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Phytophthora mississippiae sp. nov., a New Species Recovered from Irrigation Reservoirs at a Plant Nursery in Mississippi

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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 A1. Ornamented oogonia with amphigynous antheridia and plerotic oospores were produced when this novel species was paired with A2 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.

Keywords: *Phytophthora* mississippiae; Irrigation reservoir; Ornamented oogonia

Introduction

The genus of *Phytophthora* was first described by Heinrich Anton de Bary in 1876 [1]. "*Phytophthora*" was from the Greek word "φυτόνφθορά" which means "the plant-destroyer". The name evidences that *Phytophthora* genus includes a group of destructive plant pathogens. This genus was divided into 10 clades following phylogenetic analyses [2-4]. Among these clades, clade 6 has a strong association with forest and riparian environments [5]. It currently consists of 18 formal species. All were described after the year 2000, except for *P. gonapodyides* [6], *P. humicola* [7] and *P. megasperma* [8]. Clade 6 also includes a number of provisional species such as *P. taxon* forest soil, *P. taxon* oaksoil, and *P. taxon* Pgchlamydo [9], as well as many other undescribed taxa (Hong et al. unpublished).

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 [10]. 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* species complex after sequence analyses [11]. *P.* sp. O-group isolated in the 1990s was formally named as *P. inundata* [12]. *P. taxon* Salixsoil first isolated in the 1970s was assigned as *P. lacustris* [13]. Many newly isolated species in clade 6 such as *P. bilorbang* [14] and *P. gemini* [15] 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 [16,17] and forest declines caused by several *Phytophthora* species in other countries [18,19] 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* [20], 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 [21,22]. The surveys in irrigation systems also recovered a number of novel *Phytophthora* species [23-25] and many new taxa in clade 6 (Hong et al. unpublished).

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 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 [26,27]. These baits were deployed in the surveyed reservoirs for 7 days then transferred to a laboratory. They were then cut into approximately 3×3 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 [1]. 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°C. The holotype isolate

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 1: Origin and GenBank accession numbers of *Phytophthora mississippiae* isolates and reference species.

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°C 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°C. Two perpendicular measurements of each colony were taken after 8 days. This test was repeated. Following the analysis of variance using R statistical software v. 2.15.0 [28], 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 [29] 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°C) under cool-white fluorescent light.

Mating type of these isolates was determined by placing each with an A1 or A2 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 [30,31]. Several heterothallic species including *P. cinnamomi*, *P.*
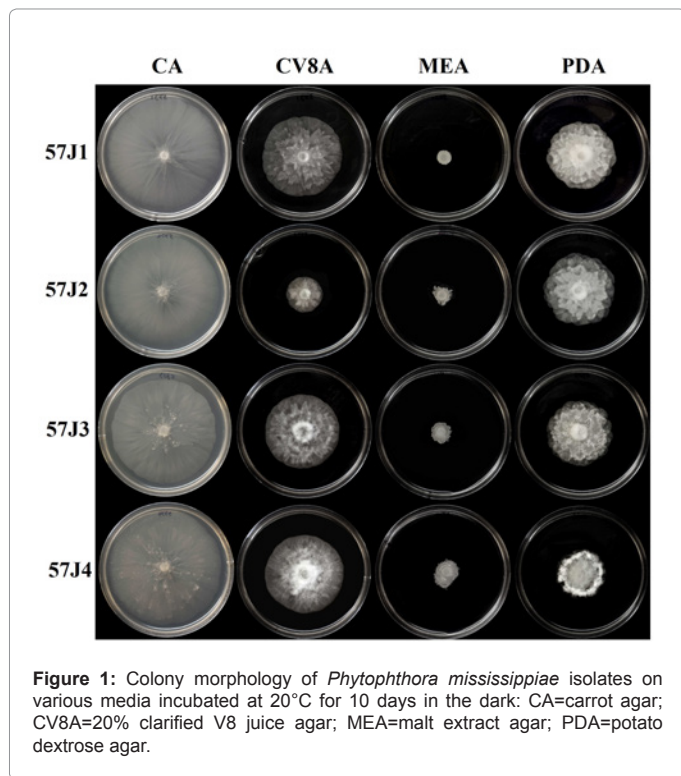
Figure 1: Colony morphology of *Phytophthora mississippiae* isolates on various media incubated at 20°C for 10 days in the dark: CA=carrot agar; CV8A=20% clarified V8 juice agar; MEA=malt extract agar; PDA=potato dextrose agar.
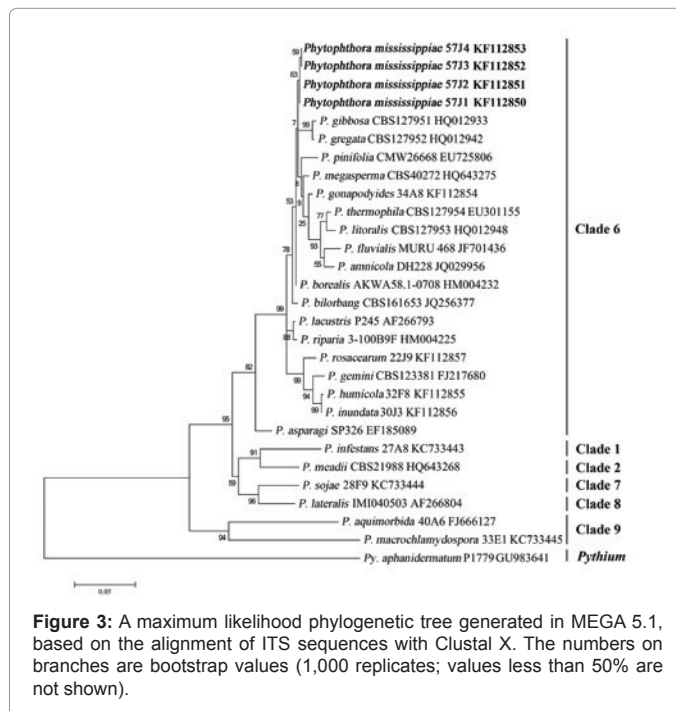

Figure 3: A maximum likelihood phylogenetic tree generated in MEGA 5.1, based on the alignment of ITS sequences with Clustal X. The numbers on branches are bootstrap values (1,000 replicates; values less than 50% are not shown).
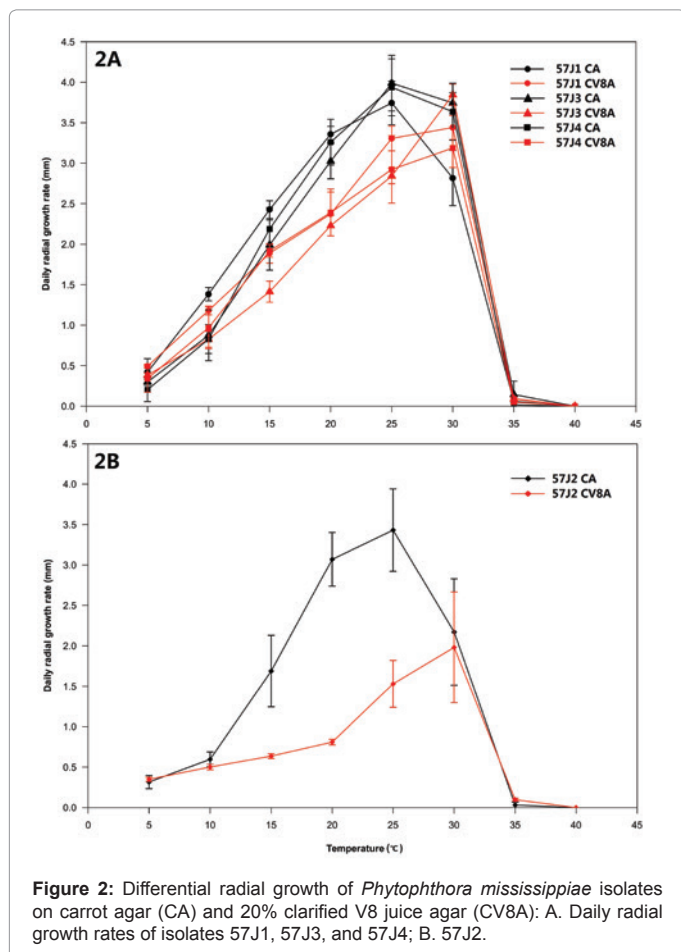

Figure 2: Differential radial growth of *Phytophthora mississippiae* isolates on carrot agar (CA) and 20% clarified V8 juice agar (CV8A): A. Daily radial growth rates of isolates 57J1, 57J3, and 57J4; B. 57J2.

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 [2] and reverse primer ITS4 [2] for the internal transcribed spacer (ITS) region covering ITS1, 5.8S rRNA gene, and ITS2, following previously described reaction mix recipe and PCR program [32]. Primer pair COX4FR was used to amplify the mitochondrial cytochrome c oxidase 1 (*cox* 1) gene [3]. 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 1). Sequences were aligned using Clustal W. Phylogeny reconstruction was conducted in MEGA 5.1 [33] using the Maximum Likelihood method based on the Tamura-Nei model [34] with 1,000 replications of bootstrap.

Results

Colony morphology

The four isolates of *P. mississippiae* had a similar growth pattern at

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: 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.

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)	47.3-70.0×21.1-43.3	48.8-64.3×26.8-38.6	55.4-71.9×28.6-41.9	47.3-77.3×20.4-37.1	47.3-77.3×20.4-43.3
Average size (μm)	61.5 ± 7.2×34.0 ± 3.5	57.4 ± 3.9×31.1 ± 2.5	62.6 ± 3.8×34.9 ± 3.2	60.3 ± 7.2×26.8 ± 3.4	60.4 ± 6.0×31.3 ± 4.5
L:W ratio	1.82	1.85	1.80	2.27	1.96

Table 3: Morphological variations of sporangial characters among isolates of Phytophthora mississippiae in this study.
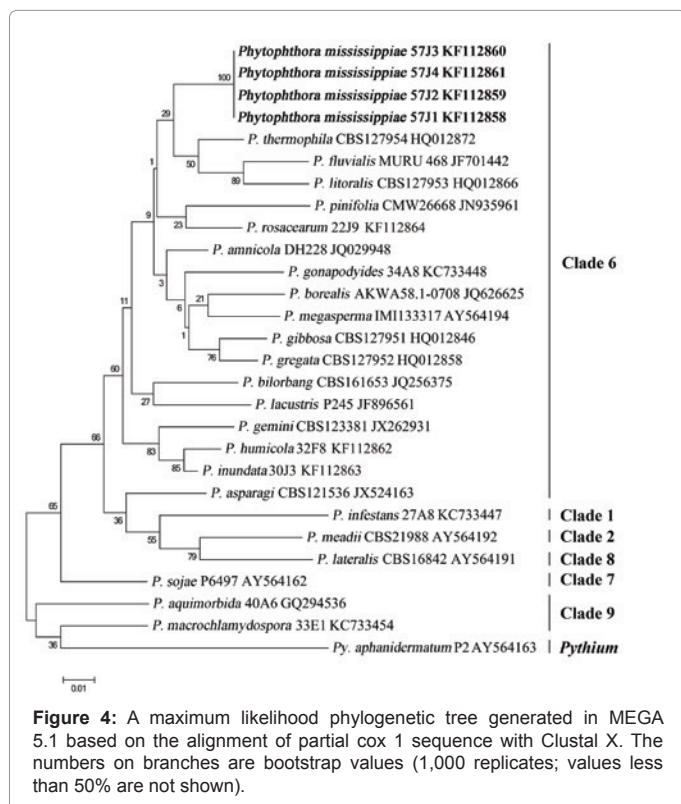

Figure 4: A maximum likelihood phylogenetic tree generated in MEGA 5.1 based on the alignment of partial cox 1 sequence with Clustal X. The numbers on branches are bootstrap values (1,000 replicates; values less than 50% are not shown).

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 2A). Isolate 57J2 grew more slowly than the other isolates at 10 to 30°C on both CV8A and CA (Figure 2B). The optimum temperature for the growth of *P. mississippiae* was 25°C on CA and 30°C on CV8A. Limited but notable growth was observed at 5 and 35°C. No growth occurred at 40°C. After the experiments were completed, cultures from all temperatures were relocated to room temperature. Additional growth occurred on plates previously maintained at 5°C but not those maintained at 40°C.

Sequence analyses and phylogenetic position

GenBank accession numbers of sequences generated in this study and used in the sequence analyses are shown in table 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). 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
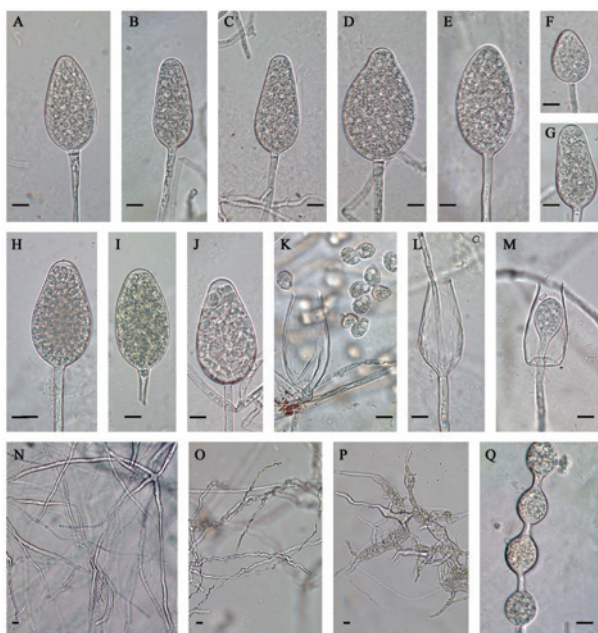
20°C on each medium (Figure 1). Colonies had none to sparse aerial mycelia and grew at the greatest rates on CA. The colony pattern on

Figure 5: Morphology of asexual structures of *Phytophthora mississippiae*: A-G) Nonpapillate, noncaducous sporangia in various shapes; A) An obpyriform to ovoid sporangium; B, C:0Obpyriform 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.
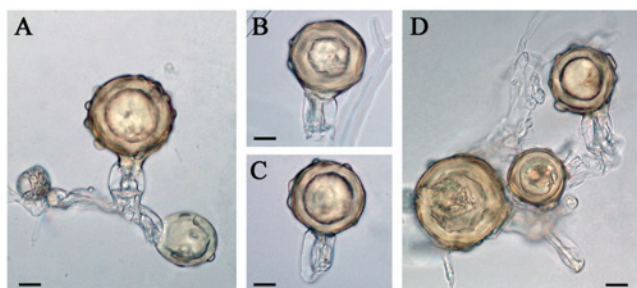
Bars=10 μm.

Figure 5: 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.

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).

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* [5,9]. The four *P. mississippiae* isolates form a distinct taxon in the phylogenetic trees based on ITS (Figure 3) and cox 1 (Figure 4) sequences.

Taxonomy

Phytophthora mississippiae X. Yang, W. E. Copes, and C. X. Hong., sp. nov.—MycoBank MB804659; Figures 1, 5A-Q, 6A-D.

Phytophthora mississippiae produced abundant sporangia in 1.5% SWE after 15 hours under light. Sporangia were mostly ovoid to obpyriform (Figures 5A-D, F-H, J). It occasionally produced slight ellipsoid sporangia (Figures 5E, I). Sporangia were noncaducous, mostly nonpapillate (Figures 5A-G) and sometimes semipapillate (Figures 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 (Figures 5L, M). Sporangial characteristics among four *P. mississippiae* isolates are summarized in table 3. Mycelia were flat (Figure 5N), coiled (Figure 5O), or swollen (Figure 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 5Q). Chlamydospores were not observed.

Phytophthora mississippiae is self-sterile. Gametangia were produced in dual cultures where *P. mississippiae* isolates were paired with an A2 mating type tester of *P. cinnamomi* suggesting that all four isolates examined in this study are A1. In the polycarbonate membrane test, gametangia were produced by isolates 57J3 and 57J4 after 50-day-breeding when paired with A2 mating type testers of *P. cryptogea* (Figures 6A, B) and *P. nicotianae* (Figure 6C, D). Oogonia had characteristic ornamented protuberances on the surface and oogonial wall was pigmented to golden-brown with maturation (Figures 6A-D). Many oogonia had a tapered base (Figures 6A, B, D). Oogonial diameter averaged 38.2 μm. Plerotic oospores averaged 34 μm in diameter (Figures 6A-D). All antheridia were amphigynous (Figures 6A-D) and averaged 19.5 μm depth and 14.3 μm width. Sometimes bi-cellular antheridia were produced (Figure 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* [17] and *P. kernoviae* [35].

Phytophthora mississippiae can be readily distinguished from all known *Phytophthora* species by its morphological and molecular characters. Within the genus *Phytophthora*, only 5 species, *P. alni* [36], *P. cambivora* [6], *P. gibbosa* [5], *P. katsurae* [37], 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* [5,31,36,37] 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. *P. mississippiae* produces both nonpapillate and semipapillate sporangia, while *P. cambivora* produces only nonpapillate sporangia [31]. *P. 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 [38]. Only a few clade 6 species have been found to cause diseases on agricultural and horticultural plants [5]. Examples include *P. asparagi* which causes root rot of asparagus [39] and *P. megasperma* which causes crown rot of hollyhock [8]. A saprophytic lifestyle for many species in this clade may play an important role in decomposing plant debris [38]. 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 2 years. Nevertheless, investigations into its origin, pathogenicity and host range are warranted.

Acknowledgments

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