Characterization of novel *Chrysosporium morrisgordonii* sp. nov., from bat white-nose syndrome (WNS) affected mines, northeastern United States

Tao Zhang[1, 2], Ping Ren[1, 4], XiaoJiang Li[1], Sudha Chaturvedi[1, 3*], and Vishnu Chaturvedi[1, 3*]

[1]Mycology Laboratory, Wadsworth Center, New York State Department of Health, Albany, New York, USA

[2] Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, PR China

[3] Department of Biomedical Sciences, School of Public Health, University at Albany, Albany, New York, USA

[4]Department of Pathology, University of Texas Medical Branch, Galveston, Texas, USA

[*]Corresponding authors:

Sudha Chaturvedi, sudha.chaturvedi@health.ny.gov;

Vishnu Chaturvedi, vishnu.chaturvedi@health.ny.gov.

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Abstract

A new fungal species, *Chrysosporium morrisgordonii sp.* nov., is described from bat's white-nose syndrome afflicted Graphite mine in Warren county, New York. This taxon is characterized by its ability to grow at low temperature spectra from 6°C to 25°C and by the presence of tuberculate and thick-walled arthroconidia and aleurioconidia, spherical or broadly ellipsoidal, unicellular, intercalary, 3.5-4.6 μm ×3.5-4.6 μm, sessile or borne on side branches or at the ends of narrow stalks. The phylogenetic tree inferred from the analysis of the sequences of the large subunit D1/D2 and ITS1-5.8S-ITS2 regions confirm the separation of this new species from other taxa of the genus *Chrysosporium*. *Chrysosporium morrisgordonii* is distinguished by morphology and physiology characteristics from the closest phylogenetic species or taxa. It is described here as a new species in *Chrysosporium* genus.

Introduction

During a survey of mycobiome from bat's White-nose syndrome affected caves and mines in northeastern region of United States [1], we isolated one interesting psychorotolerant hyphomycete which belong to the genus *Chrysosporium*. This isolate was recovered from Graphite mine in Warren County, New York, USA.

The genus *Chrysosporium*, belonging to the subphylum Pezizomycotina, comprises a large collection of cosmopolitan saprotrophs, which are commonly recovered from soil, marine sediments, decaying wood, skin or feet of reptiles [2-5]. The genus *Chrysosporium* was first introduced by Corda in 1833. Currently, a total of seventy species have been reported[3]. *Chrysosporium* species are often keratinolytic, although occasionally cause skin infections and onychomycosis in humans and animals [2, 6-10]. The resemblance of *Chrysosporium* to dermatophytes, and their pathogenic potential, displaying similarities to *Emmonsia*, *Botryotrichum*, *Trichosporiella*, *Myceliophthora*, *Sepedonium*, *Ovadendron*, *Zymonema*, *Sporotrichum* and associated teleomorhps, is of immense diagnostic importance [11-15]. The genus is readily identified by mostly white to yellowish colonies, tree-like conidiophores structure, and terminal or intercalary conidia [16]. The conidia are usually subglobose, pyriform, or clavate and are released rhexolytically [2, 16].

In this study, based on the mentioned isolate, we propose a new species of *Chrysosporium*. A comparative study employing phenotypic, microscopic, physiologic, molecular of this taxa and other members of *Gymnoascaceae* provide further support for this proposal.

Materials and methods

Sample origin and isolation of fungus

Site description and sample collection were described previously [1]. Environmental samples including sediment or swab sample were performed according to the method described previously [1]. Culture and purification process were followed the studies performed previously [1].

Fungal strains and media preparation

A total of five strains from different reference culture collections were included in the study. Among them, three including *Neogymnomyces demonbreunii* (Ajello et Cheng) CBS 122.67 (ATCC®18028™), *Renispora flavissima* (Sigler *et al*.,) CBS 708.79 (ATCC®38504™), and *Amauroascus volatilis-patellus* (Eidam) Schroeter CBS 249.72 (ATCC®18898™) were provided by the Centraalbureau voor Schimmelcultures (CBS) Utrecht, Netherlands. *Chrysosporium speluncarum* CCF 3760 and *Neogymnomyces virgineus* JN038186 were generous gifts from Prof. Alena Novakova (Institute of Microbiology, Czech Republic) and Prof. Sabrina Sarrocco (Università di Pisa, Italy), respectively. The strains were cultured on potato dextrose agar (PDA),

sabouraud dextrose agar (SDA; peptone 10 g, dextrose 40 g, agar 20 g L^{-1}), and malt extract agar (MEA; malt extract 20 g, peptone 1.0 g, dextrose 20 g, agar 20 g L^{-1}), respectively. The basal medium (1 L volume) constituents included $(NH_4)_2SO_4$ 3.0 g, K_2HPO_4 1.0 g, KH_2PO_4 1.0 g, $MgSO_4.7H_2O$ 0.5 g, 10 mL trace elements solution [17]. All strains were generally incubated at 20°C or 30°C for 14 days.

Morphological identification

The growth rate of the isolate was determined at 6°C, 10°C, 15°C, 20°C, 25°C, 30°C on SDA and MEA. The petri dishes were inoculated in the center and the colony diameters (in millimeters) were measured daily. The strains were identified using the traditional methods based on morphological characteristics [2]. The morphological structures were measured in warmed lactophenol mounts. Photomicrographs were obtained with an Olympus Provis AX70 microscope. Scanning electron microscopy techniques were described previously by Figueras and Guarro [18]. The pure culture is deposited in the Mycology Laboratory Culture Collection Repository, Wadsworth Center, New York State Department of Health, Albany, New York, USA.

DNA extraction and PCR amplification

Genomic DNA from fungal mycelium grown on SDA plates were extracted according to the manufacturer's instructions (Epicentre, Madison, WI, USA, Cat. No. MC85200). Complete ITS1-5.8S-ITS2 (ITS) and partial 28S nrDNA

were amplified using universal primers. ITS1 and ITS4 [19] were used to amplify ITS, and NL1 and NL4 [20] were used for partial 28S (large subunit, LSU) amplification. Each PCR reaction mixture (30 μL) contained 3.0 μL 10×PCR buffer, 0.9 μL sense primer (10 μM), 0.9 μL antisense primer (10 μM), 1.0 μL dNTP (10 mM each), 2.0 μL DNA template, 0.3 μL Q5® High-Fidelity DNA Polymerase (2.0 U/ μL) (New England Biolabs, Inc.) and 21.9 μL sterile water. The PCR reactions were performed in an C1000 Touch™ Thermal Cycler (Bio-Rad) with cycling conditions of an initial activation at 98°C for 30 sec, followed by 28 cycles of denaturation at 98°C for 10 sec, annealing at 55°C (ITS) and 58°C (28S) for 30 s, and elongation at 72°C for 25 sec, with a final extension step at 72°C for 2 min. PCR products were purified with the Qiaquick® PCR Purification Kit (Qiagen) and sequenced with the ABI BigDye terminator chemistry in an ABI 3730 DNA sequencer (Applied Genomic Technologies Core, Wadsworth Center). The same primers used in PCR were employed as sequencing primers. The PCR amplicons were assembled, edited using Sequencher 4.6 software software (Gene Codes Corp., Ann Arbor, MI, USA) and BLAST searched against two databases, *i.e.* GenBank (www.ncbi.nlm.nih.gov/) and CBS-KNAW (www.cbs.knaw.nl/).

Phylogenetic analysis

All sequences were aligned with Clustal X version 1.8 [21] and BioEdit v7.0.9 [22], and the multiple alignments were adjusted manually following visual inspection and the areas of sequence ambiguity were eliminated when

necessary. Databases of ITS and 28S were combined for sequence analyses. Phylogenetic analyses using the maximum parsimony analysis (MP) was performed with the MEGA 5.1 computer program [23]. The robustness of branches was assessed by bootstrap analysis with 1,000 replicates. The Dictionary of the Fungi and UniProt (http://www.uniprot.org/taxonomy/) served as the source of taxonomic references for fungal species [24].

Physiological study

Growth on dermatophyte test medium agar (DTM) and color changes from yellow (acidic) to red (basic) were recorded. Lipase activity was tested by growing on Tween 80 opacity test medium (TOTM) according to Slifkin [25]. Production of urease was determined on Christensen's urea agar after incubation for 7 d [26]. Amylase, chitinase, proteinase, cellulase, and β-glucosidase activity was verified by growing strains on induction media including basal medium with 0.5% starch, 0.5% chitin, 0.5% casein, 0.5% CM-cellulose, and 0.5% D-(+)-cellobiose, respectively. Cycloheximide tolerance was evaluated by growing strains on SDA supplemented with 0.2% and 0.5% of cycloheximide (Sigma, USA). Tolerance of NaCl was evaluated by growth of strains on SDA, amended with 3% and 5% NaCl. Hemolysis was evaluated by culturing strains on blood agar. All detections were performed at 20°C for 14 d. Temperature sensitivity tests are incubated at 6°C and 30°C, respectively.

Nucleotide Sequence Accession Numbers

All nucleotide sequences of the case isolate were deposited in GenBank under accession numbers: ITS sequence (accession number: KM273791); for 28S nrRNA gene partial sequence (accession number: KM273792).

Results

Morphology

Colonies were moderately fast growing, attaining a diameter of 38-42 mm on MEA, and 32-36 mm on PDA medium after 20-day. Optimal growth was observed at 20°C. At 25°C colonies reached 10-14 mm on MEA, and 9-12 mm on PDA in 20 days. Although no growth was observed at 30°C, initial growth was detected at 6°C after 14 days of incubation with colonies attaining diameters of 17-22 mm after four weeks. Colonies on MEA at 20 °C were white to yellowish white, powdery and dense, with a thin margin, slightly cottony at center, with reverse pale yellow to brown (Figure 1A, B). On PDA medium, colonies were yellow to sand colored, powdery, suede-like or slightly granular, raised in the center, with a poorly defined margin with reverse brown to grey in age (Figure 1C, D).

Microscopically, the isolate was distinguishable. Conidia were tuberculate and thick-walled arthroconidia (intercalary conidia) and aleurioconidia, globose to subglobose, unicellular, 3.5-4.6 μm ×3.5-4.6 μm, intercalary, sessile or borne on side branches or at the ends of narrow stalks (Figure 2A, 2B). The stalks

occur at right angles to the hypha and are straight-sided. Arthroconidia were sometimes formed by swelling and enlargement within hyphae. Hyphae septate, hyaline, smooth-walled, 1-3 µm wide, straight or branched (Figure 2C, 2D, 2E). The presence of aleurioconidia and arthroconidia were observed in all the cultures examined (Figure 2F, 2G).

Phylogeny

The ITS1-5.8S-ITS2 region sequenced from the fungal isolate included 557 base pairs. A search on GenBank database using BLAST revealed only insignificant hits from GenBank, the closest match to an identified *Chrysosporium* strain was *Chrysosporium speluncarum* CCF 3760 with 94% identity (accession no. AM949568), followed by *Gymnoascus demonbreunii* CBS 122.67 (accession no. AJ315842, 84% coverage/84% identity), *Amauroascus volatilis-patellus* CBS 249.72 (accession no. AJ390378, 73% coverage/85% identity), and *Renispora flavissima* CBS 708.79 (accession no. AF299348, 61% coverage/86% identity). None of which were considered significant for a conspecific isolate (>97% identity). The sequence of the strain *C. morrisgordonii* differed from the sequence of *C*. *speluncarum* CCF 3760 at 37 nucleotide positions. The ITS phylogenetic tree inferred from the analysis showed this isolate was grouped in highly supported clade with *C*. *speluncarum* CCF 3760 as included in the study, representing a clear genetic separation from other *Chrysosporium* spp. (Figure 3).

The D1/D2 region (partial sequence) also did not return a significant BLAST hit. The closest sequence in GenBank was *Chrysosporium speluncarum* CCF 3760 (accession no. AM949568, 94% coverage/96% identity) sequence using BLAST search, followed by *Neogymnomyces virgineus* JN038186 (accession no. JN038186), *Amauroascus volatilis-patellus* CBS 249.72 (accession no. AB075324), *Gymnoascus demonbreunii* CBS 122.67 (accession no. AY176716) all displaying 95% similarity. The phylogenetic tree inferred from the analysis of the D1/D2 sequences showed a similar topology, confirming that the isolate formed a well-supported monophyletic group with *Chrysosporium speluncarum* CCF 3760 as the closest neighbor (Figure 4).

Physiology

The esterase test was strain dependent, however the majority of the isolates were positive. All the fungi tested grew on DTM changing the color of the medium from yellow to red (data not shown), while the strain *Chrysosporium morrisgordonii* displays weak activity. The results of the other physiological tests are summarized in Table 1. The urease and glucosidase test were positive for all the isolates. All the strains in the study produced hemolysis on blood agar, showed lipolytic, proteolytic, amylatic, cellulytic, chitinolytic activity. On SDA medium with 3% and 10% NaCl, growth was scarce for *A. volatilis-patellus* and *R. flavissima*, while the ohter four isolates showed good growth (Table 1). Cycloheximide tolerance and different temperature

tolerance are listed in the Table 1, *Chrysosporium morrisgordonii* and *C*. *speluncarum* CCF 3760 are psychrotolerant strains, which could not grow above 30°C.

Phylogenetic analysis showed that *C. morrisgordonii* is closely related to *C*. *speluncarum* CCF 3760, *G*. *demonbreunii* CBS 122.67, *A*. *volatilis-patellus* CBS 249.72, *R*. *flavissima* CBS 708.79, and *N*. *virgineus* JN038186. However, *C. morrisgordonii*can is distinguished from *A*. *volatilis-patellus*, *R*. *flavissima*, and *N*. *virgineus* by its ability to grow at 6°C and exhibited no growth at 30°C. In addition, the *C*. *speluncarum* could produce much more soluble pigments in media (Figure 5).

Taxonomy

The combined morphological, molecular and physiological features of the strain isolated from the Graphite mine soil samples does not correspond to any of the species described to date within the genus *Chrysosporium* or closely related genera. Thus, we propose here as new species.

***Chrysospor*ium *morrisgordonii* Zhang, Ren, Li, Chaturvedi, Chaturvedi, sp. nov. MycoBank MB 830215**

Etymology: The epithet is derived from late Dr. Morris A. Gordon, eminent mycologist and ex-director of Mycology Laboratory, New York State Department of Health, where the isolate *Chrysosporium morrisgordonii* was characterized.

Holotypus: USA, New York, Warren County, soil of Graphite mine, January 2011, ex Morrisgordonii, Isotypus 6762-S5.

Colonies on MEA at 20°C attaining a diameter of 35-42 mm after 7 d, whitish to yellowish white, powdery and dense, with rich aerial mycelium, elevated at the center and radially folded, compact; reverse pale yellow to brown. *Hyphae* hyaline, septate, smooth-walled, straight or branched, 1-3 μm in diameter. *Conidia* (aleurioconidia) unicellular, tuberculate and thick-walled, globose to subglobose, sessile or borne on side branches or at the ends of narrow stalks, 3.5-4.6 μm ×3.5-4.6 μm; arthroconidia (intercalary conidia), formed by swelling and enlargement within hyphae, tuberculate and thick-walled, globose to subglobose, 3.5-4.6 μm ×3.5-4.6 μm.

Colonies on PDA showed features similar to those on MEA, but was more floccose and sand colored, with more sporulation. The optimum growth temperature was 20°C, and the minimum was 6°C. The fungus grew at 25°C did not grow at 30°C.

Discussion

Considering the morphological, physiological and phylogenetic species concepts, we present evidence for a novel species within *Chrysosporium*, *Chrysosporium morrisgordonii sp.* nov. *C. morrisgordonii* is distinguished by its ability to grow at low temperature range from 6°C-25°C and by the combined presence of arthroconidia and globose or subglobose aleurioconidia,

sessile or borne at the ends of narrow stalks. In the nrLSU and nrITS phylogentic trees, *Chrysosporium morrisgordonii*, *Chrysosporium speluncarum*, *Renispora flavissima*, together with two species, *Neogymnomyces virgineus* and *Gymnoascus demonbreunii* are belong to a well-defined group also known from analyses of previous studies [14, 27-30]. *C*. *speluncarum* and *R*. *flavissima* were recovered from cave samples, have affinity to bat guano, and exhibit a similar phenotype to *C. morrisgordonii* with yellowish colonies and similar tuberculate conidia [14]. The strain *C. morrisgordonii* differs from *C*. *speluncarum* by the presence of the much less pigment, powdery growth, and two different conidia formation (Figure 4). In contrast, *R*. *flavissima* differentiates from *C. morrisgordonii* for conidial size (6-8×5-8 μm) and better growth at 30°C [31, 32]. *N*. *virgineus* and *G*. *demonbreunii* have a broader ecological distribution and a *Chrysosporium* state with smooth conidia [33, 34].

There are few reports of the ability of *Chrysosporium* to adapt to low temperature, in which *C*. *magnasporum* and *C*. *oceanitesii* grow in Antarctic soil [35], *C*. *evolceanui* in alpine soil [36], and *C*. *longisporum* from snake [3], but can be easily differentiated from the new species by their morphology.

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Figure legends

Fig. 1 *Chrysosporium morrisgordonii* Zhang, Ren, Li, Chaturvedi, Chaturvedi, sp. nov. A, B) Colony on MEA agar (surface and reverse); C, D) Colony and PDA agar (surface and reverse).

Fig. 2 *Chrysosporium morrisgordonii* Zhang, Ren, Li, Chaturvedi, Chaturvedi, sp. nov. A-D) Fertile hyphae bearing sessile conidia; E) Two singly intercalary conidia and a terminal chain of arthroconidia (white arrows show the septa); F) sessile (some 2-celled) conidia. — Scale bars = 10 μm (A–F, differential interference contrast) or 1 μm (G).

Fig. 3 Neighbor-joining tree based on Kimura 2-p corrected nucleotide distances among ITS1-5.8S-ITS2 rRNA gene sequences of the species related with *Chrysosporium morrisgordonii*. Branch lengths are proportional to distance. Bootstrap replication frequencies over 60% (1,000 replications) are indicated at the nodes. The sequences were retrieved from CBS (www.cbs.knaw.nl/) or GenBank (www.ncbi.nlm.nih.gov). *Aspergillus niveoglaucus* CBS 101750 and *Aspergillus alliaceus* NRRL 20602 were used as outgroup.

Fig. 4 Neighbor-joining tree based on Kimura 2-p corrected nucleotide distances among the D1 and D2 domains of the 28S rRNA gene sequences of the species related with *Chrysosporium morrisgordonii*. Branch lengths are proportional to distance. Bootstrap replication frequencies over 60% (1,000 replications) are indicated at the nodes. The sequences were retrieved from CBS (www.cbs.knaw.nl/) or GenBank (www.ncbi.nlm.nih.gov). *Aspergillus acristata* NRRL 2394 and *Aspergillus fumigatus* CBS 286.95 were used as outgroup.

Fig. 5 Radial growth determination at temperatures ranging from 6°C to 30°C on Sabouraud dextrose agar (SAB). Type strains of *C*. *speluncarum* CCF 3760, *G*. *demonbreunii* CBS 122.67, *A*. *volatilis-patellus* CBS 249.72, *R*. *flavissima* CBS 708.79, and *N*. *virgineus* DCDSL7716.

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Table 1 Key physiological features of fungi included in this study

Fungi / Physiology	*Chrysosporium morrisgordonii*	*C. speluncarum* CCF 3760	*G. demonbreunii* CBS 427.70	*N. virgineus* DCDSL7716	*A. volatilis-patellus* CBS 249.72	*R. flavissima* CBS 708.79
casein agar	++	-	++	++	+++	+
BHI agar	+++	+++	+++	+++	++	+++
lipase	++	+	++	++	+++	+
ureae	+++	+++	++	+++	+++	+
chitinase	++	++	+	+++	+++	+
cellulase	++	+++	+++	++	++	+
β-Glucosidase	++	+	++	++	+	+
DTM agar	R/B, +	R/B, +++	R/B, ++	R/B, ++	R/B, +++	R/B, ++
SDA+3% NaCl	+++	+++	+++	+++	+	+
SDA+10% NaCl	+	+	+	+	-	-
SDA+0.2% Cyc	+++	++	++	+++	++	++
SDA+0.5% Cyc	+++	++	+	++	+	++
SDA at 6°C	++	++	-	-	-	-
SDA at 30°C	-	-	+++	+++	+++	++

Note: *C*, *Chrysosporium*; *N*, *Neogymnomyces*; *G*, *Gymnoascus*; *A*, *Amauroascus*; *R*, *Renispora*. BHI agar, Brain Heart Infusion agar; Cyc, cycloheximide; SDA, sabouraud dextrose agar; +++, positve or strong activity; +, scarse positive, moderate activity; -, absence or no activity. Y/A, yellow (acidic); R/B, red (basic).

Figure 1

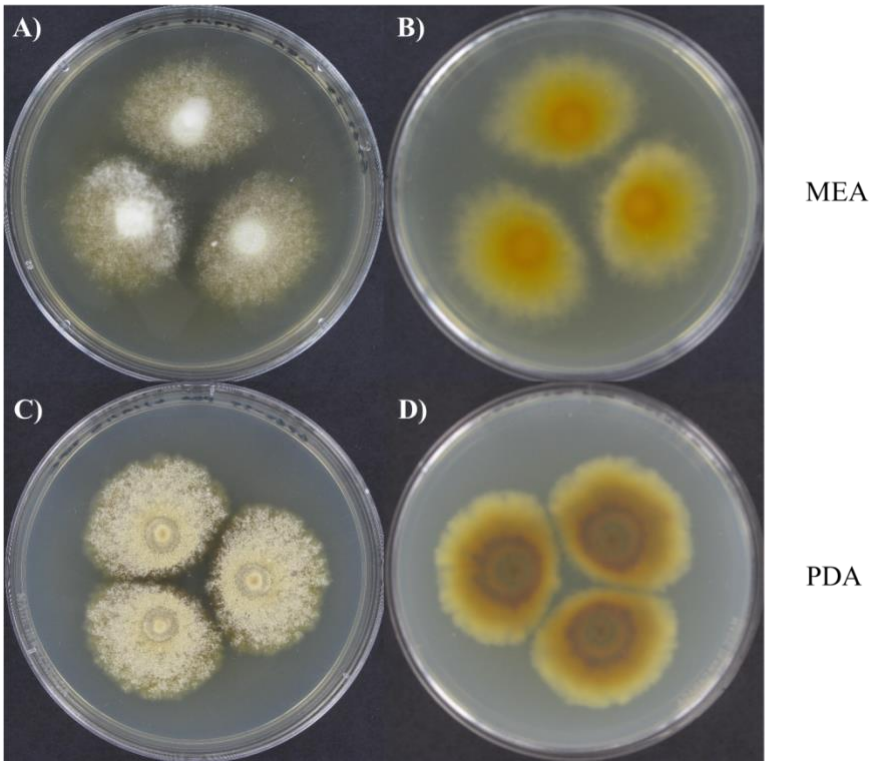


Figure 2

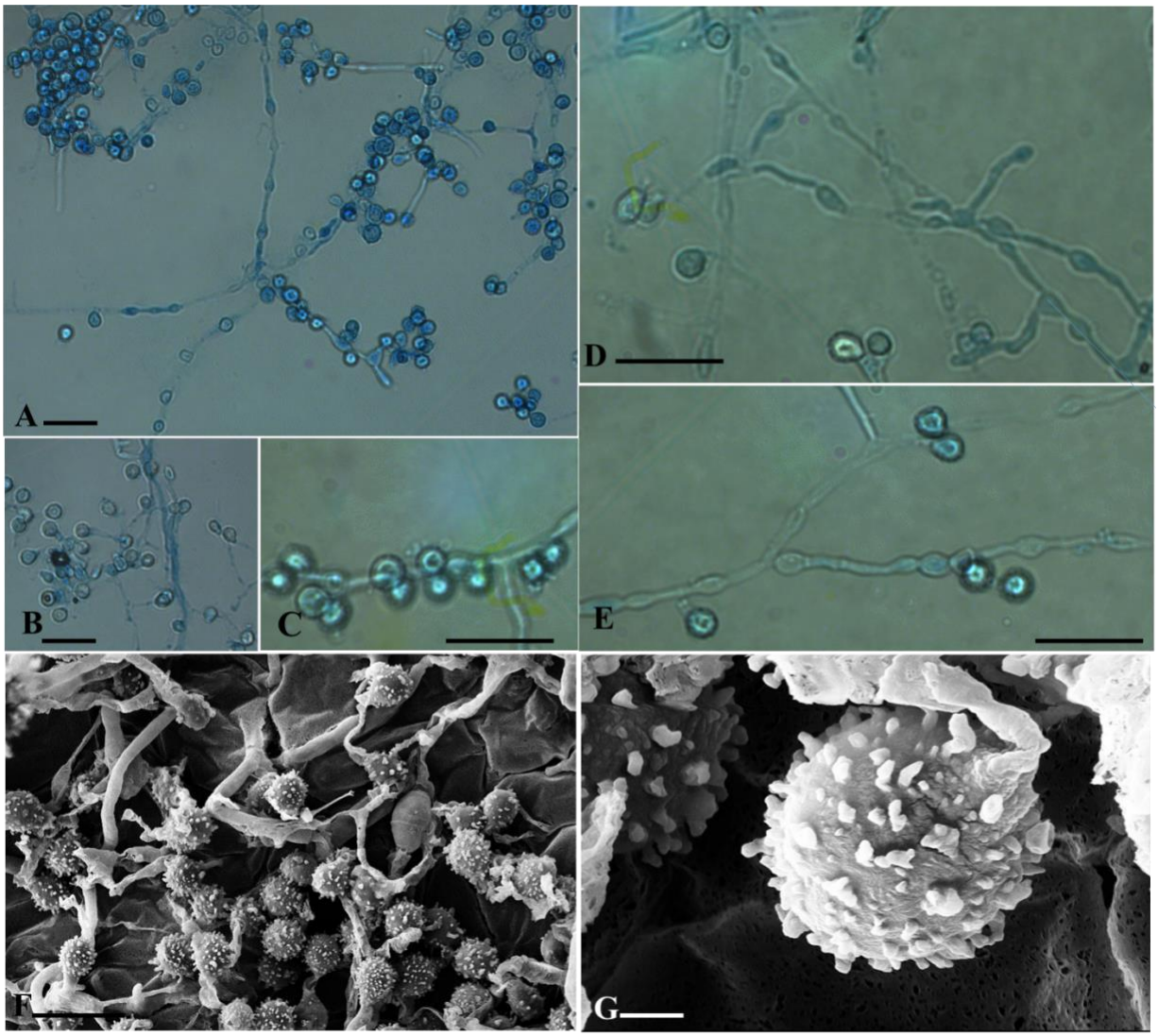
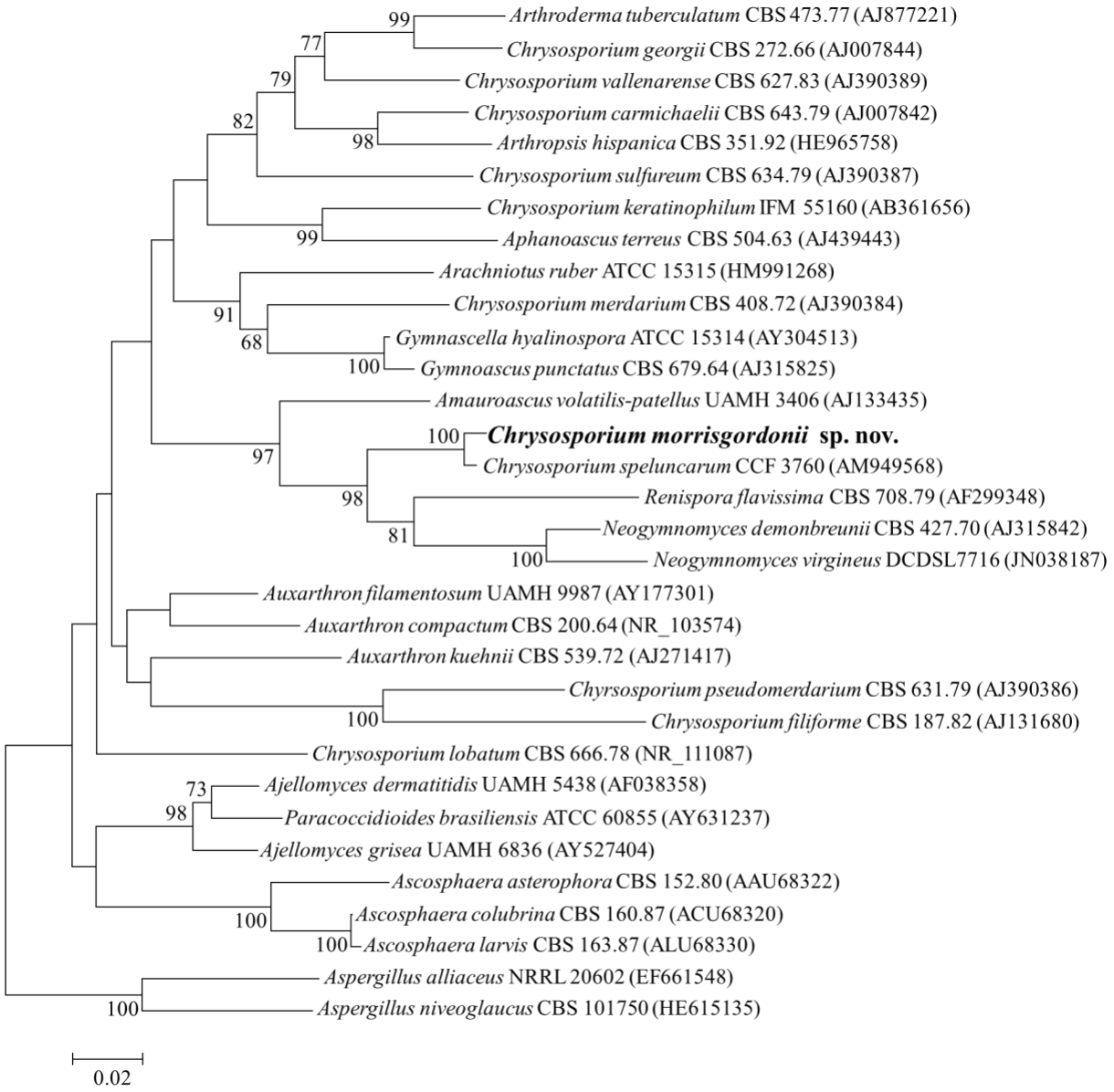


Figure 3


Arthroderma tuberculatum CBS 473.77 (AJ877221)
Chrysosporium georgii CBS 272.66 (AJ007844)
Chrysosporium vallenarense CBS 627.83 (AJ390389)
Chrysosporium carmichaelii CBS 643.79 (AJ007842)
Arthropsis hispanica CBS 351.92 (HE965758)
Chrysosporium sulfureum CBS 634.79 (AJ390387)
Chrysosporium keratinophilum IFM 55160 (AB361656)
Aphanoascus terreus CBS 504.63 (AJ439443)
Arachniotus ruber ATCC 15315 (HM991268)
Chrysosporium merdarium CBS 408.72 (AJ390384)
Gymnascella hyalinospora ATCC 15314 (AY304513)
Gymnoascus punctatus CBS 679.64 (AJ315825)
Amauroascus volatilis-patellus UAMH 3406 (AJ133435)
Chrysosporium morrisgordonii sp. nov.
Chrysosporium speluncarum CCF 3760 (AM949568)
Renispora flavissima CBS 708.79 (AF299348)
Neogymnomyces demonbreunii CBS 427.70 (AJ315842)
Neogymnomyces virgineus DCDSL7716 (JN038187)
Auxarthron filamentosum UAMH 9987 (AY177301)
Auxarthron compactum CBS 200.64 (NR_103574)
Auxarthron kuehnii CBS 539.72 (AJ271417)
Chyrsosporium pseudomerdarium CBS 631.79 (AJ390386)
Chrysosporium filiforme CBS 187.82 (AJ131680)
Chrysosporium lobatum CBS 666.78 (NR_111087)
Ajellomyces dermatitidis UAMH 5438 (AF038358)
Paracoccidioides brasiliensis ATCC 60855 (AY631237)
Ajellomyces grisea UAMH 6836 (AY527404)
Ascosphaera asterophora CBS 152.80 (AAU68322)
Ascosphaera colubrina CBS 160.87 (ACU68320)
Ascosphaera larvis CBS 163.87 (ALU68330)
Aspergillus alliaceus NRRL 20602 (EF661548)
Aspergillus niveoglaucus CBS 101750 (HE615135)
99
77
79
82
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68
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97
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81
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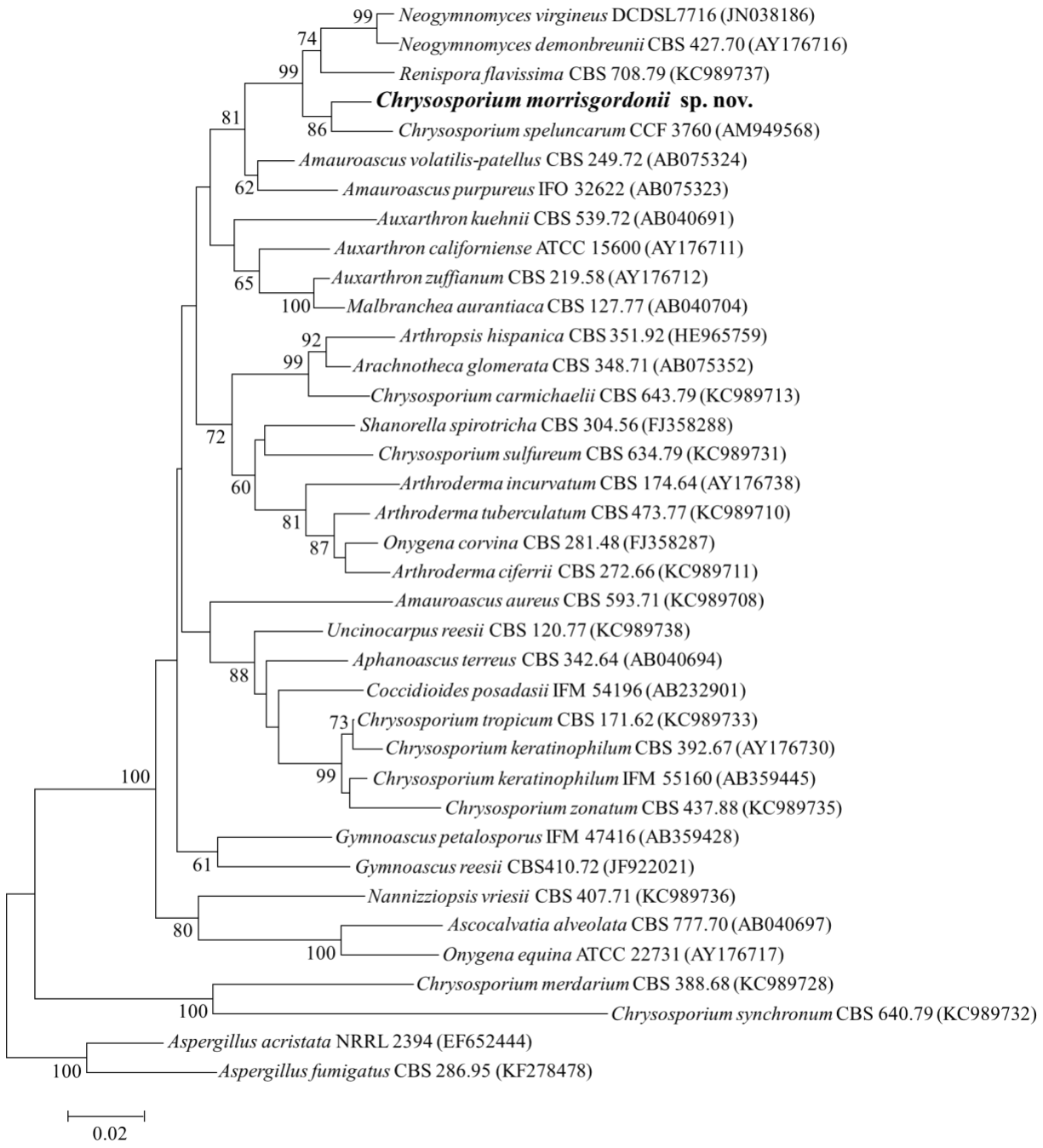
100
73
98
100
100
100
0.02

Figure 4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Chrysosporium morrisgordonii sp. nov.
81
86
Chrysosporium speluncarum CCF 3760 (AM949568)
Amauroascus volatilis-patellus CBS 249.72 (AB075324)
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Amauroascus purpureus IFO 32622 (AB075323)
Auxarthron kuehnii CBS 539.72 (AB040691)
Auxarthron californiense ATCC 15600 (AY176711)
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Auxarthron zuffianum CBS 219.58 (AY176712)
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Malbranchea aurantiaca CBS 127.77 (AB040704)
92
Arthropsis hispanica CBS 351.92 (HE965759)
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Arachnotheca glomerata CBS 348.71 (AB075352)
Chrysosporium carmichaelii CBS 643.79 (KC989713)
Shanorella spirotricha CBS 304.56 (FJ358288)
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Chrysosporium sulfureum CBS 634.79 (KC989731)
60
Arthroderma incurvatum CBS 174.64 (AY176738)
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Arthroderma tuberculatum CBS 473.77 (KC989710)
87
Onygena corvina CBS 281.48 (FJ358287)
Arthroderma ciferrii CBS 272.66 (KC989711)
Amauroascus aureus CBS 593.71 (KC989708)
Uncinocarpus reesii CBS 120.77 (KC989738)
Aphanoascus terreus CBS 342.64 (AB040694)
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Coccidioides posadasii IFM 54196 (AB232901)
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Chrysosporium tropicum CBS 171.62 (KC989733)
Chrysosporium keratinophilum CBS 392.67 (AY176730)
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99
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Gymnoascus petalosporus IFM 47416 (AB359428)
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Gymnoascus reesii CBS410.72 (JF922021)
Nannizziopsis vriesii CBS 407.71 (KC989736)
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Ascocalvatia alveolata CBS 777.70 (AB040697)
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Onygena equina ATCC 22731 (AY176717)
Chrysosporium merdarium CBS 388.68 (KC989728)
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Chrysosporium synchronum CBS 640.79 (KC989732)
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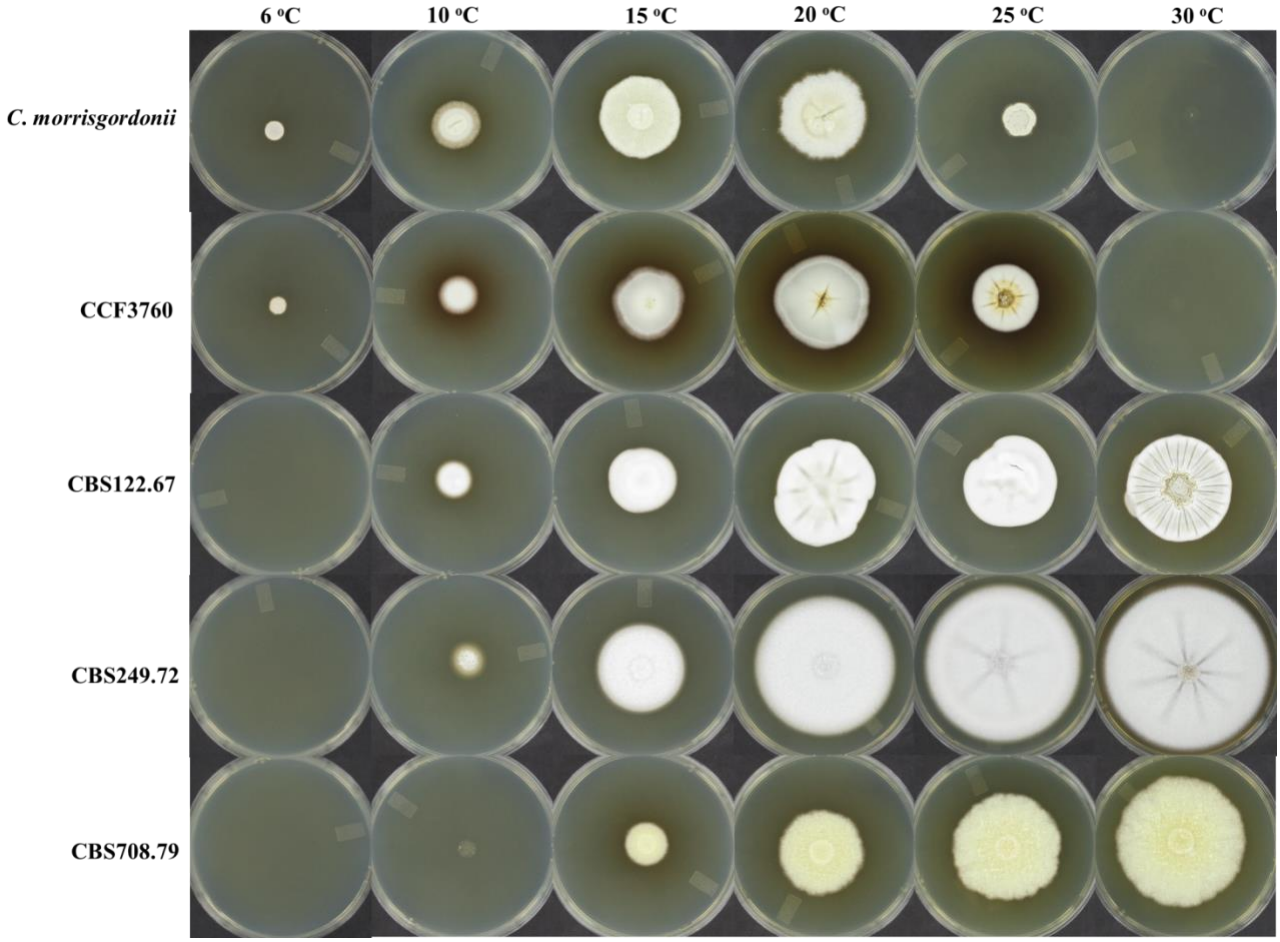
Figure 5


6 °C
10 °C
15 °C
20 °C
25 °C
30 °C
C. morrisgordonii
CCF3760
CBS122.67
CBS249.72
CBS708.79