25 *Chrysosporium*

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CONTENTS

25.1 INTRODUCTION

25.1.1 Classification and Morphology

The genus *Chrysosporium* (obsolete synonym: *Glenosporella*) belongs to the mitosporic Onygenales group, order Onygenales, class Eurotiomycetes, subphylum Pezizomycotina, phylum Ascomycota, and kingdom Fungi. The mitosporic Onygenales group consists of nine genera: *Blastomyces*, *Chrysosporium*, *Coccidioides*, *Emmonsia*, *Geomyces*, *Locazia*, *Malbranchea*, *Myriodontium*, and *Paracoccidioides*. In turn, the genus *Chrysosporium* is separated into 28 recognized species: *Chrysosporium articulatum*, *Chrysosporium carmichaelii*, *Chrysosporium chiropterorum*, *Chrysosporium europa*, *Chrysosporium evolceanui*, *Chrysosporium filiforme*, *Chrysosporium fluviale*, *Chrysosporium fluviale*, *Chrysosporium indicum*, *Chrysosporium keratinophilum*, *Chrysosporium lobatum*, *Chrysosporium lucknowense*, *Chrysosporium mephiticum*, *Chrysosporium merdarium*, *Chrysosporium minutisporosum*, *Chrysosporium ophiodiicola*, *Chrysosporium pannicola*, *Chrysosporium pilosum*, *Chrysosporium pseudomerdarium*, *Chrysosporium queenslandicum*, *Chrysosporium siglerae*, *Chrysosporium submersum*, *Chrysosporium sulfureum*, *Chrysosporium synchronum*, *Chrysosporium tropicum*, *Chrysosporium undulatum*, *Chrysosporium vallenarense*, *Chrysosporium xerophilum*, and *Chrysosporium zonatum*, and 18 unassigned species [1–3]. The teleomorphs of *Chrysosporium* spp. are found in the genera *Aphanoascus*, *Nannizziopsis*, and *Uncinocarpus*, family Onygenaceae, and order Onygenales [4,5].

The obsolete species in the genus include *Chrysosporium dermatitidis* (→ *Blastomyces dermatitidis*), *Chrysosporium pannorum* (→ *Geomyces pannorus*), *Chrysosporium pruinosum* (→ *Sporotrichum pruinosum*), and *Chrysosporium thermophilum* (→ *Myceliophthora thermophila*) [6]. Not discussed here are *Chrysosporium parvum* (or *Chrysosporium parvum* var. *parvum*) and *Chrysosporium parvum* var. *crescens*, which are considered as synonymous for *Emmonsia parva* and *Emmonsia crescens*, respectively. To simplify the presentation and reduce confusion, this chapter focuses on *Chrysosporium* spp. that are keratinophilic, causing subcutaneous infections, whereas Chapter 29 deals with *Emmonsia* spp. that mainly cause pulmonary adiaspiromycosis.

Chrysosporium colonies grow moderately at 25°C, and may appear granular, woolly, or cottony, flat, or raised, and folded. Colonies are white cream, yellow, or tan to pale brown on the front, and white to brown on the reverse. Hyphae are septate. Conidia (aleuriconidia) are hyaline, broad based, one celled, and smooth or rough walled. Conidia are broader than vegetative hyphae and occur terminally on pedicels, along the sides of the hyphae, or in intercalary positions. Conidia usually have an annular frill that is the remnant of the hyphal wall that remains after detachment from the hypha. Arthroconidia are abundant and larger than parent hyphae in diameter.

C. tropicum colonies are moderately fast growing, flat, white to tan to beige, often with a powdery or granular surface texture. Reverse pigment is absent or pale brownish yellow with age. Ameroconidia are hyaline, one celled, and produced directly on vegetative hyphae by nonspecialized conidiogenous cells. Conidia (6–7 μm × 3.5–4 μm) are typically pyriform to clavate with truncate bases and are formed either intercalary (arthroconidia), laterally (often on pedicels), or terminally.

Chrysosporium zonatum (synonym: *C. gourii*; teleomorph: *Uncinocarpus orissi*) colonies on potato dextrose agar (PDA) at 37°C are flat and coarsely powdery, and appear yellowish white initially but darken by 14–21 days to buff

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(grayish or brownish orange) with a light brown reverse. Colony topography and color on PDA are similar at 37°C and 25°C, but darkening of the colony obverse and growth rate are slightly faster at 37°C (73 mm in diameter after 14 days) than at 25°C (66 mm in diameter). Microscopically, the fungus forms solitary aleurioconidia that are borne at the ends of short, typically curved stalks or that are sessile (borne on the sides of the hyphae). Conidia (3.5–13 µm × 2.5–5 µm) are single celled, rarely two celled, smooth to slightly roughened, and clavate (club shaped) to broadly obovoid (egg shaped) and have a rounded tip and a broad, flat basal scar. Intercalary arthroconidia may be formed but are uncommon. Racquet hyphae (hyphae showing swellings near the septa) are common [4]. The teleomorph of *Chrysosporium zonatum* is a heterothallic ascomycete *Uncinocarpus orissi* (synonyms, *Pseudoarachniotus orissi* and *Gymnoascus arxii*) (family Onygenaceae). *U. orissi* ascocarps are solitary, globose, and reddish brown and are composed of pale reddish brown ascospores surrounded by thin-walled hyaline racket hyphae and conidia. Pairing of *C. zonatum* with *U. orissi* produces ascoma-containing ascospores that are oblate (like flattened disks) with truncate ends and appear smooth to slightly pitted (punctate) [4].

Chrysosporium ophiodiicola colonies attain diameters of 27–29 mm in 14 days at 25°C on potato carrot agar (PCA; 20 g potato, 20 g carrot, 15 g agar, 1 L water). Colonies are white from front and uncolored on reverse, felty, plane, and fimbriate, with a poorly defined margin. Sparse tufts of aerial mycelium are present on the submarginal zone. Vegetative hyphae (1.5–2.5 µm wide) are hyaline, branched, septate, smooth, and thin walled. They are often disarticulated at maturity to form cylindrical arthroconidia (7.5–10 µm × 2–3 µm) adjacent to each other. Fertile hyphae arise as lateral branches. Terminal and lateral conidia are borne on straight or flexuous side branches of variable length (4.5–16 µm) or are sometimes sessile. Conidia (4–9 µm × 2–3 µm) are unicellular, solitary, thin walled, smooth, hyaline to pale yellow, and cylindrical to slightly clavate and are released by rhexolytic dehiscence, with broad and long basal scars. Intercalary, solitary conidia are often present, similar to the terminal and lateral ones. Racquet hyphae are scarce, and chlamydospores are not observed. On potato flake agar at 23°C, colonies are white to pale yellow, with a similarly colored reverse side, are velvety to granular with age, and produce a strong, pungent odor. Conidia are borne on stalks as well as arthroconidia. On PDA (Difco Laboratories), *C. ophiodiicola* grows more quickly and produces denser colonies of 31–35 mm in diameter in 14 days at 25°C. Colonies are white to pale yellow, buff after 1 month, and powdery, with droplets of colorless or light yellow exudates at the periphery. On phytone-yeast extract agar (BBL), colonies measure 32–39 mm in diameter in 14 days at 25°C and are white and light yellow at the center, powdery, and dense, with the presence of droplets of colorless exudate at the center and a light brown on the reverse side. On oatmeal agar (30 g oat flakes, 1 g $MgSO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, 15 g agar, 1 L water), colonies are similar to those on PCA, with a very restricted growth at 15°C (5 mm in diameter in 14 days). At 37°C, there is no growth. Colonies produce a strong, pungent (skunk-like) odor after 1 month of incubation in all the media tested. The fungus shows a strong keratinolytic activity. Phenotypically, *C. ophiodiicola* is separated from the *Chrysosporium* anamorph of *Nannizziopsis vriesii* by the absence of asperulate fertile hyphae and globose-to-pyriform conidia sometimes grouped in clusters and the presence of an odor in the colonies by *C. ophiodiicola* [3].

Chrysosporium species are differentiated from each other by the texture of the colony and morphology, location, and size of the conidia. *Chrysosporium zonatum* differs from other members of the genus *Chrysosporium* by its faster growth at 37°C than at 25°C and by forming darken to buff colonies, and clavate, broadly truncate aleurioconidia typically borne on short, curved stalks. *Chrysosporium queenslandicum* (teleomorph: *Uncinocarpus queenslandicus*, or *Apinisia queenslandica* and *Brunneospora reticulata*) is similar, but its colonies do not darken, and intercalary arthroconidia are common [4].

C. ophiodiicola is separated from *C. mephiticum* by the narrow, cylindrical-to-slightly clavate conidia of *C. ophiodiicola*, and the pyriform-to-subglobose conidia of *C. mephiticum*; *C. ophiodiicola* is differentiated from *A. mephitalis* by the production of teleomorph during the culture of *A. mephitalis*; and *C. ophiodiicola* is distinguished from *Chrysosporium europae* by the characteristic vinaceous, buff-pigmented colonies on phytone-yeast extract agar and the absence of a strong, pungent odor of/from *C. europae*. Some species such as *Chrysosporium pannicola* do not grow at 37°C. *Chrysosporium* differs from *Blastomyces* by being nondimorphic, from *Microsporum* and *Trichophyton* by lacking macroconidia, from *Geomyces* by lacking branched, fertile hyphae on erect conidiophores, and from *Sepedonium* by having hyaline conidia.

25.1.2 Clinical Features

Chrysosporium spp. are soil saprophytes with broad distribution. They have been isolated in soil, plant material, dung, and birds [6–8]. These organisms may enter hosts through airborne conidia and exposure to soil. Many *Chrysosporium* spp. are keratinophilic filamentous fungi involved in the breakdown of shed keratinous substrates, and may cause skin infections and onychomycosis in humans [9–12]. In addition, *Chrysosporium* species have been occasionally associated with disseminated human mycosis, affecting the brain, lungs, sinuses, liver, and kidneys and leading to sinusitis, pneumonia, pleuritis, pericarditis, and osteomyelitis [13–15].

Roilides et al. [4] reported the first case of *Chrysosporium zonatum* infection in a 15-year-old boy with X-linked chronic granulomatous disease. The patient developed a lobar pneumonia and tibia osteomyelitis after a 2 month prophylactic therapy with γ-interferon. The patient

presented with pain in his right shoulder and distal part of his right tibia, infrequent cough, and fever. A computed tomography (CT) scan of the chest showed a well-demarcated large mass in the right lower lobe, enlarged left hilar lymph nodes, and lingular pneumonitis. A tibia x ray was diagnostic for osteomyelitis. A biopsy of the tibia lesion revealed granulomatous tissue and a few short and thick hyphae. *Chrysosporium zonatum* was grown from sputum and biopsy specimens, which was identified by its thermotolerance, darkening colonies (yellowish white to buff) and club-shaped terminal aleurioconidia borne at the ends of short, curved stalks. Therapy with amphotericin B, itraconazole, and then liposomal amphotericin B subdued the osteomyelitis, pneumonia, pericarditis, and pleuritis.

Guerrero Palma et al. [15] described a case of invasive sinusal mycosis due to *Chrysosporium tropicum* in a patient with acquired immunodeficiency. Levy et al. [16] also reported a case of aggressive fungal rhinosinusitis caused by *Chrysosporium* sp. in a patient with acute lymphocytic leukemia. Histopathological and microbiological studies permitted the identification of the culprit organism. In addition, Warwick et al. [17] presented an invasive *Chrysosporium* infection in an 18-year-old patient after allogeneic sibling bone marrow transplant for T lineage acute lymphoblastic leukemia. The infection started as a facial swelling and extended into the central nervous system. Despite antifungal treatment, the disease spread rapidly, and an autopsy revealed fungal invasion of brain, lungs, liver, and kidneys.

25.1.3 Diagnosis

Conventional techniques for the identification of *Chrysosporium* spp. and other fungi rely on microscopic observation of mycotic elements in clinical specimens followed by in vitro culture. *Chrysosporium* colonial morphology and growth rate are assessed on PDA at 25°C and 37°C, and tolerance to cycloheximide is tested by measuring the growth rate at 25°C on Mycosel agar (Becton Dickinson). Microscopic features of *Chrysosporium* isolates are examined in slide culture preparations with pablum cereal agar (pablum precooked mixed cereal 10%, agar 1.5%). The ability of *Chrysosporium* isolates to digest hairs in the hair perforation assay is diagnostic.

Molecular methods for the determination of *Chrysosporium* identity include sequencing analysis of small subunit (SSU) and large subunit (LSU) of ribosomal RNA genes as well as internal transcribed spacer (ITS) regions [3,18,19]. PCR amplification of ITS2 using primers ITS86 (5′-GTGAATCATCGAATCTTTGAAC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), restriction digestion with *Bst*UI (CG/CG), and restriction fragment length polymorphism (RFLP) assessment by capillary electrophoresis permit discrimination of *Chrysosporium* from dermatophyte fungi such as *Arthroderma*, *Epidermophyton*, *Microsporum*, and *Trichophyton* [20].

25.2 METHODS

25.2.1 Sample Preparation

Chrysosporium and other fungal isolates are revived from either freeze-dried or frozen (vapor phase of liquid nitrogen) stock and grown at 25°C on petri plates containing pablum cereal agar for 14–21 days. Blocks (1 × 1 cm) of mycelium and agar from cultures of *Chrysosporium* species are excised from the culture plates and transferred to sterile snap-cap polypropylene tubes (12 mm × 75 mm; Fisher Scientific). The mycelial blocks are freeze-dried by using an Edwards Moduylo freeze-dryer. Freeze-dried blocks of agar and *Chrysosporium* mycelium (100 mg) are placed in 1.5 mL tubes and ground to a fine powder by using a 200-µL capacity pipettor tip. The fungal material is rehydrated with 500 µL of DNA extraction buffer (50 mM Tris, 10 mM EDTA, 1% sarcosyl, pH 8.0) with gentle agitation for 10 min. An equal volume of 1:1 chloroform–phenol is added to each tube, and mixed by shaking for 20 min. Then the aqueous and organic phases are separated in a microcentrifuge for 5 min at 14,000 × *g*. The aqueous phase is pipetted into a clean tube, and 0.1 volume of 3 M sodium acetate (pH 6.0) and 1.3 volumes of ethanol are added. The tube is sealed, and the contents are mixed by inverting the tube several times. Precipitated nucleic acids are pelleted by centrifugation at 14,000 × *g* for 1 min. Ethanol is decanted, and the pellet is dried by inverting the tube over absorbent paper for 5 min. Nucleic acids are dissolved in 100 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and 250 µL of a saturated NaI solution and 10 µL of glass milk (Gene Clean kit, Bio 101) are added to the tube. The tube is inverted periodically, and DNA is adsorbed to the glass milk for 20 min. The glass milk is pelleted and rinsed, and the genomic DNA is eluted into 50 µL of 1/10-strength TE. DNA is stored at –20°C until used [18].

Alternatively, a loopful of *Chrysosporium* mycelium is transferred from SDA to a 1.5 mL tube containing 2.5 mg zymolase (Sigma–Aldrich) in 250 µL zymolyase lysis buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA] and incubate for 45 min at 37°C. Subsequently, 200 µL is transferred to another tube for DNA extraction using Qiagen kit, resulting in 100 µL of DNA extract [20].

25.2.2 Detection Procedures

Pounder et al. [19] described 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′). 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 [3].

25.3 CONCLUSION

The genus *Chrysosporium* contains a large number of filamentous saprophytic fungi classified in the mitosporic Onygenales group, order Onygenales. The teleomorphs of the *Chrysosporium* genus are found in the genera *Aphanoascus*, *Nannizziopsis*, and *Uncinocarpus*, family Onygenaceae, and order Onygenales. Many members of the genus *Chrysosporium* are thermotolerant and keratinophilic, and occasionally implicated in human diseases, producing clinical symptoms ranging from skin infections, onychomycosis, sinusitis, pneumonia, pleuritis, pericarditis, osteomyelitis, to other disseminate infections.

Microscopic observation of mycotic elements in clinical specimens and in vitro isolation of related fungal strains have been the main approaches for identification and diagnosis of *Chrysosporium* infections. More recently, molecular techniques such as PCR, sequencing, and RFLP analysis have been developed and applied for improved determination of *Chrysosporium* and other fungal organisms. Through examination of nucleotide sequences in the SSU and LSU of ribosomal RNA genes as well as ITS regions, the identity of *Chrysosporium* spp. can be rapidly and unequivocally ascertained.

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AUTHOR QUERIES

[AQ1] Please check if "is separated into" can be changed to "consist of" in the sentence beginning "In turn, the genus…"
[AQ2] Please check the insertion of spelled out form of PDA, that is, potato dextrose agar.
[AQ3] Please check that intergenic spacer has been replaced by internal transcribed spacer.
[AQ4] Please provide accessed date for Ref. [1].