50 *Cylindrocarpon*

Dongyou Liu

CONTENTS

50.1 INTRODUCTION

50.1.1 Classification and Morphology

The genus *Cylindrocarpon* (obsolete synonyms: *Allantospora*, *Coleomyces*, *Fusidium*, and *Moeszia*) belongs to the family Nectriaceae, order Hypocreales, class Sordariomycetes, subphylum Pezizomycotina, phylum Ascomycota, and kingdom Fungi. Depending upon the presence or absence of chlamydoconidia and microconidia, the 35 species in the genus *Cylindrocarpon* are arranged into four groups [1].

Cylindrocarpon spp. inhabit a wide range of woody and herbaceous plants and are commonly present in soil [2]. Several *Cylindrocarpon* spp. also infect humans and animals, resulting in keratitis, mycetoma, athlete's foot, peritonitis, and disseminated infection in human patients [3]. The human-infecting species include *Cylindrocarpon cyanescens* (obsolete synonym: *Phialophora cyanescens*), *Cylindrocarpon destructans*, *Cylindrocarpon lichenicola* (synonym: *Fusarium lichenicola*; obsolete synonym: *C. tonkinense*), and *Cylindrocarpon vaginae* [4–6].

Morphologically, all *Cylindrocarpon* species produce slimy macroconidia in basipetal succession (the youngest conidium at the base), which do not adhere in chains. At the species level, *Cylindrocarpon lichenicola* (synonym: *Fusarium lichenicola*) colonies grow rapidly on potato flakes agar (PFA), reaching a diameter of 35 mm in 6 days at 25°C. Colonies are velvety to floccose, initially white, then yellow, and finally pale brown at maturity. The reverse ranges from buff at the periphery to a darker brown centrally, with a brown-diffusing pigment. Hyphae are septate and hyaline, and conidiophores are long and simple or poorly branched. Subulate (slender and tapering to a point) conidiogenous cells (phialides) (of 38–50 μm × 3–5 μm) sometimes have a distinct collarette at the apices. Macroconidia (19.6–32 μm × 5 μm) are borne singly and in clusters at the apices of phialides, predominately three septate but occasionally up to five septate, hyaline, straight or curved, cylindrical to fusiform, rounded at apex, and distinctly truncate at the base with offset basal pedicels. Septations appear as rings around the macroconidia. Microconidia may be absent or one celled if present. Chlamydospores (chlamydoconidia) (of 8–15 μm in diameter) may form in old cultures, occurring singly, in chains or in clumps, intercalary, or terminal, and appear distinct hyaline to pale brown, smooth to spinulose, globose cells within the multicellular macroconidia. *C. lichenicola* grows poorly at 35°C and shows no growth at 42°C [7].

Cylindrocarpon lichenicola (*Fusarium lichenicola*) produces relatively long and narrow, several-celled, cylindrical conidia with typical rounded apices, which deviate only slightly from the macroconidia of other *Cylindrocarpon* species with straight conidia by having a conspicuously protuberant, symmetrical, truncate base. The type species of the genus *Cylindrocarpon*, *Cylindrocarpon cylindroides* (teleomorph *Neonectria neomacrospora*) is phylogenetically distinct from *C. lichenicola* (*F. lichenicola*) and other members of the genus *Fusarium*. The medically important and plant-pathogenic *Cylindrocarpon destructans* (teleomorph *Neonectria radicicola*) also clusters phylogenetically in a group of organisms well segregated from the *F. solani* and *Gibberella* clades [6]. *Cylindrocarpon* (*Phialophora*) *cyanescens* is characterized

by its phialidic conidia, chlamydospores in aggregations, and intense diffusing pigment [8].

Cylindrocarpon species resemble *Fusarium* species morphologically and taxonomically, with both sharing teleomorphs in the genus *Nectria*; and *Cylindrocarpon* differs from *Fusarium* by lacking an asymmetrical foot cell on the macroconidia [9]. Through sequencing analysis of the ribosomal large subunit (LSU), it was shown recently that despite their deviating conidial morphologies, *Cylindrocarpon lichenicola* and *Acremonium falciforme* constitute members of the *Fusarium solani* species complex (i.e., the *F. solani* clade containing *F. solani*, *Haematonectria*, and *Neocosmospora*) [10]. Thus, the original name *Fusarium lichenicola* is reestablished for *Cylindrocarpon lichenicola*, and the new combination *Fusarium falciforme* is proposed for *Acremonium falciforme* [6].

While *F. lichenicola*, *F. falciforme*, and members of the *F. solani* clade all produce long, slender, cylindrical conidiophores and integrated, terminal phialides, clearly reflecting their biological unity, other *Fusarium* groups tend to form shorter, discrete monophialides (phialides with only a single fertile opening) that are subulate (awl or candle shaped, i.e., rigid looking, tapered, and narrow at the apex) or inflated, or form polyphialides (with each phialide containing several fertile openings). In addition, *F. lichenicola*, *F. falciforme*, and *F. solani* have two- to several-celled conidia that are significantly blunter at the apex and proportionately broader than the macroconidia of fusaria in the *Gibberella* clade. However, *F. solani* conidia demonstrate a classical fusarial macroconidial form (curved phragmoconidia with somewhat pointed apices and basal foot cells), whereas *F. lichenicola* conidia do not; *F. falciforme* conidia may vary from strain to strain. Furthermore, despite showing varied colony colorations, *F. lichenicola*, *F. falciforme*, and *F. solani* colonies show similar chestnut red-brown to purplish reverse pigments. The violet-purple reverse color in *F. falciforme* overlaps with that in some *F. solani* isolates [6].

50.1.2 Clinical Features

50.1.2.1 *Cylindrocarpon lichenicola* (*Fusarium lichenicola*)

Resembling *Fusarium solani*, a common agent of keratomycoses and disseminated disease in neutropenic and/or immunocompromised hosts, *C. lichenicola* (*F. lichenicola*) has been shown to cause keratitis, localized cutaneous invasive disease, disseminated infection, and peritonitis in humans [3,11–16]. Patients from areas with warm climates may develop a distinctive fusarial intertrigo caused by *Fusarium solani*, *Cylindrocarpon lichenicola* (*Fusarium lichenicola*), or *Fusarium oxysporum* [6].

Iwen et al. [7] reported a case of trauma-related invasive *Cylindrocarpon lichenicola* infection localized to the right hand in a 53-year-old male patient with acute myelogenous leukemia (AML). Histological examination of skin biopsy showed septate, branching hyphae along with globular structures (8–11 μm in diameter) resembling conidia. As invasive molds rarely produce conidia in tissues and only in lesions exposed to ambient air, the presence of the fungus on the cutaneous surface and therefore exposure to ambient air in this case may have allowed the *Cylindrocarpon* organisms to produce conidial structures. Multiple positive fungal cultures from the biopsied tissue permitted subsequent microscopic and macroscopic identification of the fungus as *C. lichenicola*. The infection resolved following marrow regeneration, aggressive debridement of the affected tissue, and treatment with amphotericin B.

Kaliamurthy et al. [17] described a fungal keratitis due to *Cylindrocarpon* (*Fusarium*) *lichenicola* in a farmer, who presented with pain, redness, and irritation of the left eye following ocular injury caused by hay 5 days earlier. Slit-lamp examination of the affected eye showed a corneal ulcer (6×5 mm) with irregular margins, raised, with necrotic slough and infiltration. The lens was cataractous and visual acuity was restricted to "hand movements" only. Scrapings from the base and edges of the corneal ulcer contained septate fungal hyphae as examined by microscopy after staining with lactophenol cotton blue and Gram stain. Histopathological examination of the infected corneal button removed at therapeutic penetrating keratoplasty (TPK) also showed septate fungal hyphae. Scrapings and corneal button are inoculated onto Sabouraud glucose-neopeptone agar, and sheep blood agar grew cottony, white-red fungal colonies within 48 h, with brown pigmentation on reverse. The fungus was identified as *Cylindrocarpon lichenicola* since it displayed branched, septate, hyaline hyphae; phialides on simple or sparsely branched conidiophores; cylindrical to fusiform, smooth-walled macroconidia, each with three to six septa, a blunt rounded apex, and distinctly truncate base; and smooth-walled chlamydoconidia on short branches.

50.1.2.2 *Cylindrocarpon destructans*

Cylindrocarpon destructans is one of the fungal species (e.g., *Acremonium* [*Fusarium*] *falciforme*, *Acremonium kiliense*, *Acremonium recifei*, *Cylindrocarpon destructans*, *Fusarium moniliforme*, *Fusarium solani*, *Neotestudina rosatii*, *Polycytella hominis*, and *Pseudallescheria boydii*) that are known to cause white grain eumycetoma. Being a slowly progressing infection, eumycetoma develops in individuals usually after traumatic implantation of fungus-contaminated plant material. Zoutman and Sigler [18] documented a *Cylindrocarpon destructans*-related eumycetoma in a 39-year-old male, originally from Antigua, West Indies. The patient presented with a 12-year history of swelling of the left foot with pus and grains expressed from a draining sinus tract, from which *Cylindrocarpon destructans* was isolated in pure culture. The fungus was identified by its microconidial morphology, the presence of chlamydospores, and an intense brown diffusible pigment.

50.1.2.3 *Cylindrocarpon cyanescens* (*Phialophora cyanescens*)

Cylindrocarpon (*Phialophora*) *cyanescens* has also been shown to cause white grain eumycetoma [5,18].

Cylindrocarpon cyanescens produces cylindrical phialides with nonflaring collarettes. Hemashettar et al. [19] reported a case of white grain eumycetoma on the foot of a 57-year-old Indian male, who sustained a trauma by a thorn 10–12 years earlier and developed a pustule at the site after thorn removal. The pustule discharged serosanguineous fluid and eventually healed. Then, the patient was admitted to the hospital with slight swelling of the left foot, pain, and draining sinuses. Histologic examination of a biopsy tissue specimen revealed oval, lobular, white granules (0.5–1.0 mm in diameter) composed of hyaline, septate hyphae and thick-walled chlamydospores (up to 15 µm in diameter). Culture of granules from a draining sinus on Sabouraud dextrose agar (SDA) with chloramphenicol (SDA+C) at room temperature (25°C–30°C) after 12 days produced compact, very-slow-growing, and poorly sporulating colonies with a strong reddish brown pigment that diffused into the medium. Colonies on Sabouraud dextrose agar were raised in the center, downy, and white at first, becoming grayish buff after 2 weeks, measuring 6–7.5 mm in diameter at 25°C and 3.5–4.5 mm in diameter at 37°C. The isolate failed to grow at 40°C. Microscopic examination of the slide culture preparations on potato dextrose agar after 3 weeks of incubation at 25°C showed sterile, septate, hyaline hyphae (2.5–5.0 µm wide), producing chlamydospores but devoid of conidia. Conidiation occurred sparsely and best on Takashio and oatmeal salts agars after prolonged incubation (3–14 weeks at room temperature) and consisted of zero- to one-septate, cylindrical, sometimes slightly curved conidia produced from unbranched septate phialides without collarettes. Single-celled conidia measured 9–14 µm long and 2–2.5 µm wide, and two-celled conidia measured 17–25 µm long × 2–3 µm wide. Chlamydospores were mostly solitary, intercalary, or terminal and were subglobose and smooth to slightly roughened. The fungus was identified as a *Cylindrocarpon* sp. on the basis of the development of rare cylindrical conidia borne from solitary phialides lacking collarettes, in addition to chlamydospores formed singly or in short chains. It appeared highly similar to *C. cyanescens* in its strong production of reddish brown diffusible pigment and restricted growth. While *C. cyanescens* did not invade bone, *C. destructans* caused lytic bone destruction of the tarsals and the base of the third metatarsal.

50.1.3 Diagnosis

Like their *Fusarium* relatives, *Cylindrocarpon* species are anamorphs of ascomycetes belonging to the Hypocreaceae (teleomorph *Nectria*) and are cosmopolitan soil- and plant-associated fungi. Rapid and accurate determination of *Cylindrocarpon* species is vital for implementing appropriate treatment and control strategies for these organisms [20].

Both *Cylindrocarpon* (*Fusarium*) *lichenicola* and members of the *F. solani* clade produce elongate, filiform, often several-celled conidiophores incorporating terminal monophialides (up to 40 µm in length) with a relatively broad, blunt apex. The elongate conidiophores in *F. solani* isolates are mainly associated with microconidia (which are often absent in *C. lichenicola*), and its macroconidia-forming conidiophores are somewhat shorter. On the other hand, the macroconidia-forming conidiophores in *C. lichenicola* are typically elongate. The conidiophores in *A. falciforme* may be similar in shape to the conidiophores of other members in the *F. solani* clade but are relatively strongly septate [6].

Cylindrocarpon (*Fusarium*) *lichenicola* is further differentiated macroscopically from *F. solani* by forming macroconidia, which are predominately straight rather than curved, by having apical cells that are rounded rather than tapering, by having basal cells with truncate and offset rather than attenuated pedicels (foot cells), by lacking microconidia, by having pigmented chlamydoconidia, and by having a brown color rather than cream color on reverse of SDA medium [6].

Granules produced by *Cylindrocarpon* species cannot be distinguished from each other, nor from those of *Acremonium* spp. and *Fusarium verticillioides* (*F. moniliforme*) without cultural or immunofluorescence studies. However, granules of *Cylindrocarpon* species may be differentiated from those of *P. boydii* by having less prominent, fewer, and smaller chlamydospores.

Molecular methods have offered an alternative means to identify fungal organisms including *Cylindrocarpon* species. Amplification and sequencing analysis of the internal transcribed spacer (ITS) ITS1-5.8S-ITS2 region allows differentiation of *Cylindrocarpon* species from other fungi [21–26].

50.2 METHODS

50.2.1 Sample Preparation

Clinical samples are cultured on Sabouraud dextrose agar or other mycological media. Portions of samples are examined under microscope for mycotic elements using fungal stains. Colonies are grown on synthetic nutrient agar, oatmeal agar, and malt extract agar for 7–10 days at room temperature (22°C). To induce conidiation, isolate is subcultured onto PFA plates and PFA slide cultures, both incubated at 25°C. The microscopic morphology is examined from colonies on PFA plates and slide culture mounts after 6 days of incubation. Temperature studies are performed on PFA slants to evaluate the growth rate at 25°C, 35°C, and 42°C [7].

For fungal DNA extraction, 50 mL of Sabouraud dextrose (SAB) broth is inoculated by needle with conidia from a 7 day culture in SAB agar and incubated for 72 h at 30°C. The hyphae are recovered on a 0.45 µm-pore-size filter and washed with sterile saline. Aliquots of the fungal hyphae are stored frozen at −70°C until use. Prior to lysis, the hyphae are thawed and suspended in 400 µL of DNA extraction buffer (1 mM EDTA pH 8.0, 1% sodium dodecyl sulfate, 10 mM Tris–HCl pH 7.6, 100 mM NaCl, and 2% Triton X-100). Microcentrifuge tubes (1.5 mL) containing hyphae and buffer are sonicated in a water bath (Branson; model 2210) for 15 min, followed by heating at 100°C for 5 min. Following lysis, DNA is purified using the QIAmp blood kit (Qiagen) and protocols for crude cell lysates supplied by the manufacturer. The purified DNA is stored at 4°C until tested [6].

Alternatively, DNA is extracted with a FastDNA kit (Qbiogene) from mycelium grown for 3–5 days in liquid complete medium.

50.2.2 Detection Procedures

50.2.2.1 Sequencing Analysis of ITS Regions

Iwen et al. [7] described the use of primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT G-3′) from the conserved regions of the 18S (ITS1) and the 28S (ITS4) ribosomal RNA (rRNA) genes, respectively, to amplify the intervening 5.8S rRNA gene, ITS1 region, and ITS2 noncoding regions [27]. Subsequent sequencing analysis of the resulting amplicon enables identification of *Cylindrocarpon* species.

Procedure

1. PCR mixture (50 μL) is made up of 5 μL sample DNA, 20 mM Tris–HCl pH 8.4, and 50 mM KCl; 0.1 mM (each) dNTP, 1.5 mM $MgCl_2$, and 0.3 μM (each) primer; and 1.5 U of Platinum *Taq* high-fidelity DNA polymerase (Gibco BRL, Life Technologies).
2. Amplification is conducted in a Stratagene Robocycler model 96 thermocycler with initial denaturation at 95°C for 4.5 min; 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 3 min.
3. Amplicons are separated by agarose gel electrophoresis, purified, and ligated into the PCR 2.1 plasmid vector (Invitrogen). Competent INVαF′ One Shot cells are transformed using standard protocols. Colonies are isolated and purified with a Qiagen miniprep spin kit. An aliquot of purified plasmid is digested with *Eco*RI endonuclease (New England Biolabs) and screened by agarose gel electrophoresis for a 300 bp doublet, indicating the presence of the *Eco*RI cleavage site GAATTC within the 5.8S sequence.
4. Selected plasmids are analyzed by automated dye termination sequencing procedure on a Perkin-Elmer/ABI model 373 DNA sequencer. For the sequencing of cloned fragments, both strands of the plasmid containing the fungal insert are sequenced with universal M13 forward and reverse sequencing primers. For direct sequencing of noncloned amplicons, PCR products are directly sequenced using the ITS1 and ITS4 PCR primers.
5. The resultant nucleotide sequences are aligned with the MacVector sequence analysis software, version 6.5 (Oxford Molecular Group, Inc., Campbell, CA), and the Clustal W alignment algorithm. Intraspecies sequence similarity and variation are determined by the MacVector software and are visually confirmed using pairwise nucleotide alignments. Sequences from referenced isolates are aligned to complete or partial ITS sequences available in GenBank. Comparison of sequences from referenced isolates, clinical isolates, and GenBank sequences is performed using a nongapped, advanced BLAST search. The similarities of the sequences are determined with the expectation frequency minimized to 0.0001 [28].

50.2.2.2 Sequencing Analysis of rRNA LSU

Summerbell and Schroers [6] employed primers V9G and LR5 to amplify the LSU region (encompassing domain D1 and most of domain D2, nucleotides 116–615) of rRNA followed by sequencing for identification and phylogenetic analysis of *Cylindrocarpon* and *Fusarium* species.

The PCR cycling program consists of an initial denaturation at 94°C for 1 min; 35 cycles of 94°C for 35 s, 55°C for 50 s, and 72°C for 3 min; and a final elongation at 72°C for 6 min. (final elongation). The PCR products are purified with a GFX purification kit (Amersham Pharmacia Biotech) and visualized on an electrophoresis gel after ethidium bromide staining. The rRNA is sequenced with a BigDye terminator cycle sequencing kit (Applied Biosystems) and analyzed on an ABI Prism 3700 instrument (Applied Biosystems). The primers used in the sequence reaction are ITS1 and ITS4, NL1 and NL4, and LR5.

Sequence chromatographs are assembled and edited with SeqmanII software (DNAStar) and aligned with sequences downloaded from GenBank. The alignment is initially performed with the ClustalX program (version 1.8; ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX) and adjusted manually with the Megalign program (DNAStar). The phylogenetic analysis is performed with a part of the LSU rRNA available for all accessions. A parsimony analysis is performed with the software package PAUP (version 4.0b8). Gaps are coded as missing data; characters are defined as unordered and equally weighted; characters not informative to the parsimony analysis are excluded; heuristic searches of parsimonious trees are performed for all sequences with random sequence addition and 1000 replicates, using the starting trees from the stepwise addition, tree bisection–reconnection as the swapping algorithm, and all optimal trees for the next swapping round; and branch robustness is tested by the use of 100 replications of such searches based on bootstrapped data sets with random sequence addition and 10 replicates per search.

50.3 CONCLUSION

Cylindrocarpon spp. are common plant pathogens that are widely distributed in the environment. Some *Cylindrocarpon* spp. may infect humans and animals occasionally, resulting in keratitis, mycetoma, athlete's foot, peritonitis, and disseminated infection. Among the human-infecting *Cylindrocarpon* species, *Cylindrocarpon* (*Fusarium*) *lichenicola* is the only one known to induce invasive diseases besides localized infections, whereas *Cylindrocarpon cyanescens* (obsolete synonym: *Phialophora cyanescens*) and *Cylindrocarpon destructans* are mainly responsible

for white grain eumycetoma. As conventional laboratory identification of *Cylindrocarpon* species is largely based on macroscopic and microscopic characteristics of these organisms, it is not only time-consuming but also technically challenging. Application of molecular techniques such as PCR and sequencing analysis of rRNA genes and ITS regions offers a rapid, precise, and sensitive tool for *Cylindrocarpon* determination.

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