Halophytophthora fluviatilis sp. nov. from freshwater in Virginia

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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 Halophytophthora vesicula was discovered in Vancouver, British Columbia, Canada, and initially described as Phytophthora vesicula (Anastasiou & Churchland, 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. polymorpha, H. spinosa var. lobata, H. spinosa var. spinosa, and H. vesicula (Anastasiou & Churchland, 1969; Fell & Master, 1975; Pegg & Alcorn, 1982; Gerrettson-Cornell & Simpson, 1984). Thereafter, six new species H. kandeliae (Ho et al., 1991), H. cypripelora (Ho et al., 1992), H. mast-
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 *H. 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 (Fig. 1). The baits were deployed in the surveyed streams from 7 to 14 days then transferred to a laboratory. They were then cut into 10 × 10 mm sections and plated onto PARP selective media (contains pimaricin, 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 (diH2O) for long-term storage at 15 °C. 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 × 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). DNA was extracted using a DNeasy® Plant Mini kit (Qiagen, Valencia, CA). 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). Sequences in both directions were visualized with Finch TV v. 1.4.0. (GeoSpiza Inc., Seattle, WA) 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 (http://www.ncbi.nlm.nih.gov/genbank/). These sequences were aligned using MAFFT online version 7 (Katoh & Standley, 2013) and the Q-INS-I algorithm (Katoh & Toh, 2008). 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 (Rambaut, 2012). Neighbor-joining (NJ) inference was carried out with MEGA5 (Tamura et al., 2011) using the Tamura–Nei model (Tamura & Nei, 1993) with 1000 bootstrap replicates.

**Physiology: cardinal temperatures and the optimum salinity**

Three isolates, 57A9, 59B8, and 59J1, recovered from different locations (Fig. 1) in 2011 and 2012 and 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 °C. 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 °C. The salinity levels of non-amended 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 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% diH2O) 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 diH2O. Then, a sporangium-inducing solution containing 80% nonsterile soil water extract (15 g of sandy loam soil/1 L diH2O) and 20% filtered, nonsterile seawater was added. After continuously exposed under cool-white fluorescent lamps at room temperature for 2–3 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 with 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. 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 472-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 (Fig. 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 (Fig. 3). The optimum temperature for the mycelial growth was 25°C with the minimum < 5°C. Little growth was observed at 29°C. No growth occurred at 30°C. After exposing to 30°C 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 (Fig. 4). The optimum salinity for *H. fluviatilis* was 6.1 PPT.
PPT (Fig. 4) with an average daily radial growth > 2.9 mm. However, the average growth rate decreased significantly ($P < 0.05$) with increasing salinity (Fig. 4).

**Taxonomy**

*Halophytophthora fluviatilis* X. Yang & C. X. Hong, sp. nov. (Figs 5 and 6). MycoBank: MB807647.

**Etymology**

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

**Description**

Mycelia hyaline irregularly branched. Sporangiophores in a simple sympodium, occasionally in a compound sympodium. Abundant sporangia produced by mycelial mats submerged in the sporangium-inducing solution under light within 48–72 h. Immature sporangia mostly globose, nonpapillate (Fig. 5a). Sporangial apex rose and swollen with increasing maturity (Fig. 5b). Mature sporangia mostly globose to ovoid (Fig. 5b–d), sometimes limoniform (Fig. 5e) to obovoid (Fig. 5f), and rarely in distorted shapes (Fig. 5g and 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 sporangial apex (Fig. 5c and d). Zoospores released directly to the environment (Fig. 5i–l) instead of into a vesicle. External proliferations common, formed a new hypha or 1–2 sporangia (Fig. 5l). Most mature sporangia have a conspicuous basal plug (2.1–5.6 μm; ave. 3.2 ± 0.7 μm thickness) (Fig. 5b, c, e–g, i, j, l–n). Some sporangia on top of a swollen base cell to which a basal plug attached (Fig. 5m and n). Intercalary hyphal swellings (Fig. 5a and k) and sporangiophore swellings common.

Gametangia produced in HSA after 20 days. Oogonia lateral (Fig. 6a, d and e) or terminal (Fig. 6b and c); globose, golden-pigmented with maturing (Fig. 6a and f), 23.4–35.1 μm (ave. 28.2 ± 2.6 μm) diameter, sometimes formed in a cluster (Fig. 6f). Oospores plerotic, 21.8–29.3 μm (ave. 25.8 ± 2.1 μm) diameter, sometimes aborted (Fig. 6c). Antheridia paragynous (Fig. 6a, c and e), usually small (< 5.0 μm long and wide); rarely swollen, surrounding the oogonial stalk, and appeared amphigynous (Fig. 6b).

**Holotype**

ATCC MYA-4961 (exo-type: 57A9; GenBank KF734964) recovered from Flint Run Stream, Virginia, USA, October, 2011. Other isolates examined as follows: 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 (Fig. 1). This study, along with previous observations of...
Halophytophthora fluviatilis presents 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 & Churchland, 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. polymorpha* frequently retain zoospores in vesicles, although 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 5.0–8.0 µm hyphae in width (Blackwell, 1949), except some *Phytophthora* species produce relatively narrow hyphae (~5.0 µm) such as *P. rhizophyllacea* (Hong et al., 2010), *P. irrigata* (Hong et al., 2008), and *P. parisianna* (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. fluviatilis* 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. kaliediae* (Ho et al., 1991), *H. epistomium* (Fell & Master, 1975), and *H. exaprolifera* (Ho et al., 1992) are more closely related to the genera such as *Phytophthora*, *Phytopythium*, and *Pythium* than the *Halophytophthora* s. str. clade (Fig. 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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**References**


