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Phialocephala urceolata, sp. nov., from a commercial, water-soluble heparin solution

Wei Wang

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

Diane McGhee

Tyco Healthcare/Kendall, 444 McDonnell Boulevard, Hazelwood, Missouri 63042, USA

Connie Fe C. Gibas

University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Garden, Edmonton, Alberta, Canada T6G 2E1

Akihiko Tsuneda

Randolph S. Currah[1]

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

Abstract: *Phialocephala urceolata* sp. nov. was isolated from a black film that had developed on a water-soluble proprietary heparin solution (pH 2.5). Morphological and enzymatic characters, along with phylogenetic analyses of rDNA sequence data, indicated that the conidial fungus is closely related to species of *Phialocephala* known primarily as endophytes in the roots of vascular plants (e.g. *Acephala applanata*, *P. fortinii* and *P. sphaeroides*) or as associates of persistent plant organs such as the stems and needles of woody plants (e.g. *P. compacta*, *P. dimorphospora* and *P. scopiformis*). *Phialocephala urceolata* is distinctive in having urn-shaped phialides that are sparsely distributed along the conidiophore axis, a slow growth rate in culture and in exhibiting a unique combination of reactions on enzymatic test media (i.e. it acidifies casamino acids medium and is gelatinase negative). Partial sequence data from the small subunit (SSU) rDNA indicated that *P. urceolata* is among the Helotiales and close to the type species of *Phialocephala*. Sequence data from the internal transcribed spacer (ITS) region places *P. urceolata* closest to *P. sphaeroides*. The source of this contaminant is unknown but its taxonomic relationship with other root endophytic species and its ability to produce polyphenol oxidases suggest that the natural habitat of this species is possibly woody plant tissues or soil enriched with lignocellulose.

Key words: dark septate endophytes, Helotiales, rDNA sequences, urn-shaped phialide

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[1] Corresponding author. E-mail: r.currah@ualberta.ca

INTRODUCTION

Species of the polyphyletic genus *Phialocephala* W.B. Kendr. are dematiaceous hyphomycetes that produce penicillate clusters of phialides on branched, mononematous conidiophores. Analyses of ribosomal DNA sequences show that *P. dimorphospora* W.B. Kendr., the type, and several other species (e.g. *P. fortinii* C.J.K. Wang and H.E. Wilcox, *P. sphaeroides* B.J. Wilson, *P. botulispora* [Cole and Kendrick] C.R. Grünig and T.N. Sieber, *P. glacialis* C.R. Grünig and T.N. Sieber, *P. lagerbergii* [Melin and Nannf.] C.R. Grünig and T.N. Sieber) are closely related to each other among the Helotiales (Wilson et al 2004, Grünig et al 2008) in the Leotiomycetes. The helotialean species of *Phialocephala* are best known as endophytes of roots and these when isolated grow readily on agar media where they produce olive to dark brown or gray colonies. Many such isolates will not sporulate unless incubated at approximately 5 C for some months and represent a large proportion of the fungi generally known as the ''dark septate (or sterile) endophytes'' or DSE (Addy et al 2005). The genus also hosts culturally similar species that are known as saprobes or endophytes of woody tissues (e.g. *P. lagerbergii* and *P. compacta* Kowalski & Kehr).

We recently obtained a dark sterile mold that had appeared as a black film on a water-soluble proprietary heparin solution (pH 2.5), which had been refrigerated 3–4 mo. Heparin is a naturally occurring, acidic polymer of sulfated glucosamine and glucuronic acid that is used as an injectable anticoagulant in the treatment of thrombosis (Goth 1970). Despite the unusual habitat, preliminary observations indicated that the fungus was closely related to the helotialean species of *Phialocephala*. More detailed analyses of cultural features, microscopic morphology and rDNA sequences confirmed the relationship and further revealed that it represented a unique and hitherto undescribed species in the genus. Here we provide a name for this new species and compare its morphological characteristics, phylogenetic position, and enzymatic profile to similar species affiliated with genus *Phialocephala* in the Helotiales.

MATERIALS AND METHODS

Isolation and culture.—The fungus (UAMH 10827) was isolated Aug 2005 from a contaminated heparin solution by transferring some of the black film to tryptic soy agar (TSA;

40 g [Becton Dickinson, Sparks, Maryland] in 1 L distilled water) and subculturing later on cornmeal agar (CMA; 17 g cornmeal agar [Difco-Becto, Detroit, Michigan] in 1 L distilled water), potato dextrose agar (PDA; 39 g potato dextrose agar [Becton Dickinson, Sparks, Maryland] in 1 L distilled water), and oatmeal agar (OA; 20 g rolled oats, 20 g agar, 1 L distilled water). Plates were incubated in the dark at 5 C for several months to encourage sporulation.

Scanning electron microscopy.—Agar disks, 5 mm diam, were cut from cold-incubated, 3 mo old cultures, washed in phosphate buffer (pH 7.0), and fixed in 2% glutaraldehyde in buffer 2 h at room temperature. After rinsing with distilled water, disks were immersed in a 2% tannic acid-2% guanidine hydrochloride solution 4–5 h, rinsed thoroughly in distilled water and postfixed overnight in 2% OsO_4 at 5 C. The fixed material was dehydrated in an ethanol series, taken to amyl acetate and critical point dried in a Polaron E-3000 unit (Polaron Controls Ltd, Watford, Hertfordshire) with carbon dioxide. The dried samples were coated with gold and examined with a Hitachi S-510 scanning electron microscope (Hitachi, Tokyo, Japan).

DNA sequencing and phylogenetic analysis.—Sequences for the SSU and ITS of the nuclear rRNA gene were determined for the ex-type strain of *P. urceolata*. Other sequences representing a selection of taxa in the Helotiales were obtained from GenBank (TABLE I). *Cryptosporiopsis brunnea* Sigler was used as outgroup taxon in phylogenetic analysis. Note that ITS sequences were not available for the ex-type strain of *P. dimorphospora*, the type of *Phialocephala* or for *P. lagerbergii*. *Phialocephala urceolata* was grown on PDA overlaid with a cellophane membrane (Innovia Films, Atlanta, Georgia). Approximately 100 mg of fresh mycelium was scraped from the surface of the membrane, placed in a precooled sterile porcelain mortar containing a small amount of acid sterilized sand, frozen with liquid nitrogen and ground to a powder. DNA extraction followed Wang et al (2007). Crude DNA material was purified with the QIAquick PCR Purification Kit (QIAGEN Inc., Mississauga, Ontario, Canada) and stored at −20 C.

PCR was done to amplify regions of the nuclear SSU rDNA gene and the ITS region. The SSU rDNA was amplified with primers NS1 and NS8 (synthesized by CyberSyn Inc., Aston, Pennsylvania) (White et al 1990). PCR amplification (Perkin Elmer GeneAmp 9700 Thermal cycler, Applied Biosystems, Foster City, California) was performed with these cycling parameters: denaturation at 94 C for 2 min; annealing at 55 C for 1 min and extension at 72 C for 2 min. Initial denaturation was at 94 C for 2 min, and the final extension was at 72 C for 7 min. There were 30 cycles. Cycle sequencing was done with the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, California) with forward primers NS1 (White et al 1990), NS11, NS13, and NS151 developed by K.N. Egger (University of Northern British Columbia, Prince George, British Columbia) and reverse primers NS2, NS4, NS6, and NS8 (White et al 1990). The sequencing products were cleaned with G50 Superfine Sephadex columns (Amersham Pharmacia Biotech Inc., Arlington Heights, Illinois) and run on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, California). The ITS rDNA (i.e. ITS1, ITS2 and 5.8S) were amplified with primers BMBC-R (Lane et al 1985) and ITS4 (White et al 1990). Cycle Sequencing was done with forward and reverse primers BMBC-R (Lane et al 1985), ITS1, ITS2, ITS3, and ITS4 (White et al 1990). Consensus sequences were determined with Sequencher™ for Windows 4.0.5 (Gene Codes Corp., Ann Arbor, Michigan) and alignment was done manually with Se-Al v2.0a11 Carbon (Rambaut 1996). Phylogenetic analysis was done with PAUP v.4.0b10 (Swofford 2003). Heuristic searches were performed with parsimony with tree bisection reconnection as the branch swapping algorithm. Gaps were treated as missing characters. Bootstrap analysis was performed with 1000 replicates (Felsenstein 1985).

Enzymatic profile.—An agar block (1 × 1 cm) cut from the margin of an actively growing colony of *P. urceolata* was placed on three plates each of a selection of enzymatic test media: casamino acids-bromocresol medium, tannic acid medium, wood powder medium, a medium solidified with gelatin (Wilson et al 2004) and Mycosel (Becton Dickinson, Sparks, Maryland). Uninoculated media served as controls.

TAXONOMY

Phialocephala urceolata Wang and McGhee, sp. nov. FIGS. 1–9

Post 21 dies ad fere 20 C, coloniae ad 2.4 cm diam in agaro farinae avenae (OA). Mycelium olivaceum ad nigrum, lucidius ad marginem, leve, plane, vel cristatum. Conidiogenesis post incubationem longam ad 5 C in agaro OA. Conidiophora erecta, fusce pigmentata, septata, cum ramis brevibus et lateralibus, 2–5 numero, in ordine alterna, vel contraria, vel verticillata. Phialides sessiles in axi conidiogenosa, praecipue ad apicem conidiophoram, vel portatae in glomeribus penicillatis in ramis lateralibus, 2–8 phialides continentibus. Rami tumescentes vel intus curvati in axim conidiophoram. Phialides ampulliformes vel urniformes, cum monili discreto, 3 μm latae et ad 4 μm altae. Conidium primum teliformum, 3–3.5 × 2 μm, conidia sequentia globosa, pedicillata, 2 μm diam, singula vel adligata ad apicem phialidam in glomeribus parvis.

Colonies after 21 d at 22 C, up to 1.6 cm diam on CMA and PDA, and 2.4 cm on OA (FIG. 1). Mycelium olivaceous to black, lighter toward the margin, smooth, plane to tufted. Vegetative hyphae regularly septate and dematiaceous, smooth to coarsely warted (FIG. 2), occasionally forming strands. Conidiogenesis occurring only on OA after prolonged incubation (3 mo or longer) at 5 C. Conidiogenous apparatus consisting of an upright, hyaline to darkly pigmented, septate, mononematous conidiophore, (13–)30–39 (–50) × 2–4.2 μm (FIGS. 3, 4), bearing 2–5 short, lateral branches, 5–7 × 2–2.5 μm, in an alternate, opposite, or verticillate pattern (FIGS. 3–5). Phialides sessile on the conidiogenous axis, especially toward

TABLE I. Isolates obtained from GenBank and included in the analysis of the internal transcribed spacer (ITS) rDNA sequences

Species	Source	Isolate number[a]	GenBank accession number (ITS)
Acephala applanata	Root of *Picea abies*, Switzerland	CBS 109318	AY078151
A. applanata	Root of *P. abies*, Switzerland	CBS 109321	AY078145 (ex-type)
Cryptosporiopsis brunnea	Root of *Gaultheria shallon*, Canada	UAMH 10106	AF149074 (ex-type)
Phaeomollisia piceae	Needle of *P. abies*, Switzerland	UAMH 10851	EU434836 (ex-type)
Phialocephala botulispora	Wood of *P. banksiana*, Canada	DAOM 75261	AF083198 (ex-type)
P. compacta	Bark of a living branch of *Alnus glutinosa*, Germany	CBS 507.94	AF486125 (ex-type)
P. dimorphospora	Wood pulp, Canada	CBS 300.62	AF486121
P. fortinii	Root of *P. sylvestris*, Finland	CBS 443.86	AY249076 (ex-type)
P. fortinii	Root of *P. sylvestris*, Finland	n/a[b]	AF011326
P. fortinii	Root of *P. abies*, Switzerland	n/a	AY078129
P. fortinii	Root of *P. abies*, Switzerland	n/a	AY347403
P. fortinii	Root of *P. abies*, Switzerland	n/a	AY664501
P. glacialis	Root of *Vaccinium myrtillus*, Switzerland	UAMH 10852	EU434843 (ex-type)
P. lagerbergii	Blue-stained wood of *Pinus sylvestris*, Sweden	CBS 266.33	AF083197 (ex-type)
P. scopiformis	A living branch of *P. abies*, Germany	CBS 468.94	AF486126 (ex-type)
P. sphaeroides	Root of *Aralia nudicaulis*, Canada	UAMH 10279	AY524844 (ex-type)

[a] CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; DAOM = National Mycological Herbarium, Canada Department of Agriculture; UAMH = University of Alberta Microfungus Collection and Herbarium, Alberta, Canada.
[b] n/a = not available.

the apex of the conidiophore, or borne in small penicillate clusters of 2–8 phialides on lateral branches. Basal cells (5–11 × 2.5–3.3 μm) of branches arising just below a septum, markedly narrow at the point of origin (0.9–2.1 μm wide), and increasing in breadth toward the terminus of the cell, swollen toward the outside and curving in toward the conidiophore axis (FIG. 5). Phialides flask- to urn-shaped, 5.2–10.4 × 2.6–3 μm, each with a prominent cylindrical, hyaline collarette, 3 μm wide and up to 4 μm deep (FIGS. 3, 6, 7). First-formed conidium ellipsoidal to bullet-shaped, 3–3.5 × 2 μm (FIG. 8), subsequent conidia globose, pedicellate, 2 μm diam (FIG. 9), single or adhering in small clusters at the phialide apex.

Specimen examined. USA. MISSOURI: Hazelwood. From a water-soluble heparin solution, collected by D. McGhee, Aug 2005. Holotype, a dried culture, UAMH 10827. Deposited DNA sequences, 18S rDNA, GenBank EU155146; ITS, GenBank EU155145.

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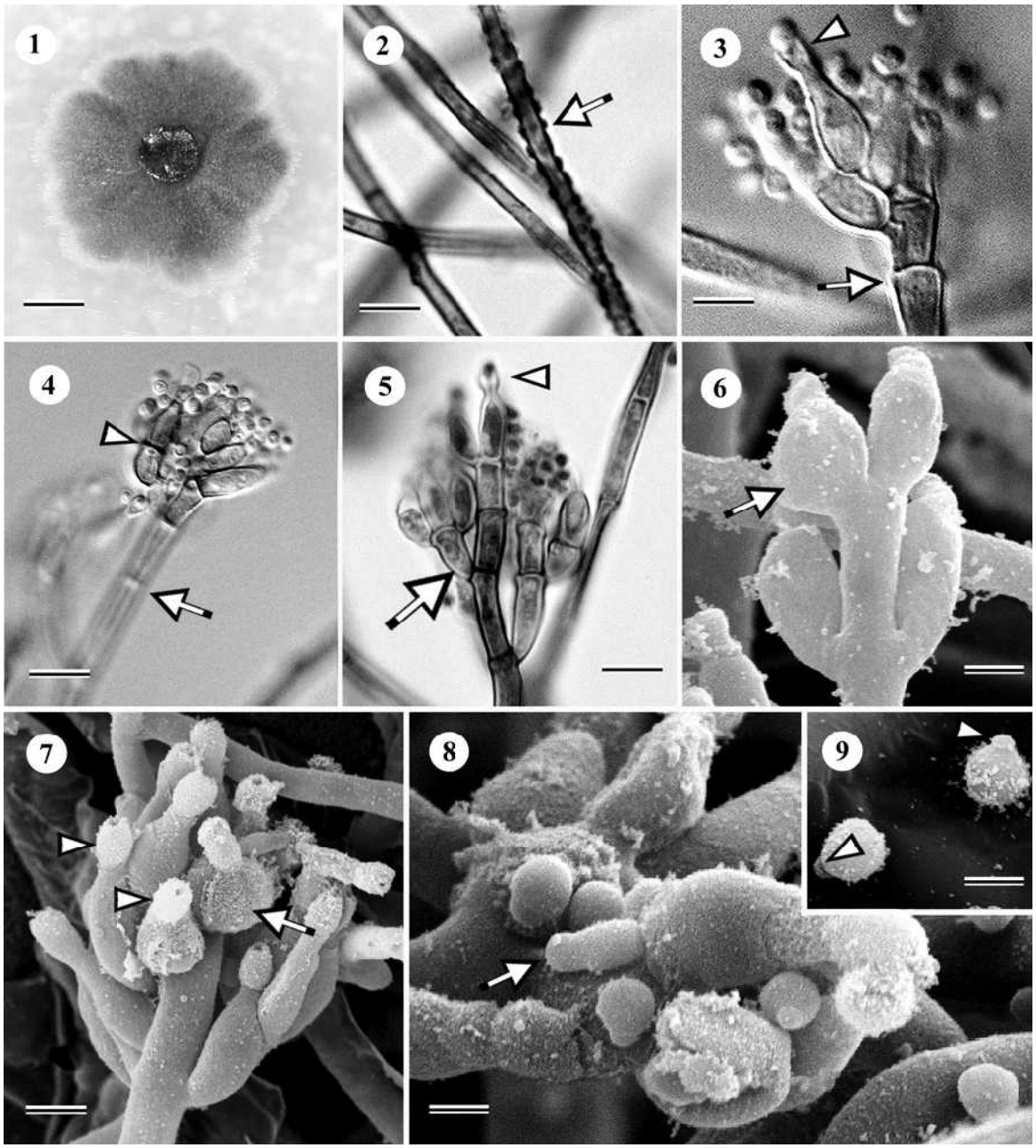
DISCUSSION

Phialocephala urceolata exhibits morphological characteristics that are similar to the dark septate endophyte species of *Phialocephala*. Its dark olivaceous colony resembles those formed by isolates of *P. fortinii*, although the growth rate of *P. urceolata* is much slower, in fact among the slowest recorded for species in the genus (i.e. reaching 1.6 cm diam on CMA after 21 d at 22 C, compared to 4–6 cm reported for *P. fortinii* 15–25 C [Currah and Tsuneda 1993] and 2.3 cm and 2.8 cm after 14 d at 21 C in *P. scopiformis* and *P. compacta* respectively [Kowalski and Kehr 1995]). The vegetative hyphae of *P. urceolata* and *P. fortinii* are similar in being smooth to blistered although our isolate of *P. urceolata* does not form the minute, scattered sclerotia that are characteristic of many isolates of *P. fortinii* (Currah and Tsuneda 1993). *Phialocephala urceolata* is close to *P. sphaeroides* phylogenetically, but *P. sphaeroides* differs in producing a distinctive orange-brown diffusible pigment and in having smooth-walled instead of blistered hyphae (Wilson et al 2004).

The conidiogenous apparatus of *P. urceolata* is morphologically distinctive among the helotialean species of *Phialocephala* because of its curved, urn-shaped phialides and their irregular and sometimes sparse arrangement on the conidiophore axis. In *P. dimorphospora* and *P. fortinii* phialides are mostly cylindrical and usually arranged in obconic, penicillate clusters (Kendrick 1961, Wang and Wilcox 1985). In *P. sphaeroides* phialides are long (up to 20 μm) and cylindrical and form a large spherical head (Wilson et al 2004). *Phialocephala compacta*, which has its affinities with *P. fortinii* (Grünig et al 2008), is somewhat similar to *P. urceolata* in having phialides that can be flask-shaped and curved but these are usually borne in deeply pigmented, ''rounded'' compact heads, which can resemble sclerotia (Kowalski and Kehr 1995). Phialides in *P. scopiformis* are



FIGS. 1–9. *Phialocephala urceolata* sp. nov. 1. A colony of *P. urceolata* on oat meal agar after 21 d of incubation. Bar = 0.5 cm. 2. Septate and warted (arrow) vegetative hyphae. Bar = 10 μm. 3. Conidiophore (arrow) and deep and wide collarette (arrowhead). Nomarski interference contrast. Bar = 5 μm. 4. Conidiophore (arrow) and phialides with globose conidia. The arrowhead indicates a septum separating basal cell and a phialide. Nomarski interference contrast. Bar = 10 μm. 5. Basal cells (arrow) bearing phialides. The arrowhead indicates a conidium exiting the collarette at the phialide apex. Bar = 10 μm. 6–9. Scanning electronic micrographs. 6, 7. Urn-shaped phialides (arrows) and collarettes (arrowheads). Bars 6 = 3 μm, 7 = 6 μm. 8. First-formed ellipsoidal conidium (arrow) and globose subsequent conidia. Bar = 3 μm. 9. Globose subsequent conidia with a pedicel (arrowheads). Bar = 2 μm.

also long and narrow and borne in complex, branched and broom-like conidiogenous heads. The sparse arrangement of phialides along the conidiophore axis in *P. urceolata* is somewhat reminiscent of *P. lagerbergii* (≡ *Cadophora lagerbergii*), a taxon shown by Harrington and McNew (2003) and Grünig et al (2008) to be phylogenetically close to *P. dimorphospora*.

The SSU sequence (GenBank EU155146) shows that *P. urceolata* fits well among the Helotiales (data

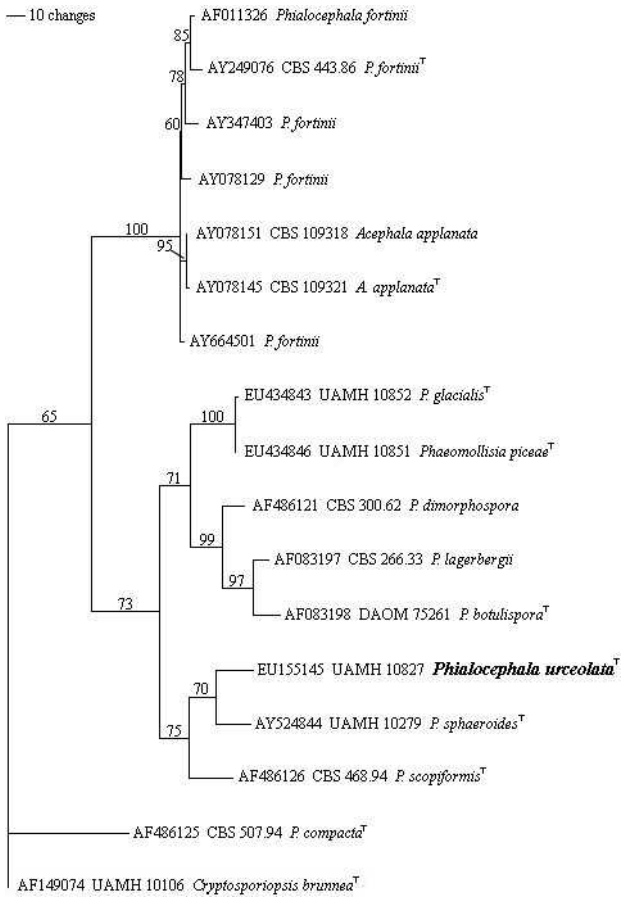


FIG. 10. One of three most parsimonious trees based on the sequences of the ITS1, ITS2 and the 5.8s genes of the rDNA of *Phialocephala urceolata* sp. nov. and related species in *Acephala*, *Phialocephala* and *Phaeomollisia piceae* (Helotiales). Bootstrap values greater than 50% calculated from 1000 replicates are given above the branches. (Source and accession information are summarized in TABLE I).

not shown). Furthermore ITS sequence data along with cultural, morphological and enzymatic criteria all indicate that *P. urceolata* is distinctive and placed appropriately among other species allied to the type, *P. dimorphospora*. One of three most parsimonious trees (MPT) of 370 steps (FIG. 10) was derived from analysis of the ITS sequence (GenBank EU155145) from the ex-type strain of *P. urceolata* and similar sequences from eight *Phialocephala* species, the teleomorph taxon *Phaeomollisia piceae* T.N. Sieber & C.R. Grünig and the non-sporulating, monotypic genus *Acephala*. The consistency index (CI), homoplasy index (HI) and retention index (RI) are respectively 0.746, 0.254, and 0.739. The tree consists of two large clusters, one of which has 100% support and includes five strains of *P. fortinii* and two of *A. applanata*. The sister clade, which has relatively weak support, shows a close relationship between *P. glacialis* and *Ph. piceae* (100% bootstrap support) and a strong relationship among *P. dimorphospora*, *P. lagerbergii* and *P. botulispora* (99%). *Phialocephala urceolata* and *P. sphaeroides* appear in a clade with 70% support, which in turn forms a clade with *P. scopiformis*. The position of *P. compacta* is unresolved in this analysis, which is consistent with reports by Grünig et al (2002, 2008).

According to Wilson et al (2004), *P. sphaeroides*, like *P. urceolata*, causes a shift in color from purple to yellow on the casamino acids-bromocresol medium but *P. urceolata* differs in being unable to liquefy gelatin. Like *P. sphaeroides*, *P. urceolata* is cycloheximide sensitive and also produces polyphenol oxidases, as indicated by the production of a red halo on wood powder medium and a brown halo around the block of inoculum on tannic acid media. The source of this contaminant in the heparin solution is unknown but the presence of polyphenol oxidases activity, in common with *P. fortinii* and *P. sphaeroides*, both well known as associates of roots, suggests that the native habitat of *P. urceolata* likely would be a substrate rich in lignocellulose, such as woody roots or stems. The requirement for several months of incubation at low temperatures before conidiogenesis will occur is well known in *P. fortinii* and *P. sphaeroides* and also might be evidence of a root endophytic habit in that the production of conidia after a prolonged cold period (i.e. during spring) would ensure the availability of young roots for colonization. The ability of *P. urceolata* to grow in the low pH heparin solution might indicate its native habitat is acidic such as might be found in a bog environment. An accession of *P. dimorphospora* (UAMH 8326 n.s.) from a pediatric chemotherapy patient is the only clinical record of a species of *Phialocephala* that we are aware of so it seems unlikely that the helotialean species of *Phialocephala* have appreciable medical significance.

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