42 *Phialophora*

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42.1 INTRODUCTION

42.1.1 CLASSIFICATION, MORPHOLOGY, AND BIOLOGY

The genus *Phialophora* is a dematiaceous (dark-walled) filamentous fungus belonging to the mitosporic Magnaporthaceae group, family Magnaporthaceae, order Magnaporthales, class Sordariomycetes, subphylum Pezizomycotina, phylum Ascomycota. The obsolete synonyms of the genus include *Cadophora* and *Margarinomyces*, and teleomorphs of this genus are found in *Chaetosphaeria*, *Gaeumannomyces*, and *Mollisia*.

Currently, the genus *Phialophora* contains 16 recognized and over 69 unassigned species, the most notable of which are *Phialophora bubakii*, *Phialophora europaea*, *Phialophora gregata*, *Phialophora repens* (obsolete synonym *Cadophora repens*), *Phialophora reptans*, *Phialophora richardsiae* (obsolete synonyms: *Cadophora brunnescens*, *Cadophora richardsiae*, *P. brunnescens*, and *P. caliciformis*), and *Phialophora verrucosa* (synonym: *P. americana*; obsolete synonym: *Cadophora Americana*; and teleomorph: *Capronia semiimmersa*) [1].

A number of former *Phialophora* species have been reclassified: *Phialophora compactum* (synonym: *Fonsecaea compacta*), *Phialophora cyanescens* (synonym: *Cylindrocarpon cyanescens*), *Phialophora dermatitidis* (synonyms: *Exophiala dermatitidis* and *Wangiella dermatitidis*), *Phialophora hoffmannii* (synonym: *Lecythophora hoffmannii*), *Phialophora jeanselmei* (synonym: *Exophiala jeanselmei var. jeanselmei*), *Phialophora mutabilis* (synonym: *Lecythophora mutabilis*), *Phialophora parasitica* (synonym: *Phaeoacremonium*

parasiticum), *Phialophora pedrosoi* (synonym: *Fonsecaea pedrosoi*), and *Phialophora spinifera* (synonym: *Exophiala spinifera*).

Phialophora is a ubiquitous organism that inhabits the soil, plants, and decaying food [2,3]. Several *Phialophora* species are the causative agents of human infections such as chromoblastomycosis, phaeohyphomycosis, and eumycetoma. *P. verrucosa* and *P. richardsiae* are often involved in phaeohyphomycosis, with clinical presentations ranging from cutaneous infections, subcutaneous cysts, keratitis, endocarditis, arthritis, osteomyelitis, cerebral infection, fatal hemorrhage, and disseminated infection. *Phialophora verrucosa* is the principal causative agent of chromoblastomycosis in tropical and subtropical areas, particularly in Japan and South America. *Phialophora europaea* has been isolated from cutaneous and nail infections in North-Western Europe.

Phialophora has short conidiophores (sometimes reduced to phialides) and unicellular conidia, although conidia are sometimes absent. *Phialophora* colonies grow moderately slowly and reach a diameter of 2–3 cm after 7 days at 25°C. Colonies are gray to olivaceous black and often become dark gray-green, brown, or black with age. From the reverse, colonies appear iron-gray to black; colony texture is cottony to velvety and may be heaped and granular.

Phialophora hyphae (up to 5 μm wide) are branched and hyaline to brown. If present, condiophores are short and pale brown; conidiogenous cells (phialides) have characteristic flask-shaped or cylindrical phialides with distinctive collarettes, which are terminally or laterally located on the hyphae;

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conidia are one celled, hyaline to olivaceous brown, smooth walled, ovoid to cylindrical or allantoid, and often aggregate in slimy heads at the apices of the phialides, giving the appearance of a vase of flowers; phialides may be solitary or in a brush-like arrangement. In tissues infected with *Phialophora* species, spherical or polyhedral, dark brown, thick-walled sclerotic bodies (muriform cells) and phaeoid hyphae are often present, although absence of muriform cells is noted in cases involving immunosuppressed or debilitated patients.

At the species level, *Phialophora ripens* produces intercalary phialides without basal septa or cylindrical to lageniform phialides (<20 µm in length) with narrow collarettes; *Phialophora richardsiae* conidia are globose to cylindrical and phialides with saucer- or vase-shaped collarettes; and *Phialophora verrucosa* produces flask-shaped phialides with vase-shaped collarettes; *Phialophora europaea*—a member of the *P. verrucosa* complex—shows reduced, flaring phialidic collarettes [4].

42.1.2 Clinical Features and Pathogenesis

The term dematiaceous (dark-pigmented) fungi refers to a large and heterogeneous group of molds belonging to several different genera that are capable of causing a diverse range of clinical diseases such as phaeohyphomycosis, chromoblastomycosis, and eumycetoma. The most notable human pathogens in the dematiaceous fungal group include *Alternaria*, *Bipolaris*, *Cladophialophora*, *Curvularia*, *Exophiala* (*Wangiella*), *Fonsecaea*, *Madurella*, *Phialophora*, *Rhinocladiella*, *Scedosporium*, and *Scytalidium*. Being widely distributed in the environment including soil, wood, and decomposing plant debris, these organisms have the capacity to take advantage of weakened defense mechanism of human hosts, inducing cutaneous, subcutaneous, and corneal infections, invasive and allergic sinusitis, arthritis, endocarditis, and systemic and disseminated infections. Traumatic implantation, injury, and inhalation represent the main routes of entry, and host immune deficiency (suppression) plays a critical role in the development of systemic and disseminated infections [5,6].

Pheohyphomycosis affects the skin and subcutis, paranasal sinuses, or central nervous system and is often categorized into four types by the region of the body in which the infection occurs: superficial, cutaneous, subcutaneous, and systemic [2]. Chromoblastomycosis is a cutaneous and subcutaneous mycotic disease (mostly of the feet and legs) with the appearance of pigmented sclerotic bodies, commonly called "copper pennies" [5]. Eumycetoma is a subcutaneous fungal infection (mostly of the feet and legs) showing a mass with multiple sinuses draining pus and "grains." The grains (0.5–1.5 mm in diameter) in eumycetoma may appear white (due to infection with *Acremonium*, *Cylindrocarpon*, *Fusarium*, *Neotestudina*, or *Pseudallescheria*) or black (due to infection with *Exophiala*, *Madurella*, *Leptosphaeria*, *Pyrenochaeta*, or *Phlenodomus*).

Phialophora spp. have been shown to cause chromoblastomycosis, mycotic keratitis, cutaneous infections,

osteomyelitis, and prosthetic valve endocarditis as well as disseminated infections [7–13], with *P. verrucosa* and *P. richardsiae* being most commonly reported [9,14–17].

42.1.2.1 *Phialophora verrucosa*   AQ1

A case of fatal hemorrhage due to *Phialophora verrucosa* occurred in a patient with prolonged neutropenia undergoing autologous bone marrow transplant (BMT) for acute myelogenous leukemia (AML). Autopsy uncovered granulation tissue on the tracheal wall, which showed fungal hyphae by histopathological examination and grew *P. verrucosa* in culture [18].

In a case of *Phialophora verrucosa*-related multifocal subcutaneous phaeohyphomycosis, an 85 year-old woman presented with purulent multifocal subcutaneous nodules on the right forearm and hand. The patient reported a history of gardening and recalled the occurrence of a painless, soybean-sized nodule on the dorsal site of the right forearm 3 years earlier, which gradually extended peripherally to the dorsum of the right hand. The pustules and nodules (ranging from 5×5 mm to 20×40 mm in size) showed a yellow   AQ2 crust on the surface and were surrounded by erythema and discharged a purulent fluid upon pressure. Examination of the biopsied specimen revealed a subcutaneous granuloma (containing faint brown septate hyphae with rare branching) with central abscess and necrosis, consistent with phaeomycotic cyst. Culture of the discharged purulent fluid on potato dextrose agar (PDA) at room temperature grew a nearly black fungus, attaining a diameter of 20 mm in 14 days and 50 mm in 35 days. Under light microscope, flask-shaped phialides (conidiogenous cells) with cup-shaped collarettes and a bunch of elliptical conidia at the apices of the collarettes were noted along with brown to light-brown hyphae. These morphologic features confirmed the fungus as *P. verrucosa*, which responded to oral itraconazole treatment [19].

A cutaneous fungal infection due to *P. verrucosa* was reported in a 53 year-old woman who had a history of systemic corticosteroid treatment for Evans' syndrome. The patient presented asymptomatic multiple nodules on her gluteal region. A smear from the purulent exudate of the nodules showed brown-colored hyphae, spores, and large dark-brown cells; and a dark-brown-colored colony grew on Sabouraud glucose agar, showing *Phialophora*-type conidia formation. The fungus was diagnosed as *Phialophora verrucosa* [20].

Phialophora verrucosa was also shown to be responsible for a severe, verrucous facial mycosis and sinusitis in a 12 year-old girl. The disease began with the development of verrucous, hyperkeratotic plaques, and subcutaneous violet nodules on face and upper extremities and later evolved into severely progressive tumorous cutaneous and nasal lesions. Microscopic examination of scale samples from the upper extremities and the face showed brown, thick-walled fungal elements, which were subsequently identified as *Phialophora verrucosa*. Oral treatment with itraconazole and fluorocytosin resulted in significant improvement of the lesions [17].

A case of polymicrobial keratitis caused by *Phialophora verrucosa*, *Candida tropicalis*, and *Propionibacterium acnes*

has also been described. Histopathologic evaluation revealed penetration of the crystalline lens by fungus at the site of synechiae between the intact lens capsule and iris [13].

42.1.2.2 *Phialophora richardsiae*

A case of subcutaneous phaeohyphomycosis due to *Phialophora richardsiae* was described in renal transplant recipient. The patient developed dark-colored nodules that subsequently ulcerated after transplantation. Histopathologic examination showed dematiaceous fungal hyphae with a surrounding granulomatous reaction, and the fungus was identified as *P. richardsiae* [21].

Diseases due to dematiaceous fungi require antifungal and surgical treatments. Current antifungal therapy for chromoblastomycosis and subcutaneous phaeohyphomycosis relies on itraconazole, and for eumycetoma is ketoconazole. Surgical debridement in combination with amphotericin B treatment is useful for chronic invasive sinusitis [6,9].

42.1.3 Laboratory Diagnosis

Phialophora represents one of the dematiaceous fungi responsible for causing phaeohyphomycosis and chromoblastomycosis in humans. There is a need to differentiate among the other dematiaceous fungi in order to implement appropriate treatment regimens as these organisms demonstrate varied sensitivity to the currently available antifungal drugs [6].

Dematiaceous fungi including *Phialophora* spp. have been traditionally identified by macroscopic and microscopic characteristics as well as biochemical properties. For example, phospholipase production, induced by egg yolk substrate, offers a useful means for the differentiation of the taxonomically related dematiaceous fungal species on the basis of their distinct enzymatic profiles [22]. Nevertheless, these methods are laborious and sometimes incapable of distinguishing species with polymorphic conidiogeneses or lacking conidia formation.

For these reasons, molecular techniques such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and DNA sequencing have been adopted increasingly for identification [23]. In particular, sequencing analysis of small subunit (SSU) 18S and large subunit (LSU) 28S rRNA genes and internal transcribed spacer regions (ITS) has proven valuable. Indeed, many *Phialophora* species can be correctly identified by examining the nucleotide sequence of the D1/D2 domains in the 28S rRNA gene [24].

42.2 METHODS

42.2.1 Sample Preparation

Clinical specimens suspected of containing dematiaceous fungi are treated with 10% KOH or stained with calcofluor white and observed by microscopy for septate hyphal elements. Samples are then inoculated onto inhibitory mold agar, modified Sabouraud agar, or PDA at 25°C, 35°C, and 50°C, respectively. Preliminary identification of the colonies is based on macroscopic and microscopic features [25].

After the growth for 1–7 days on PDA slants, approximately 1 cm^2 of mycelium is collected by scraping the slant with a sterile stick that is placed in 1 mL of sterile H$_2$O. The material is transferred to a 2 mL screw-cap tube, and the tube is centrifuged for 1 min at 6000 × *g*. If the mycelium does not pellet, the material is passed through a pediatric blood serum filter. After removal of the supernatant, the material is resuspended in 200 μL of IDI sample buffer (IDI lysis kit, BD) and transferred to the lysis tube containing glass beads. The lysis tube is vortexed on the highest setting for 5 min, and the tube is placed in a boiling water bath for 15 min. Following centrifugation for 5 min at 16,000 × *g*, the supernatant is stored at −20°C until PCR is undertaken [25].

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Alternatively, about 1 cm^2 of fungal material is transferred to a 2 mL Eppendorf tube containing a 2:1 (wt/wt) mixture of silica gel and Celite (silica gel H, Merck 7736/Kieselguhr Celite 545; Machery) and 300 μL of TES buffer (2 g Tris [hydroxymethyl]-aminomethane, 0.38 g Na-EDTA, and 2 g sodium dodecyl sulfate in 80 mL of ultrapure water pH 8). The fungal material is ground with a micropestle for 1–2 min. The volume is adjusted by adding 200 μL of TES buffer. After vigorous shaking and the addition of 10 μL of a 10 mg/mL concentration of proteinase K to the tube, the mixture is incubated at 65°C for 10 min. With the addition of 140 μL of 5 M NaCl solution, the mixture is combined with 1/10 volume (~65 μL) of cetyltrimethylammonium bromide (CTAB) buffer 10%, followed by incubation for another 30 min at 65°C. One volume (~700 μL) of chloroform–isoamyl alcohol (vol/vol = 24/l) is added and mixed by inversion. After incubation for 30 min at 0°C (on ice water) and centrifugation at 14,000 rpm at 4°C for 10 min, the top layer is transferred to a clean Eppendorf tube. The sample is added with 225 μL of 5 M NH$_4$–acetate and incubated for at least 30 min (on ice water) and centrifuged. The supernatant is transferred to a clean sterile Eppendorf tube and mixed with a 0.55 volume (~510 μL) of ice-cold isopropanol. After centrifugation for 7 min at 14,000 rpm and 4°C (or room temperature), the supernatant is decanted. The pellet is washed with ice-cold ethanol 70% twice and air dried. The powder is resuspended in 48.5 μL of Tris–EDTA buffer with 1.5 μL of 10 mg of RNase/mL, incubated at 37°C for 15–30 min, and stored at −20°C until use [26].

In addition, fungal strains can be cultured in 20 mL of RPMI 1640 medium with L-glutamine but without sodium bicarbonate (Sigma-Aldrich) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich). After 3–14 days of growth with agitation at 30°C (100 rpm), the mycelium is transferred into a tube and washed in 40 mL of sterile distilled water. Mycelium is then stored at −20°C until use. For DNA extraction, 100 mg of mycelium is homogenized for 1 min in a tube containing 1 mL of lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 10 mM Tris–HCl pH 8, 100 mM NaCl, and 1 mM EDTA pH 8), three 0.5 cm diameter glass beads (Sigma), and approximately 500 mg of 425–600 μm glass beads (Sigma). The homogenized

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mycelium is snap-frozen in liquid nitrogen, thawed, and refrozen once. DNA is then extracted using a DNeasy plant kit (Qiagen) [27].

Also, fungal DNA may be prepared by suspending 50 mg of mycelium in 600 μL extraction buffer (200 mM Tris–HCl, pH 7.5, 25 mM EDTA, 0.5% w/v sodium dodecyl sulfate, and 250 mM NaCl). The mixture is vortexed for 15 s, incubated at 100°C for 15 min, kept on ice for 60 min, and then centrifuged at $14,000 \times g$ for 15 min. Supernatants are transferred to new tubes and extracted with phenol–chloroform–isoamyl alcohol (25:24:1 v/v). Each sample DNA is precipitated with cold isopropanol (–20°C), dried, and resuspended in 100 μL distilled water [24].

42.2.2 Detection Procedures

42.2.2.1 Pan-Fungal Real-Time PCR and Sequencing Analysis

Pounder [25] reported a real-time PCR with SYBR green DNA-binding dye and amplicon-melting temperature analysis for fungal detection using pan-fungal primers ITS1 forward (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′) [28]. The identity of the fungus is confirmed by sequencing.

Procedure

1. PCR mixture consists of 1× Lightcycler FastStart DNA Master Hybridization Probes mixture (Roche Applied Science) containing deoxynucleoside triphosphates, FastStart *Taq* DNA polymerase, and 1 mM MgCl₂ (additional MgCl₂ is added to a final concentration of 4.6 mM), 0.4 μM each of ITS1 forward and ITS4 reverse primers, 1× SYBR green (Molecular Probes), and 3 μL template DNA.
2. Thermal cycling parameters include 95°C for 10 min; 50 cycles of 95°C for 5 s, 60°C for 20 s, and 72°C for 30 s; and a final 72°C for 2 min.
3. The quality of the amplicon is determined using the derivative of the melt analysis curve (55°C–99°C, 45 s hold at 55°C, and 5 s/°C) using the RotorGene 3000 (Corbett Robotics, Inc).
4. The amplified product is purified for bidirectional sequencing using ExoSAP-IT (USB Corp). Five microliters of Big Dye Terminator Ready Reaction Mix v. 1.1 (Applied Biosystems) is added to 4 μL of each primer (0.8 pmol/μL) and 3 μL of purified PCR product. Cycle sequencing is performed with a 9700 thermal cycler (ABI), using 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing reaction products are passed through a Sephadex G-50 fine column to remove unincorporated dye terminators. Purified sequencing reaction products are run on an ABI Prism 3100 Genetic Analyzer with a 50 cm capillary array.
5. Sequences are analyzed with the SmartGene Integrated Database Network software version

3.2.3 vr. SmartGene is a web-based software and database system with reference sequences derived from the National Center for Biological Information (NCBI) GenBank repository.

Note. Sequence-based identifications are defined by percent identity: species, ≥99%; genus, 93%–99%; and inconclusive, ≤93%.

42.2.2.2 Sequencing Analysis of the ITS1-5.8S-ITS2 Region

Zeng et al. [26] utilized primers V9G (5′-TTA CGT CCC TGC CCT TTG TA-3′) and LS266 (5′-GCAT TCC CAA ACA ACT CGA CTC-3′) for PCR amplification and sequence analysis of the ITS-5.8S-ITS2 region.

Procedure

1. PCR mixture (50 μL) is composed of 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl·6H₂O, 0.01% gelatin, 200 mM of each dNTP, 25 pmol of each primer, 0.5 U of *Taq* DNA polymerase (Sigma), and 10–100 ng of extracted DNA.
2. The mixture is subjected to an initial 95°C for 4 min, 35 cycles of 94°C for 45 s, 52°C for 30 s, and 72°C for 2 min, and a final 72°C for 7 min in a GenAmp PCR System 9700 thermocycler (Applied Biosystems).
3. Amplicons are purified with GFX columns (GE Healthcare), and both strands are sequenced with each of the two primers separately. The sequencing PCR mixture consists of 1 μL of template DNA (1–10 ng), 3 μL of dilution buffer, 1 μL of BigDye v3.1, and 1 μL of 4 pmol primer filled with 4 μL of MilliQ water to a final volume of 10 μL. The amplification is performed with initial 95°C for 1 min, 30 cycles of 95°C for 10 s, 50°C for 5 s, and 60°C for 2 min. Reaction products are cleaned with Sephadex G-50 Fine (GE Healthcare Bio-Sciences) and analyzed by using an ABI Prism 3730xl DNA analyzer (Applied Biosystems).
4. The sequences are adjusted by using the Lasergene software program SeqMan Π (DNASTAR, Inc.) and aligned iteratively using Ward's averaging in the Bionumerics package v. 4.0 (Applied Maths). Nearest neighbors are identified by local BLAST searches. The distance trees are based on a realigned file using the DCSE program and calculated by the neighbor-joining (NJ) method of the Treecon package with Kimura-2 correction; only unambiguously aligned positions are taken into account. A total of 100 bootstrap replicates are used for analysis. Bootstrap values of >90 from 100 resampled datasets are shown.

Note. If the similarity of sequences of the ITS region is more than 99% between a studied strain and its nearest neighbor

and they are distributed in the same branch of the phylogenetic tree, the strain is regarded as belonging to the same species as its nearest neighbor.

42.2.2.3 Sequencing Analysis of the D1/D2 Domains of 28S rRNA Gene

Abliz et al. [24] utilized primers NL-1 (5′-GCATATCAAT AAGCGGAGGAAAAG-3′) and NL-4m (5′-GGTCC GTGTTTCAAGACG-3) to amplify the conserved D1/D2 domains of the 28S rRNA from *Phialophora* and other dematiaceous fungi. Subsequent analysis of the amplicon with primers NL-1 and NL-4m and internal primers NL-2m (5′-CTTGTGCGCTATCGGTCTC-3′) and NL-3m (5′-GAGACCGATAGCGCACAAG-3′) permitted the correct identification of *Phialophora* spp.

Procedure

1. PCR mixture (50 μL) is made up of 5 μL of template DNA, 5 μL (2 pmol) each of primers NL-1 and NL-4m, 4 μL (2.5 mM) dNTP mixture, 0.25 μL (5 U/μL) *Taq* polymerase, and 5 μL 10× reaction buffer.
2. Amplification is conducted with an initial 95°C for 4 min; 30 cycles of 94°C for 1 min, 55°C for 2.5 min, and 72°C for 2.5 min; and a final 72°C for 10 min.
3. The amplified products are first checked on 1.5% agarose gel, purified with SUPREC™-02 (TaKaRa) and sequenced with an ABI Prism 3100 sequencer after labeling with BigDye™ Terminator Cycle Sequencing Ready Reaction (Applied Biosystems) using external primers, NL-1 and NL-4m, and the internal primers, NL-2m and NL-3m.
4. The sequence data are aligned with CLUSTAL W (version 1.6). Phylogenetic trees are constructed with the NJ method.

42.3 CONCLUSION

The genus *Phialophora* is a dematiaceous fungus that represents one of the causes for human phaeohyphomycosis and chromoblastomycosis. The most common *Phialophora* spp. identified in human infections are *P. verrucosa* and *P. richardsiae*, with clinical symptoms ranging from cutaneous and subcutaneous infections, mycotic keratitis, osteomyelitis, endocarditis, and disseminated infections.

Because different dematiaceous fungal genera/species demonstrate varied sensitivity and resistance to antifungal reagents, it is important to determine their species identity with the aim of implementing most effective treatment measures for these diseases [6]. While traditional macroscopic, microscopic, and biochemical methods provide a useful approach for the differentiation of the taxonomically related dematiaceous *Phialophora* spp., they suffer from the shortcomings of being time consuming, laborious, and incapable of distinguishing species with polymorphic conidiogeneses

or lacking conidia. Exploiting the nucleotide variations within the ITS by PCR followed by sequencing offers a valuable tool for precise determination of *Phialophora* species.

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[AQ2]   Please check if the insertion of the word 'were' in the sentence beginning "The pustules and nodules (ranging from 5×5 mm …" is ok.

[AQ3]   Please provide expansion for "IDI, PCR, and DCSE" if needed.

[AQ4]   Please check if the edit made to the sentence beginning "After 3–14 days of growth with agitation at …" is ok.

[AQ5]   Please provide accessed date for reference [1].