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Clohesyomyces symbioticus sp. nov., a fungal endophyte associated with roots of water smartweed (*Persicaria amphibia*)

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Abstract. The widespread aquatic plant *Persicaria amphibia* (water smartweed, *Polygonaceae*) occurs in both flooded aquatic habitats and moist terrestrial environments. Its physiological versatility and wide geographic range highlight its resilience to stress and make the species intriguing for the study of fungal endophytes. Endophytes occur within living plant tissues and are known from diverse aquatic, marine, and terrestrial plants, where they often mitigate plant responses to stress. As part of a study evaluating endophyte communities associated with aquatic plants in lentic waters of Arizona, USA, we isolated a distinctive clade of endophytes from healthy, living roots of seasonally inundated *P. amphibia*, which we describe here on the basis of morphology and evidence from four loci as new species *Clohesyomyces symbioticus* (*Lindgomycetaceae*, *Pleosporales*, *Dothideomycetes*, *Ascomycota*). *Clohesyomyces* has long been considered a monotypic genus comprising the saprobic species *C. aquaticus,* presently known from submerged wood in freshwater systems in Asia and Australia. Description of *Clohesyomyces symbioticus* highlights the occurrence of endophytism in this genus and expands its geographic scope to the western hemisphere.

Key words: Endophytic, freshwater, *Hongkongmyces, Lindgomyces,* macrophyte, *Polygonum,* symbiosis

Introduction

Aquatic plants that persist under non-inundated conditions often have physiology and root traits that may attract distinctive fungal symbionts relative to purely aquatic and purely terrestrial plants (Sandberg et al. 2014; Moora et al. 2016; Stevens et al. 2018). Although it is primarily aquatic, water smartweed (*Persicaria amphibia*, *Polygonaceae*) is an emergent macrophyte that can survive in moist locations or when stranded by receding waters (Mitchell 1968; Costea 2012). It typically is found in shallow, still water in ponds and lakes, along the margins of rivers and streams, and in moist locations such as meadows (Flora of North America, www.efloras.org). Water smartweed has a wide geographic range, occurring natively in Europe, Asia, North America, and northern Africa (USDA-ARS GRIN-Global, https://npgsweb.ars-grin.gov) and as an introduced species in Mexico, southern Africa, and South America (Partridge 2001). In the United States, it is recorded in at least 46 states (Flora of North America, www.efloras.org), including Arizona, where it occurs in natural and impounded waters.

We previously characterized communities of fungal symbionts associated with leaves and roots of *P. amphibia* in lentic systems in central Arizona, with a special focus on fungal endophytes (Sandberg et al. 2014). Fungal endophytes (hereafter, endophytes) occur within living tissues without causing symptoms of disease (Rodriguez et al. 2009). Endophytes of aquatic plants are often highly diverse and distinctive relative to the endophyte communities found in plants in nearby terrestrial environments (e.g., Kohout et al. 2012; Sandberg et al. 2014; You et al. 2015; Duan et al. 2019).

As part of our surveys, we detected a distinctive group of isolates with unusual greyish and olivaceous growth. Sequence data from the internal transcribed spacers and 5.8S gene (ITS rDNA) suggested an affiliation with *Lindgomycetaceae* (Hirayama et al. 2010), but no strong affiliation with any previously described species. Here we characterize three representative strains based on morphology and molecular analyses as a new species of *Clohesyomyces.* The genus was previously considered monotypic, with *C. aquaticus* described as a saprobe that lives on submerged wood in Asia and Australia (Hyde 1993; Zhang et al. 2012). Our study

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highlights the large geographic range of *Clohesyomyces*, now including southwestern North America, and expands the ecological scope of the genus by including endophytism in living roots.

Materials and methods

Focal strains (DM0144, DM0177 and DM0192) were isolated from surface-sterilized, submerged, healthy roots of *Persicaria amphibia* (syn. *Polygonum amphibium*) collected in June 2012 at Willow Creek Reservoir (also known as Willow Lake) in Prescott, Yavapai County, Arizona, USA (34.602067, −112.437950). Roots were collected by gently digging up healthy plants in shallow water in areas of the reservoir separated by about 30–40 m from one another. Collection details were presented in Sandberg et al. (2014).

Plant tissues were washed and surface-sterilized as described by Sandberg et al. (2014) prior to plating on 2% malt extract agar (MEA) under sterile conditions. Axenic isolates were preserved as living vouchers in sterile water at room temperature prior to subsequent characterization.

Culturing and assays

Isolates were regrown from vouchers by transferring small hyphal plugs to 2% MEA supplemented with 50 µg/mL kanamycin sulfate, 40 µg/mL ciprofloxacin, 100 µg/mL ampicillin salt, and 15 µg/mL tetracycline HCl (see Hoffman and Arnold 2010). These plates were used to provide fresh mycelia for culturing, assays, and molecular analyses, per below.

A plug of actively growing mycelium was transferred from each isolate to 2% MEA in a 60 mm Petri dish. After ~10 days of growth at ~22°C with natural light/dark cycles, each isolate was transferred under sterile conditions to 100 mm Petri dishes containing either 2% MEA, potato dextrose agar (PDA), water agar, water agar amended with thrice autoclaved pine needles (Harrington et al. 2019), cellulose medium (Gazis et al. 2012), or lignin (indulin) medium (Gazis et al. 2012). The plates were incubated under the above laboratory conditions for 14 d. Growth of each isolate on each medium was characterized and cellulase and ligninase assays were performed as in Gazis et al. (2012).

Microscopy was conducted following Harrington et al. (2019) with images captured on Leica DM4000b and Nikon Eclipse Ni compound microscopes and a Nikon SMZ18 dissecting microscope with stacking and adjustment in Helicon Focus 7. We compared morphology with records for representative *Lindgomycetaceae* as published by Hyde (1993) (original description of the genus *Clohesyomyces* and the type species, *C. aquaticus*), as well as Hirayama et al. (2010), Raja et al. (2011, 2013, 2015, 2017), Raudabaugh et al. (2018), and Zhang et al. (2012).

Molecular analyses

Total genomic DNA was extracted from each isolate with the Sigma RedExtract-n-Amp plant PCR kit (Sigma-Aldrich, St. Louis, MO, USA) per the manufacturer's protocol. The following primers were used to amplify ITS rDNA, the nuclear ribosomal small subunit (SSU rDNA), the nuclear ribosomal large subunit (LSU rDNA) and translation elongation factor 1 alpha (*TEF-1a*): ITS rDNA: ITS1F, ITS5, ITS4; LSU rDNA: LROR, LR3, LR7; SSU rDNA: NS1, NS4; *TEF-1a*: EF1-983F, EF1-2281R (Vilgalys & Hester 1990; White et al. 1990; Gardes & Bruns 1993; Hopple & Vilgalys 1994; Carbone and Kohn 1999; Rehner & Buckley 2005).

The PCR reaction mixture for each nuclear ribosomal region consisted of 10 µl of REDExtract-N-Amp PCR Ready Mix, 0.8 µl of each primer, 7.9 µl of molecular grade water, and 0.65 µl of total genomic DNA template. The reaction mixture for *TEF-1a* consisted of 10 ul of DreamTaq Hot Start Green Master Mix (Thermo Scientific, Waltham, MA, USA), 0.8 µl of each primer, 6.9 µl of molecular grade water, and 2 µl of total genomic DNA template. When amplification did not occur for *TEF-1a* with the EF1-983F/EF1-2218R primer set, products from the initial first round of amplification were diluted 1:10 with molecular grade water and used as the template for a second PCR under the same reaction and cycle conditions as the first round. We used negative controls in all PCRs. All amplifications were performed in a MJ Research PTC200 thermocycler (Waltham, MA). Cycle conditions for each region are given in Table 1.

After visualization on a 1% agarose gel, PCR products were cleaned with ExoSAP-IT (Thermo-Fisher, Waltham, MA) and sequenced at the University of Arizona Genetics core (bidirectional Sanger sequencing with the primers used in PCR). The software applications *phred* and *phrap* (http://phrap.org) were used to call bases and assemble contigs with automation provided by the ChromaSeq package in Mesquite v. 1.06 (http://mesquiteproject.org). Base calls were checked by eye against chromatograms in Sequencher v. 4.5 (Gene Codes, Anne Arbor, MI).

Phylogenetic analyses

Comparison of ITS rDNA sequences with GenBank records via BLAST revealed top matches for the focal strains in the *Lindgomycetaceae*, a family of primarily

Table 1. PCR conditions following Arnold et al. (2009) and Harrington et al. (2019).

Gene	Initial denature	Denature	Anneal	Extend	Cycles	Final extension
ITS rDNA	94°C/3 min	94°C/30 sec	54°C/30 sec	72°C/1 min	34	72°C/10 min
LSU rDNA	94°C/3 min	94°C/30 sec	50°C/1 min	72°C/1 min	34	72°C/10 min
SSU rDNA	94°C/3 min	94°C/1 min	51°C/30 sec	72°C/1 min	34	72°C/10 min
TEF-1a	95°C/5 min	95°C/1:30 min	55°C/90 sec	72°C/1 min	35	72°C/10 min

Table 2. Growth of focal strains on 2% MEA. Color codes follow Kornerup & Wanschen (1967).

	Diameter at 14 d (mm)	Color, above: Central colony	Color, above: Colony edge	Growth visible as rings from above	Colony texture	Color, below: Central colony	Color, below: Colony edge	Growth visible as rings from below	Medium color, after 14 d
DM0144	39	3E2	3E3	Yes	Dense, velvety	3F2	3D2	No	Natural
DM0177	34	3E2	3C2	Yes	Dense, velvety	3F2	3C2	No	Mild reddish pigment
DM0192	39	3B1	3C2	Yes	Dense, velvety	8E4	8C2	No	Diffuse, dark red pigment

aquatic, wood-decaying fungi. Top matches included *Hongkongmyces, Lindgomyces,* and *Clohesyomyces,* including unidentified strains within these genera or affiliated with them. Based on Hirayama et al. (2010), Hyde et al. (2013), Raudabaugh et al. (2018), Raja et al. (2011, 2013, 2015, 2017), Tsang et al. (2014), and Zhang et al. (2012, 2014), as well as scrutiny of available sequence data in GenBank, and preliminary analyses designed to confirm outgroup taxa and clarify sequence quality, we generated the following alignments: (1) to confirm placement in *Lindgomycetaceae*, SSU rDNA (with *Aquasubmersa* and *Didymosphaeria* as outgroup taxa per Zhang et al. 2012) and LSU rDNA (with outgroups as above); and (2) to confirm placement in *Clohesyomyces* and confirm species placement, ITS rDNA and *TEF-1a*. Each dataset was aligned in MUSCLE (Edgar 2004) and verified by eye. Alignments are deposited at TreeBASE (study number 28770).

Each dataset was analyzed via maximum likelihood (ML) and maximum parsimony in GARLI (Zwickl 2006) and PAUP* 4.0a169 (Swofford 2003; https://paup.phylosolutions.com), respectively, as described in Harrington et al. (2019). For ML analyses, we used PAUP* 4.0a169 for automated model selection based on the Akaike Information Criterion, implementing GTR+I+gamma for SSU rDNA, LSU rDNA, and ITS rDNA and TIM+I for *TEF-1a*. Topological support was evaluated with 1000 bootstrap replicates per Harrington et al. (2019).

Results

The focal strains grew readily under laboratory conditions on water agar, water agar amended with autoclaved pine needles, 2% MEA, PDA, cellulose medium, and lignin medium. Details of growth on MEA and PDA are given in Table 2 and Table 3, respectively.

Analyses of the SSU rDNA and LSU rDNA placed the strains within *Lindgomycetaceae* with strong support (Fig. 1, Fig. 2). Analyses of ITS rDNA and *TEF-1a* confirmed placement within *Clohesyomyces* with strong support, and highlighted the distinctiveness of the focal isolates from *C. aquaticus* and other not-yet-named strains collected previously from Europe (Fig. 3, Fig. 4).

On MEA and PDA, gross colony morphology (Fig. 5, Fig. 6) was broadly consistent with that of *C. aquaticus* (Hyde 1993; Zhang et al. 2012), including the generally velvety to woolly, grey to greyish white to olivaceous appearance. Growth of the strains on MEA and PDA was relatively fast, with colony diameters of 34–39 mm and 44 mm after 14 d, respectively (Table 2, Table 3). In comparison, Zhang et al. (2012) reported that *C. aquaticus* reached a colony diameter of 30 mm after 25 d at 25°C, referring to it as slow-growing. However, Hyde (1993) reported a colony diameter of 90 mm in 7 d on PDA. Thus, growth rate of the type species of *Clohesyomyces* appears to be variable.

Hyde (1993) noted that *C. aquaticus* did not color PDA, but we observed reddish pigments from DM0177 and especially DM0192 on MEA and PDA (Tables 2, 3; Fig. 6). We also noted marked variation in colony appearance among the focal strains and observed that individual strains, when subcultured, could appear quite distinct from their source culture for at least several weeks, even on the same medium.

Hyde (1993) reported that *C. aquaticus* produced large numbers of conidiomata after 7 d on PDA. However, we only observed pycnidia development in the focal strains on water agar with pine needles (rarely on the pine needles themselves) and, after four weeks, on cut edges of MEA where material had been removed for subculturing (Fig. 5).

Table 3. Growth of focal strains on PDA. Color codes follow Kornerup & Wanschen (1967).

	Diameter at 14 d (mm)	Color, above: Central colony	Color, above: Colony edge	Growth visible as rings from above	Colony texture	Color, below: Central colony	Color, below: Colony edge	Growth visible as rings from below	Medium color, after 14 d
DM0144	44	1B1	1B2	No	Dense, velvety	3F2	3D2	No	Natural
DM0177	44	1B1	3E2	Yes	Dense, velvety	3F3	3F3	No	Mild reddish pigment
DM0192	44	1B1	1B1	Yes	Dense, velvety	3F2	1B1	Yes	Mild reddish pigment

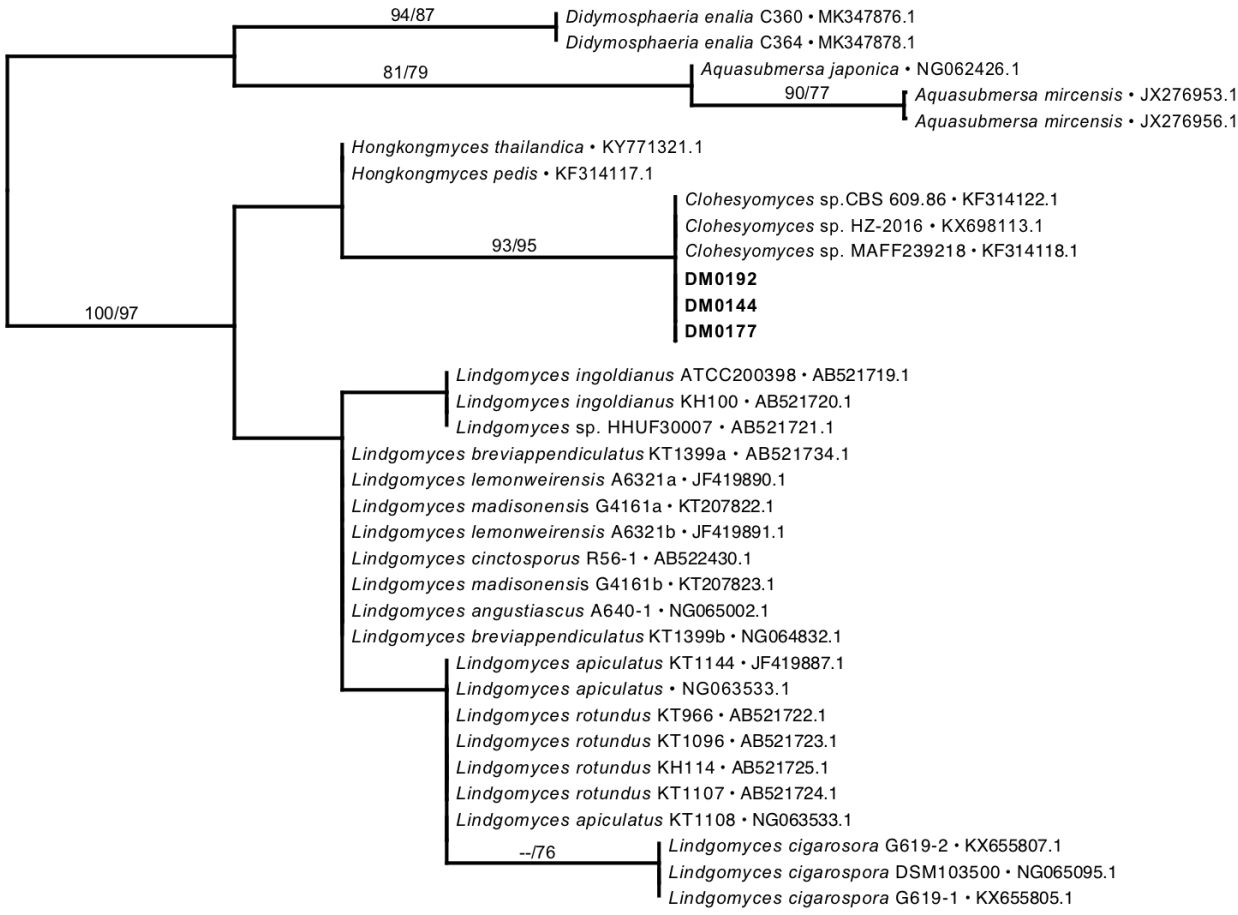

Figure 1. Maximum likelihood analysis of SSU rDNA data places the focal clade in *Lindgomycetaceae*. Bootstrap values ≥70 are shown (maximum parsimony/maximum likelihood). Representative sequences were chosen for each genus. The final alignment consisted of 869 characters, of which 16 were variable and parsimony-informative.

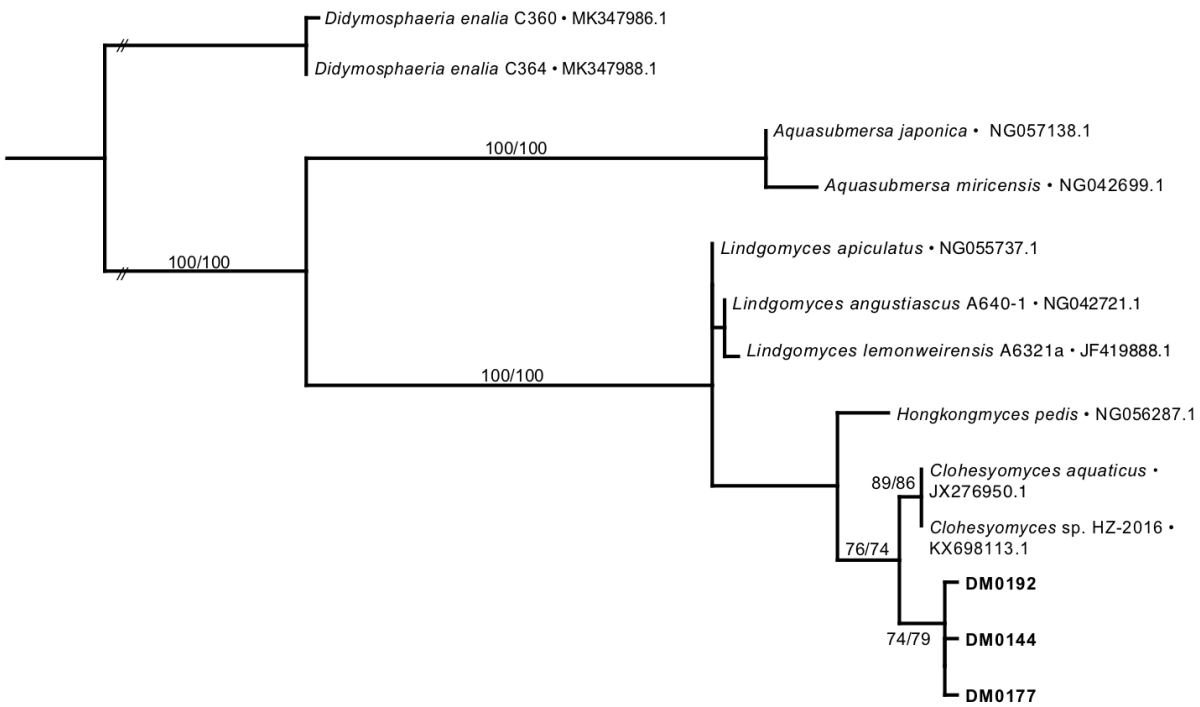

Figure 2. Maximum likelihood analysis of LSU rDNA data supports placement of the focal clade in *Lindgomycetaceae*. Bootstrap values ≥70 are shown (maximum parsimony/maximum likelihood). The final alignment consisted of 836 characters, of which 75 were variable and 70 were parsimony-informative.

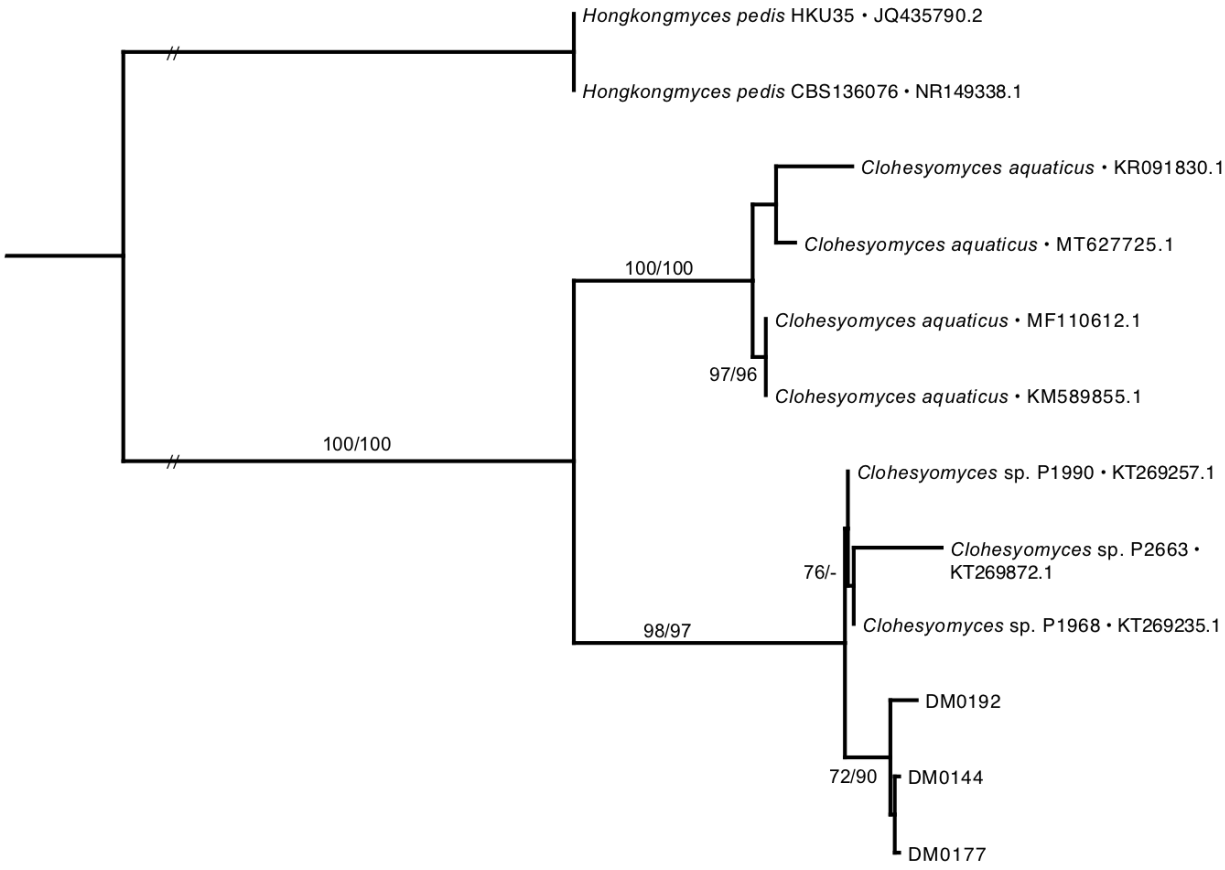

Figure 3. Maximum likelihood analysis of ITS rDNA data places the focal clade in *Clohesyomyces* and supports the description of *C. symbioticus* sp. nov. Bootstrap values ≥70 are shown (maximum parsimony/maximum likelihood). The final alignment consisted of 591 characters, of which 134 were variable and 120 were parsimony-informative.

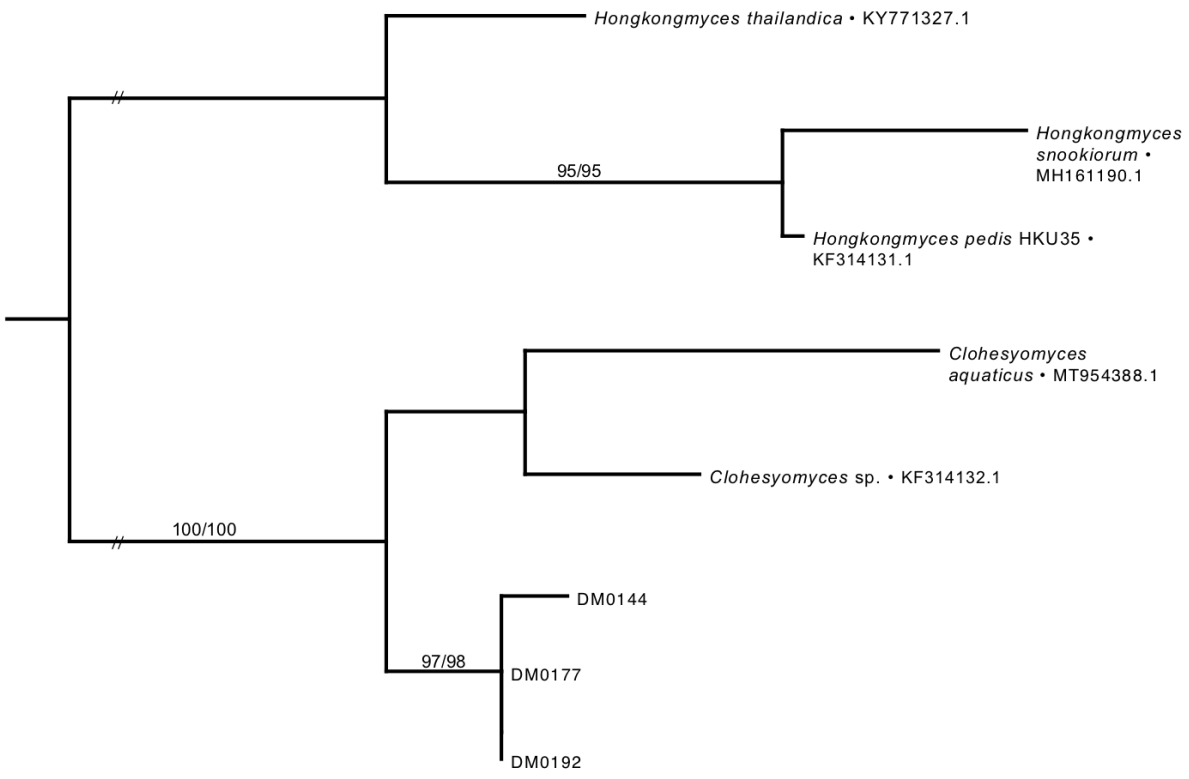

Figure 4. Maximum likelihood analysis of *TEF-1a* data places the focal clade in *Clohesyomyces* and supports the description of *C. symbioticus*. Bootstrap values ≥70 are shown (maximum parsimony/maximum likelihood). The final alignment consisted of 454 characters, of which 55 were variable and 33 were parsimony-informative.

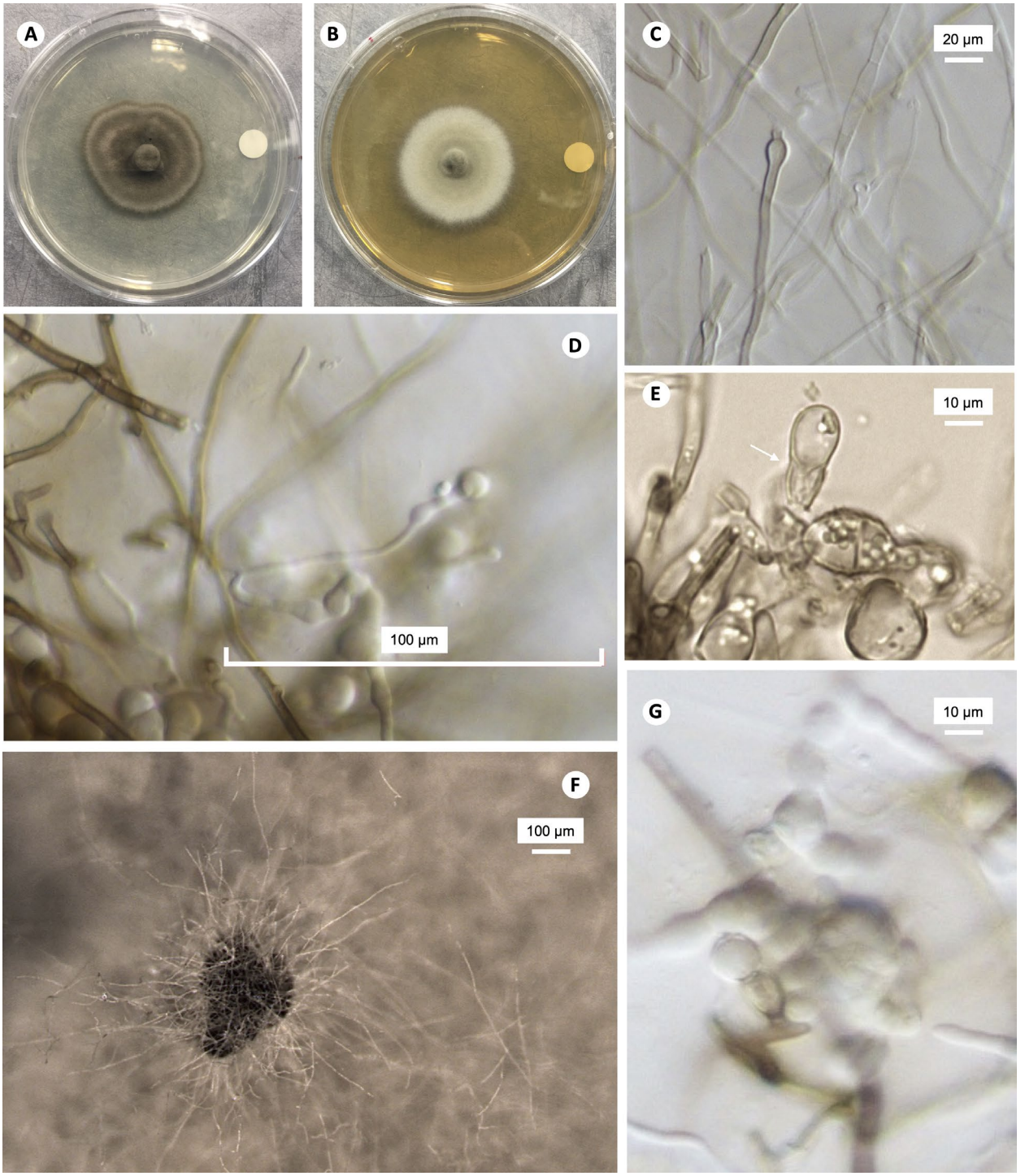

Figure 5. Morphology of *Clohesyomyces symbioticus*, illustrated by the type, ARIZ-AEADM0144. A – Isolate on 2% MEA (100 mm plate); B – Isolate on PDA (100 mm plate). White circles on A and B are for white balance calibration; C – Conidiogenous cell; D – Details of hyphae and conidiogenous cells; E – Conidia and conidiogenous cell, illustrating ephemeral collarette (arrow); F – Pycnidium, formed on water after in proximity to thrice-autoclaved pine needles; G – Details of conidia and conidiogenous cells, with a nearly mature euseptate conidium. Images C, D, and G were captured on a Nikon Eclipse Ni; E was captured on a Leica DM4000b.

Colony diameter on water agar averaged 53 mm, 57 mm, and 69 mm in 14 d for DM0144, DM0177, and DM0192, respectively. Diameter on cellulase medium averaged ~33 mm in 14 d (DM0144, DM0177) and 42 mm (DM0192). On ligninase medium, colony diameters ranged from 38 mm in 14 d (DM0177, DM0144) to 47 mm (DM0192). No cellulase or ligninase activity was observed.

Taxonomy

Clohesyomyces symbioticus A. E. Arnold and D. C. Sandberg, sp. nov.

MycoBank MB 841143

Type: USA, Arizona, Yavapai County, Prescott, Willow Creek Reservoir (Willow Lake), endophytic in roots of healthy *Persicaria amphibia* (*Polygonaceae*) growing in standing water (16.8 and 20.4 cm depth at the time of collection), collected in

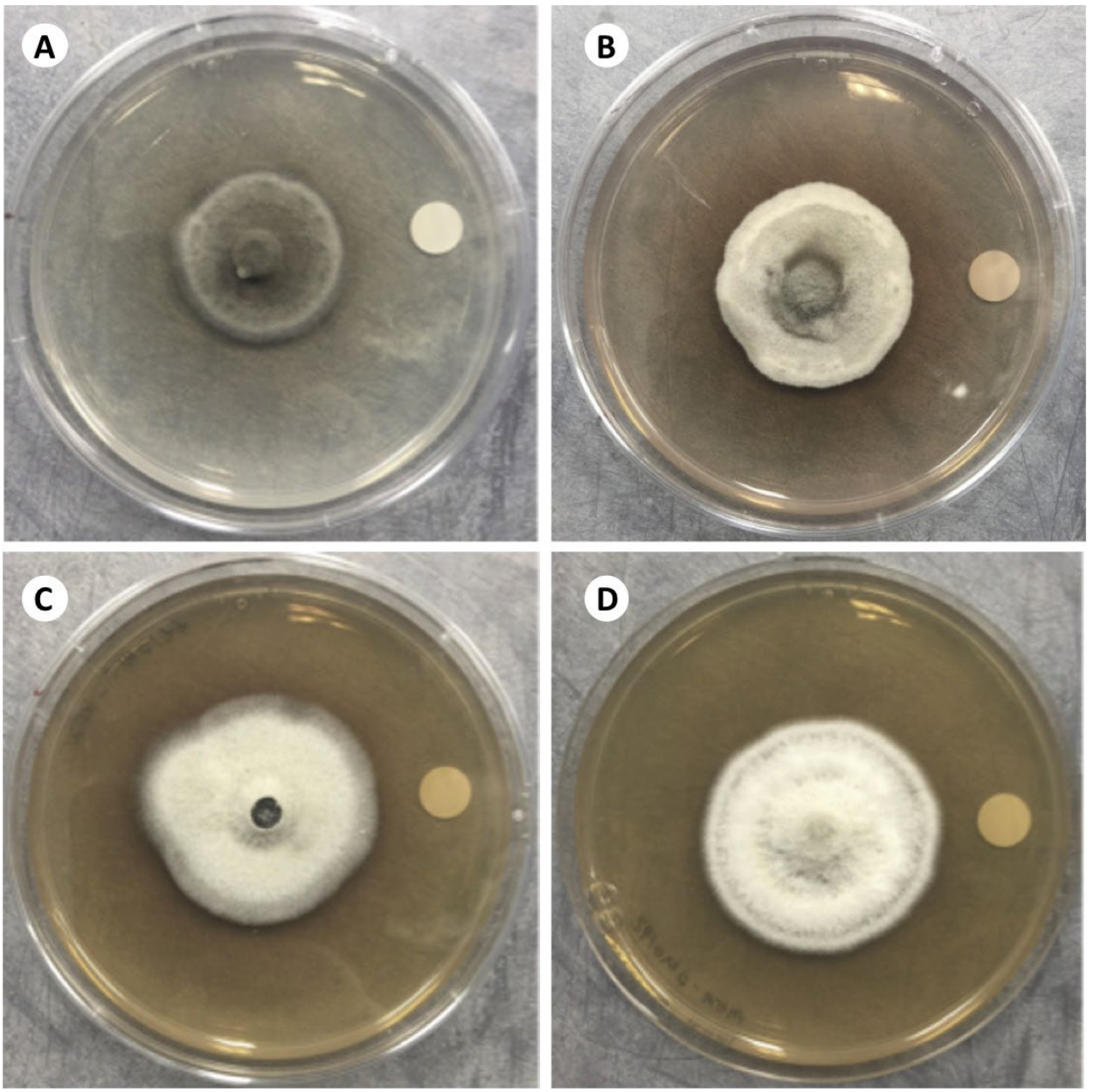

Figure 6. Colony morphology of paratypes of *Clohesyomyces symbioticus* (all on 100 mm plates): A – DM0177 on 2% MEA; B – DM0192 on 2% MEA; C – DM0177 on PDA; D – DM0192 on PDA. White dots on the undersides of plates are for white balance. Red pigment is visible in all plates, especially panels B and C.

June 2012, isolated by D. C. Sandberg (ARIZ-AEADM0144 – holotype; preserved in a metabolically inactive state (lyophilized) at the Robert L. Gilbertson Mycological Herbarium, University of Arizona).

Description. Colonies on PDA attained a diameter of 44 mm in 14 d at 22°C, with a largely pale grey (1B1) to greyish white appearance from above, with dense and velvety aerial hyphae and rings of hyphal growth visible in some cultures; from below, typically olive grey (3F2) to goose turd (per Kornerup and Wanschen 1967, 3F3) at the colony center, and pale grey (1B1) to yellowish grey (3D2) to goose turd (3F3) from below. Margin variable. PDA remains colorless or may be colored red, either mild or dark, by a diffusible pigment that becomes more visible as colonies age past 1 month; not present in all subcultures. Colonies on 2% MEA were similar in having a dense, velvety appearance, but they grew somewhat more slowly than on PDA (34–39 mm diameter in 14 d at 22°C), with a generally darker grey aspect (from above, olive grey, 3E2) to grey (3B1), at times with a mildly yellowish grey aspect at the colony edge (3C2). From below, colonies on MEA were typically yellowish white (3F2) to olive (3F3) at the center (but at times reddish brown, when red diffusible pigment was present); and at the edge, yellowish grey (3C2) to olive grey (3D2) to brownish grey (8C2, when red pigment was present). On MEA concentric growth rings were more visible than on PDA from above, but they were not evident from below. Diffusible red pigment was observed on MEA, ranging from mild to dark, but sometimes absent, as on PDA. Cellulase and ligninase negative; moderate and colorless growth on water agar, cellulose medium, and indulin medium. Vegetative hyphae brown with age to hyaline at growing edge of colony; with microscopy, typically olivaceous with obvious septation.

Pycnidia 150–310 µm long, 60–130 µm diam., subglobose to ellipsoidal, irregular, very dark brown to black, infrequent, appearing on the medium where cuts were made in hyphal transfers; semi-immersed, solitary, unilocular, with a wall approximately 10–20 µm thick, comprised of thick-walled cells. Conidiogenous cells 8.5–12.5 µm long, hyaline, and as described by Zhang et al. (2012) for *C. aquaticus,* with marginal periclinal thickening and a short collarette. Conidia blastic, measuring 10.0–18.5 × 10.5–20.0 µm, becoming clearly delimited by a single septa at maturity, circular to ovoid, hyaline, smooth, thin-walled. Sexual structures undetermined.

Etymology. Named for its occurrence within healthy roots of living plants.

Notes. Pycnidia and conidia of *C. symbioticus* are smaller than those described for *C. aquaticus* (per Hyde 1993: pycnidia, 247–390 µm long and 156–260 µm in diameter; conidia, 21–31 × 9.5–12 µm). The conidia of *C. symbioticus* also are generally more spherical at maturity than those of *C. aquaticus.* The reddish pigment produced by DM0192, and especially DM0177, was less commonly visible in subcultures of DM0144, appearing only periodically. Pycnidia were rarely produced by all strains. *Clohesyomyces symbioticus* was detected in roots of individuals in several different areas of shallow water at Willow Creek Reservoir, with subsequent review of the Gilbertson endophyte collection highlighting the occurrence of the species also in roots of *P. amphibia* in shallow water at Lower Lake Mary (Flagstaff, Coconino County, Arizona, USA).

Other specimens examined. USA. Willow Creek Reservoir (Willow Lake), Prescott, Yavapai County, Arizona, DM0192 and DM0177, as described in Sandberg et al. (2014).

Vouchers and data deposition. Lyophilized vouchers of the paratypes DM0192 and DM0177 are deposited in the publicly accessible culture collection of the Robert L. Gilbertson Mycological Herbarium at the University of Arizona (ARIZ; accession numbers match isolate numbers; see above for holotype information), with data available at MyCoPortal.org. Sequence data for SSU rDNA, LSU rDNA, ITS rDNA, and *TEF-1a* are deposited in GenBank (Table 4).

Table 4. GenBank accessions for molecular data generated for this study.

Isolate	SSU rDNA	LSU rDNA	ITS rDNA	TEF-1a
DM0144	OK139667	OK135170	OK139670	OK094526
DM0177	OK139668	OK135171	OK139671	OK094527
DM0192	OK139669	OK135172	OK139672	OK094528

Discussion

Clohesyomyces symbioticus was identified from multiple strains isolated as endophytes from healthy, submerged roots of water smartweed in impounded lentic waters in central Arizona. *Clohesyomyces* thus aligns with the observation of Shearer (1993) that *Ascomycota* occurring in freshwater systems generally fall into two functional groups based on their nutrition: saprophytes, consuming dead plant material, such as *C. aquaticus*; and endophytic parasites of living algae and macrophytes, such as *C. symbioticus*.

A subsequent review of the culture collection of endophytes at the Robert L. Gilbertson Mycological Herbarium revealed that *C. symbioticus* also was detected in roots of *P. amphibia* in a second reservoir in the same region (Lower Lake Mary, Flagstaff, Coconino County, Arizona, USA; depth at collection, 11.9 cm). In both locations, water smartweed was found in shallow water where seasonal drought occasionally exposes the substrate to drying (see Arizona Department of Environmental Quality 2015). In extensive surveys of terrestrial plants in the same region (e.g., Lau et al. 2013; Massimo et al. 2015; Huang et al. 2018; Bowman & Arnold 2021), we did not detect this species, suggesting that it may be specialized to aquatic or periodically flooded habitats. Surveys by Sandberg et al. (2014) in 2011 and 2012 detected *C. symbioticus* only rarely, and only in submerged tissues of plants from relatively shallow and seasonally dry areas.

Our analyses expand the scope of *Clohesyomyces* in terms of taxonomy, ecology, and geography. *Clohesyomyces* has long been considered a monotypic genus comprising the saprobic species *C. aquaticus* (Hyde 1993), presently known from freshwater in Thailand, China, and Australia (see Zhang et al. 2012). The addition of *C. symbioticus* in North America highlights an endophytic association with roots of a widespread aquatic plant. Our analyses also suggest the potential existence of a third species in *Clohesyomyces* (Fig. 3), which includes root-endophytic strains from *Microthlaspi perfoliatum* (perfoliate pennycress, *Brassicaceae*; now *Noccaea perfoliata*; Al-Shehbaz 2014) that were detected in Europe (e.g., Greece and Germany) (Macia-Vicente et al. 2020). Thus the capacity to live endophytically in association with roots, including in both aquatic and terrestrial environments, may be more important in the genus than was suspected previously.

The genera *Lindgomyces* and *Hongkongmyces* are closely related to *Clohesyomyces.* Both occur in the United States, with records in Pennsylvania, Wisconsin, Florida, and North Carolina. Thus, our study expands the range of the family to include the southwestern USA. We anticipate that further exploration of aquatic habitats globally, especially across the wide range of *P. amphibia,* would reveal additional *Clohesyomyces* strains. In future work, we will characterize the interaction of *C. symbioticus* with diverse plants under flooded and non-flooded conditions to evaluate the physiological tolerance and symbiotic contributions of this newly described species.

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