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Calcarisporium cordycipiticola sp. nov., an important fungal pathogen of *Cordyceps militaris*

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Abstract

Production of the medicinal and edible fungus *Cordyceps militaris* has dramatically increased in China because of its alleged medicinal properties. During industrial production, a fungal parasite was found to infect the fruiting bodies causing significant quality and yield losses. Two isolates were obtained from fruiting bodies of *C. militaris* in Beijing and Shanghai mushroom farms and were characterized by morphological and phylogenetic analyses of SSU and ITS sequence data. The phylogenetic analyses showed that they belong to the genus *Calcarisporium* and are related to the type species, *C. arbuscula* with 91% ITS sequence similarity. We introduce this taxon as *Calcarisporium cordycipiticola* sp. nov., and compare it morphologically with other taxa in the genus. The interaction between this taxon, its host, and methods to control this fungicolous taxon need further studies.

Keywords: Fungicolous fungi, Hyphomycetes, Caterpillar fungus, Ascomycota, Medicinal mushroom

Introduction

Cordyceps militaris (L.) Fr., commonly known as the orange caterpillar fungus, has a worldwide distribution (Yang *et al*. 2014; Wen *et al*. 2014), and is the type species of *Cordyceps* in the family Cordycipitaceae (Sung *et al*. 2007; Maharachchikumbura *et al*. 2015). *Cordyceps militaris* is an important medicinal and edible species (Wen *et al*. 2014), and extensive research is being carried out on its cultivation using liquid or solid media (Kang *et al*. 2014; Wen *et al*. 2014). The fruiting bodies or extracts from *C. militaris* are used as nutraceuticals to improve human immunity (De Silva *et al*. 2013; Chou *et al.* 2014). The fruiting body of *C. militaris* is, however, more valuable as it contains metabolites not present in the vegetative mycelium (Shrestha 2012). Thus, production of the fruiting bodies of *C. militaris* has increased in China. However, when *C. militaris* was cultivated in the State Key Laboratory of Mycology in Beijing and in the Shanghai Guobao Enterprise Development Center, the developing fruiting bodies were colonized by white cottony colonies. We isolated these fungicolous organisms and morphological study indicated that they belonged to *Calcarisporium.*

The genus *Calcarisporium* was introduced by Preuss (1851), and is typified by *C. arbuscula* Preuss. Species in this genus are hyphomycetous Sordariomycetes (Ascomycota) (Hausner & Reid 2004). The diagnostic morphological features of *Calcarisporium* are the formation of hyaline, erect, verticillate conidiophores and sympodial conidiation (Hughes 1951, de Hoog 1974).

de Hoog (1974) treated *Calcarisporium* as monotypic with *C. arbuscula* as the type species, and synonymized *C. antibioticum* Haller & Loeffler with the type species. *Calcarosporium abietis* B. Sutton, *C. parasiticum* H.L. Barnett, *C. setiphilum* Deighton & Piroz., and *C. thermophilum* H.C. Evans were transferred to *Acrodontium*, *Hansfordia*, *Sporothrix*, and *Calcarisporiella*, respectively; and *C. echinosporum* Deighton & Piroz., *C. griseum* Speg., *C. indicum* D. Rao & R. Rao, and *C. pallidum* Tubaki were treated as doubtful species. Later, *C. acerosum* Matsush., *C. ovalisporum* (Petch) de Hoog and *C. phaeopodium* Somrith. & E.B.G. Jones were added to the genus. Somrithipol & Jones (2006) provided a key to the four accepted species of the genus. The classification of species of *Calcarisporium* has previously

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relied on morphological characters, its taxonomic position at a higher rank was uncertain due to lack of phylogenetic analysis. Hausner & Reid (2004) provided small ribosomal subunit (SSU) sequence data for *C. arbuscula* and showed that *Calcarisporium* belongs to the Hypocreales, in family *incertae sedis*. Maharachchikumbura *et al.* (2015) listed it under Sordariomycetes, genera incertae sedis.

Colony growth rate, morphological characters, and phylogenetic analyses of the combined SSU and ITS sequence data, showed that our taxon is a new species of *Calcarisporium*. The purpose of this paper is to describe and illustrate the new species *C. cordycipiticola*, and compare it with the four known species of the genus.

Materials & methods

Collection and isolation

Samples were collected from *Cordyceps militaris* growing on sterilized silkworm chrysalis in the State Key Laboratory of Mycology, Beijing (N 40° 0′ 9.94″, E116° 22′ 39.86″), in June 2014 and on sterilized rice medium in Shanghai Guobao Enterprise Development Center, Shanghai (N31° 14′ 24.71″, E121° 28′ 42.12″) in September 2012. Single-spore isolates were obtained as detailed in Chomnunti *et al*. (2014). Germinating spores were aseptically transferred to malt extract agar (MEA) plates and grown at 20 °C. Growth rates were determined on PDA. The holotype is deposited in the Herbarium of Mycology, Chinese Academy of Science (HMAS). Ex-type living cultures are deposited in in the Culture Collection in Mae Fah Luang University (MFLUCC) and China General Microbiological Culture Collection Center (CGMCC). Facesoffungi and Index Fungorum numbers are provided as explained in Jayasiri *et al*. (2015).

Morphology

All isolates were grown on PDA in Petri-dishes incubated at 20 °C for 10 d in darkness. Morphological features of colonies were recorded on PDA. Microscopic observations and measurements were made from preparations mounted in 50% lactic acid. The statistics presented here are based on the measurement of 30 mature conidia (± S.D.) and 30 conidiogenous cell (± S.D.) at 100 × magnification. Conidiophores were taken from the edge of conidiogenous pustules or fascicles to describe their structure and morphology.

DNA extraction, PCR amplification, and DNA sequencing

Fresh mycelia (50–100 mg) were harvested from 10-day-old cultures and placed in 1.5 mL Eppendorf tubes for genomic DNA extraction. DNA was extracted following the protocol of Porebski *et al.* (1997). Sequences of the internal transcribed spacer (ITS) regions and SSU were amplified by polymerase chain reaction (PCR) with the primer pairs ITS5–ITS4 and NS1–NS4 (White *et al*. 1990). Each amplification reaction included 0.2 mM of each dNTP, 0.4 mM of each primer, 0.5 U of Taq polymerase (TransGen, China), 2 µL of genomic DNA solution, 10 × Easy Taq buffer (TransGen, China) in 50 µL reaction volume. A typical reaction included an initial denaturation at 95 °C for 5 min; followed by 38 cycles of denaturation at 95 °C for 50 s, annealing at 52 °C for 50 s, extension at 72 °C for 60 s and a final extension at 72 °C for 10 min. Reactions were run with positive and negative controls to ensure accuracy and to detect contamination. Automated sequencing was performed by Sino Geno Max Co., Ltd. (Beijing, China).

Phylogenetic analyses

The SSU and ITS sequence data from isolates MFLUCC 15-0685, MFLUCC 15-0686 and reference sequences downloaded from GenBank (Table 1) were aligned by MAFFT ver.7.03 using the Q-INS-I strategy individually and in combination (Katoh and Standley 2013). Ambiguous areas of alignment were located and removed using Gblocks 0.91b (Castresana 2000). Single and combined genes analyses of SSU and ITS sequence data were carried out using Maximum-likelihood (ML) analysis performed in RAxML (Stamatakis 2006) implemented in raxmlGUI v.1.3 (Silvestro & Michalak 2012) with rapid bootstrap analysis with 1,000 replicates and "GTR+GAMMA+I" model. For the model-based Bayesian analyses, the evolutionary model that best fitted the data set was estimated using MrModeltest2.3 (Nylander 2008). Posterior probabilities (Rannala & Yang 1996; Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (MCMC) in MrBayes v. 3.2 (Ronquist *et al*. 2012).

TABLE 1. Strains and sequences used in the phylogenetic analyses

Species	Access numbers[a]	GenBank accession No.	
		SSU	ITS
Bionectria ochroleuca	IAM 14569	AB003950	
Bionectria pityrodes	CBS 246.78	AY249900	
Calcarisporium arbuscula	CBS 221.73	AY271796	AY271809
C. arbuscula	CBS 900.68	KT945002	KT945003
Calcarisporium cordycipiticola	CGMCC 3.17905, MFLUCC 15-0686	KT944998	KT944999
C. cordycipiticola	CGMCC 3.17904, MFLUCC 15-0685	KT945000	KT945001
Calcarisporium sp.	KF 525		KC800713
Claviceps fusiformis	ATCC 26019	DQ522539	JN049817
Claviceps purpurea	MAFF 240419	AB490177	
Cordyceps brongniartii	BCC 16585	JF415951	JN049867
Cordyceps militaris	OSC 93623	AY184977	JN049825
Drechmeria coniospora	CBS 596.92	AF106012	AF106018
Elaphocordyceps ophioglossoides	NBRC 106332	JN941732	JN943322
Hansfordia nebularis	CBS 115626		KF893290
Hansfordia pulvinata	CBS 254.59		KF893288
Hansfordia sinuosae	OUCMBI 121190		JX014299
Haptocillium sinense	CBS 567.95	AF339594	AJ292417
Hirsutella citriformis	ARSEF 8378	KJ803255	
Hirsutella longicolla	Strain 1458		FJ973071
Lecanicillium attenuatum	CBS 402.78	AF339609	AJ292434
Lecanicillium lecanii	IMI 304807	AF339604	JN049836
Lecanicillium psalliotae	CBS 532.81	AF339614	JN049846
Metacordyceps atrovirens	TNMF 10184	JF415950	JN049882
Metacordyceps martialis	TTZ 070716-04	JF415955	JN049871
M. martialis	HMAS 197472(S)	JF415956	JN049881
Myrothecium inundatum	IMI 158855	AY489699	
Myrothecium roridum	ATCC 16297	AY489676	
M. roridum	CGMCC 3.3682	FJ235932	FJ235932
Myrothecium verrucaria	ATCC 9095	AY489681	
Nectriopsis violacea	CBS 424.64	AY489687	
Neonectria faginata	CBS 217.67	HQ840413	AY677277
Neonectria neomacrospora	CBS 118984	HQ840406	HQ840388
Neonectria ramulariae	CBS 182.36	HQ840408	HM054157
Ophiocordyceps heteropoda	EFCC 10125	EF468957	JN049852
Ophiocordyceps sinensis	EFCC 7287	EF468971	JN049854
Ophiocordyceps stylophora	OSC 111000	DQ522552	JN049828
Pochonia gonioides	CBS 891.72	AF339599	AJ292409
Sordaria fimicola	MUCL 937	X69851	
S. fimicola	Caroline Biological Supply company 15-6291	AY545724	
Tolypocladium inflatum	SCALT 1007-002		KC963032
T. inflatum	C-1	KC963035	
Tolypocladium parasiticum	ARSEF 3436	EF468993	FJ973068
'Verticillium' epiphytum	CBS 154.61	AF339597	AJ292404

[a]ARSEF = ARS Collection of Entomopathogenic Fungal Cultures; ATCC = American Type Culture Collection; BCC = BIOTEC Culture Collection, Thailand; CBS = Centraalbureau voor Schimmelcultures, Netherlands; CGMCC = China General Microbiological Culture Collection Center, China; EFCC = Entomopathogenic Fungal Culture Collection, USA; HMAS = Herbarium of Mycology, Chinese

Academy of Sciences; IAM = Institute of Applied Microbiology, Japan; IMI = International Mycological Institute, England; MAFF = Ministry of Agriculture, Forestry and Fisheries, Japan; OSC = Oregon State University Herbarium, USA; NBRC = Distribution and Deposit of Biological Resources, Japan; OUCMBI = Ocean University of China, Microbiology Institute.

Six chains of 385,000 Markov chain Monte Carlo generations were run, sampling every generation resulted in 3,850 total trees (in two simultaneous analyses). An average standard deviation < 0.01 for split frequencies was used to suggest a convergence between parallel runs. The initial 770 trees (20%) were discarded as burn-in, and the remaining trees in each analysis were used to calculate posterior probabilities in the majority rule consensus tree (Cai *et al*. 2006). Bayesian posterior probabilities were estimated by constructing a 50% majority rule consensus tree of all trees sampled after burn-in. Trees were figured in FigTree v1.4.2 (Rambaut 2014). Bootstrap values higher than 70 % from maximum parsimony analysis (BSMP) and from RAxML (BSML), and Bayesian posterior probabilities (BYPP) greater than 0.95 are given at the nodes.

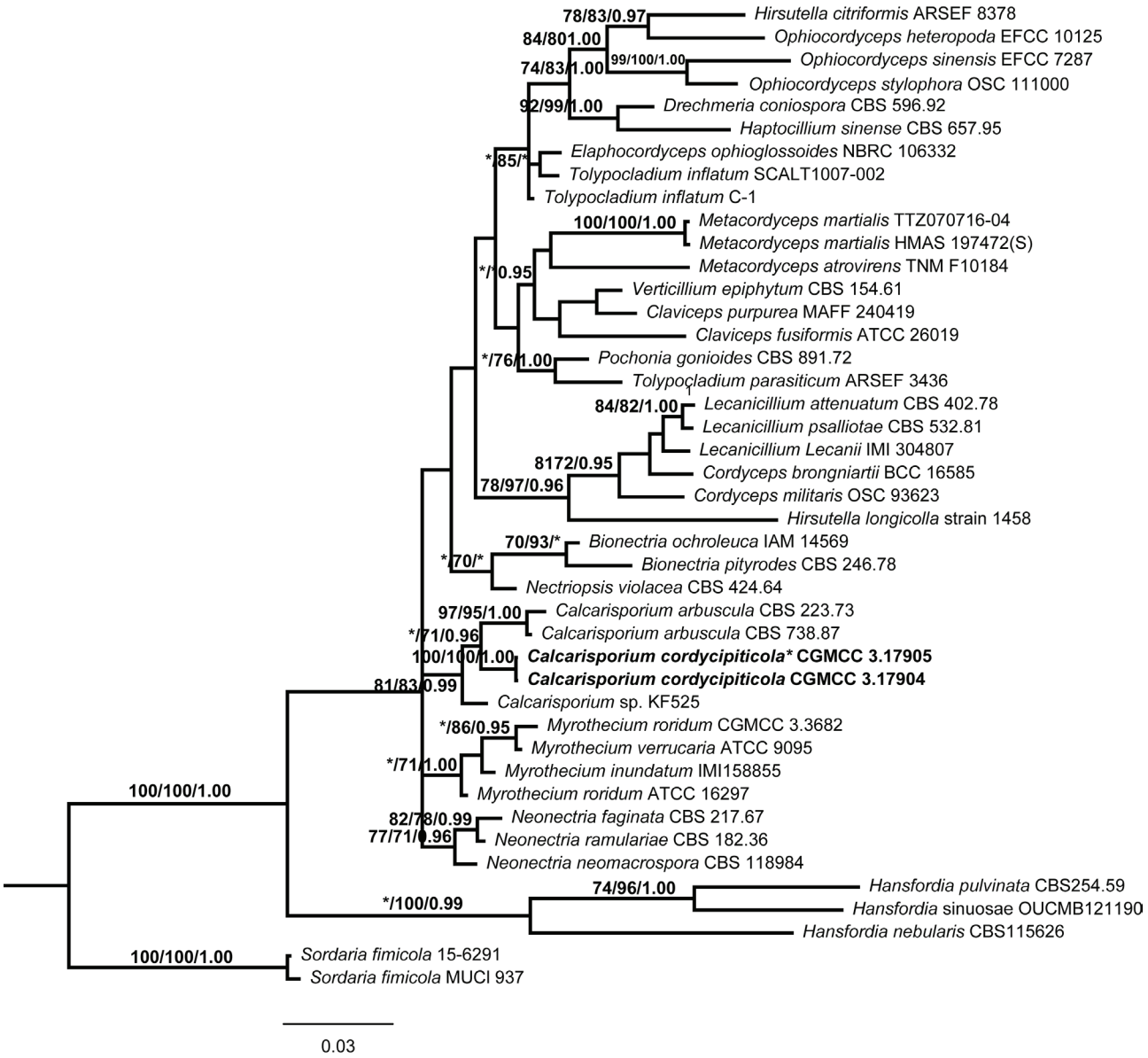

FIGURE 1. Phylogenetic analysis of *Calcarisporium cordycipiticola* and related species based on combined partial SSU and ITS sequence data. The tree is rooted with *Sordaria fimicola*. Bootstrap values higher than 50 % from maximum parsimony analysis (BSMP) (left) and from RAxML (BSML) (middle) are given above the nodes respectively. Bayesian posterior probabilities greater than 0.95 are indicated (BYPP) (right). Asterisks indicate bootstrap values of less than 70% or Bayesian posterior probabilities lower than 0.95 for a lineage. *denotes an ex- type strain.

Results

Phylogenetic analyses

Nucleotide sequence blasts of the NCBI Nucleotide Database showed that the SSU sequence of *C. cordycipiticola* exhibited 99% similarity with *Calcarisporium arbuscula* (AY271796), *Aphanocladium album* (Preuss) W. Gams (AF339568) and *Tolypocladium* spp. (Hypocreales). ITS sequence data had 91% similarity with *C. arbuscula* (AY271809).

The combination matrix included 41 in group taxa with two *Sordaria fimicola* (Roberge ex Desm.) Ces. & De Not. strains as outgroup taxon. This data matrix comprised 1436 characters, of which 1072 (71%) are constant, 94 (6.4%) are parsimony-uninformative, and 270 (12.2%) are parsimony-informative. In the parsimony analyses, 30 equally parsimonious trees were generated and the first of the most parsimonious trees is selected (tree length = 967, CI = 0.567, RI = 0.671, RC = 0.380, HI = 0.380) with parsimony bootstrap and Bayesian posterior probability values shown in Fig. 1. *C. cordycipiticola* (MFLUCC 15-0685, MFLUCC 15-0686) formed a clade with *C. arbuscula* (CBS 221.73, CBS 900.68) and *Calcarisporium* sp. (KF 525) with strong support (81% and 83% bootstrap value for MP and ML, respectively, and 99% Bayesian posterior probability). However, the *C. cordycipiticola* isolates clustered as a distinct lineage in the *Calcarisporium* clade, it is well supported by the bootstrap probabilities for both MP (100%) and ML (100%) and Bayesian posterior probability (100%) (Fig. 1).

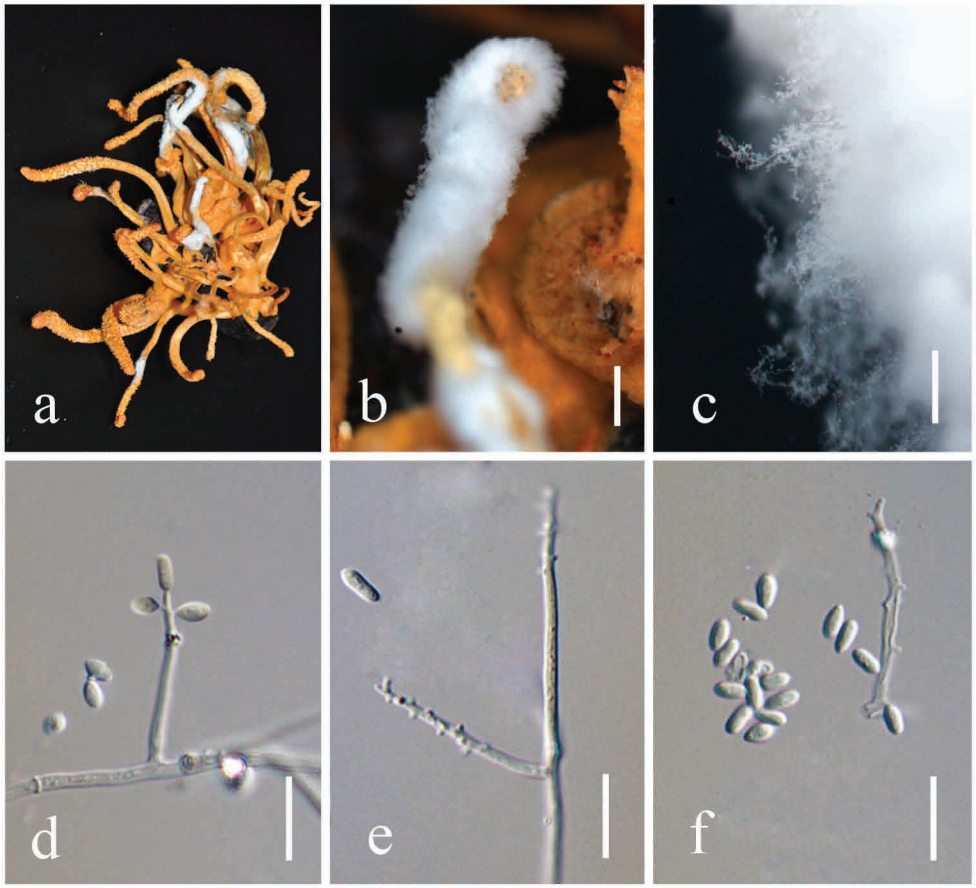

FIGURE 2. *Calcarisporium cordycipiticola* (Holotype HMAS 253931) a, b. *Calcarisporium cordycipiticola* on a fruiting body of *Cordyceps militaris*. c. Colony of *Calcarisporium cordycipiticola*; d, e. Conidiophore with and without conidia; f. Conidia. Bars: b = 5 mm; c =1000 mm; d, e, f = 10 μm.

Taxonomy

Calcarisporium cordycipiticola Jing Z. Sun, Cai H. Dong, Xing Z. Liu & K.D. Hyde, ***sp. nov.***
Figs. 2–3

Index Fungorum number: IF551382
Faces of Fungi number: FoF 00753

Etymology. *cordycipiticola*, the stem *cordycipit-* refers the host fungus *Cordycpes militaris*, the ending *-cola* means "dweller, inhabit".

Parasitic with white mycelium forming a cottony layer on the stromata of *Cordyceps militaris*. Colonies on PDA reaching 18–22 mm diam. at 20 °C after 10 days. Hyphae 2–3 µm wide. Conidiophores 18–70 × 1–3 µm, erect, hyaline, verticillate, with 1–5 verticils, each verticil with 1–15 conidiogenous cells. Conidiogenous cells 15–70 × 1.2–3 µm, gradually tapering near the apex, conidiogenous denticles 2–2.5 µm long. Conidia 3–5 × 1.5–2.5 µm, holoblastic, unicellular, hyaline, smooth-walled, thin-walled, ovoid, navicular or fusiform, base acuminate.

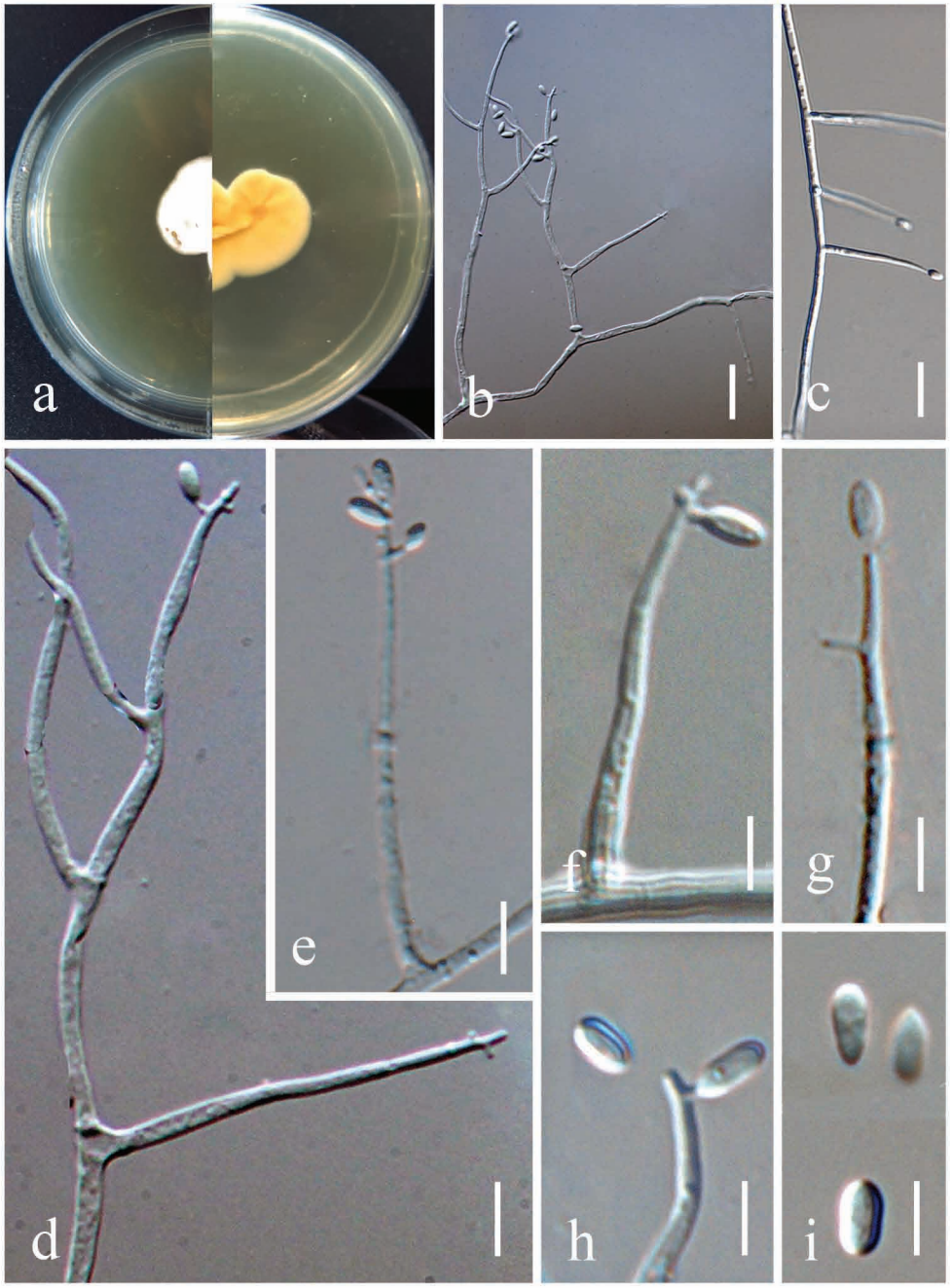

FIGURE 3. *Calcarisporium cordycipiticola* (ex-type CGMCC 3.17905, MFLUCC 15-0686) a. Surface (left) and reverse (right) of colony of *Calcarisporium cordycipiticola* on PDA medium 10 days at 20 °C. b, c, d, e, f, g. Conidiophores with conidia; h. i. Conidia. Bars: b, c, d, e, f, g = 10 µm; h, i = 5 µm.

Holotype. CHINA. Beijing: on fruiting body of *Cordyceps militaris*, 28 June 2014, Cai-Hong Dong, HMAS 253931, ex-type living culture at CGMCC 3.17905, MFLUCC 15-0686.

Other material examined. CHINA. Shanghai: on fruiting body of *C. militaris*, 11 Sep. 2012, Xing-Zhong Liu, HMAS 253930, CGMCC 3.17904, MFLUCC 15-0685;

Notes. *Calcarisporium cordycipiticola* grows on the young and mature fruiting bodies of *Cordyceps militaris*. It can be distinguished from the known species of the genus by morphology and phylogeny. It has similar sized conidia to *C. arbuscula* (4–6 × 1.7–2 μm) and *C. ovalisporum* (3–5 × 1.2–1.8 μm). However, the length/width ratio of conidia of the new species is lower than that of the above species, and *C. cordycipiticola* grows more slowly on PDA at 20 °C.

Discussion

Calcarisporium cordycipiticola has morphological features similar to *C. arbuscula*, as it produces discrete, flask-shaped conidiogenous cells, with a sympodial and denticulate apex, in whorls on primary unbranched conidiophores (Somrithipol & Jones 2006). *Calcarisporium cordycipiticola* differs markedly from *C. phaeopodium* and *C. acerosum*, mainly in the shape of the conidia. *Calcarisporium acerosum* and *C. phaeopodium* have acereose to narrowly obclavate conidia, whereas in *C. cordycipiticola* conidia are oval to ellipsoidal (Table 2). The conidia of *C. arbuscula*, *C. cordycipiticola* and *C. ovalisporum* are, however, oval to ellipsoidal (de Hoog 1974; Somrithipol & Jones 2006). There are only a few differences between these species in the size of conidiophores and conidia (Table 2). *Calcarisporium cordycipiticola* has relatively long conidiophores and a lower conidial length/width ratio. Additionally, *C. cordycipiticola* grows more slowly than *C. arbuscula* and *C. ovalisporum* and has a dense cottony context on PDA (de Hoog 1974). Phylogenetic analyses also show the distinctness of *C. cordycipiticola* with other species in the genus.

TABLE 2. Comparison of characters of *Calcarisporium* species in culture. All with verticillate conidiophores and holoblastic unicellular hyaline conidia.

Species	Source	Colony on PDA	Conidiophores	Conidia	Reference
Calcarisporium arbuscula	Diverse fungal fruitbodies	Woolly-felty texture, mycelium, white when young and greyish-pink when mature, 25–29 mm diam in 10 days at 20 °C	Hyaline, well differentiated, conidiogenous cells 12–26 × 2–3 μm	Oval to ellipsoidal (length/width ratio < 5/1), 4–6× 1.4–1.8 μm	de Hoog (1978)
C. acerosum	dead bark of *Fagus crenata*	Not described	Hyaline, basal part moderately brown, densely verticillate	Acerose to narrowly obclavate (length/ width ratio > 8/1), 10.5–18× 1.2–1.8 μm	Matsushima (1975)
C. cordycipiticola	Fruiting body of *Cordyceps militaris*	Dense villous texture, mycelium purely white, 18–22 mm diam in 10 days at 20 °C	Hyaline, less differentiated, conidiogenous cells 18–70 × 1.2–3.0 μm	Oval to ellipsoidal (length/width ratio < 5/1), 3–5 × 1.5–2.5 μm	This study
C. ovalisporum	Rust fungi	Cottony texture, mycelium yellowish-white, 33–49 mm diam in 10 days at 20 °C	Hyaline, less differentiated, conidiogenous cells 5–12 × 1.4–1.8 μm	Oval to ellipsoidal (length/width ratio < 5/1), 3–5 × 1.2–1.8 μm	de Hoog (1978)
C. phaeopodium	Dead leaf	Not described	Hyaline, basal part brown to dark, with proliferation; conidiogenous cells discrete, subulate, 6–14.5 × 2–3 μm	acerose to narrowly obclavate (length/ width ratio > 8/1), 7.5–12.5 × 0.7–1 μm	Somrithipol and Jones (2006)

Species of *Calcarisporium* may be fungicolous, caulicolous, or foliicolous. *Calcarisporium arbuscula* is common on taxa of Russulaceae (Anke & Sterner 1988) and agarics as well as Xylariaceae and other ascomycetes and is

also found on the cucumber powdery mildew fungus *Sphaerotheca fuliginea* (Schltdl.) Pollacci (Hijwegen 1989; Carrión & Rico-Gray 2002), occasionally on wood (de Hoog 1974) and is rarely isolated from soil (Barron 1968). *Calcarisporium ovalisporum* was originally reported growing on *Hirsutella citriformis* Speare, an entomopathogenic taxon growing on the brown plant hopper (Rombach and Roberts 1987). *Calcarisporium acerosum* was isolated from dead bark (Matsushima 1975), while *C. phaeopodium* was found on dead leaves (Somrithipol & Jones 2006).

Ophiocordyceps sinensis (Berk.) G.H. Sung *et al*., is one of the most valuable medicinal fungi, and is endemic to the alpine regions on the Tibetan plateau. This species has yet to be cultivated and the huge commercial demand has led to excessive harvest and a dramatic decline in its number, and the price for natural *O. sinensis* has increased dramatically (Zhang 2009). *Cordyceps militaris* has similar chemical and medicinal properties as *O. sinensis* (Zheng *et al*. 2011; Dong *et al.* 2012; De Silva et al 2013) and is relatively easily cultured in both solid and liquid media and with a variety of carbon and nitrogen sources (Shrestha 2012). This fungus has been increasingly viewed as a substitute for *O. sinensis* (Zheng *et al*. 2011; Dong *et al*. 2012). However, *C. cordycipiticola* may be a new and widespread problem in the cultivation of *C. militaris* on sterilized silkworm chrysalis, rice, wheat and other available substrate, as it grows on fruiting bodies affecting quality and yield (Cai H. Dong, personal communication). Since we only found this fungus on cultivated *C. militaris*, the origin and interaction of this taxon with its host, and its control should be further studied. In any case strict hygienic discipline is required in production of *C. militaris* in order to prevent spread of this new parasite.

Acknowledgments

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