2006; Blair *et al.*, 2008; Lara & Belbahri, 2011; Robideau *et al.*, 2011; Martin *et al.*, 2014). In 2000, Cooke *et al.* developed the first phylogeny for the genus *Phytophthora* by analyzing sequences of the internal transcribed spacer region (ITS) of 51 species (Cooke *et al.*, 2000). Kroon *et al.* constructed a phylogeny in 2004 based on sequences of four nuclear and mitochondrial genes of 48 species (Kroon *et al.*, 2004). In 2008, Blair *et al.* constructed a sophisticated phylogeny based on sequences of seven nuclear genetic markers. This multi-locus phylogeny divided 82 *Phytophthora* species into 10 phylogenetically well-supported clades (Blair *et al.*, 2008). In 2014, Martin *et al.* analyzed sequences of seven nuclear and four mitochondrial genes of 90 formal and 17 provisional species. General grouping and clade-structure of the 2014 phylogeny was

similar to those of the 2008 phylogeny, except that *P. quercina* and *P.* sp. ohioensis were excluded from clade 4 and grouped into a potentially new clade (Martin *et al.*, 2014).

Although sequence analyses have greatly advanced the phylogeny-based taxonomy of the genus *Phytophthora*, many problems and challenges have occurred. First, a number of new *Phytophthora* species have not been included in previous phylogenetic studies. Although the most recent phylogeny included 90 formal and 17 provisional species (Martin *et al.*, 2014), at least 25 formal and many provisional species were not included. Second, many new species have challenged the 10-clade phylogeny. For example, *P. quercina* and *P.* sp. ohioensis were grouped in a potential new clade (Martin *et al.*, 2014). Yang *et al.* (2014) found that a novel species, *P. stricta* represented a new clade in a ITS phylogeny that included approximately 120 *Phytophthora* species (Figure 5.1) (Yang *et al.*, 2014). Third, limited numbers of types and authentic cultures were used in previous phylogenies. For example, only 9, 18, 16, and 26 type cultures were included in the 2000, 2004, 2008 and 2014 phylogenies, respectively (Cooke *et al.*, 2000; Kroon *et al.*, 2004; Blair *et al.*, 2008; Martin *et al.*, 2014). This has led to questions about the accuracy and representativeness of individual species in these previous phylogenies. Obviously, a new phylogeny including a majority of type or authentic *Phytophthora* cultures is warranted. Fourth, although the general clade-grouping and phylogenetic structures among different analyses were similar, a variety of individual genes or combinations of genetic markers were used in previous phylogenies and variations have been found among their results (Martin & Tooley, 2003; Kroon *et al.*, 2004; Villa *et al.*, 2006; Blair *et al.*, 2008; Lara & Belbahri, 2011; Robideau *et al.*, 2011; Martin *et al.*, 2014). Fifth, the relationship

between traditional morphology- and phylogeny-based taxonomy has been rarely studied. This makes the taxonomy of *Phytophthora* species completely dependent on molecular technologies while the still meaningful morphology-based classification has not concurrently kept up with changes in the *Phytophthora* taxonomy.

A few studies have reviewed the relationships between morphological, physiological, and molecular characters of *Phytophthora* species. Ersek and Ribeiro (2010) reviewed morphological classification and ITS phylogenetic positions of 88 *Phytophthora* species and found no consistent correlation between the Waterhouse's six groups and the 10 ITS clades. Species in each ITS clade were morphologically classified into 2 to 4 Waterhouse's groups, except that two clade-5 species, *P. heveae* and *P. katsurae*, belonged to Waterhouse's group II (Ersek & Ribeiro, 2010). In two multi-locus phylogenetic analyses, no obvious correlation was found between the Waterhouse system and phylogenetic (sub)clades, except all species in subclade 1a, 1b and 1c were respectively grouped in Waterhouse's groups I, II, and IV, as well as the two clade-5 species being in group II (Blair *et al.*, 2008; Martin *et al.*, 2014). Two recent reviews listed morphological and physiological characters of species in individual (sub)clades and indicated no correlation between phylogenetic classification and morphology of sexual organs including homothallism and antheridial configuration (Kroon *et al.*, 2012; Martin *et al.*, 2012). However, species in individual phylogenetic groups were generally similar in sporangial papillation and optimum and maximum temperatures (Kroon *et al.*, 2012; Martin *et al.*, 2012).

In this study, a new phylogeny including more than 120 *Phytophthora* species was constructed. Sequences of five genetic markers were used for phylogenetic reconstruction. Also, correlations between morphological and physiological characters, and phylogeny were studied and discussed.

Materials and Methods

Isolate collection

Isolate numbers and GenBank accession numbers of 128 *Phytophthora* isolates used in this study are listed in table 7.1. Ninety-seven isolates are types or authentic cultures which were obtained from original collectors of the species. These isolates represent more than 120 formal species and variants plus 6 provisional species.

DNA extraction

Isolates were grown in 20% clarified V8 broth at room temperature (*c*. 23℃) for seven days to produce mycelial masses which were then dried and lysed using a FastPrep®-24 system (MP Biomedicals, Santa Ana, CA, USA). DNA was extracted using the DNeasy® Plant Mini kit (Qiagen, Valencia, CA, USA).

Sequencing and phylogenetic analyses

Sequences of five genetic markers including 60S ribosomal protein L10 (60S), beta-tubulin (Btub), elongation factor 1 alpha (EF1α), enolase (Enl), and 28S Ribosomal DNA (28S) genes were analyzed. PCR amplifications were performed using the primer pairs 60SL10_for and 60SL10_rev for 60S, Btub_F1 and Btub_R1 for Btub, EF1A_FL and EF1A_RL for EF1α, Enl_for and Enl_rev for Enl, and LROR-O and LR6-O for 28S (Blair *et al.*, 2008). Sequences in both directions were visualized with Finch TV *v.* 1.4.0 (Geospiza Inc., Seattle, WA, USA), aligned using Clustal W and edited manually to correct obvious errors.

Five phylogenetic inferences were constructed based on sequences of individual genetic markers. Most sequences were generated in this study and some from previous phylogenetic analyses (Blair *et al.*, 2008) were obtained from GenBank (Table 7.1). Phylogeny reconstruction was conducted in MEGA 5.1 (Tamura *et al.*, 2011) using the maximum likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993) with 500 replications of bootstrap.

Results

Phylogeny of individual genetic markers

The 60S locus was consistently amplified for all *Phytophthora* isolates. However, due to its short length (496 bp) and limited variable characters among species, it provided limited value in both species identification and phylogenetic reconstruction (Blair *et al.*,

2008). The 60S phylogenetic tree provided very low phylogenetic resolution. Species previously designated in individual (sub)clades were scattered in the phylogenetic tree (Figure 7.1). Although species of a few designated (sub)clades were clustered such as clade 3, subclades 6a, 6b, 8a and 8b, they were not supported by strong bootstraps (Figure 7.1).

The B-tub gene was also consistently amplified throughout the genus except a few species such as *P. cichorii* and *P. dauci* in clade 8 and *P.* sp. lagoariana in clade 9 (Table 7.1). An approximately 1136-bp DNA fragment was amplified which provided abundant variable characters. The B-tub phylogeny provided the highest phylogenetic resolution among the selected genetic markers (Blair *et al.*, 2008). The (sub)clade grouping of individual species in the B-tub phylogeny (Figure 7.2) was almost identical to that in previous phylogenetic analyses (Blair *et al.*, 2008; Martin *et al.*, 2014) with a few exceptions. *Phytophthora quercina* was not grouped in any known phylogenetic group. It was placed basal to both clades 2 and 4 in the B-tub phylogeny while it was basal to clade 4 in the previous analysis (Martin *et al.*, 2014). Also, *P. cinnamomi* and *P. parvispora*, usually considered as subclade-7b species, were not grouped in any (sub)clade in the B-tub phylogeny (Figure 7.2). *Phytophthora stricta* was also not grouped in any known clade and was basal to clade 8 (Figure 7.2). Among all clades, structure of clade 9 in the B-tub phylogeny was the most different from that in previous multi-locus phylogenies (Martin *et al.*, 2014). Yet, all the high-temperature tolerant species (Yang *et al.*, 2014) were clustered in the B-tub phylogeny and a ITS phylogeny (Figure 5.1) (Yang *et al.*, 2014). However, the cluster that included *P.* sp. lagoariana and *P.* sp. cuyabensis were

separated from the cluster that included *P. insolita* and *P. polonica* in a previous multi-locus phylogeny (Martin *et al.*, 2014). The multi-locus phylogeny did not include any formally described species of the high-temperature tolerant cluster, whereas the B-tub phylogeny in this study included all known species in the cluster and was only based on B-tub sequences. The exact phylogenetic structure of clade 9 warrants further analysis.

The EF1α gene was reported to provide moderate phylogenetic resolution and was able to resolve relationships within most clades (Blair *et al.*, 2008). However, the EF1α phylogeny in this study, which included 129 species, provided poor phylogenetic resolution of both inter- and intra-clade relations (Figure 7.3). Only subclades 7b, 8b, and 8d were supported with relatively high bootstrap values (Figure 7.3), while most species of individual phylogenetic groups were scattered. Conversely, many species of clades 1, 2, 4, 5 and 6 were inseparable and grouped in a poorly resolved cluster (Figure 7.3).

Approximately 120 *Phytophthora* species were included in the Enl phylogeny (Figure 7.4). This gene was relatively difficult to amplify for some species such as *P. fluvialis* in clade 6, *P. pisi* in clade 7, and the outgroup species such as *Halophytophthora fluviatilis* (Table 7.1). As a result, an Enl sequence of *Apodachlya brachynema* (GenBank Accession AY430415) was used as the outgroup for the Enl phylogeny (Blair *et al.*, 2008). This genetic marker provided abundant variable codons which were meaningful in both species identification and phylogenetic construction. The Enl phylogeny provided relatively high genetic resolutions among species. The general subclade-grouping of Enl phylogeny was similar to that of multi-locus analyses. However, subclades of clades 1, 2

and 7 were scattered (Figure 7.4). Especially, *P. bisheria* was excluded from any known clade and placed basal to clades 1-8. Additionally, *P. polonica* and *P. insolita*, recognized as clade-9 species, were clustered with species of clade 10 in the Enl phylogeny (Figure 7.4).

The 28S locus provided higher resolution of (sub)clade-grouping and species relationships than other selected genetic markers except B-tub. Inter- and intra-clade structures of the 28S phylogeny were similar to those designated by multi-locus analyses (Blair *et al.*, 2008; Martin *et al.*, 2014) with a few exceptions. For example, clades 6 and 7 were inseparable (Figure 7.5). Also, *P. polonica* and *P. insolita* were placed basal to clades 1-8 (Figure 7.5). Additionally, *P. quercina* was basal to clade 1, *P. asparagi* was basal to clades 1-7, and *P. stricta* was basal to clade 8 in the 28S phylogeny (Figure 7.5).

Correlation between morphology, physiological characters and phylogeny

The B-tub phylogeny was used for mapping important morphological and physiological characters including sporangial papillation, caducity, homothallism, antheridial configuration, and maximum growth temperature (Figure 7.2). The result showed general correlations between classifications of phylogenetic subclades and papillation as well as maximum growth temperature (Figure 7.2). Correlation between phylogeny and caducity, homothallism, or antheridial configuration was not obvious. However, several phylogenetic groups had general trends in characters of sexual organs, such as consistent homothallism in clades 3, 10 and the '*P. citricola*' complex, as well as common sterility

in part of subclades 6a and 6b (Figure 7.2). The morphological, physiological and phylogenetic characters and their relationships of species in individual (sub)clades are reviewed in detail below.

1. Clade 1

Clade 1 currently contains 12 species in three subclades, 1a, 1b, and 1c, plus *P. nicotianae* which is not grouped in any subclade. Subclade 1a only has papillate and homothallic species. They produce either caducous or non-caducous sporangia, and paragynous or a mix of amphigynous and paragynous antheridia. Subclade-1a species are relatively low-temperature species, which cannot sustain growth at > 30℃. All three species in subclade 1b produce papillate sporangia and are homothallic with a mix of amphigynous and paragynous antheridia. The maximum growth temperatures for subclade-1b species are 30 to 35℃, higher than those of subclade-1a species. Subclade 1c currently has five species which produce papillate and caducous sporangia. They are either homothallic or heterothallic species, but all produce amphigynous antheridia. This is also a relatively low-temperature group. The maximum growth temperature for *P. ipomoeae* is only 25℃ (Flier *et al.*, 2002). *Phytophthora nicotianae* produces papillate and non-caducous sporangia, and is heterothallic with amphigynous antheridia. It is the only species sustaining growth at > 35℃ in clade 1.

2. Clade 2

Clade 2 contains three well-supported phylogenetic groups: subclades 2a and 2b and the '*P. citricola*-complex' (Figure 7.2). Subclade 2a has mostly papillate species with the only exception that *P. colocasiae* being semi-papillate (Erwin & Ribeiro, 1996; Gallegly & Hong, 2008). No consistency of caducity has been found in this subclade. Also, all subclade-2a species are heterothallic with producing amphigynous antheridia except *P. himalsilva*, which is homothallic and produces both amphigynous and paragynous antheridia (Vettraino *et al.*, 2011). Subclade 2a is a relatively high-temperature group. The maximum growth temperatures of the species in subclade 2a are consistently between 30 to 35℃. Subclade 2b only has papillate species with either caducous or non-caducous sporangia. Except *P. glovera*, all species are heterothallic, produce amphigynous antheridia and have maximum growth temperatures at > 30℃. *Phytophthora glovera* is a homothallic species producing both amphigynous and paragynous antheridia. Its maximum growth temperature is 30℃ (Abad *et al.*, 2011). The '*P. citricola*-complex' in clade 2 consists of a group of phylogenetically and morphologically similar species. All members are semi-papillate and noncaducous. They are also all homothallic and mostly producing paragynous antheridia. Except two sister species *P. capensis* (not included in the B-tub phylogeny) and *P.* taxon emzansi, all other species in the complex sustain growth at > 30℃. Species in clade-2 but not clustered in the above three groups include *P. bisheria*, *P. elongata*, *P. frigida*, *P. multivesiculata* and *P.* taxon aquatilis. They have various traits in sporangial morphology as well as vary in homothallism and antheridial morphology. The only common characteristic of these species is that they all have maximum growth temperature between 30 to 35℃. The wide

diversity in morphological and physiological characters among clade-2 species indicates that clade 2 is likely a paraphyletic clade.

3. Clade 3

Clade 3 consists of five phylogenetically close and morphologically similar species. All species are semi-papillate, caducous, homothallic with amphigynous antheridia, and have maximum growth temperatures between 21 to 25℃. The only exception is that *P. pseudosyringae* (not included in figure 7.2) produces paragynous antheridia (Jung *et al.*, 2003).

4. Clade 4

Clade 4 has five species, which produce papillate and either caducous or non-caducous sporangia. No correlation was observed between phylogeny and homothallism or antheridial morphology. Except *P. megakarya*, all species have the maximum growth temperatures above 30℃.

5. Clade 5

Only two described species, *P. heveae* and *P. katsurae* are grouped in clade 5. Both species produce papillate and non-caducous sporangia. They are homothallic with amphigynous antheridia. Maximum growth temperature is about 32℃ for both species (Erwin & Ribeiro, 1996; Gallegly & Hong, 2008).

6. Clade 6

Clade 6 is one of the quickly expanding clades of the genus *Phytophthora.* Only six species were included in the multi-locus phylogeny in 2008 (Blair *et al.*, 2008). Nineteen taxa were included in the B-tub phylogeny and approximately 30 taxa are included in an ongoing study of building a multi-locus phylogeny for the genus. The general grouping into subclades was identical to that in a previous review (Kroon *et al.*, 2012), except that *P. lacustris* (= *P.* taxon Salixsoil) was grouped in subclade 6a of the B-tub phylogeny. Another important feature of clade 6 is that it contains abundant species commonly recovered from aquatic environments. These aquatic species can sustain high temperature > 30℃ and some can even grow at >35℃ (also see 9. Clade 9). The only three species that cannot grow at >30℃ are *P. asparagi*, *P. megasperma* and *P. pinifolia*, which are important pathogens that usually infect terrestrial agricultural and forestry plants (Erwin & Ribeiro, 1996; Duran *et al.*, 2008; Crous *et al.*, 2012). The relationship between phylogenetic position, ecological habitat, and maximum growth temperature is interesting and warrants further investigation.

Subclade 6a only has non-papillate and non-caducous species. Species are homothallic, heterothallic, or sterile. Subclade 6b contains mostly non-papillate and non-caducous species, except that *P. gibbosa* and *P. mississippiae* produce a mix of non-papillate and semi-papillate sporangia (Jung *et al.*, 2011; Yang *et al.*, 2013), and *P. pinifolia* produces slightly caducous sporangia (Duran *et al.*, 2008). *Phytophthora mississippiae* and *P.* ×

stagnum are the only two heterothallic species in subclade 6b (Yang *et al.*, 2013). Subclade 6c only has a single species *P. asparagi*, which is basal to all other clade-6 species. It produces non-papillate and non-caducous sporangia and is homothallic with amphigynous antheridia.

7. Clade 7 (including *P. cinnamomi* and *P. parvispora*)

Clade 7 was divided into two subclades 7a and 7b (Blair *et al.*, 2008; Martin *et al.*, 2014). With addition of several new species including the very recently described *P. asiatica*, *P. fragariaefolia* and *P. nagaii* (Rahman *et al.*, 2014; Rahman *et al.*, 2014), it currently contains 20 species. All species in clade 7a produce non-papillate and non-caducous sporangia. *Phytophthora cambivora* is the only heterothallic species in this subclade (Erwin & Ribeiro, 1996). There is no consistency in antheridial configuration within this subclade. Except for *P. cambivora* and *P. europaea*, all subclade-7a species are relatively low-temperature species which cannot grow at > 30℃. In contrast, all species of subclade 7b can grow at > 30℃. Some species such as *P. melonis*, *P. pistaciae*, and *P. pisi* sustain growth at > 35℃ (Mirabolfathy *et al.*, 2001; Ho *et al.*, 2007; Heyman *et al.*, 2013). All species of subclade 7b are non-papillate and non-caducous. The B-tub phylogeny also indicates that *P. cinnamomi* and *P. parvispora* are clustered with but not grouped in clades 7a or 7b. Previous multi-locus phylogenies (Blair *et al.*, 2008; Martin *et al.*, 2014) indicated that *P. cinnamomi* and *P. parvispora* were basal to other species of subclades 7a and b. The exact phylogenetic positions of *P. cinnamomi* and *P. parvispora* warrant further investigation by the multi-locus phylogeny.

8. Clade 8

Clade 8 contains approximately 22 *Phytophthora* taxa, which are well classified by evidences of phylogeny, morphology of sporangia, and cardinal temperatures. Subclade 8a contains only non-papillate and non-caducous species. Also, subclade-8a species sustain higher maximum growth temperatures, from 31 to > 35℃, than species in subclades 8b-d. Subclade 8a contains both heterothallic and homothallic species, which mostly produce amphigynous and paragynous antheridia, respectively. Subclade 8b contains only semi-papillate and mostly non-caducous species. Most species are homothallic, except that *P. cichorii* is heterothallic and *P.* taxon castitis is sterile (not shown in Figure 7.2) (Bertier *et al.*, 2013). No correlation has been observed with the antheridial configuration. Subclade 8b is well known for containing pathogenic species of winter-grown vegetables (Bertier *et al.*, 2013). The maximum growth temperatures of subclade-8b species are between 21 to 26℃ and many cannot sustain growth > 25℃. Subclade 8c also contains a low-temperature species *P. hibernalis* and a couple of relatively low-temperature species including two notorious pathogens, *P. lateralis* and *P. ramorum* (not shown in Figure 7.2). All subclade-8c species except *P. lateralis* produce semi-papillate and caducous sporangia. *Phytophthora ramorum* is the only heterothallic species producing amphigynous antheridia in this subclade (not shown in Figure 7.2) (Werres *et al.*, 2001). Subclade 8d contains homothallic species producing semi-papillate and non-caducous sporangia as well as sustaining growth < 30℃.

9. Clade 9

Clade 9 is another quickly expanding group within the genus *Phytophthora*. Only eight clade-9 taxa were included in previous phylogenetic analyses (Blair *et al.*, 2008; Martin *et al.*, 2014). Approximately 15 taxa are included in the B-tub phylogeny. The phylogenetic grouping of clade 9 has not been formally determined. Here it is divided into three subclades 9a, 9b and 9c in the B-tub phylogeny. Subclade 9a includes species in the high-temperature tolerant cluster. All these species tolerate extremely high temperature at 35℃ and some can sustain growth at 40℃ (Yang & Hong, 2013; Yang *et al.*, 2014; Yang *et al.*, 2014). Subclade-9a species uniformly produce non-papillate and non-caducous sporangia. Subclade 9b includes three described species. *Phytophthora richardiae* (not shown) and *P. quininea* are identical in producing non-papillate and non-caducous sporangia as well as being homothallic and having maximum growth temperatures at < 30℃, while *P. macrochlamydospora* is semi-papillate, sterile, and has maximum growth temperature at > 30 ℃ (Erwin & Ribeiro, 1996). Subclade 9c also contains three species. *Phytophthora captiosa* and *P. fallax* are sister species sharing common features including non-papillate and non-caducous sporangia as well as homothallism (Dick *et al.*, 2006). *Phytophthora constricta* is basal to these two species. It is unique in producing a mix of non-papillate and semi-papillate, and slightly caducous sporangia (Rea *et al.*, 2011). Phylogenetic evidence and variations in morphological characters of clade-9 species support that clade 9 is paraphyletic.

10. Clade 10

All species of clade 10 except *P. gallica* are papillate, caducous, and homothallic while producing amphigynous antheridia. *Phytophthora gallica* produces non-papillate and non-caducous sporangia and its sexual organs are unknown (Jung & Nechwatal, 2008).

11. *Phytophthora quercina*

Phytophthora quercina was grouped in clade 4 (Blair *et al.*, 2008; Kroon *et al.*, 2012), but recently excluded from the clade and grouped in a unique cluster along with *P.* sp. ohioensis (Martin *et al.*, 2014). In the B-tub phylogeny, *P. quercina* is basal to clades 1, 2 and 4. *Phytophthora quercina* produces papillate and non-papillate sporangia and is a homothallic species producing paragynous antheridia.

12. *Phytophthora stricta*

Phytophthora stricta is basal to clade 8 in the B-tub phylogeny (Figure 7.2) and 28S phylogeny (Figure 7.5), while it was placed basal to clades 1-8 in a prior ITS phylogenetic tree of 120 species (Figure 5.1). Also interesting, it was placed close to subclade 8a based on *cox* 1 (Figure 5.2) and grouped in clade 8 in the Enl phylogeny (Figure 7.4). The exact phylogenetic position of *P. stricta* needs further investigation. This species is non-papillate and heterothallic. It is morphologically unique in producing slightly caducous sporangia with 1 to 3 constrictions on sporangiophore (Yang *et al.*, 2014).

13. *Phytophthora* sp. e1

Phytophthora sp. e1 is a novel species frequently recovered from irrigation water, rivers and streams in Connecticut, Maryland, North Carolina and Virginia (Yang & Hong, unpublished data; Brazee and Ivors, personal communication). A formal description of this novel species is in process. In the B-tub phylogeny, it is close to, but not clustered in clade 10 (Figure 7.2). It produces non-papillate sporangia, which are distinct from the papillate sporangia produced by all clade-10 species except *P. gallica* (Jung & Nechwatal, 2008). Therefore, it is not grouped in clade 10 and its exact phylogenetic position warrants further investigations.

Discussions and Conclusions

Five genetic markers including 60S, B-tub, EF1α, Enl, and 28S were used to construct phylogenies for more than 120 species of the genus *Phytophthora*. The results showed that B-tub provided the highest phylogenetic resolution and was the most informative among selected genetic markers. Enl and 28S also provided high phylogenetic resolutions. However, the phylogenies done in this study were based on sequences of single genes. Although the general structure of the B-tub phylogeny was almost identical to that in previous multi-locus phylogenies (Blair *et al.*, 2008; Martin *et al.*, 2014), some exceptions, exemplified by the exclusion of *P. cinnamomi* and *P. parvispora* from clade 7, indicated potential errors in phylogenies based on sequences of single genes.

Relationships between the B-tub phylogeny and morphological and physiological characters were investigated. The results showed generally correlation between phylogeny and morphology of papillation and maximum growth temperature. Correlations between phylogeny and caducity, homothallism and antheridial configuration were not obvious. These findings were similar to those in previous reviews (Ersek & Ribeiro, 2010; Martin *et al.*, 2012). However, two reasons have challenged the accuracy of these findings. First, only a single-gene phylogeny, the B-tub phylogeny was used as the framework to study the correlation. Due to potential errors in a single-gene phylogeny, the investigation in correlations for some species may be based on an incorrect framework. Second, the data of morphological characters and maximum growth temperatures used in this study were obtained from previous morphological keys and species descriptions. Various methods may have been used by different studies to generate these data. Also, many well-known species in the traditional morphological keys and descriptions have been divided into several new species. It is possible that the morphological characters described in the morphological keys belong to the new species, but not the well-known species. Due to these two reasons, the findings in correlations between morphology and maximum growth temperature, and phylogeny may be inaccurate for some species. In the future, the investigation in relationships between the phylogeny and morphological and physiological characters will be built on the more reliable, multi-locus phylogeny. Also, the morphological and physiological data will be obtained from types and authentic cultures through standard methods.

Currently, a new multi-locus phylogeny of the genus is in progress. Phylogenetic inferences are based on seven nuclear and four mitochondrial genetic markers (Blair *et al.*, 2008; Martin *et al.*, 2014). Approximately 146 *Phytophthora* species and at least 103 type and authentic cultures are included in the new multi-locus phylogeny, which represent almost all formal *Phytophthora* species and most provisional taxa. This new multi-locus phylogeny aims to validate new clades and clusters, as well as investigate detailed phylogenetic and evolutionary relations among species in this quickly expanding, taxonomically complex group, the genus *Phytophthora*.

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Table 7.1 Isolates and sequence accession numbers used in phylogenetic analyses

Clade	Species.	Type [x]	Isolate ID			60S ribosomal protein L10	Beta-tubulin	Elongation factor 1a	Enolase	28S ribosomal DNA
			Local	Original	International					
1	*Phytophthora andina*	A	60A2	EC3394		TBD [y]	TBD	TBD	TBD	TBD
1	*P. cactorum*	R	22E6	S814		TBD	TBD	TBD	TBD	TBD
1	*P. clandestina*	T	32G1		ATCC 58713	EU079866	EU079867	EU079868	EU079869	EU079871
1	*P. hedraiandra*	A	33F4	RBMNT63		TBD	TBD	TBD	TBD	TBD
1	*P. idaei*	T	34D4	P6767	ATCC MYA-4065	EU080129	EU080130	EU080131	EU080132	EU080134
1	*P. infestans*	R	27A8	2C		TBD	TBD	TBD	TBD	TBD
1	*P. ipomoeae*	T	31B4	P10225	CBS 109229	EU080837	EU080838	EU080839	EU080840	EU080842
1	*P. iranica*	T	33D4	DCSCRP386	ATCC60237	EU080111	EU080112	EU080113	EU080114	EU080116
1	*P. mirabilis*	R	30C1	JRP3006		TBD	TBD	TBD	TBD	TBD
1	*P. nicotianae*	R	22G1			TBD	TBD	TBD	TBD	TBD
1	*P. phaseoli*	R	23B4			TBD	TBD	TBD	TBD	TBD
1	*P. pseudotsugae*	T	29B3	333		EU079988	EU079989	EU079990	TBD	EU079992
1	*P. tentaculata*	A	30D5	WMVPD97/5552		TBD	TBD	TBD	TBD	TBD
2	*P. acerina*	T	61H1	B057	CBS 133931	TBD	TBD	TBD	TBD	TBD
2	*P. bisheria*	T	31E6	Cg.2.3.3	CBS122081	EU080741	EU080742	EU080743	EU080744	EU080746
2	*P. botryosa*		46C2		ATCC26481	TBD	TBD	TBD	TBD	TBD
2	*P. capsici*	R	22F4		ATCC15399	TBD	TBD	TBD	TBD	TBD
2	*P. citricola*	T	33H8		CBS22188; IMI021173	TBD	TBD	TBD	TBD	TBD
2	*P. citrophthora*			P6310		EU080537	EU080538	EU080539	EU080540	EU080542
2	*P. colocasiae*	R	22F8		ATCC MYA-4159	TBD	TBD	TBD	TBD	TBD
2	*P. elongata*	T	55C4		CBS 125799	TBD	TBD	TBD	TBD	TBD
2	*P. frigida*	A	47G6	CMW19433		TBD	TBD	TBD	n/a	TBD
2	*P. glovera*	A	31E5	11091.1		TBD	TBD	TBD	TBD	TBD
2	*P. himalsilva*	T	61G2	isolate 44	CBS 128753	TBD	TBD	TBD	TBD	TBD
2	*P. inflata*	T	28D1		IMI342898	TBD	TBD	TBD	TBD	TBD
2	*P. meadii*	R	22G5		ATCC MYA-4043	TBD	TBD	TBD	TBD	TBD
2	*P. mengei*	T	42B2		ATCC MYA-4554	TBD	TBD	TBD	TBD	TBD
2	*P. mexicana*	T	45G4		ATCC46731	TBD	TBD	TBD	TBD	TBD
2	*P. multivesiculata*	T	29E3	95/8679	CBS 545.96	EU080065	EU080066	EU080067	EU080068	EU080070
2	*P. multivora*	T	55C5	WAC13201	CBS 124094	TBD	TBD	TBD	TBD	TBD
2	*P. pini*	T	45F1		ATCC 64532	TBD	TBD	TBD	TBD	TBD
2	*P. plurivora*	R	22E9			TBD	TBD	TBD	TBD	TBD
2	*P. siskiyouensis*	T	41B7	WA-030403		TBD	n/a [z]	TBD	TBD	TBD
2	*P.* taxon aquatilis	T	38J5			TBD	TBD	TBD	TBD	TBD
2	*P.* taxon emzansi	A	61F2	STE-U 6272		TBD	TBD	n/a	TBD	TBD
2	*P. tropicalis*	T	35C8	JUH245	CBS 434.91	TBD	TBD	TBD	TBD	TBD
3	*P. ilicis*	R	23A7		ATCC MYA-2897	TBD	TBD	TBD	TBD	TBD
3	*P. nemorosa*	T	41C4	P13	ATCC MYA-2948	TBD	TBD	TBD	TBD	TBD
3	*P. pluvialis*	T	60B3	LC-9.2-020508		TBD	TBD	TBD	TBD	TBD
3	*P. pseudosyringae*	T	30A8	PSEU 6		TBD	TBD	TBD	TBD	TBD
3	*P. psychrophila*	T	29J5		CBS80395	TBD	TBD	TBD	TBD	TBD

Clade	Species.	Type [x]	Isolate ID			60S ribosomal protein L10	Beta-tubulin	Elongation factor 1a	Enolase	28S ribosomal DNA
			Local	Original	International					
4	*P. alticola*	T	47G4	CMW19417	CBS 121937	TBD	TBD	TBD	TBD	TBD
4	*P. alticola*	A	47G5	CMW19425		TBD	TBD	TBD	TBD	TBD
4	*P. arenaria*	T	55C2	ENA 3	CBS127950	TBD	TBD	TBD	TBD	TBD
4	*P. megakarya*	R	22H7			TBD	TBD	TBD	TBD	TBD
4	*P. palmivora*	R	22G8		ATCC-MYA4039	TBD	TBD	TBD	TBD	TBD
4	*P. quercetorum*	R	15C7	SJAF.018		TBD	TBD	TBD	TBD	TBD
5	*P. heveae*	R	22J1			TBD	TBD	TBD	TBD	TBD
5	*P. katsurae*	A	30E7	FFM 10-5C		TBD	TBD	TBD	TBD	TBD
6	*P. amnicola*	T	61G6	DH228	CBS131652	TBD	TBD	TBD	TBD	TBD
6	*P. asparagi*	A	33D7	SCRP20	IMI384046	TBD	TBD	TBD	TBD	TBD
6	*P. bilorbang*	T	61G8	SA262	CBS161653	TBD	TBD	TBD	TBD	TBD
6	*P. borealis*	T	60B2	AKWA58.1-0708	ATCC MYA-4881; CBS 132023	TBD	TBD	TBD	TBD	TBD
6	*P. fluvialis*	T	55B6	MURU 468	CBS 129424	TBD	TBD	TBD	n/a	TBD
6	*P. gemini*	T	46H1		CBS 123381	TBD	TBD	TBD	TBD	TBD
6	*P. gibbosa*	A	55B7	VHS22007		TBD	TBD	TBD	TBD	TBD
6	*P. gonapodyides*	T	34A8		CBS 554.67; ATCC 60351	TBD	TBD	TBD	TBD	TBD
6	*P. gregata*	A	55B8	VHS21992		TBD	TBD	TBD	TBD	TBD
6	*P. humicola*	T	32F8		CBS 200.81	TBD	TBD	TBD	TBD	TBD
6	*P. inundata*	T	30J3	SKP894		TBD	TBD	TBD	TBD	TBD
6	*P. lacustris*	A	61D6	Ph1		TBD	TBD	TBD	TBD	TBD
6	*P. litoralis*	T	55B9	MURU 463	CBS 127953	TBD	TBD	TBD	TBD	TBD
6	*P. megasperma*	R	23A1	EH61		TBD	TBD	TBD	TBD	TBD
6	*P. mississippiae*	T	57J3		ATCC MYA-4946	TBD	TBD	TBD	TBD	TBD
6	*P. pinifolia*	T	47H1	CMW26668	CBS 122924	TBD	TBD	TBD	TBD	TBD
6	*P. riparia*	T	60B1	VI 3-100B9F		TBD	TBD	TBD	TBD	TBD
6	*P. rosacearum*	T	47J1	EH52		TBD	TBD	n/a	TBD	TBD
6	*P. thermophila*	T	55C1	VHS13530	CBS127954	TBD	TBD	TBD	TBD	TBD
6	*P.* ×*stagnum*	T	43F3			TBD	TBD	TBD	TBD	TBD
7	*P. alni* subsp. *alni*	T	47A7	P772	IMI 392314	TBD	TBD	TBD	TBD	TBD
7	*P. asiatica*	A	45G1	HHLu625		TBD	TBD	TBD	TBD	TBD
7	*P. cajani*	R	33D9	DCSCRP231		TBD	TBD	TBD	TBD	TBD
7	*P. cambivora*	R	22F6	Tsao's P592		TBD	TBD	TBD	TBD	TBD
7	*P. cinnamomi*	R	23B2			TBD	TBD	TBD	TBD	TBD
7	*P. europaea*		34C2	EHAmance 1		TBD	TBD	TBD	TBD	TBD
7	*P. melonis*	T	45F3		CBS 582.69	TBD	TBD	TBD	TBD	TBD
7	*P. parvispora*	A	30G9	KK65507	ATCC MYA-4078	TBD	TBD	TBD	TBD	TBD
7	*P. pisi*	T	60A4	97603		TBD	TBD	TBD	n/a	TBD
7	*P. pistaciae*	T	33D6	DCSCRP533	IMI386658	TBD	TBD	TBD	TBD	TBD
7	*P. rubi*	R	22G6		ATCC 11374	TBD	TBD	TBD	n/a	TBD
7	*P. sojae*	R	28F9	P6497		TBD	n/a	TBD	TBD	TBD
7	*P. uliginosa*	T	62A3		CBS 109054	EU080011	EU080012	EU080013	ts	EU080015
7	*P. vignae*	T	45G6	GZP606 (901)		TBD	TBD	TBD	TBD	TBD

Clade	Species.	Type [x]	Isolate ID			60S ribosomal protein L10	Beta-tubulin	Elongation factor 1a	Enolase	28S ribosomal DNA
			Local	Original	International					
8	*P. austrocedrae*	T	41B6	AG203	ATCC MYA-4074	TBD	TBD	TBD	TBD	TBD
8	*P. brassicae*	A	29D8		CBS 686.95	TBD	TBD	TBD	TBD	TBD
8	*P. cichorii*	A	61E3		CBS133815	TBD	n/a	TBD	TBD	TBD
8	*P. dauci*	T	61E5	BorfSP370	CBS127102	TBD	n/a	TBD	TBD	TBD
8	*P. erythroseptica*		22J4		ATCC MYA-4041	TBD	TBD	TBD	TBD	TBD
8	*P. foliorum*	T	49J8	LT192		TBD	TBD	TBD	TBD	TBD
8	*P. hibernalis*	T	22H1		CBS27031	TBD	TBD	TBD	TBD	TBD
8	*P. lactucae*	T	61F4	BPIC 1985		TBD	TBD	TBD	TBD	TBD
8	*P. lateralis*	R	29A9		ATCC 201856	TBD	TBD	TBD	TBD	TBD
8	*P. medicaginis*	R	28F1	EP1057		TBD	TBD	TBD	TBD	TBD
8	*P. obscura*	T	60E9	BBA 2/94-IIB	CBS 129273	TBD	TBD	TBD	TBD	TBD
8	*P. porri*	R	32E7	= DB148-1		TBD	TBD	TBD	TBD	TBD
8	*P. primulae*	R	29E9	WMVPD97/875		TBD	TBD	TBD	TBD	TBD
8	*P. ramorum*	R	32G2	SJ04-4398		TBD	TBD	TBD	TBD	TBD
8	*P. richardiae* II	A	31E8			TBD	TBD	TBD	TBD	TBD
8	*P. sansomeana*	T	47H3	1819B	ATCC MYA-4455	TBD	TBD	TBD	n/a	TBD
8	*P.* sp. kelmania	A	22G2		CBS30862; IMI325907	TBD	TBD	TBD	TBD	TBD
8	*P. syringae*	A	21H9		ATCC 34002	TBD	TBD	TBD	TBD	TBD
8	*P. trifolii*	A	29B2	EH33		TBD	TBD	TBD	TBD	TBD
9	*P. aquimorbida*	T	40A6		ATCC MYA-4578	TBD	TBD	TBD	TBD	TBD
9	*P. captiosa*	T	46H7	310c		EU079658	EU079659	EU079660	EU079661	EU079663
9	*P. chrysanthemi*	T	61F1	GF749	CBS123163	TBD	TBD	TBD	TBD	TBD
9	*P. constricta*	T	55C3	MURU 454	CBS125801	TBD	TBD	TBD	TBD	TBD
9	*P. fallax*	T	46J2	310L		TBD	TBD	TBD	TBD	TBD
9	*P. hydrogena*	T	46A3		ATCC MYA-4919	TBD	TBD	TBD	TBD	TBD
9	*P. hydropathica*	T	5D1		ATCC MYA-4460	TBD	TBD	TBD	TBD	TBD
9	*P. insolita*	T	38E1	PMC5-1	ATCC 38789	EU080175	EU080176	EU080177	EU080178	EU080180
9	*P. irrigata*	T	23J7		ATCC MYA-4457	TBD	TBD	TBD	TBD	TBD
9	*P. macilentosa*	T	58A7		ATCC MYA-4945	TBD	TBD	TBD	TBD	TBD
9	*P. macrochlamydospora*		31E9			EU080658	EU080659	EU080660	n/a	EU080662
9	*P. parsiana*	T	47C3	SUC25		TBD	TBD	TBD	TBD	TBD
9	*P. polonica*	T	49J9		CBS 119650	EU080256	EU080257	EU080258	EU080259	EU080261
9	*P. quininea*	T	45F2		CBS406.48	EU080107	EU080108	EU080109	n/a	EU080110
9	*P.* sp. lagoariana	A	60B4	P8220		EU080358	n/a	EU080359	EU080360	EU080362
9	*P. virginiana*	T	46A2		ATCC MYA-4927	TBD	TBD	TBD	TBD	n/a
10	*P. boehmeriae*	T	45F9	P6950	CBS 291.29	EU080161	EU080162	EU080163	EU080164	EU080166
10	*P. gallica*	T	50A1	GAL 1	CBS 111474	TBD	TBD	TBD	TBD	TBD
10	*P. kernoviae*	A	38E4	P1920		TBD	TBD	TBD	TBD	TBD
new	*P. quercina*	T	30A5		CBS 784.95	TBD	TBD	TBD	TBD	TBD
new	*P.* sp. e1		45B7			TBD	TBD	TBD	TBD	TBD
new	*P. stricta*	T	58A1		ATCC MYA-4944	TBD	TBD	TBD	TBD	TBD
outgroup	*Halophytophthora fluviatilis*	T	57A9			TBD	TBD	TBD	TBD	TBD
outgroup	*P.* sp. PK2	A	PK113			TBD	TBD	n/a	TBD	TBD

Clade	Species.	Type [x]	Isolate ID			60S ribosomal protein L10	Beta-tubulin	Elongation factor 1a	Enolase	28S ribosomal DNA
			Local	Original	International					
outgroup	*Phytopythium vexans*	A		IFAPA-CH835		EU080483	EU080484	EU080485	n/a	EU080487
outgroup	*Pythium undulatum*	A		P10342		EU080440	EU080441	EU080442	n/a	EU080444

[x] T = type culture; A = authentic culture used in original species description; R = representative culture validated by multiple sequences analyses

[y] TBD = sequences generated in this study and to be deposited

[z] n/a = data not available

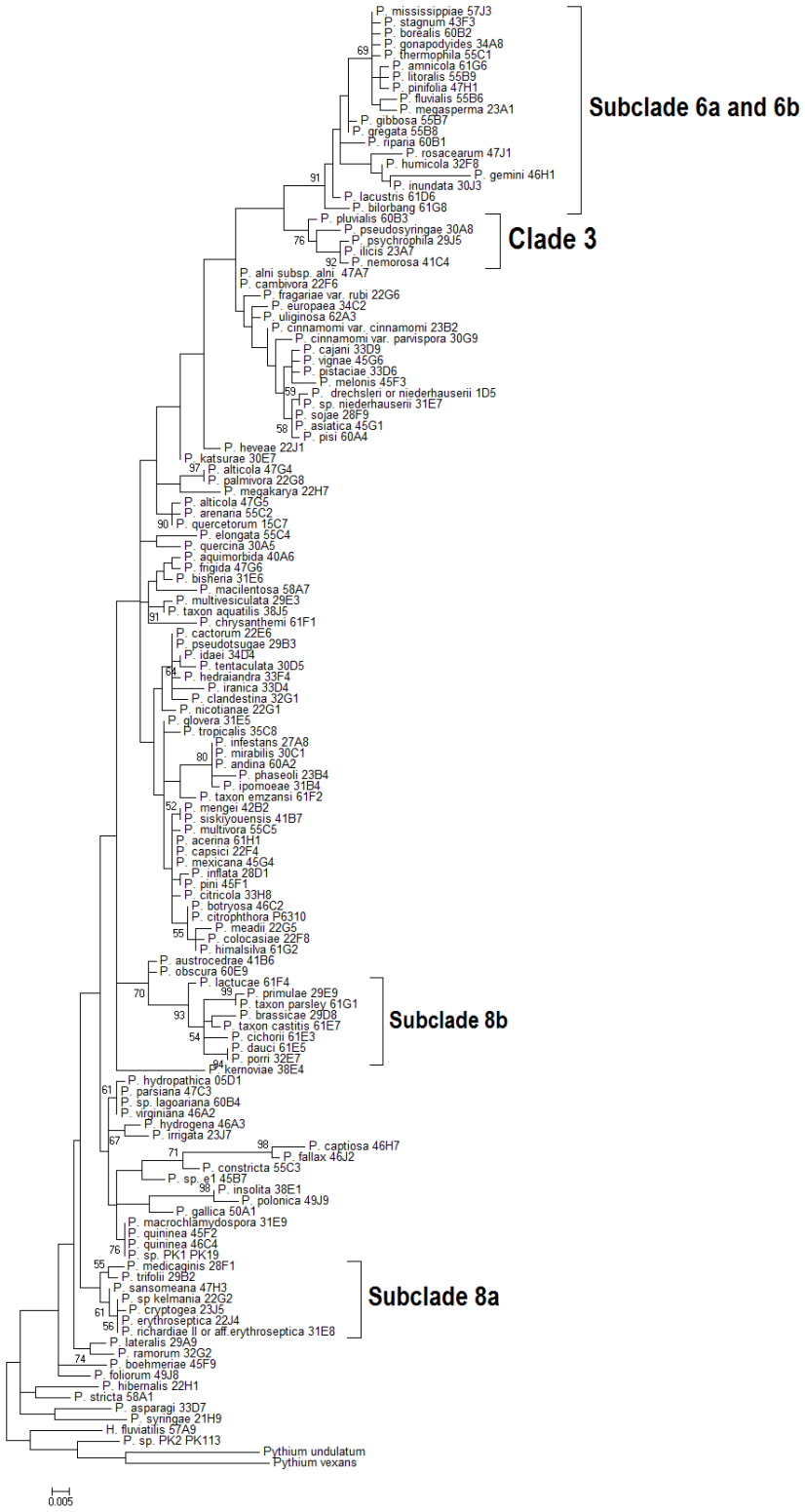

Figure 7.1 Maximum likelihood phylogenetic tree based on sequences of 60S Ribosomal protein L10 locus. Alignment was conducted with Clustal W and the phylogenetic tree was generated and compressed in MEGA 5 (500 replications of bootstrap; values < 50% not shown).

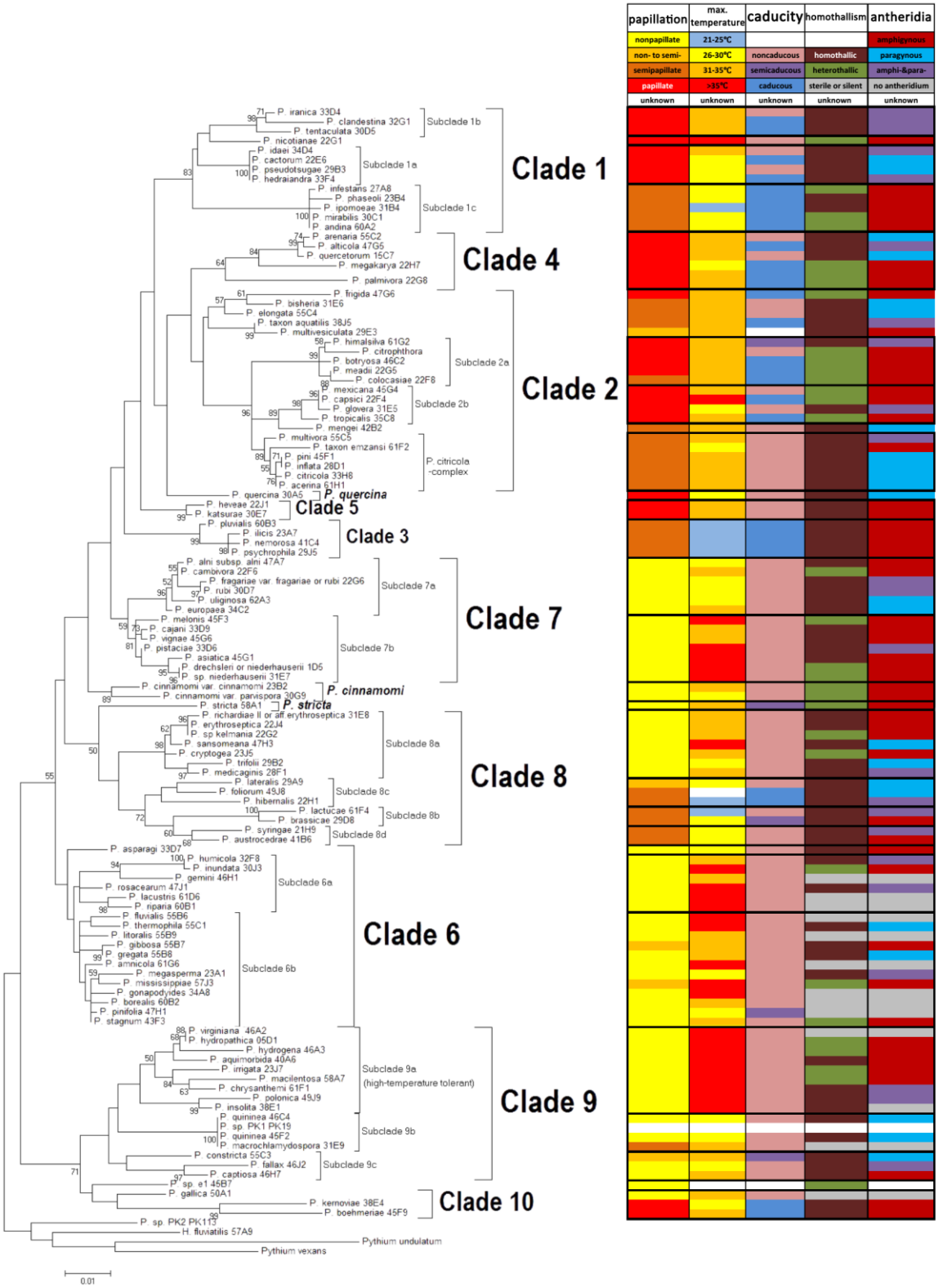

Figure 7.2 Maximum likelihood phylogenetic tree based on sequences of beta-tubulin gene with features of sporangial papillation, caducity, gametangia, and maximum growth temperature.

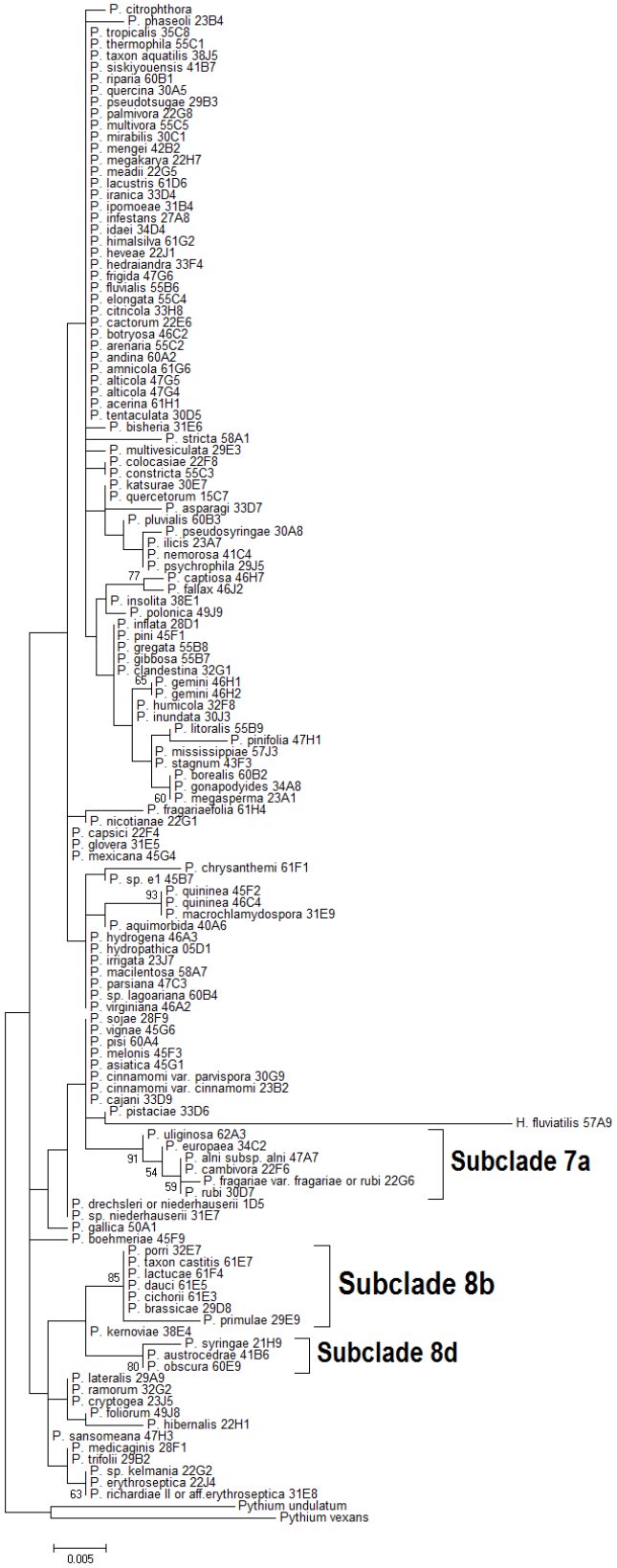

Figure 7.3 Maximum likelihood phylogenetic tree based on sequences of elongation factor 1α gene.

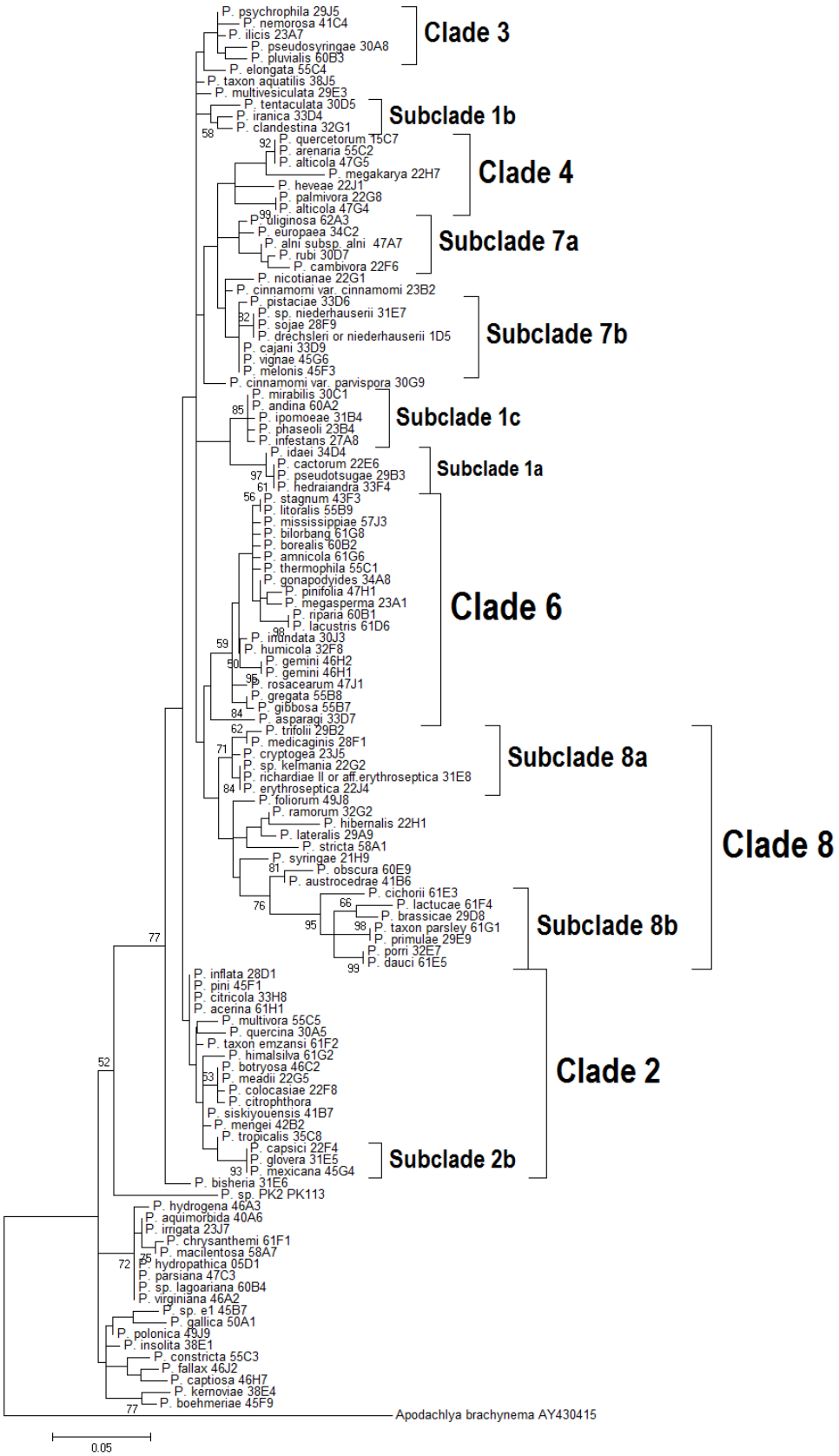

Figure 7.4 Maximum likelihood phylogenetic tree based on sequences of enolase gene.

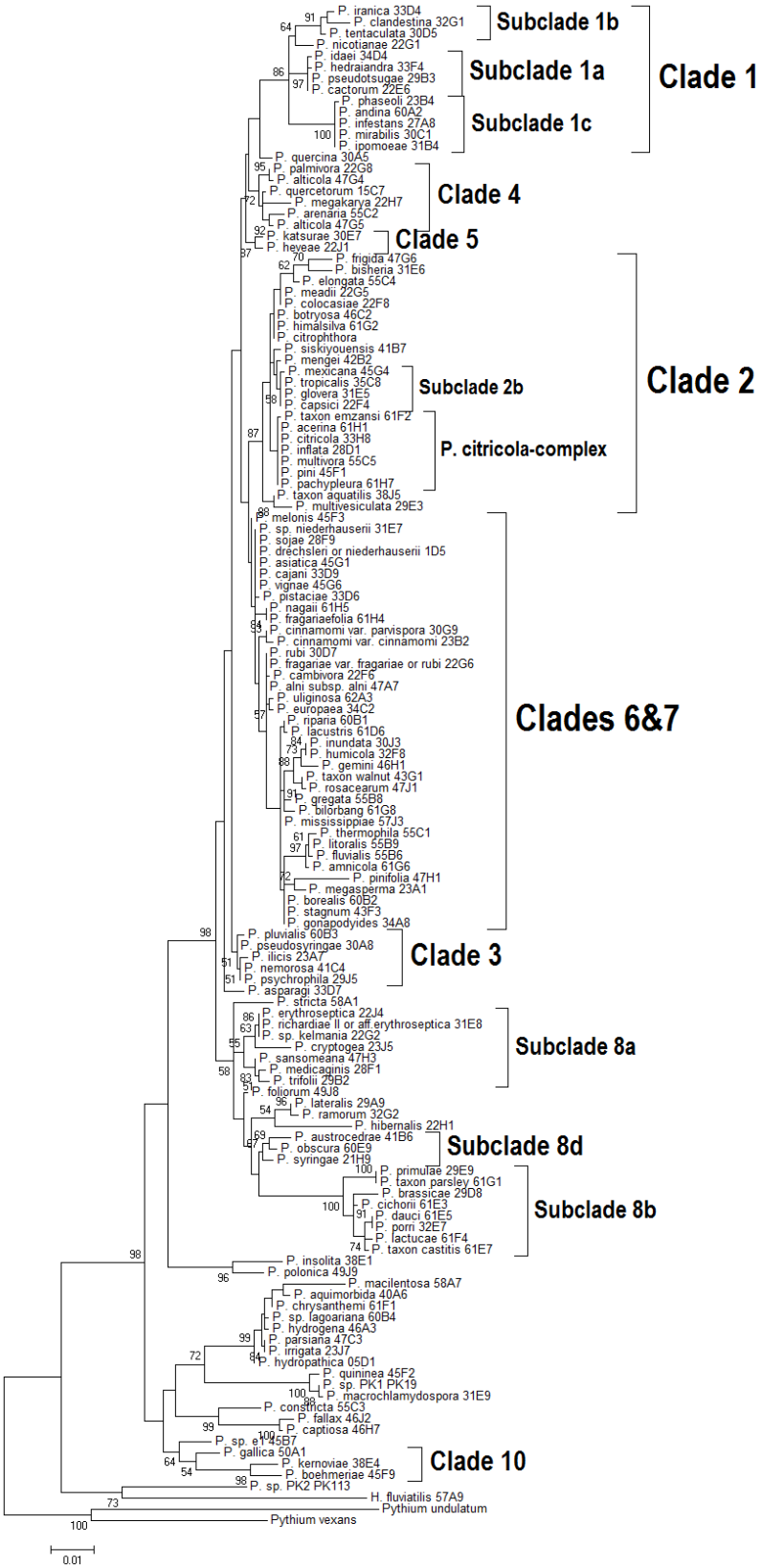

Figure 7.5 Maximum likelihood phylogenetic tree based on sequences of 28S Ribosomal DNA locus.

Appendix 1 *Halophytophthora fluviatilis* sp. nov. from freshwater in Virginia

Yang X & Hong CX (2014) *Halophytophthora fluviatilis* sp. nov. from freshwater in Virginia. *FEMS Microbiology Letters* **352**: 230-237. http://dx.doi.org/10.1111/1574-6968.12391

Abstract

Halophytophthora fluviatilis, a novel species from inland freshwater in Virginia, is characterized and described in this study. This homothallic species produced ovoid to globose sporangia which release zoospores directly through exit pores. It grew well in a relatively wide range of salinity from 1.8 to 19.0 parts per thousand. Sequence analysis of the rRNA internal transcribed spacer region placed this new species in the *Halophytophthora sensu stricto* clade. Description of this new species expanded the habitat to include geographically distinct inland freshwater ecosystems for the genus *Halophytophthora*, challenging the notion that this genus is marine or brackish. The need to construct a molecular-based taxonomy for the genus *Halophytophthora* is also discussed.

Introduction

The genus *Halophytophthora* includes a group of *Phytophthora*-like oomycetes that have been perceived to inhabit marine ecosystems. The type species, *H. vesicula* was discovered in Vancouver, British Columbia, Canada, and initially described as *Phytophthora vesicula* (Anastasiou & Churchalnd, 1969). Likewise, several other groups from tropical, subtropical and temperate seawater were initially described as *Phytophthora* species (Fell & Master, 1975; Pegg & Alcorn, 1982; Gerrettson-Cornell & Simpson, 1984).

The genus *Halophytophthora* was established in 1990 to separate nine marine *Phytophthora* species from those freshwater and terrestrial species (Ho & Jong, 1990). These nine species were *H. avicennae*, *H. bahamensis*, *H. batemanensis*, *H. epistomium*, *H. mycoparasitica*, *H. operculata*, *H. polymorphica*, *H. spinosa* var. *lobata*, *H. spinosa* var. *spinosa*, and *H. vesicula* (Anastasiou & Churchalnd, 1969; Fell & Master, 1975; Pegg & Alcorn, 1982; Gerrettson-Cornell & Simpson, 1984). Thereafter, six new species: *H. kandeliae* (Ho *et al.*, 1991), *H. exoprolifera* (Ho *et al.*, 1992), *H. masteri*, *H. tartarea* (Nakagiri *et al.*, 1994), *H. porrigovesica* (Nakagiri *et al.*, 2001), and *H. elongata* (Ho *et al.*, 2003) from the Atlantic and Pacific marine environments, have been added to this genus. These species were described primarily by morphological features. The dichotomous key for identifying *Halophytophthora* species was based on the morphology of sporangium, sporangiophore, and the presence or absence of vesicles and dehiscence plugs (Nakagiri *et al.*, 2001). In the year 2010, *H. tartarea* was reexamined and subsequently transferred to the genus *Salisapilia* following sequence analyses (Hulvey *et al.*, 2010). Therefore, the genus *Halophytophthora* currently includes 14 species plus two

varieties. Isolates representing new *Halophytophthora* taxa have recently been recovered (Hulvey *et al.*, 2010; Nigrelli & Thines, 2013), but they are yet to be formally described.

Although all known *Halophytophthora* species have been almost exclusively found in saline water, there is evidence that some of these species may survive at a range of salinity levels. *Halophytophthora* species have a wide tolerance to salinity from fresh to saline water (Nakagiri *et al.*, 1994; Nakagiri *et al.*, 2001). This physiological characteristic may be an indication that some *Halophytophthora* species live in freshwater environments. Specifically, *H. batemanensis*, *H. spinosa*, and *H. vesicula* have been collected from upstream of a river flowing into the sea, which contained brackish water with a very low salinity (Nakagiri, 2000).

The genus *Halophytophthora* includes a genetically diverse group. Several recent studies have indicated that this genus is paraphyletic. Five *Halophytophthora* species including *H. avicenniae*, *H. bahamensis*, *H. batemanensis*, *H. polymorphica*, and the type species *H. vesicula* form a distinct clade which is commonly referred as the *Halophytophthora sensu stricto* clade (Lara & Belbahri, 2011; Nigrelli & Thines, 2013). A phylogenetic analysis based on mitochondrial sequences showed that *H. exoprolifera* and *H. tartarea* (current name *Salisapilia tartarea*) are closely related to other genera instead of the *Halophytophthora s. str.* clade (Robideau *et al.*, 2011). These observations are supported by more recent studies (Lara & Belbahri, 2011; Nigrelli & Thines, 2013).

This study examined the molecular, morphological and physiological traits of a group of freshwater isolates that belong to the *Halophytophthora s. str.* clade, but not to any known species. This previously unknown species is named as *Halophytophthora fluviatilis* sp. nov.

Materials and Methods

Isolate collection and maintenance

Isolates of *Halophytophthora fluviatilis* were recovered from rhododendron leaf baits during a stream survey for *Phytophthora ramorum* by the Virginia Department of Forestry. These isolates were recovered from several distinct inland freshwater locations in Virginia, USA (Figure A1.1). The baits were deployed in the surveyed streams for 7 to 14 days then transferred to a laboratory. They were then cut into 10 mm × 10 mm sections and plated onto PARP selective media (contains pimarcin, ampicillin, rifampicin, and pentachloronitrobenzene). Pure cultures were obtained from hyphal tips of emerging colonies from the edge of leaf pieces. Cultures were maintained and routinely subcultured onto 20% clarified V8 juice agar (CV8A) or seawater V8 juice agar (SV8A, containing 20% seawater) in the study. Blocks of fresh cultures growing in CV8A were transferred into microtubes with sterile distilled water (diH_2O) for long-term storage at 15℃. The holotype was deposited at the American Type Culture Collection (MYA-4961) in Manassas, Virginia. Seawater used in the salinity tests and sporangial production in this study was collected from the Chesapeake Bay in Virginia Beach, Virginia in September,

2013 then filtered through two layers of Whatman #1 filter paper (Particle retention: 11 µm; Whatman International Ltd., Maidstone, England). The filtrate had a salinity of 22.2 parts per thousand (PPT) and was stored at 4°C until use.

DNA extraction, amplification and sequencing

To produce mycelia for DNA extraction, a 10 mm × 10 mm agar plug from an actively growing culture of each *H. fluviatilis* isolate was placed in 20% V8 juice broth and incubated at room temperature (*c*. 23°C) for 10 days (Erwin & Ribeiro, 1996). The mycelial mass was dried and lysed using a FastPrep®-24 system (MP Biomedicals, Santa Ana, CA, USA). DNA was extracted using a DNeasy® Plant Mini kit (Qiagen, Valencia, CA, USA). The ITS region was amplified using the forward primer ITS6 and reverse primer ITS4 (Cooke *et al.*, 2000). PCR products were sequenced in both directions using the same primers at the University of Kentucky Advanced Genetic Technologies Center (Lexington, KY, USA). Sequences in both directions were visualized with Finch TV *v.* 1.4.0. (Geospiza Inc., Seattle, WA, USA) and edited manually to correct obvious errors.

Sequence analysis

The ITS sequences of *H. fluviatilis* were compared to those of selected species of *Halophytophthora*, *Phytophthora*, *Phytopythium*, *Pythium*, and *Salisapilia* in GenBank (www.ncbi.nlm.gov/genbank/). These sequences were aligned using MAFFT online version 7 (Katoh & Standley, 2013) and the Q-INS-i algorithm (Katoh *et al.*, 2005).

Maximum Likelihood (ML)-based phylogeny was constructed using RAxML web servers (Stamatakis *et al.*, 2008) with default setting. The ML phylogenetic tree was visualized using FigTree *v*. 1.4.0. Neighbor Joining (NJ) inference was done with MEGA5 (Tamura *et al.*, 2011) using the Tamura-Nei model (Tamura & Nei, 1993) with 1,000 bootstrap replicates.

Physiology: cardinal temperatures and the optimum salinity

Three isolates 57A9, 59B8, and 59J1, recovered from different locations (Figure A1.1) in 2011 and 2012, were assessed for their cardinal temperatures and the optimum salinity. The cardinal temperature test was conducted at 5, 10, 15, 20, 25, 27, 28, 29, and 30℃. Agar blocks (5 mm in diam.) were taken from actively growing cultures (~10-day-old) and placed mycelial side down at the center of 10-cm Petri dishes containing freshly-made CV8A. Triplicate dishes per isolate were placed in the dark at each temperature. Two perpendicular diameters of each colony were measured after 9 days, when the fastest growing colonies were about 1 cm away from the dish edge.

The salinity test used the same base medium (CV8A) incorporating five treatments of 0, 20%, 40%, 60% and 80% of seawater, all at 25℃. The salinity levels of nonamended and seawater-amended V8 broths were measured using a Horiba U-10 Water Quality Checker (Horiba Ltd., Kyoto, Japan) before adding agar and autoclaving. The salinity in nonamended, 20%, 40%, 60% and 80% seawater-amended V8 broths was 1.8, 6.1, 10.4, 14.7 and 19.0 PPT, respectively. Colony diameters were measured after 9 days. Both

temperature and salinity tests were repeated once. Analysis of variance of the radial growth rates in both tests and among isolates of *H. fluviatilis* was performed in R statistical software *v.* 2.11.0 (R Core Team, 2012).

Morphology

Sporangia were produced by using a modified method of Ho *et al.* (2003). Three mycelial agar plugs (1 mm in diam.) were taken from the edge of an actively growing culture on CV8A using a sterile Pasteur pipette, placed in a 6-cm plastic Petri dish, and submerged in sterile seawater V8 broth (20% clarified V8 juice, 20% filtered, seawater, and 60% diH_2O) for 3 days at room temperature in the dark until the diameter of mycelial mats reached ~ 20 mm. The seawater V8 broth was removed. The mycelial mats were rinsed twice with diH_2O. Then a sporangium-inducing solution containing 80% non-sterile soil water extract (15 g of sandy loam soil / 1 L diH_2O) and 20% filtered, non-sterile seawater was added. After continuously exposed under cool-white fluorescent lamps at room temperature for 2 to 3 days, sporangia were observed at the edge of mycelial mats. Fifty randomly selected mature sporangia were photographed with a Nikon Fujix Digital Camera HC-300Zi connected to a Nikon Labophot-2 microscope and measured for length and width with Image-Pro® Plus *v.* 5.1.2.53.

Halophytophthora fluviatilis did not produce sexual structures in CV8A or SV8A. In order to produce gametangia, individual isolates of *H. fluviatilis* were grown separately

on hemp seed agar (HSA). More than 30 randomly selected gametangia were measured for the size of oogonia and oospores.

Results

Sequences and phylogeny

Six isolates of *H. fluviatilis* have an identical ITS sequence, which is distinct from those of all known species. The sequence was 914 bp comprising 280-bp ITS1 region, 162-bp 5.8s rDNA, and 527-bp ITS2 region. Aligned sequences indicate that *H. fluviatilis* is divergent from its closest relatives, *H. avicenniae*, by more than 130 bp (indels and points of mutation) in the whole ITS sequence. The homologous region in ITS sequence of *H. fluviatilis* is 93% identical to that of *H. avicenniae* (GenBank Accession No. HQ643147).

In both ML and NJ phylogenetic trees based on the ITS sequences of selected *Halophytophthora*, *Phytophthora*, *Phytopythium*, *Pythium*, and *Salisapilia* species (Figure A1.2), isolates of *H. fluviatilis* form a distinct taxon and reside in the *Halophytophthora s. str.* clade.

Radial growth at various temperatures and salinities

In the temperature tests, radial growth rates were statistically the same between two repeated experiments ($P = 0.45$) and among three isolates of *H. fluviatilis* ($P = 0.99$).

Therefore, radial growth rates of repeated temperature tests were pooled and the averages of three isolates were analyzed and plotted (Figure A1.3). The optimum temperature for the mycelial growth was 25℃ with the minimum < 5℃. Little growth was observed at 29℃. No growth occurred at 30℃. After exposing to 30℃ for 9 days, mycelial plugs were not able to resume growth at room temperature.

Three examined isolates also showed similar responses to various salinities ($P = 0.19$). They grew in a relatively wide range of salinity from 1.8 to 19.0 PPT. However, colony morphology varied under different salinity levels. The edges of the cultures were relatively smooth at 1.8 and 6.1 PPT, while becoming irregular at higher salinities (Figure A1.4). The optimum salinity for *H. fluviatilis* was 6.1 PPT (Figure A1.4) with an average daily radial growth >2.9 mm. However, the average growth rate decreased significantly ($P < 0.05$) with increasing salinity (Figure A1.4).

Taxonomy

Halophytophthora fluviatilis X. Yang & C. X. Hong, sp. nov. (Figures. A1.5 & A1.6)

MycoBank: MB807647

Etymology

'*fluviatilis*' refers to the freshwater habitats where this new species was initially recovered.

Mycelia hyaline, irregularly branched. Sporangiophores simple sympodium, occasionally compound sympodium. Abundant sporangia produced by mycelial mats submerged in the sporangium-inducing solution under light within 48 to 72 hours. Immature sporangia mostly globose, nonpapillate (Figure A1.5A). Sporangial apex rose and swollen with increasing maturity (Figure A1.5B). Mature sporangia mostly globose to ovoid (Figures A1.5B, C, D), sometimes limoniform (Figure A1.5E) to obovoid (Figure A1.5F), and rarely in distorted shapes (Figures A1.5G, H). Mature sporangia 28.3-58.2 µm (ave. 38.4 ± 5.4 µm) long and 20.1-41.0 µm (ave. 28.8 ± 4.4 µm) wide. Papillae 1.8-5.5 µm (ave. 3.1 ± 0.8 µm) thickness, sometimes produced at the sporangia apex (Figures A1.5C, D). Zoospores released directly to the environment (Figures A1.5I, J, K, L) instead of into a vesicle. External proliferations common, formed a new hypha or 1-2 sporangia (Figure A1.5L). Most mature sporangia have a conspicuous basal plug (2.1-5.6 µm; ave. 3.2 ± 0.7 µm thickness) (Figures A1.5B, C, E, F, G, I, J, L, M, N). Some sporangia on top of a swollen base cell to which a basal plug attached (Figures A1.5M, N). Intercalary hyphal swellings (Figures A1.5A, K) and sporangiophore swellings common.

Gametangia produced in HSA after 20 days. Oogonia lateral (Figures A1.6A, D, E) or terminal (Figures A1.6B, C); globose, golden-pigmented with maturing (Figures A1.6A, F), 23.4-35.1 µm (ave. 28.2 ± 2.6 µm) diameter, sometimes formed in a cluster (Figure A1.6F). Oospores plerotic, 21.8-29.3 µm (ave. 25.8 ± 2.1 µm) diameter, sometimes

aborted (Figure A1.6C). Antheridia paragynous (Figures A1.6A, C, E), usually small (< 5.0 μm long and wide); rarely swollen, surrounding the oogonial stalk and appeared amphigynous (Figure A1.6B).

Holotype

ATCC MYA-4961 (exo-type: 57A9; GenBank KF734963), recovered from Flint Run Stream, Virginia, USA, October, 2011. Other isolates examined: 52G9 from Jordan River, Virginia, December, 2010; 57B5 (GenBank KF734964) from Compton Creek, Virginia, October, 2011; 59B8 (GenBank KF734965) and 59B9 (GenBank KF734966) from Stockton Creek, Virginia, October, 2012; 59H9 (GenBank KF734967) and 59J1 (GenBank KF734968) from Rappahannock River, Virginia, October, 2012.

Discussion

This study describes a new species, *H. fluviatilis*, with all examined isolates recovered from inland freshwater (Figure A1.1). This study, along with previous observations of *Halophytophthora batemanensis*, *H. spinosa*, and *H. vesicula* in upstream of a river (Nakagiri, 2000), present a strong challenge to the notion that all members of the genus *Halophytophthora* are marine or brackish (Ho & Jong, 1990).

Halophytophthora fluviatilis can be readily distinguished from the fourteen known *Halophytophthora* species by its distinct morphology. Previously described

Halophytophthora species are distinguished from *Phytophthora* species by their mode of zoospore release (Ho & Jong, 1990). *Phytophthora* species release zoospores directly through exit pores or into evanescent vesicles, while *Halophytophthora* species produce vesicles to retain zoospores or release zoospores through dehiscence tubes. Specifically, *H. masteri* (Nakagiri *et al.*, 1994) and *H. vesicula* (Anastasiou & Churchalnd, 1969) retain zoospores in semi-persistent or persistent vesicles; *H. epistomium* and *H. spinosa* (Fell & Master, 1975) release zoospores through dehiscence tubes in the sporangial apex plug; *H. elongata* (Ho *et al.*, 2003) produces both tubular vesicles and dehiscence plugs; *H. batemanensis* and *H. polymorphica* frequently retain zoospores in vesicles, though sometimes release zoospores directly (Gerrettson-Cornell & Simpson, 1984). Unlike these previously described *Halophytophthora* species, *H. fluviatilis* does not produce vesicles. Even though papillae are produced by *H. fluviatilis*, they are not as elongated as those of *H. elongata* (Ho *et al.*, 2003), *H. epistomium*, or *H. spinosa* (Fell & Master, 1975). Under morphological examination, the papillae of *H. fluviatilis* quickly vanish at the moment of zoospore release without any sign of producing dehiscence tubes. Accordingly, *H. fluviatilis* is the one species in this genus that only releases zoospores directly, which is essentially the same as most *Phytophthora* species (Erwin & Ribeiro, 1996).

Although being similar in the mode of zoospore release, *H. fluviatilis* can be easily distinguished from most *Phytophthora* species. First, this new species produces narrower hyphae (3.0-5.5 µm, ave. 4.1 µm), while most *Phytophthora* species produce 5.0 to 8.0 µm hyphae in width (Blackwell, 1949), except some *Phytophthora* species produce

relatively narrow hyphae (~ 5.0 µm) such as *P. hydropathica* (Hong *et al.*, 2010), *P. irrigata* (Hong *et al.*, 2008), and *P. parsiana* (Mostowfizadeh-Ghalamfarsa *et al.*, 2008). Second, *H. fluviatilis* has slow vegetative growth (< 3 mm daily on CV8A at optimum temperature) like other *Halophytophthora* species (Ho & Jong, 1990), unlike most *Phytophthora* species which usually have greater growth rates at optimum temperature. Both attributes are useful for differentiating this new *Halophytophthora* species from most *Phytophthora* species.

This study explicitly expanded the ecological habitat from seawater to include freshwater for the genus *Halophytophthora*, permanently changing the notion that it is a marine genus (Ho & Jong, 1990). All isolates examined in this study originated from inland freshwater. The ecological role of this new species is not known at this time. It is possible that *H. fluviatilis* decomposes plant debris fallen into streams and rivers as many other *Halophytophthora* species do in marine water. As demonstrated by the salinity tests in this study, *H. fluvialitis* is well adapted to a wide range of salinity. Investigations into whether this new species exists in saline water and its exact ecological roles are warranted.

This study also supported the previous finding that the genus *Halophytophthora* is a paraphyletic group, highlighting the importance of molecular characterization when naming a new species. Specifically, *H. porrigovesica* (Nakagiri *et al.*, 2001), *H. kandeliae* (Ho *et al.*, 1991), *H. epistomium* (Fell & Master, 1975), and *H. exoprolifera* (Ho *et al.*, 1992) are more closely related to the genera such as *Phytophthora*,

Phytopythium, and *Pythium* than the *Halophytophthora s. str.* clade (Figure A1.2). Similar observations were made in several previous phylogenetic analyses (Cooke *et al.*, 2000; Hulvey *et al.*, 2010; Lara & Belbahri, 2011; Robideau *et al.*, 2011; Nigrelli & Thines, 2013). This result along with previous studies indicated the urgent need of constructing a molecular-based taxonomy for the genus *Halophytophthora* and reevaluating the taxonomic status of those species outside the *Halophytophthora s. str.* clade. Prior to this study, identifications of *Halophytophthora* species were based primarily on morphological characters. Inclusion of sequence analysis along with morphological descriptions of new species will help to avoid further complications to the already complex taxonomy of the genus *Halophytophthora*.

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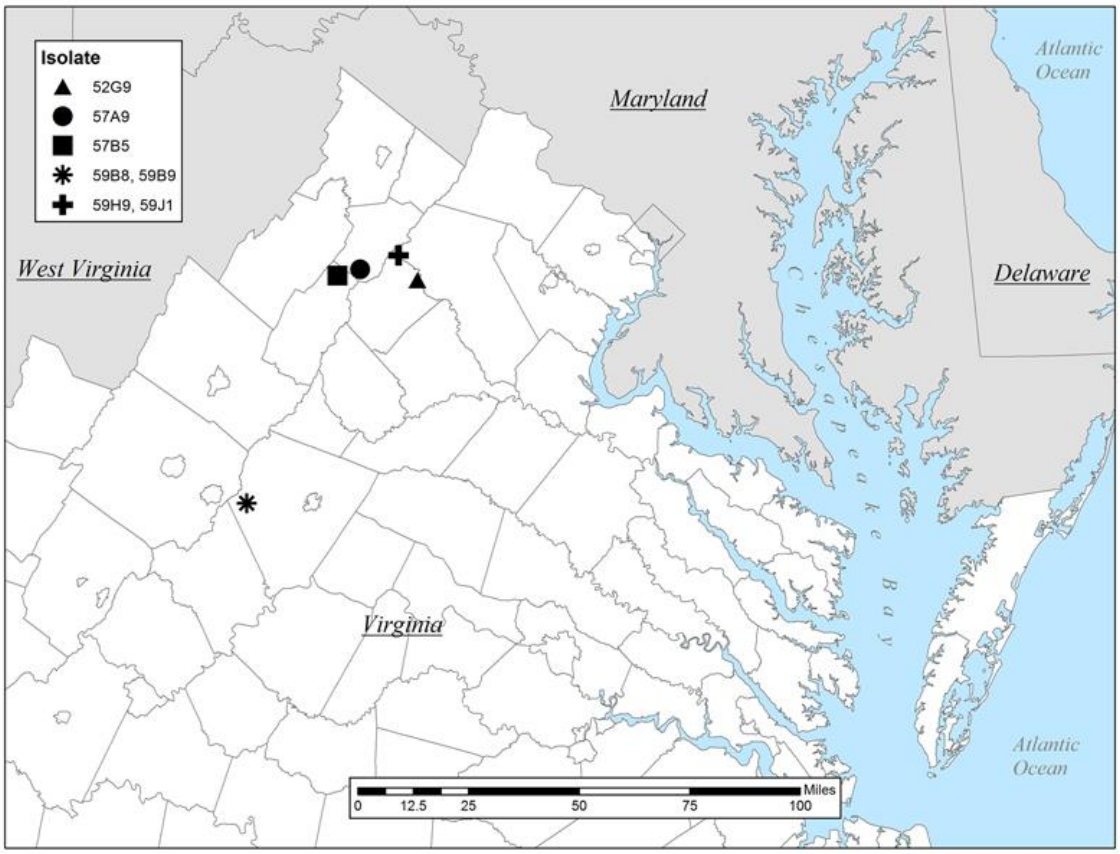

Figure A1. 1 Virginia map showing locations from which the isolates of *Halophytophthora fluviatilis* were recovered

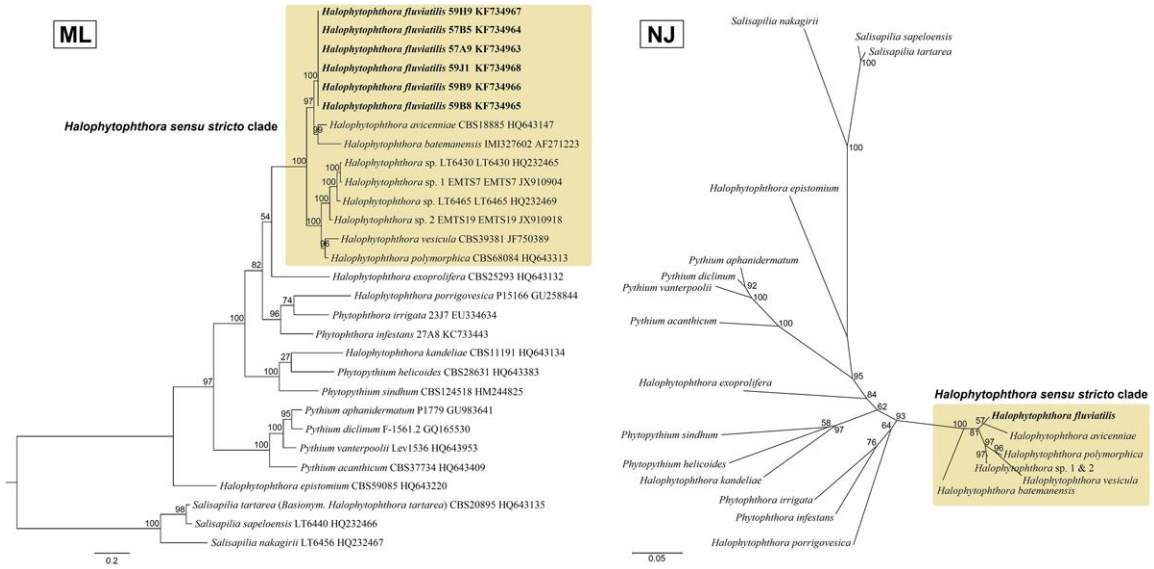

Figure A1. 2 Maximum Likelihood (ML) and radial Neighbor Joining (NJ) phylogenetic trees based on ITS sequences of selected *Halophytophthora*, *Phytophthora*, *Phytopythium*, *Pythium*, and *Salisapilia* species. Alignment was conducted with MAFFT version 7. The ML-tree was generated by RAxML and the NJ-tree in MEGA5. GenBank accession numbers of sequences are given following the species names and isolate codes in the ML-tree.

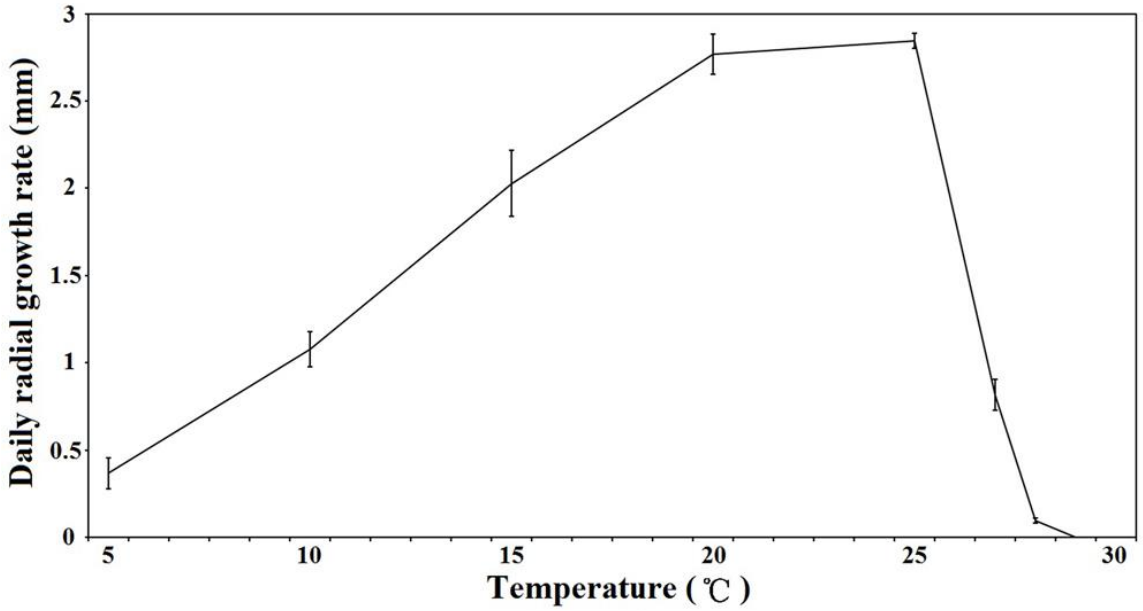

Figure A1. 3 Average daily radial growth of isolates 57A9, 59B8, and 59J1 on 20% clarified V8 juice agar at various temperatures

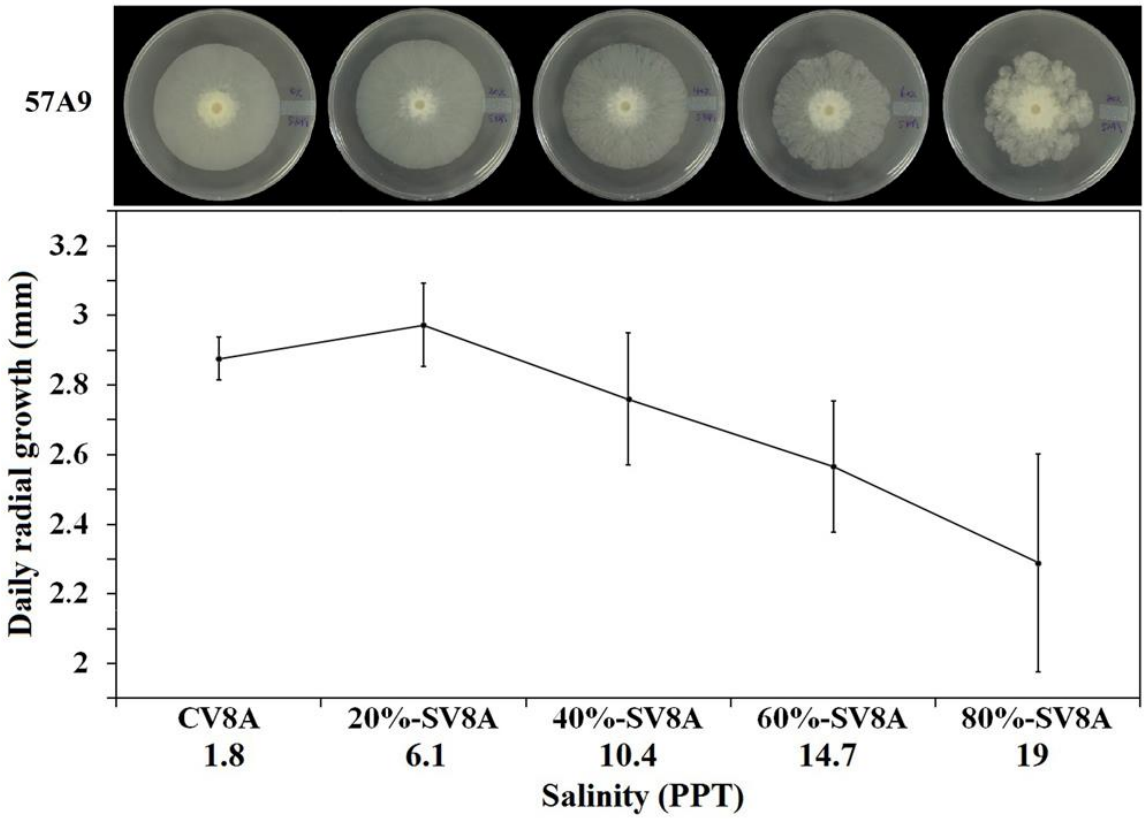

Figure A1. 4 Growth of *Halophytophthora fluviatilis* on 20% clarified V8 juice agar (CV8A) and 20-80% seawater V8 juice agar (SV8A) at 25℃ for 9 days: colony morphology of isolate 57A9 (Top) and average daily radial growth of isolates 57A9, 59B8, and 59J1 (Bottom)

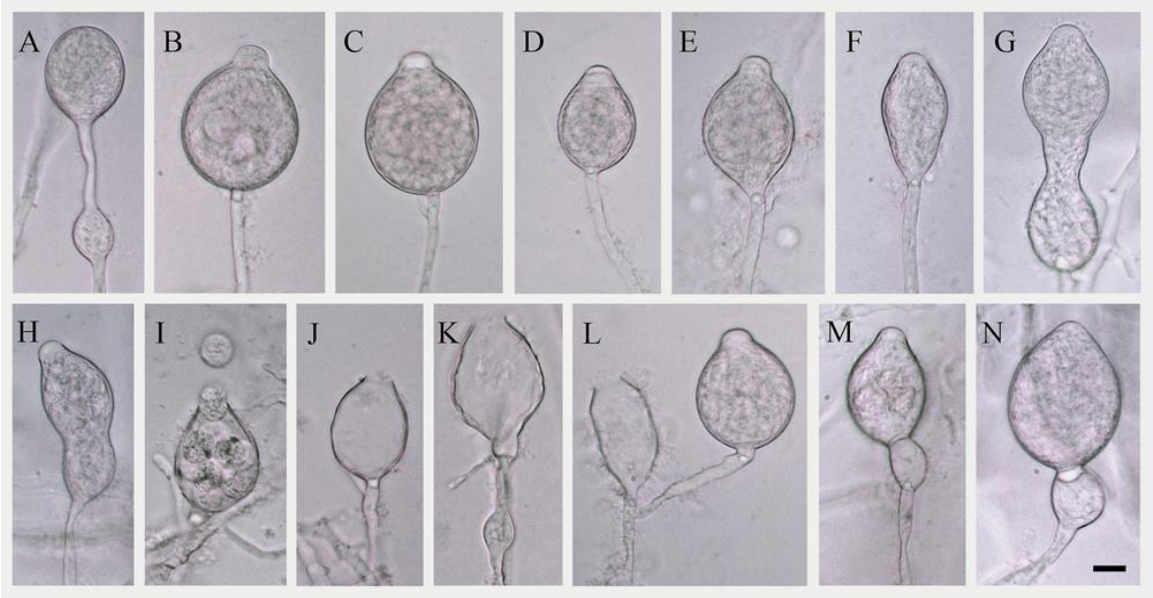

Figure A1. 5 Morphology of asexual structures of *Halophytophthora fluviatilis*. A: An immature nonpapillate sporangium and intercalary swelling on sporangiophore. B-H: Sporangia in various shapes. B: A globose to ovoid sporangium with rise of the apex and a basal plug. C: A papillate, globose to ovoid sporangium with a basal plug. D: A slightly papillate, ovoid sporangium. E: A limoniform sporangium with a basal plug. F: An obovoid sporangium with a basal plug. G-H: Sporangia in distorted shapes. I: A sporangium releasing zoospores directly through the exit pore. J: A sporangium after releasing zoospores with no sign of vesicle or dehiscence plug. K: An empty sporangium with external proliferation and intercalary swelling. L: An empty sporangium and a secondary globose sporangium by external proliferation. M-N: Sporangia with swelled tips of sporangiophores. M: Basal plug attached to the base cell. N: Basal plug attached to the sporangium. Bar = 10 μm.

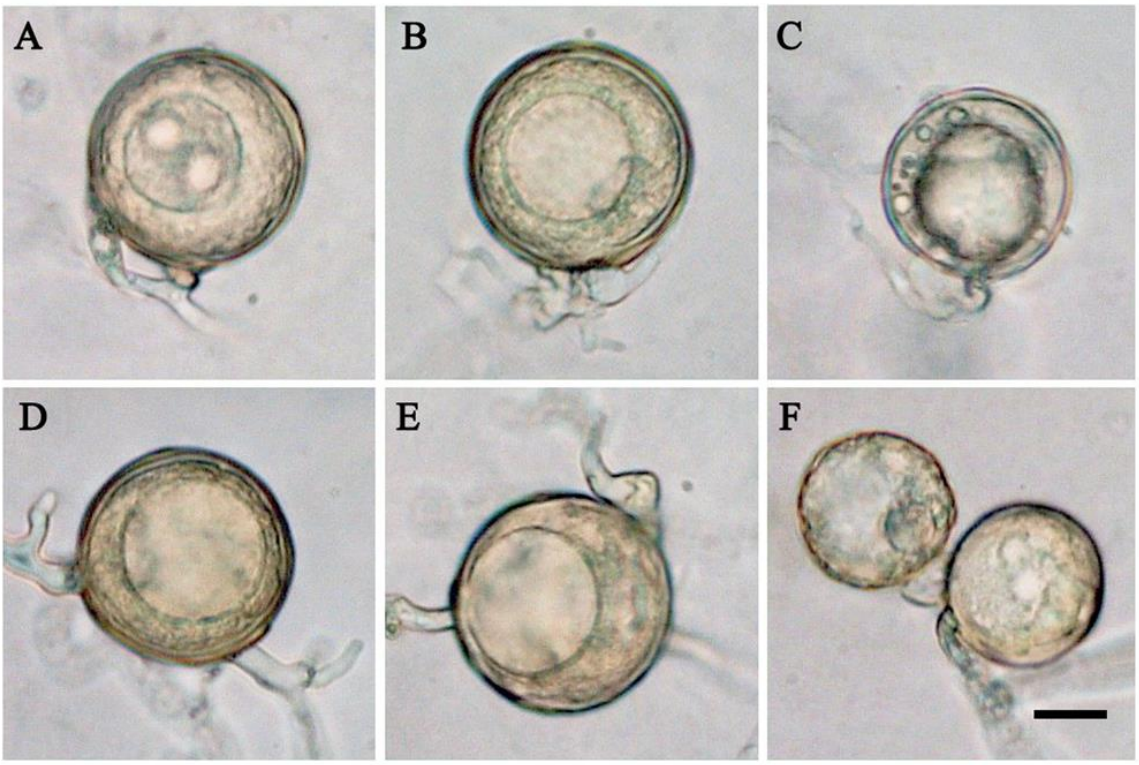

Figure A1. 6 Gametangia of *Halophytophthora fluviatilis*. A: A plerotic oogonia with a small, paragynous antheridium. B: A plerotic oogonium with a swelled, amphigynous-like antheridium. C: An aborted oogonium. D: A lateral oogonium. E: A lateral oogonium with a paragynous antheridium. F: Two immature, clustered gametangia. Bar = 10 µm.

Appendix 2 *Phytophthora hedraiandra* detected from irrigation water at a perennial ornamental plant nursery in Virginia

Yang X, Richardson PA, Ghimire SR, Kong P & Hong CX (2012) *Phytophthora hedraiandra* detected from irrigation water at a perennial ornamental plant nursery in Virginia. *Plant Disease* **96**: 915. http://dx.doi.org/10.1094/PDIS-07-11-0614-PDN

Abstract

Water survey for *Phytophthora* spp. by baiting with rhododendron leaves in April 2006 at a perennial ornamental plant nursery in Virginia detected five isolates showing a unique, previously unknown single-strand conformation polymorphism (SSCP) fingerprint (Kong *et al.*, 2003). These cultures were isolated from two reservoirs at different depths of water column from surface to 2 m. They were homothallic and produced smooth-surfaced spherical oogonia with an average diameter of 27 μm on 10% V8 agar. Oospores were aplerotic. The paragynous antheridia were averaging 12 μm in diameter. Sporangia were papillate, spherical to ovoid, averaging 39 by 28 μm (length by width). They were caducous with short (< 4 μm) pedicels (Figure A2.1). Chlamydospores and hyphal swellings were not observed. Two isolates were sequenced for the rRNA internal transcribed spacer region and cytochrome oxidase subunit 1 (*cox* 1) gene. ITS sequences of both isolates (GenBank Accession Nos. JN376065 and JN376066) were identical to that of *Phytophthora hedraiandra* type culture (GenBank Accession No. AY707987). Also, the *cox* 1 sequence of an isolate (Accession No. JN376067) had 99% homology

with that of the type culture (GenBank Accession No. AY69115). Pathogenicity of both isolates was tested on *Rhododendron catawbiense* and *Viburnum tinus*, two known hosts of *P. hedraiandra* (de Cock & Lévesque, 2004). For each isolate and host, five leaves and stems on potted plants were wounded by needles and then inoculated by placing over each wound a 5-mm^2 mycelial plug from a 7-day-old culture and securing with Parafilm. V8 agar was used instead of mycelial plugs on control plants. After inoculation, each plant was enclosed in a plastic bag for 1 day and then incubated at 22 ℃ with a 12-h photoperiod. Distilled water was sprayed daily for 5 days postinoculation (dpi) until disease symptoms were observed. At 15 dpi, 3 of the 10 inoculated rhododendron leaves and 6 of the 10 stems showed leaf lesions, wilting, dieback, and cankers, eventually leading to rhododendron death. Two of the 10 viburnum leaves and 4 of the 10 stems showed similar symptoms. Leaf lesions were approximately 3 to 5 cm in diameter (Figure A2.2). *Phytophthora hedraiandra* was recovered from diseased tissues and all resulting cultures showed an identical SSCP fingerprint to tested isolates as well as a *P. hedraiandra* isolate from Minnesota (Schwingle *et al.*, 2006). No symptom developed on control plants. To our knowledge, this is the first report of *P. hedraiandra* in Virginia. Considering neither host plant has been grown or bought for resale by this nursery, this study indicates that *P. hedraiandra* may have a wider host range than is currently known. This possibility and the importance of water dispersal for *P. hedraiandra* in disease epidemiology warrant further investigation.

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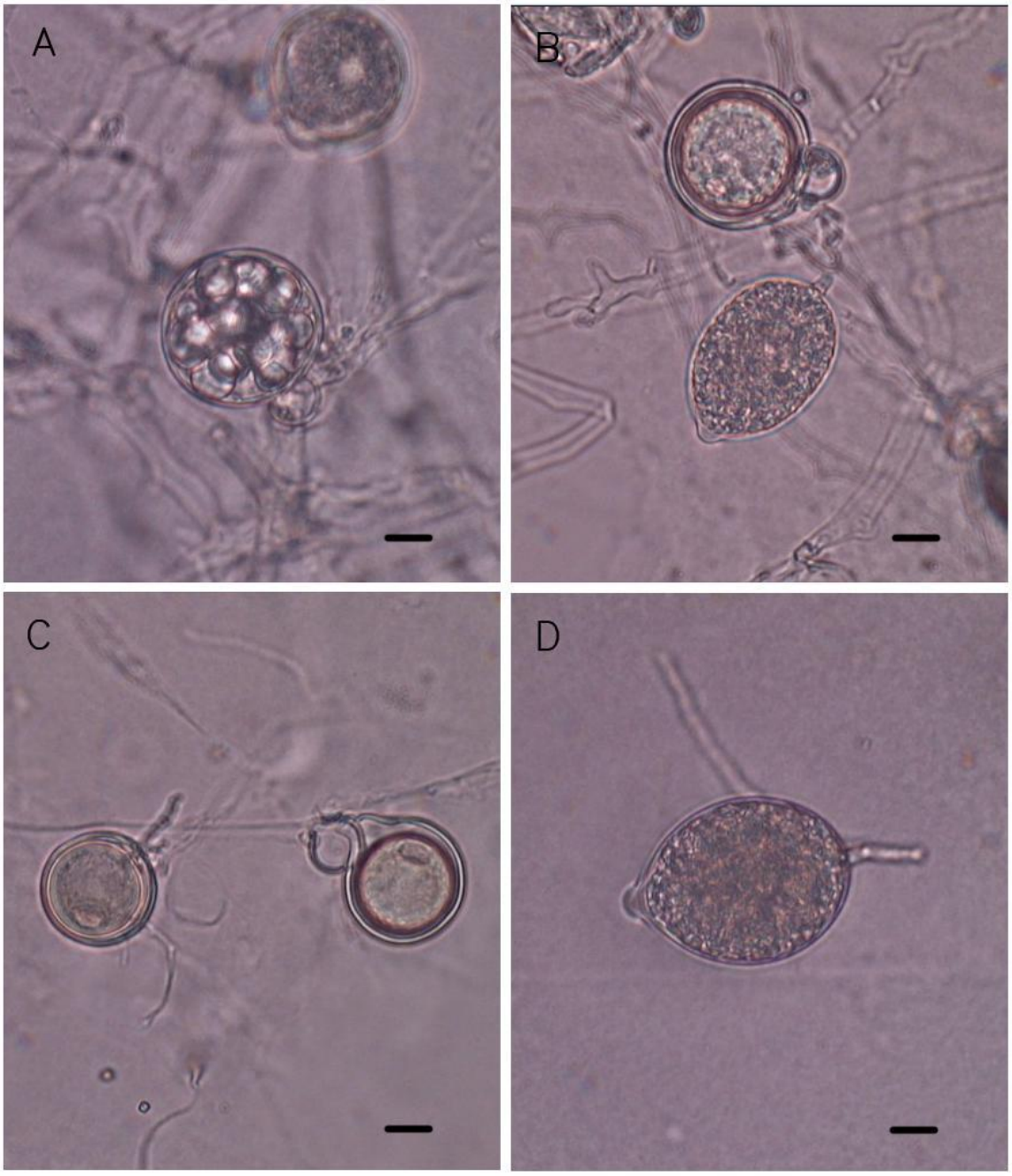

Figure A2. 1 Morphology of *Phytophthora hedraiandra*. A: An immature oogonium with a paragynous antheridium. B: An aplerotic oogonium with a paragynous antheridium and a spherical to ovoid, papillate sporangium. C: Two aplerotic oogonia. D: An ovoid, papillate, caducous sporangium with long pedicel. Bars = 10 µm.

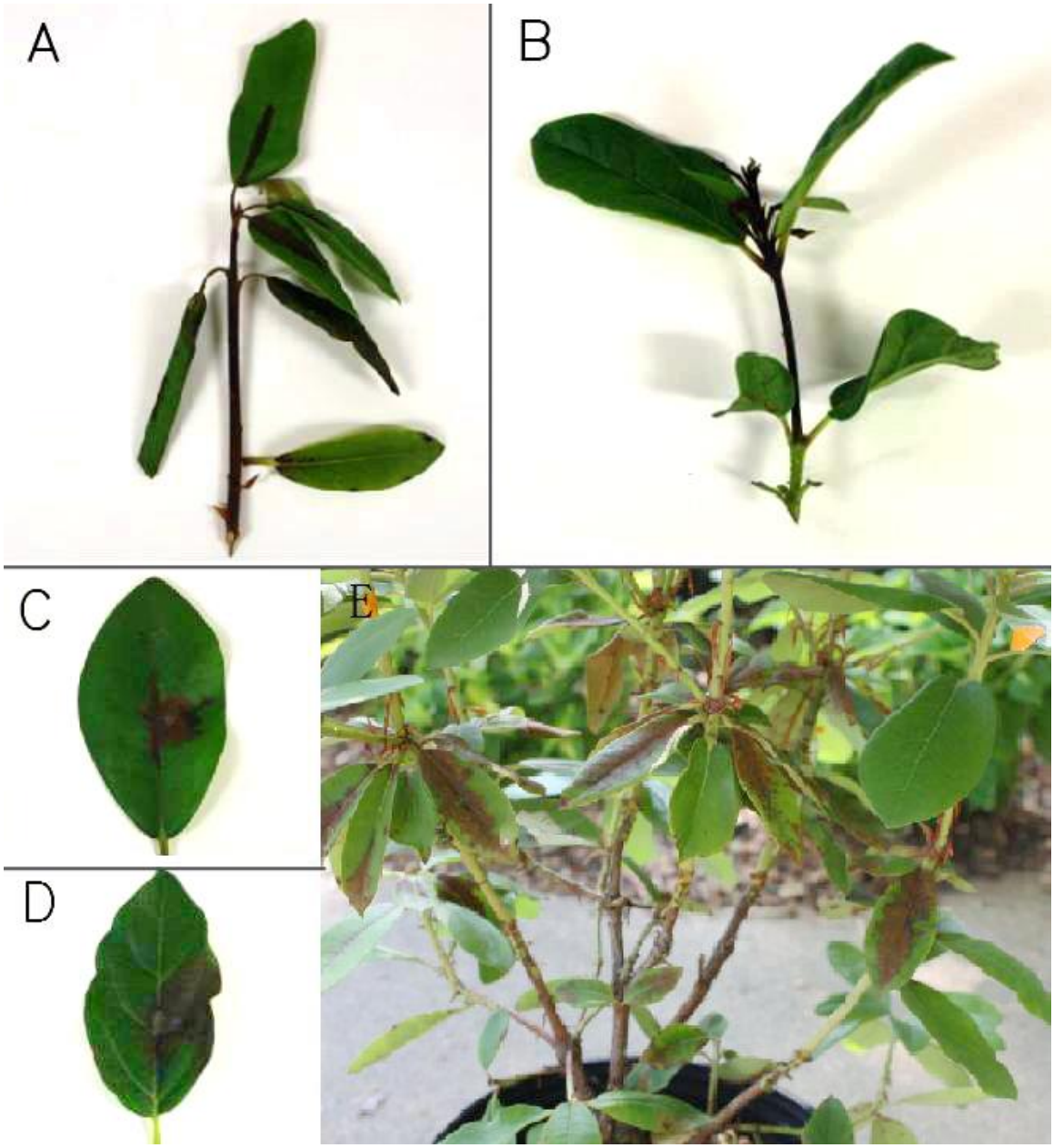

Figure A2. 2 Disease symptom on rhododendron and viburnum caused by *Phytophthora hedraiandra* on 15 days after inoculation. A: Dieback on rhododendron. B: Dieback on viburnum. C: Foliar lesion on rhododendron. D: Foliar lesion on viburnum. E: A rhododendron plant showing dieback, canker, and foliar lesions.

Appendix 3 Root and stem rot of begonia caused by *Phytopythium helicoides* in Virginia

Yang X, Richardson PA, Olson HA & Hong CX (2013) Root and stem rot of begonia caused by *Phytopythium helicoides* in Virginia. *Plant Disease* **97**: 1385.

http://dx.doi.org/10.1094/PDIS-05-13-0472-PDN

Abstract

In the summer of 2011, severe root and stem rot of begonia (*Begonia* ×*semperflorens-cultorum* cv. Vodka Dark Red) was observed during a field trial. Seventy-eight percent of the plants had symptoms included foliar blight, blackened and rotting roots, rotting stems, and collapsing crown, often leading to plant death. Isolation from the diseased plant roots consistently recovered a *Pythium*-like species and 41 isolates were subcultured for identification. These isolates produced very similar single strand conformation polymorphism (SSCP) fingerprints (Kong *et al.*, 2003), which were distinct from those of other oomycete pathogens known to attack begonia (Hong *et al.*, 2008). These isolates produced proliferous, ovoid to globose, terminal, and papillate sporangia which were 30.6 to 45.4 µm (av. 38.7 µm) in length and 20.5 to 35.4 µm (av. 28.2 µm) in width. Oogonia were produced in single culture grown in clarified V8 juice agar. These smooth-walled oogonia were mostly aplerotic and 28.9 to 36.8 µm (av. 33.1 µm) in diameter. Each contained a single oospore with a diameter of 23.7 to 34.4 µm (av. 26.9 µm). Single to multiple antheridia were attached lengthwise to each oogonium. These morphological

characteristics match the description of *Phytopythium helicoides* (= *Pythium helicoides*) (Van der Plaats-Niterink, 1981). The identity of these isolates was confirmed by sequencing the rRNA internal transcribed spacer (ITS) region. ITS sequence of the representative isolate 55C7 (GenBank Accession No. KC907734) had 97 to 99% homology with *P. helicoides* sequences in GenBank. Two isolates, 55C7 and 56A7, were tested for pathogenicity to begonia in the summer of 2012. Twelve plants per isolate were inoculated by injecting ground *P. helicoides*-colonized rice grains into the root soil using a long-neck funnel. Sterile rice grains were used on control plants. Aboveground symptoms including foliar blight, stem rot, and collapsing crown were observed 7 days after inoculation and the disease progressed for additional 6 weeks. At 7 weeks, all inoculated plants showed different symptom levels. Four and 10 plants inoculated with 55C7 and 56A7, respectively, were already dead. Begonia roots showed severe symptoms including blackening, stunted growth, and rotting. Seven of 12 control plants also had notable symptoms due to cross contamination. Isolates recovered from all symptomatic plants had identical SSCP fingerprints to those of isolates 55C7 and 56A7. To our knowledge, this is the first report of *P. helicoides* attacking begonia plants. The avenue of this pathogen entering the 2011 field trial remains unknown. The field trial in 2011 and pathogenicity test in 2012 indicate that this pathogen is potentially destructive to begonia. Additional research is warranted to identify the origin and dissemination of this pathogen to mitigate the risk to begonia production.

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Appendix 4 Evaluation of a nonregistered fungicide for control of rose downy mildew, 2012.

Yang X, Richardson PA & Hong CX (2013) Evaluation of nonregistered fungicide for control of rose downy mildew, 2012. *Plant Disease Management Reports* 7: OT001.

Bare-root canes of tea rose cultivar 'Rio Samba' were potted in 3-gallon containers on 13 Jan and drenched with Captan (2.4 g/L) the same day at a local commercial nursery. Canes were treated with Cleary's 3336 [1.4 g/L; active ingredient (a.i.): thiophanate-methyl] plus Nu-Film (40 ml/L) on 14 Jan. A mix of Heritage (0.038 g/L; a.i.: azoxystrobin), Penncozeb (0.152 g/L; a.i.: mancozeb) and Scanner (0.095 g/L; a.i.: 3-oxapentane-1,5-diol, propane-1,2,3-triol, alkylphenol ethoxylate, poldimethylsiloxane) was applied on 17 Feb after the plants began to leaf out. Test plants were delivered and placed on a gravel pad at the Hampton Roads Agricultural Research and Extension Center on 6 Mar for the trial. They were irrigated twice daily at 7 AM and 5 PM for 30 minutes. The product evaluated in this trial was A9180A (a.i.: experimental) under different application methods and schedules. The control treatment was water applied to the foliage while FenStop (a.i.: fenamidone) was used as an industry standard. Each treatment had four replicates with three containers per replicate. The six treatments were arranged in a randomized complete block design. The first treatments were applied on 16 Mar; thereafter 10 sets of treatments were performed at the intervals listed. Twenty-five roses with severe downy mildew symptoms from a production nursery were placed between each randomized block on 4 Apr, as no naturally developed downy mildew symptoms on foliage were observed. Flower buds were counted on 6 Apr. Disease

incidence was evaluated on 29 May, 4 Jun and 11 Jun. Data were analyzed using the SAS PROC ANOVA v9.2 (SAS Institute, Cary, NC) and the mean numbers of flower buds and diseased leaves from different treatments were separated by the least square difference *t*-test at $P = 0.05$.

Symptoms of downy mildew on foliage were first observed on 21 May and increased sharply in the following weeks due to cool and wet weather conditions. Four treatments with A9180A had lower disease incidence than the water treated control or FenStop on 29 May, 4 Jun and 11 Jun. Compared to the control, weekly and biweekly drenches of A9180A reduced disease incidence by 84% and 72% respectively as assessed on 29 May. Weekly sprays or an alternating spray/drench of this product reduced downy mildew by 70% or 79%. All treatments had a slight increase in disease incidence during the following two assessments on 4 Jun and 11 Jun. There was no difference among the four A9180A treatments in terms of disease incidence although the number of diseased leaves was lower in the weekly drenches than the weekly spray treatments. Plants alternately sprayed and drenched with A9180A had more flower buds than those sprayed with water or FenStop on 6 Apr. There was no difference in the number of flower buds among the four A9180A treatments. FenStop left a clear residue on test plants. A9180A provided good control of rose downy mildew during the trial and even two weeks after the final treatments.

Treatment and rate/100 gal	Disease Incidence*			Avg. no Buds		No. of	Interval
	29 May	4 Jun	11 Jun	6 Apr	Application method	Applications	(days)
Water.....	118.25 a**	119.33 a	154.83 a	4.083 b	Spray	11	7
A9180A 0.5 oz.....	35.83 b	41.58 b	56.17 b	7.083 ab	Spray	11	7
A9180A 0.25 oz/0.5 oz.....	25.42 b	34.25 b	46.50 b	7.917 a	alternate Drench/Spray	6/5	7
A9180A 0.25 oz.....	33.00 b	40.33 b	57.33 b	6.583 ab	Drench	6	14
A9180A 0.25 oz.....	18.58 b	30.42 b	37.83 b	5.250 ab	Drench	11	7
FenStop 10 fl oz.....	117.58 a	132.08 a	166.08 a	4.000 b	Drench	3	28

* Mean number of diseased leaves (12 plants).

** Means within a column followed by the same letter are not significantly different at P=0.05 as separated by the least square difference t-test (DF=71).

Appendix 5 Evaluation of fungicide drenches for control of Phytophthora root rot of petunia, 2012.

Yang X, Kong P & Hong CX (2013) Evaluation of fungicide drenches for control of Phytophthora root rot of petunia, 2012. *Plant Disease Management Reports* 7: OT002.

Petunia plants growing in 5-inch pots filled with potting mix were obtained from a commercial ornamental producer. Pre-treatments of the plants included: Actino Iron integrated into potting mix at 5 lb/yd, Heritage [4 oz/100 gall; active ingredient (a.i.): azoxystrobin] and Daconil (1.5 oz/100 gall; a.i.: chlorothalonil) in week 17, and Medallion (4 oz/100 gall; a.i.: fludioxonil) in week 20. Plants were placed on a gravel pad at the Hampton Roads Agricultural Research and Extension Center. They were irrigated twice daily at 8 AM and 6 PM for 30 to 60 minutes depending on weather conditions. The new fungicide evaluated was Plentrix (A13836B; a.i.: experimental). Comparison treatments were Heritage combined with Subdue Maxx (a.i.: mefenoxam) and FenStop (a.i.: fenamidone), along with a nontreated, non-inoculated and a nontreated, inoculated control. Each of the treatments had six replicates with three pots per replicate. The five treatments were arranged in a randomized complete block design. All treatments were made 6 days prior to inoculation by drenching at a rate of 3 fl oz/ pot on 28 June, *Phytophthora nicotianae* isolate 51A5 from petunia collected in Virginia Beach in 2010, was used for inoculation. Plants were inoculated on 4 July by placing five infested rice grains into a 1-2 cm deep hole about half way between the stem and pot edge. Non-colonized rice grains were used for the non-inoculated controls. Disease severity was

assessed weekly for 3 weeks after initial symptoms were observed on 16 July. Root quality was rated following the final disease assessment on 30 July. Means of the area under the disease progress curves (AUDPC) and root quality ratings among treatments were separated with the Waller-Duncan k-ratio *t*-test in R.

Disease pressure was severe in this trial with all the nontreated, inoculated plants dead by 30 July. The lowest AUDPCs were obtained with FenStop, followed by Heritage/Subdue. Disease severity was significantly lower in plants treated with Plentrix than in the nontreated, inoculated control, and statistically similar to Heritage/Subdue and FenStop. Plants in the non-inoculated control had excellent root quality, followed by those treated with FenStop, however, none of the treatments provided excellent root quality as non-inoculated control. Phytotoxicity was not observed from any product used in this trial.

Treatment and rate/100 gal	AUDPC [z]		Root quality [y]	
Nontreated, non-inoculated	2.528	c [x]	4.17	a
Nontreated, inoculated	44.72	a	0.11	c
Plentrix 3.66 SE 1.3 fl oz	25.08	b	1.39	bc
Heritage 50 WG 0.9 oz Subdue Maxx SL 1 fl oz	19.25	bc	0.83	c
FenStop 20 SC fl oz	10.69	bc	2.56	b

[z] Each number is the mean AUDPC calculated from the disease severity ratings for each plant. Ratings are based on a 0 to 5 rating scale (0 = healthy plant, 1 = slight wilt/infection, 2 = light infection, 3 = moderate infection: plant may not survive, 4 = severe infection: plant will not survive, and 5 = dead plant).

[y] Each number is a mean root quality rating from six replicates. Ratings are based on a 0 to 5 scale (0 = poor root quality, 3 = acceptable root quality for the plant size, and 5 = excellent root quality).

[x] Means within a column followed by the same letter are not significantly different at $P = 0.05$ as determined by Waller-Duncan k-ratio *t*-test (k-ratio=100, 88df).

Appendix 6 Fungicidal control of Phytophthora aerial blight on annual vinca in Virginia, 2012.

Yang X, Richardson PA, Kong P & Hong CX (2013) Fungicidal control of Phytophthora aerial blight on annual vinca in Virginia, 2012. *Plant Disease Management Reports* 7: OT005.

Annual vinca plants growing in 5-in. pots filled with pine bark potting mix were obtained from a commercial ornamental nursery. Prior fungicide treatment of these test plants included: Actino Iron integrated into the potting mix at 5 lb/yd^3, followed by foliar applications of Heritage [4 oz/100 gal; active ingredient (a.i.): azoxystrobin] and Daconil (1.5 oz/100 gal; a.i.: chlorothalonil) in week 17 (23 Apr) and Medallion (4 oz/100 gal; a.i.: fludioxonil) in week 20 (14 May). Upon receipt, test plants were placed on a gravel pad at the Hampton Roads Agricultural Research and Extension Center. They were irrigated two or three times daily at 8 AM, noon (optional) and 6 PM for 30 to 60 min depending on weather conditions. The experimental product evaluated in this trial was A14658C (a.i.: experimental) applied alone, or in combination with Heritage (a.i.: azoxystrobin) or Subdue MAXX (a.i.: mefenoxam). FenStop (a.i.: fenamidone) was used as a competitive standard. Untreated, uninoculated and untreated, inoculated controls were also included. Fungicides were reapplied at 14-day or 28-day (FenStop) intervals for a total of two or three applications. Each of the treatments had six replicates with three pots per replicate. The eight treatments were arranged in a randomized complete block design. All treatments were applied by spraying to runoff with approximately 1.6 fl oz of solution per

plant using a SafeSpray® Wide Mouth sprayer. Treatments were applied on 5 Jun, one day prior to the initial inoculation. *Phytophthora nicotianae* isolate 3A12, isolated from annual vinca grown in Virginia Beach in 2000, was used for inoculum. Approximately 1.69 fl oz of suspension at a concentration of 300,000 to 600,000 zoospores per fl oz was sprayed evenly onto the foliage of each plant on 6 Jun. The same amount of deionized water was used for the uninoculated controls in the same manner. A second and third inoculation was made on 13 and 20 Jun, respectively. To increase disease pressure, 80 diseased, annual vinca plants previously inoculated with isolate 3A12 were evenly placed between each randomized block on 16 Jul. Disease incidence was assessed weekly by counting blighted shoot tips on each plant. The area under the disease progress curve (AUDPC) was calculated from the disease incidence counts for each plant.

Blighted tips were first observed on 19 Jun, two weeks after the initial inoculation. Blighting progressed during the following two weeks and then ceased. Disease progression resumed after the 80 diseased plants were introduced into the trial on 16 Jul. Disease incidence was significantly lower in plants treated with A14658C alone, or in combination with Subdue MAXX or Heritage compared to the untreated, inoculated, Subdue MAXX and Heritage treatments. The experimental product provided excellent control of Phytophthora aerial blight, comparable to the FenStop treatment, throughout the duration of the trial. Phytotoxicity was not observed for any test plants during the trial.

Treatment and rate/100 gal	No. of applications	Application interval (days)	AUDPC*
Untreated uninoculated	-	-	0.00 d**
Untreated inoculated.	-	-	163.50 a
A14658C SL 64 fl oz	3	14	17.50 cd
Subdue MAXX SL 1 fl oz.	3	14	110.60 ab
Heritage 50WG 2 oz.....	3	14	94.69 ab
A14658C SL 64 fl oz + Subdue MAXX SL 1 fl oz	3	14	20.81 cd
A14658C SL 64 fl oz + Heritage 50WG 2 oz	3	14	12.64 cd
FenStop 4.13 SC 10 fl oz	2	28	75.64 bc

* Each number is the mean AUDPC calculated from the disease incidence counts of 18 plants.

** Means with a letter in common are not significantly different at *P*=0.05 as determined by Waller-Duncan k-ratio *t*-test (k-ratio=100, 142df).