20 *Ulocladium*

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CONTENTS

20.1 INTRODUCTION

The genus *Ulocladium* covers a number of saprotrophic, darkly pigmented hyphomycetes that share similar morphological characteristics with some saprotrophic *Alternaria* species. The morphological separation between the genera *Ulocladium* and *Alternaria* hinges on whether developing conidia are ovoid or obovoid, in addition to their subtle differences in the pigmentation and verrucosity of mature conidia. Although *Ulocladium* spp. have been often considered as contaminants in medical microbiology laboratories, there is increasing evidence that supports the role of these organisms in the pathogenesis of human mycoses.

20.1.1 Classification

The genus *Ulocladium* (obsolete synonym: *Pseudostemphylium*) is a dematiaceous (dark-walled) filamentous fungus belonging to the mitosporic Pleosporaceae group, family Pleosporaceae, order Pleosporales, class Dothideomycetes, subphylum Pezizomycotina, phylum Ascomycota, kingdom Fungi. The mitosporic Pleosporaceae group consists of 13 genera: *Alternaria*, *Dendryphiella*, *Dendryphion*, *Drechslera*, *Embellisia*, *Exserohilum*, *Nimbya*, *Pithomyces*, *Pyrenochaeta*, *Stagonospora*, *Stemphylium*, *Ulocladium*, and *Unifilum*. In turn, the genus *Ulocladium* is separated into 14 recognized species: *Ulocladium alternariae*, *Ulocladium atrum*, *Ulocladium botrytis*, *Ulocladium capsicum*, *Ulocladium chartarum*, *Ulocladium consortiale*, *Ulocladium cucurbitae*, *Ulocladium dauci*, *Ulocladium multiforme*, *Ulocladium obovoideum*, *Ulocladium oudemansii*, *Ulocladium septosporum*, *Ulocladium subcucurbitae*, and *Ulocladium tuberculatum*, as well as 25 unassigned species [1].

As saprophytes inhabiting the soil and decaying herbaceous plants, *Ulocladium* spp. are widely distributed in nature and have been isolated from paper, textiles, and wood. Although often considered as contaminants, some *Ulocladium* spp. (e.g., *Ulocladium chartarum*, *Ulocladium botrytis*, and *Ulocladium atrum*) are occasionally implicated in human disease process, causing subcutaneous phaeohyphomycosis, onychomycosis, keratitis, and other infections.

Ulocladium grows moderately rapidly. Colonies are wooly (suede-like) to cottony (floccose), brown to olivaceous-black or grayish on potato dextrose agar (PDA) at 25°C. Hyphae are brown, septate. Conidiophores are simple or branched, smooth, strongly geniculate (bent at the points where the conidia are produced, leading to a zigzag or bent knee appearance) and bear the conidia. Conidia (13–30 µm × 6–19 µm) are brown to black, typically obovoid (narrowest at the base), smooth or rough and verrucous. These conidia are typically muriform with transverse and longitudinal septations, appear solitary (*Ulocladium botrytis*) or form short chains (*Ulocladium chartarum*). Solitary, multicelled conidia (dictyoconidia) are formed through a pore (poroconidia) by a sympodially elongating geniculate conidiophore. When chains are produced, a tubular, short outgrowth is formed on the conidia at the point of secondary conidium formation [2,3].

Previously, *Ulocladium chartarum* had been included in the genus *Alternaria* as *A. chartarum* and *A. stemphylioides*. *Ulocladium* differs from *Alternaria* by its strongly geniculate (zigzag) conidiophores and the absence of beak-like tapered

apex of conidia. It differs from *Bipolaris*, *Curvularia*, and *Drechslera* by producing muriform conidia. *Ulocladium* is differentiated from *Stemphylium* by having geniculate, sympodial conidiophores.

20.1.2 Clinical Features

Ulocladium spp. are dematiaceous fungi that exist as saprobes on rotten plant material and soil. These organisms appear to have low pathogenicity and are occasionally associated with subcutaneous infection, keratitis, and onychomycosis in humans, especially those under immunosuppressive therapies or after a local trauma.

20.1.2.1 *Ulocladium chartarum*

Several cases of cutaneous infections due to *U. chartarum* have been reported in the literature. Because *U. chartarum* was formerly regarded as a member of the genus *Alternaria*, most of these cases were described as being alternariosis [4–6].

Duran et al. [7] documented a cutaneous mycoses caused by *Ulocladium chartarum* in a 62-year-old male heart transplant recipient under immunosuppressive therapy (consisting of tacrolimus 2 mg/day, azathioprine 100 mg/day, and prednisone 10 mg/day). The patient noticed a painless, flesh-colored cutaneous lesion on his right toe over the previous 4 weeks, with no fever, malaise, or sweating and no history of trauma. The lesion appeared as a 6 × 6 cm sharply demarcated plaque on the dorsal area of his right big toe, with a granular surface and a vermiculate consistency. In cutaneous biopsy tissue sections stained with hematoxylin–eosin, numerous rounded, refringent, hyaline or slightly eosinophilic thick-walled fungal structures, together with a few elongated budding yeast-like forms and branched septate hyphae, were observed in the granuloma and within the giant cells present. Fungal elements were also revealed by periodic acid-Schiff (PAS) and Grocott-Gomori methenamine-silver nitrate stains, but brown pigment was detected in fungal cell walls with Masson-Fontana stain. The lesion was completely removed by surgery, and culture of the lesion tissue homogenate on bacteriologic (blood, chocolate, and MacConkey agars and thioglycolate broth) and mycologic media (Sabouraud dextrose agar [SDA] with and without chloramphenicol and gentamicin and brain heart infusion agar with 5% of blood) grew a mold after 2 days at 30°C and 35°C, respectively. Subculture of the mold on PDA generated powdery to lanose and black to olivaceous black colonies of 6 cm in diameter after 7 days at 30°C, which grew more slowly at 35°C. Microscopic examination of slide cultures on PDA demonstrated a mycelium with pale yellow or brown septate hyphae. Conidiophores were erect, geniculate, simple or branched and golden brown. Conidia were brown and verrucose with transverse and longitudinal septa, solitary or in chains through apical production of short conidiophores (false beaks). The fungus was thus identified as *U. chartarum*. Treatment with oral itraconazole (400 mg/day) began after complete surgical removal of the lesion, with a reduced intake of tacrolimus and prednisone and switch of azathioprine to mycophenolate mofetil. After 6 months of antifungal therapy, the surgical wound had fully healed with no relapse of the lesion.

20.1.2.2 *Ulocladium botrytis*

Romano et al. [8] reported a *Ulocladium botrytis*-related case of disto-lateral onychomycosis of the third toe of the right foot in a 45-year-old man is reported. Culture of pathological material grew a mold that was identified as *Ulocladium botrytis* based on the macro and microscopic characteristics of the colonies. After 3 months of topical therapy with ciclopirox olamine, the lesion was completed resolved.

20.1.2.3 *Ulocladium atrum*

Badenoch et al. [9] described a case of *Ulocladium atrum* keratitis in a 43-year-old man. The patient presented with corneal ulcer in his right eye, showing photophobia, marked conjunctival injection, corneal edema, Descemet's membrane folding, a central stromal infiltrate with a feathery edge, and a reduced visual acuity (VA) (for hand motions only) in the right eye. Gram staining of corneal scrapings showed septate hyphae, and culture of the material on Sabouraud's dextrose agar (without antibiotics) grew a visible colony at 28°C after 4 days, which reached a diameter of 2 cm by day 8 and appeared grayish brown and powdery. Under microscope, conidia were dark brown, coarsely verrucose with transverse and longitudinal septa. Subcultures of the isolate on cornmeal agar at 23°C or 28°C revealed long, flexuous, or simple to short, geniculate, and branched conidiophores. Conidia appearing within 48 h of subculture were spherical to ellipsoidal and mostly single. By 72 h, septate conidia became dark brown, verrucose at either temperature, with the septa often intersecting at right angles. The isolate was identified as a *Ulocladium* species on morphological grounds, most closely resembling *U. atrum*, and internal transcribed spacer (ITS) sequence analysis confirmed its *U. atrum* identity. After receiving hourly eye drops of natamycin (5%) and fluconazole (0.2%; the neat intravenous preparation), the patient was comfortable, with clinical signs disappearing in 4 weeks and an improved VA (20/20).

20.1.3 Diagnosis

Ulocladium represents one of the dematiaceous mold genera that are ubiquitously distributed in the environment and that may transiently colonize the respiratory, integument, and gastrointestinal systems of human hosts, with the potential to take advantage of the weakened host defense mechanisms (or injury), causing a diverse range of clinical diseases (often referred to as phaeohyphomycosis, chromoblastomycosis, and eumycetoma) [10–12]. In the case of *Ulocladium* spp., the most common clinical presentations are subcutaneous infections in addition to keratitis and onychomycosis.

Preliminary diagnosis of phaeohyphomycoses including *Ulocladium* infections are through demonstration of brownish hyphal and/or yeast-like elements in tissue, often with melanin-specific Fontana-Masson stain. However, the dark pigmented elements of some dematiaceous fungi (e.g.,

Alternaria infectoria and *Ulocladium chartarum*) may not be visible histologically by Fontana-Masson staining [9,13]. This is clearly demonstrated by Badenoch et al. [9] in their dealing of *Ulocladium chartarum*, whose dematiaceous nature was only evident after examination of a cultured isolate.

Because of the close morphological and clinical similarities between *Ulocladium* and *Alternaria*, including the formation of brown multiseptate conidia and the induction of nonspecific cutaneous infections, *Ulocladium* needs to be distinguished from *Alternaria*. Morphologically, *Ulocladium* differs from *Alternaria* by having obovoid, coarsely verrucose conidia with tapered and narrow bases and with no or short, spindle-shaped apices (false beaks). Furthermore, *Ulocladium* conidia are in short chains or non-catenate. In addition, *Ulocladium* species should not be confused with other poroconidial genera such as *Stemphylium*, *Bipolaris*, *Exserohilum*, *Dreschlera*, and *Curvularia* [14,15].

Polymerase chain reaction (PCR) amplification and sequencing analysis of the rRNA internal transcribed spacer (ITS) regions allows clear separation of *Ulocladium* from *Alternaria* and other fungal genera [9,13,15–19].

20.2 METHODS

20.2.1 Sample Preparation

Clinical specimens are examined microscopically with fungal stains and also inoculated onto SDA for fungal culture [20]. Microscopic structures are observed on tease or tape preparations and slide cultures for up to 21 days.

Susceptibility of *Ulocladium* isolates to amphotericin B, flucytosine, fluconazole, itraconazole, ketoconazole, voriconazole is performed using the National Committee for Clinical Laboratory Standards M38-A method for molds. For *Ulocladium* susceptibility to natamycin and terbinafine, an agar disk method adapted from M38-A is carried out. Namely, Neo-Sensitabs tablets (Rosco Diagnostica, Taastrup, Denmark) containing either diffusible natamycin (50 µg) or terbinafine (30 µg) are incorporated into RPMI 1640 agar supplemented with glucose (0.2%) and buffered with MOPS (morpholinepropanesulfonic acid; 0.165 M). The inoculum is standardized between 0.4×10^4 and 5×10^4 CFU/mL using a spectrophotometer. The plates are incubated at 35°C and examined at 48 and 72 h. The zone sizes indicate the sensitivity of the fungus to natamycin and terbinafine [9,21].

For DNA extraction, the isolate is cultured on PDA at 30°C for 5 days. A suspension to a McFarland standard of 2.0 is prepared in saline (2 mL) and centrifuged. The pellet is resuspended in 200 µL of sorbitol buffer containing 200 U of lyticase (Sigma-Aldrich) and incubated at 37°C for 60 min and centrifuged (5400 × *g*; 5 min). Spheroplasts are resuspended in 180 µL of lysis solution T and 20 µL of proteinase K (GenElute Mammalian Genomic DNA Miniprep kit; Sigma-Aldrich) and then incubated at 55°C for 60 min. DNA is extracted according to the manufacturer's instructions with a final elution volume of 200 µL. Samples are stored at –20°C until use [9].

20.2.2 Detection Procedures

20.2.2.1 Standard PCR Amplification and Sequencing Analysis of ITS Region

Badenoch et al. [9] utilized universal fungal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) to amplify the ITS region of the rRNA gene complex, incorporating ITS1, the 5.8S gene, and ITS2 [22].

Procedure

1. PCR mixture (25 µL) is made up of 1× GeneAmp PCR buffer (Applied Biosystems), 5% glycerol, 125 µM each deoxynucleoside triphosphate, 0.5 µM each primer (ITS1 and ITS4), 1.25 U of *Taq* DNA polymerase (Applied Biosystems), and 10 µL of DNA.
2. Amplification is conducted with an initial denaturation at 94°C for 2 min; 30 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s; and a final extension of 72°C for 6 min.
3. The amplified product is purified using a GFX PCR DNA and gel band purification kit (Amersham Biosciences) and then sequenced using the ITS1 primer and a BigDye Terminator v. 3.1 cycle sequencing kit in an ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems).
4. The sequence is edited using Chromas v. 2.23 software (Technelysium Pty. Ltd.), and a 520 base sequence is compared with other sequences at GenBank (02/06) using FASTA.

20.2.2.2 Real-Time PCR Amplification and Sequencing Analysis of ITS Region

Pounder et al. [18] described a real-time PCR with SYBR green DNA binding dye and amplicon melting temperature analysis for fungal detection also using pan-fungal primers ITS1 forward (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′). The identity of the fungi is verified by subsequent sequencing analysis.

Procedure

1. PCR mixture is composed of 1× Lightcycler FastStart DNA Master Hybridization Probes mixture (Roche Applied Science) (containing deoxynucleoside triphosphates, FastStart *Taq* DNA polymerase, and 1 mM $MgCl_2$, additional $MgCl_2$ 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 76°C for 30 s; and a final extension at 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, 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%.

For strains producing discrepant identification between the methods based on phenotypic characteristics and ITS sequence analysis, the D1–D2 region of the large-subunit RNA gene is amplified with primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) and sequenced for species clarification.

20.3 CONCLUSION

The genus *Ulocladium* consists of a large number of saprotrophic, darkly pigmented hyphomycetes that occur as saprophytes in soil and decaying herbaceous plants. Several *Ulocladium* spp. (e.g., *Ulocladium chartarum*, *Ulocladium botrytis*, and *Ulocladium atrum*) are occasionally implicated in human disease process, causing subcutaneous phaeohyphomycosis, onychomycosis, keratitis, and other infections. Because *Ulocladium* spp. often appear in laboratory cultures for dermatophytes and are regarded as contaminants, confirmation of the causative role of *Ulocladium* species in human disease relies on the presence of the clinical appearance that are consistent with a fungal infection, the observation of hyphae in biopsy tissue, and the culture isolation of the organism with characteristic colonial and microscopic morphology. Application of molecular techniques especially sequence analysis of the ITS region of rRNA genes provides a rapid and precise means of identifying *Ulocladium* spp. from *Alternaria* and other fungal taxa that may potentially confuse the diagnosis based on phenotypic features.

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Pezizomycotina: Eurotiomycetes