Phytotaxa 400 (5): 257–264
https://www.mapress.com/j/pt/

Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

https://doi.org/10.11646/phytotaxa.400.5.1

Morphological traits and molecular analysis for two new *Chrysosporium* species from Fujian Province, China

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Abstract

Two new species, *Chrysosporium laterisporum* and *C. ovalisporum*, were isolated from forest soils and zoo soils in Fuzhou City, Fujian Province, China, using child hair as baits. Molecular (ITS-5.8S rDNA sequences) and morphological characters were used to identify taxonomic status. The phylogeny showed *C. laterisporum* and *C. ovalisporum* grouped into a separate subclade, closely related to *C. georgii*, *C. magnasporum*, C. *oceanitesii* and *C. vespertilium*. These previously described *Chrysosporium* species can be distinguished from the new species based on morphology alone.

Keywords: filamentous fungi, identification, morphology, phylogeny

Introduction

The genus *Chrysosporium* Corda was established in 1833 (Oorschot 1980). *Chrysosporium* spp. are mostly saprophytic and keratinolytic, widely distributed and can be isolated from various habitats such as air, sea water, sludge, and waste water (Padhye *et al*. 1967, Ulfig 1991, Ulfig & Korcz 1995). Known telemorphs that have been associated with the genus variously belong to the Gymnoasceae, Onygenaceae, Ascosphaeraceae and Sordariaceae in Ascomycetes, Ascomycota.

With the development of molecular technology, phylogenetic analysis based on sequences of internal transcribed spacer regions 1 and 2 and 5.8S rDNA (ITS1-5.8S-ITS2) of 57 *Chrysosporium* species revealed that the genus was polyphyletic with affiliations to at least two orders of the Ascomycota, and that it should be restricted to asexual states of Onygenales (Vidal *et al*. 2000). Pitt *et al*. (2013) studied *Chrysosporium* using nuclear ribosomal large subunit (nrLSU) genes and transferred an extreme xerophilic species, *C. xerophilum* Pitt, to the new genus *Xerochrysium* (Pitt) Pitt. To date, there are about 80 valid species in the genus. Twelve new species have been reported in the past 5 years, namely, *C. guarroi* J. Cabañes & Abarca (Abarca *et al*. 2010), *C. speluncarum* A. Nováková & M. Kolařík (Nováková & Kolařík 2010), *C. longisporum* Stchigel, Deanna A. Sutton, Cano & Guarro (Stchigel *et al*. 2013), *C. magnasporum* Stchigel, Cano, Mac Cormack & Guarro (Crous *et al*. 2013), *C. qinghaiense* Y.F. Han, J.D. Liang & Z.Q. Liang (Han *et al*. 2013), *C. oceanitesii* Stchigel, Cano, Archuby & Guarro and *C. sanyaense* Y.F. Han & Z.Q. Liang (Zhang *et al*. 2013), *C. hubeiense* Y.W. Zhang, Y.F. Han & Z.Q. Liang (Zhang *et al*. 2016a), *C. guizhouense* Y.W. Zhang, Y.F. Han & Z.Q. Liang (Zhang *et al*. 2016a), *C. shanxiense* Y.W. Zhang, W.H. Chen, X. Zou, Y.F. Han & Z.Q. Liang (Zhang *et al*. 2016b), *C. jingzhouense* Y.W. Zhang, Y.F. Han & Z.Q. Liang (Zhang *et al*. 2017) and *C. clavisporum* Y.W. Zhang, Y.F. Han & Z.Q. Liang (Zhang *et al*. 2017) .

We isolated *Chrysosporium* fungi from Fujian Province, and identified two new species based on molecular and morphological analyses. This paper provides a phylogenetic tree, descriptions, and illustrations of the novel species.

Accepted by Eric McKenzie: 27 Mar. 2019; published: 9 Apr. 2019

Materials & Methods

Sample collection and strain isolation

Four strains of *Chrysosporium* were isolated from forest soils and zoo soils in Fuzhou City, Hubei Province (N26°08′, E119°28′), China. Soil samples were added to sterilized child hair fragment (5 mm long) and kept moist at 26 °C for approximately one month. When fungal growth was observed, the hair fragment was mixed with sterilized water in an Erlenmeyer flask, and 1 mL suspensions were evenly spread on Martin's medium and incubated at 26 °C. The pure cultures were then transferred to potato dextrose agar (PDA) slants and stored at -70 °C. The holotypes and ex-type cultures were deposited at the Institute of Fungus Resources, Guizhou University (GZAC) (Zhang *et al*. 2016a, b).

Morphological identification

Isolates were transferred to PDA and Czapek agar, incubated at 26 °C for 14 days, and subjected to examination. Fungal micro-characteristics were examined using a Motic microscope (Guangzhou, Motic Co., China) and photographed. Diagnostic features were defined based on these observations. The fungi were morphologically identified according to colony characteristics and conidiogenous structures (Oorschot 1980, Han *et al*. 2013, Zhang *et al*. 2013, 2016a, b).

DNA extraction, PCR amplification and nucleotide sequencing

Total genomic DNA was extracted from fresh sporulating cultures after seven days at 26 °C using a Fungal DNA Mini Kit (Omega Biotech, Doraville, GA, USA) according to the manufacturer's protocol and then stored at –20 °C. ITS-5.8S rDNA region was amplified with primers ITS5 (5′-GGTGAGAGATTTCTGTGC-3′) and ITS4 (5′-TCCTCCGCTTAT TGA TATGC-3′) (Zhang *et al*. 2016a). Amplifications were carried out in 25 µL volumes, with 12.5 µL 2× Master Mix, 2 µL template DNA, 1 µL primer ITS4, 1 µL primer ITS5, and 8.5 µL ddH_2O. Amplification conditions were 5 mins at 94 °C, then 35 cycles of 94 °C for 40 s, 50 °C for 40 s, 72 °C for 1 min, and finally 10 min at 72 °C. The resulting PCR products were sequenced by Sangon Biotech (Shanghai, China) using the same primers. The generated ITS-5.8S rDNA sequences were submitted to GenBank (KY350785 to KY350788).

Molecular phylogenetic analysis

ITS-5.8S rDNA sequences of 44 *Chrysosporium* species were downloaded from GenBank. An ITS-5.8S rDNA sequence of *Myceliophthora thermophila* (Apinis) Oorschot was retrieved to use as an outgroup (Fig. 1). Alignment of the ITS-5.8S rDNA region of the downloaded sequences, and the sequences generated in this study, was achieved with MAFFT v. 7.037b (Katoh *et al*. 2013), followed by manual adjustment to maximize sequence similarity. Sequence alignment editing was done with BioEdit (Hall 1999).

Phylogenetic trees were constructed using Bayesian analysis (Zhang *et al*. 2015) and maximum likelihood (ML) (Felsenstein 1981). The GTR+I+G nucleotide substitution model were used for Bayesian analysis, as suggested by Modeltest 3.7 (Posada & Crandall 1998). Posterior probabilities were determined by Markov chain Monte Carlo (MCMC) sampling in MrBayes v. 3.2 (Ronquist *et al*. 2012) using the estimated model of evolution. Six simultaneous Markov chains were run for 1,000,000 generations, with trees sampled every 100th generation (resulting in 10,000 total trees). The first 2,000 trees, which represented the burn-in phase of the analysis, were discarded and the remaining 8,000 trees were used to calculate posterior probabilities in the majority rule consensus tree. Each run was examined with the program Tracer v. 1.5 (Drummond & Rambaut 2007) after the analysis finished, to determine burn-in and to confirm that the runs had converged.

The aligned sequences were also analyzed using maximum parsimony (MP) and maximum likelihood (ML) methods in MEGA 6 (Tamura *et al*. 2013), with gaps treated as missing data and all other parameters following the default condition. Bootstrap support for nodes in the resulting trees was assessed using 1,000 replications per analysis. The final aligned data set and the phylogenetic tree are available in TreeBASE under the submission ID 20374 (http://purl. org/phylo/treebase/phylows/study/TB2: S20374?x-access-code=59f67b932e3d4771550 f66001f9f05b&format=html) and taxonomic novelties were deposited in MycoBank (www.MycoBank.org).

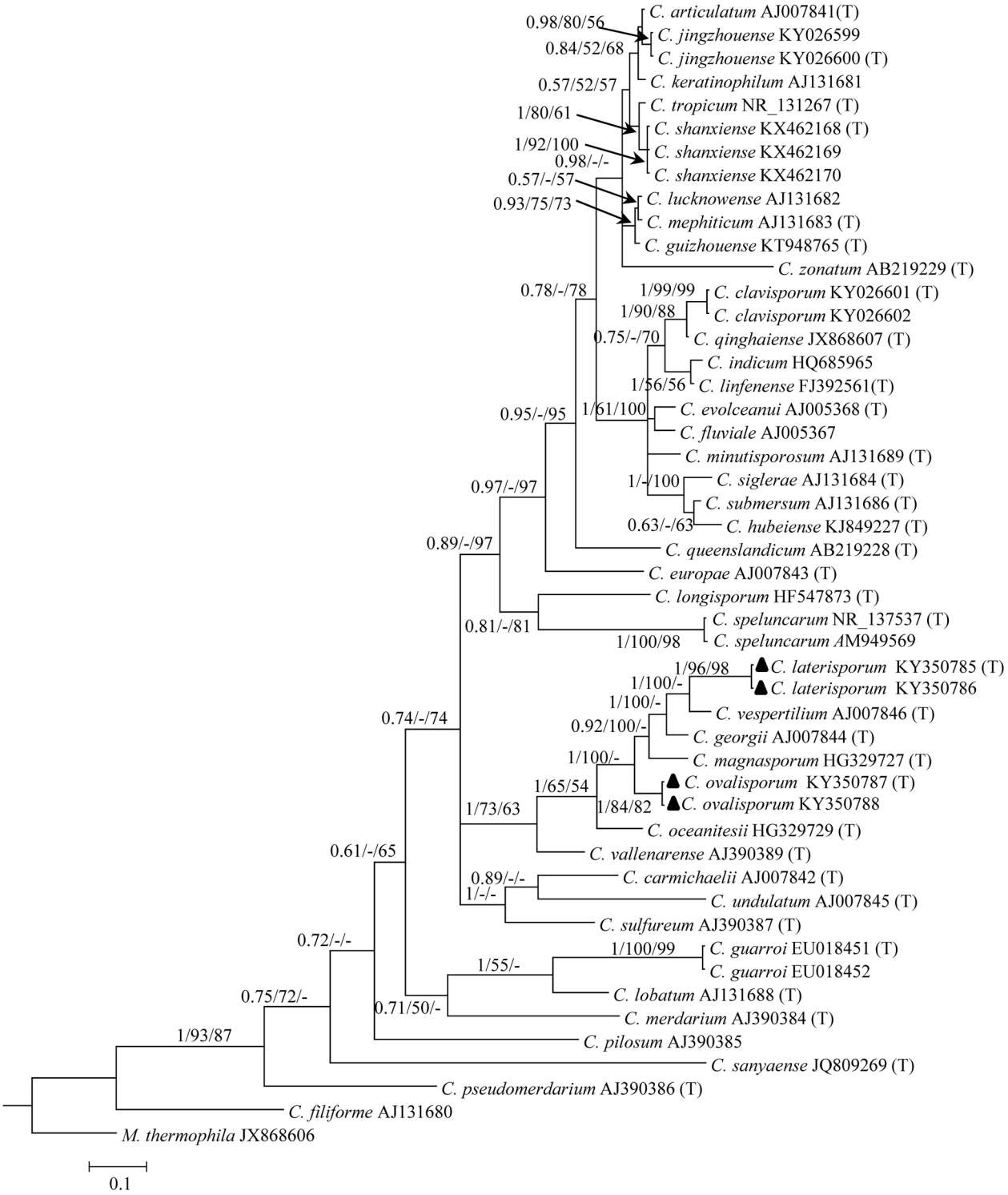

FIGURE 1. Phylogenetic tree of *Chrysosporium* spp. constructed from ITS-5.8S rDNA sequences. Statistical support values (Bayesian posterior probability/ML/MP) are shown at the nodes. The tree was rooted using *Myceliophthora thermophila* as outgroup.

Results

Phylogenetic analysis

Three methods (Bayesian inference/ML/MP) were used to phylogenetically analyze the 48 *Chrysosporium* ITS-5.8S rDNA sequences (Fig. 1). The alignment obtained for the ITS region was 456 bp long. Phylogenies obtained using Bayesian MCMC analyses and maximum parsimony were mostly congruent. Most branches were strongly supported in three analyses. Consequently, a combined tree is shown in Fig.1 with support values given at nodes for all three methods (Bayesian inference/ML/MP). In this tree, *C. laterisporum* G310.1 and G310.2 are in a separate subclade and cluster with strong support (1/96/98); *C. ovalisporum* G446.1 and G446.2 are also in a subclade with high support (1/84/82). Four strains clustered in a subclade with *C. georgii*, *C. magnasporum*, *C. oceanitesii* and *C. vespertilium* with high support value.

Taxonomy

Chrysosporium laterisporum Z. Li, Y.W. Zhang, W.H. Chen & Y.F. Han *sp. nov.* (Fig. 2)

MycoBank No.: MB 819480, GenBank: KY350785, KY350786

Type:—CHINA. Fujian Province: Fuzhou City, 26°08´N, 119°28´E, GZUIFR-G310 (dried culture) isolated from soil of forest park.

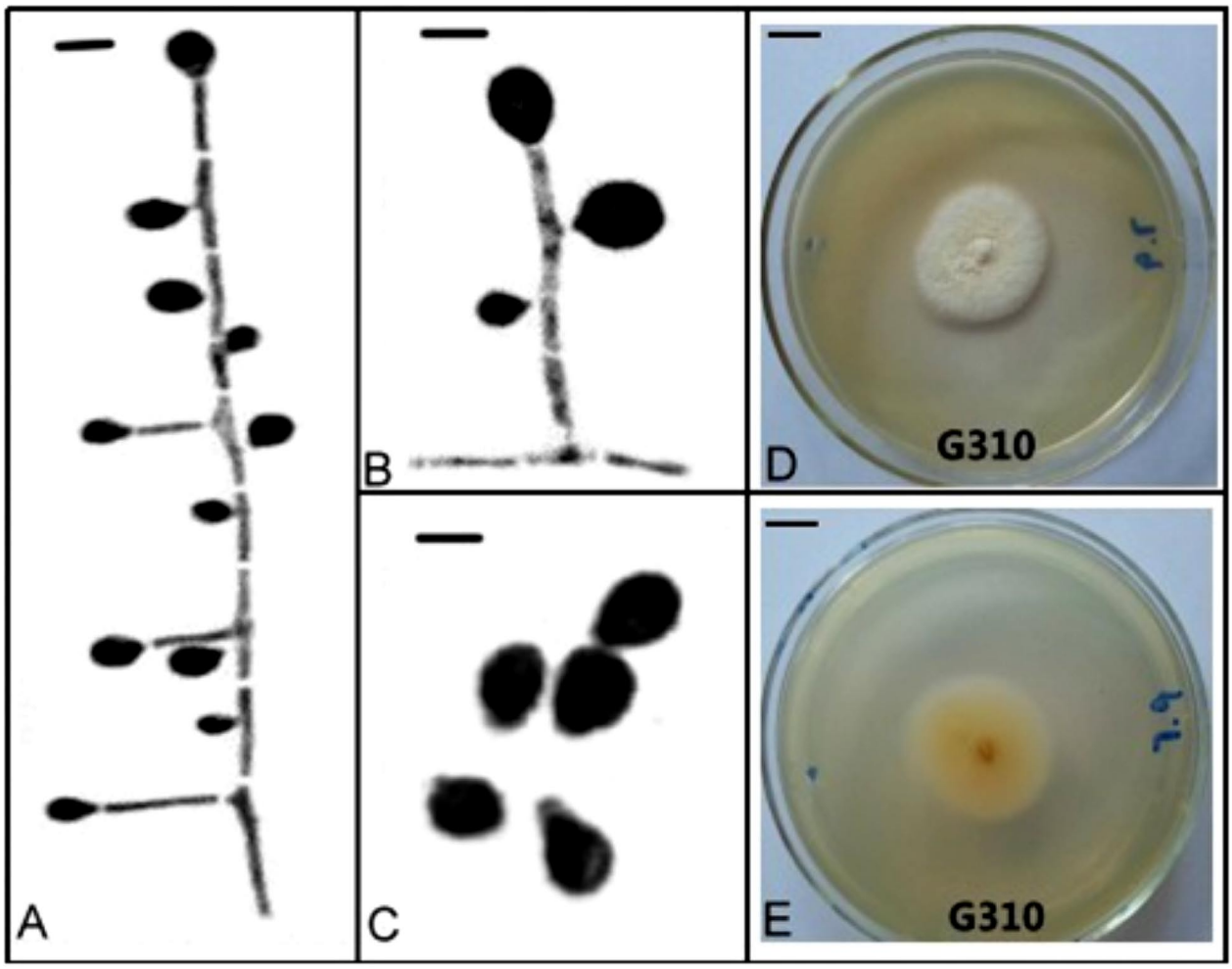

FIGURE 2. Colony and conidiogenous structures of *Chrysosporium laterisporum* (GZUIFR-G310, holotype). A, B. Conidiogenous structures. C. Conidia. D, E. Colony (top and reverse) on PDA. Bars A–C = 10 µm, D–E = 10 mm.

Colonies on PDA, attaining 32 mm in 7 d at 25 °C, white to yellowish, fluffy, ridges in the center, margin irregular; reverse yellowish. *Hyphae* hyaline, septate, smooth, 1.2–3.3 µm wide. *Racquet hyphae* absent. *Terminal and lateral*

conidia sessile or on short protrusions or side branches, solitary, hyaline, smooth, single-celled, obpyriform to ellipsoidal, 5–12.5 × 2.5–10 µm ($\overline{x}$=7.5 × 5.8, n= 60), basal scars 0.8–1.5 µm wide. *Intercalary conidia* and *chlamydospores* absent.

Etymology:–laterisporum (Latin), referring to the abundant lateral conidia.

Material examined:—The ex-type G310.1 and ex-isotype G310.2 were isolated from the soils of the forest park in November 2014 by Y.F. Han. The ex-type G310.1 culture was dried as the type GZUIFR-G310. They were deposited in the Institute of Fungus Resource, Guizhou University (GZAC).

Distribution:—Fuzhou City, Fujian Province, China.

Chrysosporium ovalisporum Z. Li, Y.W. Zhang, W.H. Chen & Y.F. Han *sp. nov*. **(**Fig. 3)

MycoBank No.: MB 819481, GenBank: KY350787, KY350788

Type:—CHINA. Fujian Province: Fuzhou City, 26°08´N, 119°28´E, GZUIFR-G446 (dried culture) isolated from soils of the zoo park.

Colonies on PDA attaining 28 mm in 14 d at 25 °C, white to yellowish, powdery, flat, margin irregular; reverse brown. *Hyphae* hyaline, septate, smooth, 2.5–3.5 µm wide. *Racquet hyphae* absent. *Terminal and lateral conidia* on long or short stalk perpendicular to hyphae, solitary, hyaline, smooth, single-celled, occasionally 2-celled, long obovate to clavate, few cylindrical, 5–15 × 2.5–7 µm ($\overline{x}$= 6.5 × 4.6, n = 60), basal scars 0.8–2.5 µm wide. *Intercalary conidia* and *chlamydospores* absent.

Etymology:—ovalisporum (Latin), referring the conidia that are long obovate.

Material examined:—The ex-type G446.1 and ex-isotype G446.2 isolated from the soils of the zoo park in November 2014 by Y.F. Han. The ex-type G446.1 culture was dried to form the type GZUIFR-G446. They were deposited in the Institute of Fungus Resource, Guizhou University (GZAC).

Distribution:—Fuzhou, Fujian Province, China.

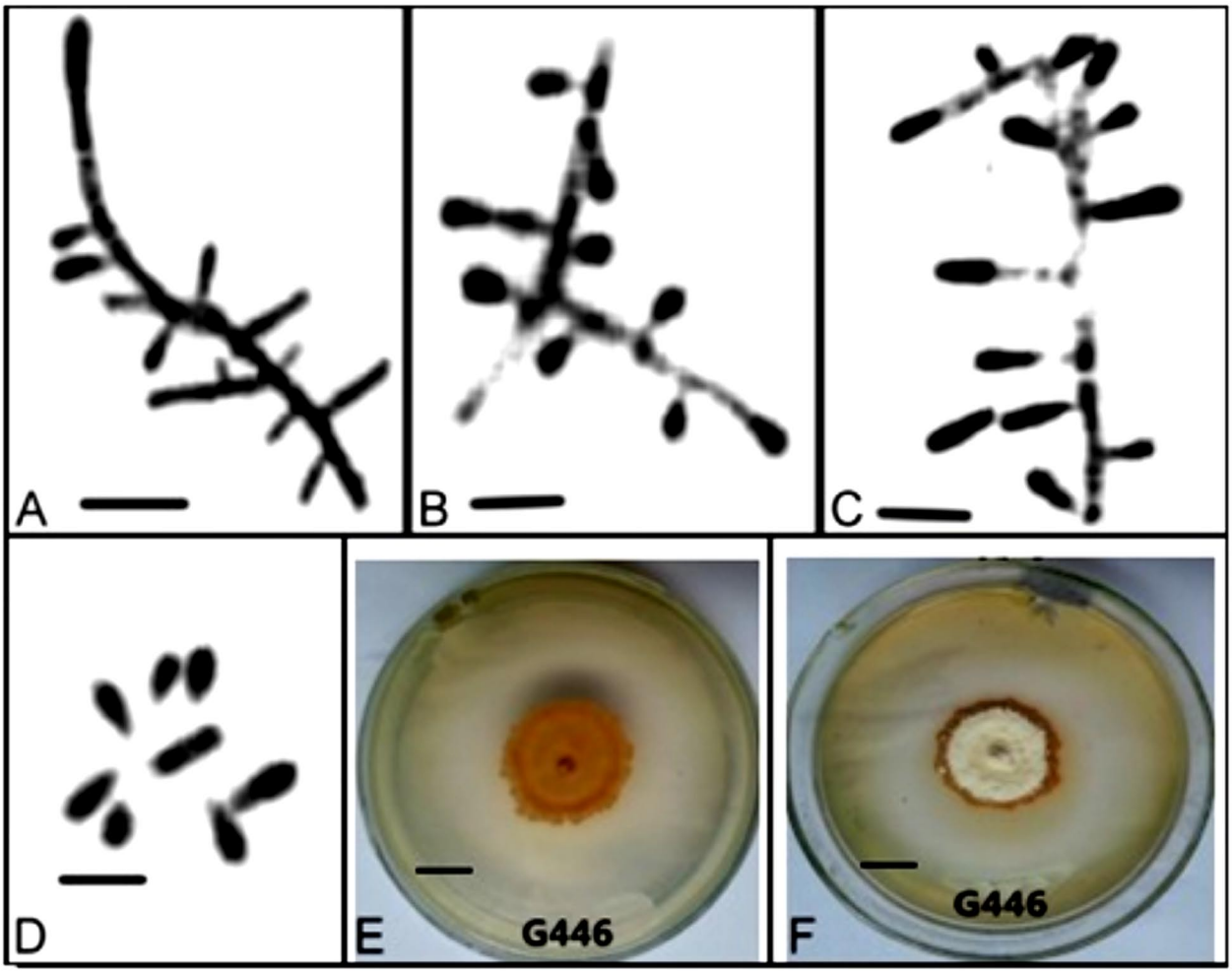

FIGURE 3. Colony and conidiogenous structures of *Chrysosporium ovalisporum* (GZUIFR-G446, holotype). A–C. Conidiogenous structures. D. Conidia. E–F. Colonies (reverse and top) on PDA. Bars A–D = 10 µm, E–F. = 10 mm.

Discussion

ITS-5.8S rDNA sequences can successfully distinguish most fungal species and have even been used as the barcode marker in fungal identification and evolutionary biology (Schoch *et al*. 2012). Although D1/D2 region of the nuclear 28S subunit (nucLSU) sequences have been used for phylogenetic analysis in the genus *Chrysosporium*, recent studies demonstrated that ITS rDNA sequences alone were able to successfully distinguish *Chrysosporium* species (Zhang *et al*. 2016a, Zhang *et al*. 2017, Stichigel *et al*. 2013). From the phylogenetic tree based on ITS-5.8S rDNA, *C. laterisporum* and *C. ovalisporum* clustered into separated subclades, related to *C. vespertilium* Guarro, P. Vidal & De Vroey (Vidal *et al*. 1996), *C. georgii* (Varsavsky & Ajello) van Oorschot (Oorschot 1980), *C. magnasporum* Stchigel, Cano, Mac Cormack & Guarro (Crous *et al*. 2013) and *C. oceanitesii* Stchigel, Cano, Archuby & Guarro (Crous *et al*. 2013). These related species can be distinguished morphologically from *C. laterisporum* and *C. ovalisporum* (Table 1). *Chrysosporium vespertilium* and *C. georgii* have obvious racquet hyphae; *C. magnasporum* has larger conidia (10–27 × 7–12 µm vs. *C. laterisporum* 5–12.5 × 2.5–10 µm, *C. ovalisporum* 5–15 × 2.5–7 µm); and *C. oceanitesii* produces intercalary conidia.

Only *C. synchronum* Oorschot and *C. sulfereum* (Fiedl) Oorschot & Samson have similar morphological characteristics to the two new species, of no racquet hyphae and no intercalary conidia (Table 1). However, these two species have rough conidia (Oorschot 1980).

TABLE 1. Comparison of the main morphological characters of allied species.

Species	Texture	Racquet hyphae	Conidia shape	Conidia size (µm)	Conidia surface
C. georgii	Felty to powdery	Present	Subglobose to obovoid	3–8 × 2–3	Smooth to rough
C. laterisporum GZUIFR-G310	Fluffy	Absent	Obpyriform to ellipsoidal	5–12.5 × 2.5–10	Smooth
C. magnasporum	Downy	Absent	Obovate to clavate	10–27 × 7–12	Smooth
C. oceanitesii	Downy	Absent	Obovate to clavate	7–17 × 4–10	Verrucose
C. ovalisporum GZUIFR-G446	Powdery	Absent	Long oval to obovate	2.2–9.7 × 1–3.2	Smooth
C. sulfereum	Felty to powdery	Absent	Subglobose to ovoid or clavate	3–7 × 3–8	Rough
C. synchronum	Fluffy to felty	Absent	Ovoid to fusiform	7.5–11 × 4–5.5	Rough
C. vesperiilium	Powdery	Present	Clavate to pyriform	5–8 × 2–4	Smooth to rough

Acknowledgements

This work was supported by Ministry of Science and Technology of China for Fundamental Research (no. 2013FY110400), the National Natural Science Foundation of China (nos. 31460010, 31860002), Construction Program of Biology First-class Discipline in Guizhou (GNYL [2017] 009) and China Scholarship Council. We thank Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

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