**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 amphigenous antheridia and plerotic oospores were produced when this novel species was paired with A2 mating type testers of *P. cypreegosa* and *P. nicotianae* in polycarbonate membrane tests. Sequence analyses of the rDNA internal transcribed spacer (ITS) region and the mitochondrial 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 “φυτόνφθορα” which means “the plant-destroyer.” The name evidences the 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. hunicola* [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* Pgcchlamydo [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 unpublished).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. rosacarum* and *P. sansomenea* 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. inudata* [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 other *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 cm² sections and plated onto PARP selective media (contains pimarinc, 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

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### 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 against temperature using the gplots package v. 2.11.0 [29] in R. Radial growths along with their standard errors were plotted together. Radial growths along with their standard errors were plotted against temperature using the ggplot package v. 2.11.0 [29] in R.

### Morphology of sporangia and gametangia

Sporangia were produced by transferring agar plugs (10×10 mm²) 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* sp. nov., a New Species Recovered from Irrigation Reservoirs at a Plant Nursery in Mississippi. J Plant Patol Microb 4: 180 doi:10.4172/2157-7471.1000180.

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

| Species          | ITS clade | Isolate | Location       | Substrate           | Date     | GenBank accession no. |
|------------------|-----------|---------|----------------|---------------------|----------|-----------------------|
| *Phytophthora mississippiae* | 6         | 57J1    | Mississippi, USA | Irrigation water    | 2012     | KF112850 KF112858     |
|                  |           | 57J2    | Mississippi, USA | Irrigation water    | 2012     | KF112851 KF112859     |
|                  |           | 57J3†   | Mississippi, USA | Irrigation water    | 2012     | KF112852 KF112860     |
|                  |           | 57J4    | Mississippi, USA | Irrigation water    | 2012     | KF112853 KF112861     |
| *P. annicola*    | 6         | DH228   | Australia       | Still water         | 2009     | JQ029956 JQ029948     |
| *P. asparagi*    | 6         | SP326   | Michigan, USA   | Asparagus officinalis | 2008     | EF185089 n/a          |
|                  |           | PBS12   | Michigan, USA   | Asparagus officinalis | 2008     | EF185089 n/a          |
|                  |           | CBS121536 | The Netherlands | Asparagus officinalis | n/a      | n/a                  |
|                  |           | CBS161653 | Australia      | Rhizosphere soil of dying Rubus sp. | 2012     | JQ256377 JQ256375     |
| *P. borealis*    | 6         | AKWA858.1-0708 | Alaska, USA | Creek water        | 2012     | HM004232 JQ026625     |
| *P. fluvialis*   | 6         | MURU4025 | Australia      | River water         | 2009     | JF701436 JF701442     |
| *P. gemeni*      | 6         | CBS123338 | The Netherlands | Zostera marina    | 1998     | FJ217680 JX262931     |
| *P. gibboosa*    | 6         | CBS127951 | Australia      | Root soil of dying Acacia pycnantha | 2009     | HQ012933 HQ012846     |
| *P. gonapodyides*| 6         | 34A8, CBS55467 | United Kingdom | Fruit bait         | 1967     | KF112854 KF112858     |
| *P. gregata*     | 6         | CBS127952 | Australia      | Root soil of dying Pternonia 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 Bankia sp. | 2008     | HQ012948 HQ012866     |
| *P. megasperma*  | 6         | CBS40272 | Washington, D.C., USA | Althea rosea | 1931     | HQ643275 n/a          |
|                  |           | IM133317 | Australia      | n/a                 | 1968     | n/a YAY564194         |
| *P. pinifolia*   | 6         | CMV266668 | Chile         | Pinus radiata      | 2007     | EU725806 JN935961     |
| *P. riparia*     | 6         | 3-10089F | Oregon, USA   | Creek water        | 2006     | HM004225 n/a          |
| *P. roaceearum*  | 6         | 22J9, OSU 62 | California, USA | Cherry            | n/a      | KF112857 KF112864     |
| *P. thermophila* | 6         | CBS127954 | Australia      | Root soil of dying Eucalyptus sp. | 2004     | EU031155 HQ012872     |
| *P. infestans*   | 1         | 27A6, KDT-2C | Mexico      | Solanum tuberosum  | 1992     | KJ173443 KJ173447     |
| *P. meadii*      | 2         | CBS21968 | India         | Hvea brasilienensis | 1987     | HQ012872 AY564192     |
| *P. sojae*       | 7         | 28F9, P6497 | Mississippi, USA | Glycine max | 1974     | KJ173444 AY564162     |
| *P. lateralis*   | 6         | M6040503, CBS16842 | Oregon, USA | Chamaecyparis lawsoniana | 1942     | AF266804 AY564191     |
| *P. aquimoribida*| 9         | 40A6    | Virginia, USA | Irrigation reservoir | 2006     | FJ666127 GQ294536     |
| *P. macrochlamydospora* | 9 | 3E1, P10264 | Australia | Glycine max | 2003     | KJ173445 KJ173454     |
| *Pythium aphani†eratum* | Pythium | P1779   | n/a           | n/a                  | 2013     | GU983641 n/a          |
|                  |           | P2      | n/a           | n/a                  | 2013     | AY564163 n/a          |

*explode-type

*n/a=not available

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 against temperature using the gplots package v. 2.11.0 [29] in R.

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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. aphanidermatum* Pythium sp. and *P. citrhorum* were also grown using the same methods and conditions.
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 [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 (cox1) 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...
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 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).

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

| Isolate | ITS1 | ITS2 |
|---------|------|------|
| 57J1    | T    | G    |
| 57J2    | C    | C    |
| 57J3    | C    | G    |
| 57J4    | T    | C    |

Table 3: Morphological variations of sporangial characters among isolates of \( Phytophthora \) mississippiae in this study.
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). Chlamydoospores 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 bicellular 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
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. ali [36], P. cambivora [6], P. gibbosa [5], P. katsurae [37], and this new species, P. mississippiae produce ornamented oogonia with bulbate protuberances. P. mississippiae is easily separated from three homothallic species, P. ali, 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.

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