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RESEARCH ARTICLE

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Two New Species in the Family Cunninghamellaceae from China

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ABSTRACT

The species within the family Cunninghamellaceae are widely distributed and produce important metabolites. Morphological studies along with a molecular phylogeny based on the internal transcribed spacer (ITS) and large subunit (LSU) of ribosomal DNA revealed two new species in this family from soils in China, that is, *Absidia ovalispora* sp. nov. and *Cunninghamella globospora* sp. nov. The former is phylogenetically closely related to *Absidia koreana*, but morphologically differs in sporangiospores, sporangia, sporangiophores, columellae, collars, and rhizoids. The latter is phylogenetically closely related to *Cunninghamella intermedia*, but morphologically differs in sporangiola and colonies. They were described and illustrated.

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1. Introduction

The family Cunninghamellaceae Naumov 1935 ex R.K. Benj. 1959 (Mucorales, Mucoromycetes, Mucoromycota, Fungi) has been under debate on how many genera should be circumscribed. Initially, it included three genera *Cunninghamella* Matr. 1903, *Sigmoideomyces* Thaxt. 1891, and *Thamnocephalis* Blakeslee 1905 [1,2]. Later, the genera *Benjaminia* S. Ahmad 1967, *Chaetocladium* Fresen. 1863, *Mycotypha* Fenner 1932 and *Phascolomyces* Boedijn 1959 ex Benny & R.K. Benj. 1976 were placed in this family [2–7]. However, Cannon and Kirk [8] and Benny [9] only listed the genus *Cunninghamella* as its member. Currently, the Encyclopedia of Life curates six genera in this family, that is, *Absidia* Tiegh. 1878, *Chlamydoabsidia* Hesseltine & J.J. Ellis 1966, *Cunninghamella*, *Gongronella* Ribaldi 1952, *Halteromyces* Shipton & Schipper 1975, and *Hesseltinella* H.P. Upadhyay 1970 (http://www.eol.org/, accessed on 16 November 2020). Among these, *Absidia* and *Cunninghamella* are commonly encountered.

The species of *Absidia* are not only potential pathogens for humans and animals [10] but also used to produce 11-α-hydroxylation of medroxyprogesterone and hydrocortisone [11,12]. The genus *Absidia* possesses stolons, rhizoids, and non-rhizoid-opposite apophysate sporangia with deliquescent walls [13,14], and most species have an apical projection on columellae [15,16]. They are frequently isolated from soil and grow optimally from 20 to 42 °C [17,18]. Currently, Catalogue of Life (http://www.catalogueoflife.org, accessed on 16 November 2020), curates the genus *Absidia* in Cunninghamellaceae and accommodates 31 species in this genus.

The species of *Cunninghamella* were usually studied in secondary metabolites, such as polyunsaturated fatty acids [19–21]. They are also mainly isolated from soil and grow optimally from 23 to 28 °C. Traditional morphologies for the classification of this genus include colonies, sporangiophores, vesicles, and sporangiola [22]. It is now comprised of 14 species and three varieties [22,23].

With the development of molecular biology, the rDNA internal transcribed spacer (ITS) has become the DNA barcode for Fungi [24]. However, *Absidia* was highly variable in ITS sequences [14,17,18]. Consequently, other molecular markers, such as the rDNA large subunit (LSU rDNA) and the gene for actin (*act1*), were combined with ITS in phylogenetic analyses [17]. For *Cunninghamella*, the ITS rDNA sequence could effectively identify species

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[25,26]. Afterward, the LSU rDNA and EF-1α sequences were also applied to reconstruct the evolutionary relationship in *Cunninghamella* [23,27]. Herein the ITS and LSU rDNA were used for reconstructing the molecular phylogenetic tree.

On the basis of a combination of morphological traits and ITS/LSU rDNA sequences, two new species within the family Cunninghamellaceae in China, each belonging to *Absida* and *Cunninghamella*, will be proposed in this study.

2. Materials and methods

2.1. Soil sample collection and strains isolation

Soil samples were collected from Xinjiang (44°43′10″N, 86°17′03″E), Beijing (40°33′50″N, 116°26′26″E) and Yunnan (102°54′30″N, 23°42′49″E) in China. A portion of the soil (1 g) was suspended in sterile water (100 mL) and shaken vigorously. Then, 100 µL of the suspension were spread evenly on a potato dextrose agar (PDA, 20 g/L glucose, 20 g/L agar, 200 g/L potato, and 1000 mL distilled water) plate with antibiotics streptomycin sulfate (100 mg/mL) and ampicillin (100 mg/mL), and incubated darkly at 25 °C. The PDA plate was examined daily with a stereomicroscope (SMZ1500, Nikon, Tokyo, Japan). Upon the presence of colonies, they were transferred to new PDA plates. The living culture was deposited in the China General Microbiological Culture Collection Center, Beijing, China (CGMCC). The dried cultures were deposited in the Herbarium Mycologicum Academiae Sinicae, Beijing, China (HMAS).

2.2. Morphology and growth temperature

Modified synthetic mucor agar (SMA: dextrose 20 g, asparagine 2 g, KH_2PO_4 0.5 g, $MgSO_4{\cdot}H_2O$ 0.25 g, thiamin chloride 0.5 mg, agar 20 g, 1000 mL distilled water, pH7) was used for morphological studies and maximum growth temperature tests [22]. For morphological observation, cultures were incubated at 28 °C for 9 d, and daily examined under a microscope (Axio Imager A2, Carl Zeiss, Oberkochen, Germany); while for determining maximum growth temperature, they were initially incubated at 30 °C for 2 d, and then the incubation temperature increased until the colonies stopped growing. Specifically, a field emission scanning electron microscope (1430VP, Carl Zeiss, Oberkochen, Germany) was used to detect the surface of the sporangia.

2.3. DNA extraction, amplification, and sequencing

Mycelia were grown at 25 °C for 5 d on PDA plates, and then cell DNAs were extracted using a kit (GO-GPLF-400, GeneOnBio Corporation, Changchun, China). The primers NS5M (5′-GGC TTA ATT TGA CTC AAC ACG G-3′) and LR5M (5′-GCT ATC CTG AGG GAA ACT TCG-3′) were used to amplify a fragment covering partial SSU, entire ITS, and partial LSU rDNA [28]. The PCR program was performed with an initial temperature at 95 °C for 5 min, then 30 cycles of denaturation at 95 °C for 60 s, annealing at 55 °C for 45 s and extension at 72 °C for 60 s, and finally an extra extension at 72 °C for 10 min. The PCR product was sequenced with the primer LR5M, as well as ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS5 (5′-GGA AGT AAA CGT AAC AAG G-3′) [29,30].

Table 1. The taxa of *Absidia* and *Cunninghamella* used in phylogenetic analyses.

		GenBank accession numbers	
Species	Strains	ITS rDNA	LSU rDNA
A. caatinguensis	URM 7156	KT308169	KT308171
A. caerulea	CBS 101.36	MH855718	MH867230
	CBS 103.28	MH854938	MH866431
A. californica	CBS 314.78	MH861141	MH872902
	FSU 4747	AY944872	EU736300
A. glauca	CBS 129233	MH865253	MH876693
	CBS 127122	MH864429	MH875867
A. koreana	EML-IFS45-2	KR030063	KR030057
	EML-IFS45-1	KR030062	KR030056
A. macrospora	FSU 4746	AY944882	EU736303
A. ovalispora sp. nov.	CGMCC 3.16018T†	MW264130	MW264071
	CGMCC 3.16019	MW264131	MW264072
A. panacisoli	SYPF 7183	MF522181	MF522180
	CBS 140959	NR_159563	NG_063948
A. pararepens	CCF 6352	MT193669	MT192308
	CCF 6351	MT193670	MT192307
A. pseudocylindrospora	CBS 100.62	NR_145276	MH869688
A. psychrophilia	FSU 4745	AY944874	EU736306
A. repens	CBS 115583	NR_103624	HM849706
A. spinosa	FSU 551	AY944887	EU736307
	FSU 552	AY944888	EU736308
A. spinosa var. *spinosa*	CBS 106.08	JN205809	JN206590
A. stercoraria	EML-DG8-2	KU168829	KT921999
	EML-DG8-1	KU168828	KT921998
C. binariae	CBS 481.66	MH858865	MH870507
	CBS 782.68	JN205869	MH870950
C. clavata	CBS 100178	JN205890	HM849696
C. echinulata	CBS 156.28	JN942997	JN939199
	CBS 766.68	JN205894	MH877699
C. elegans	EML-RUS1-2	MF806021	MF806028
	EML-RUS1-1	MF806023	MF806027
	CBS 167.53	MH857146	HM849700
C. gigacellularis	URM 7400	NR_168760	NG_068773
C. globospora sp. nov.	CGMCC 3.16020T†	MW264132	MW264073
	CGMCC 3.16021	MW264133	MW264074
C. homothallica	CBS 168.53	MH857147	MH868684
C. intermedia	CBS 347.69	MH859320	JN206606
C. phaeospora	CBS 692.68	AF254934	NG_058812
C. polymorpha	CBS 779.68	JN205874	JN206599
	CBS 693.68	JN205871	JN206600
C. vesiculosa	CBS 989.96	JN205897	HM849693
Mucor janssenii†	CBS 205.68	MH859119	MH870832

†The "T" represents ex-holotype strains. *Mucor janssenii* serves as outgroup.

2.4. Phylogenetic analyses

ITS and LSU rDNA sequences were used for de novo assembly, manual proofreading, and target extraction with Geneious 8.1 (http://www.geneious.com). For reconstructing a phylogenetic tree, BLAST research was performed in order to retrieve related sequences (Table 1). All the sequences were realigned locally using AliView version 3.0 [31]. Phylogenetic analyses were carried out following the methods by Nie et al. [32,33], including maximum-parsimony (MP), maximum-likelihood (ML), and Bayesian Inference (BI) implemented in PAUP version 4.0b10 [34], RAxML version 8 [35] and MrBayes 3.2.7a [36], respectively. MP analyses were conducted using 1000 heuristic search replicates. The best models for the ML and BI analyses were selected with Akaike Information Criterion (AIC) by using jModelTest 2.1.7 [37,38]. ML tree was reconstructed with 1000 bootstrap replicates [35]. In the BI analyses, Markov Chain Monte Carlo (MCMC) chains ran until the convergences met and the standard deviation fell below 0.01. The phylogram was viewed and modified with FigTree version 1.4.4 [39]. Sequence alignments and phylogenetic trees were deposited at TreeBase (submission ID S27294).

3. Results

3.1. Taxonomy

Absidia ovalispora **H. Zhao & X.Y. Liu sp. nov.**
MycoBank No.: 838024
Figures 1 and 2

Typification: CHINA, YUNNAN: Jianshui County, Honghe Hani and Yi Autonomous Prefecture, 102°54′30″N, 23°42′49″E, from soil sample, December 28, 2018, Min Qiao (holotype HMAS 249157, living ex-holotype culture CGMCC 3.16018. GenBank: ITS = MW264130; LSU = MW264071)

Additional culture examined: CHINA, YUNNAN: Jianshui County, Honghe Hani and Yi Autonomous Prefecture, 102°54′30″N, 23°42′49″E, from a soil sample, December 28, 2018, Min Qiao (HMAS 249158, living culture CGMCC 3.16019. GenBank: ITS = MW264131; LSU = MW264072).

Etymology: *ovalispora* (Lat.), referring to the shape of the sporangiospores.

Ecology and distribution: Soil in Yunnan Province, China.

Morphological characteristics: *Colonies* on SMA reaching 9 cm at 28 °C for 7 d, floccose, initially white, quickly grayish-white, and finally brown, irregular at the edge. *Hyphae* flourishing. *Rhizoids* always simple and fingerlike. *Stolons* present. *Sporangiophores* on stolons, erect, single or 2–6 verticillate, 27.96–212.96 µm long and 2.14–6.39 µm wide, occasionally septate or swollen 20–30 µm below the sporangia. *Apophyses* 2.14–14.92 µm long and 5.15–23.94 µm wide. *Collars* absent. Columellae globose to ellipsoidal, 7.99–20.80 µm long and 7.01–23.88 µm wide, always with an apical projection. *Sporangia* globose to ellipsoidal, 12.54–40.80 µm long and 12.47–38.54 µm wide. *Sporangiospores* ovoidal to ellipsoidal, 2.13–5.38 µm long and 1.73–4.57 µm wide. *Chlamydospores* not observed. *Zygospores* not observed.

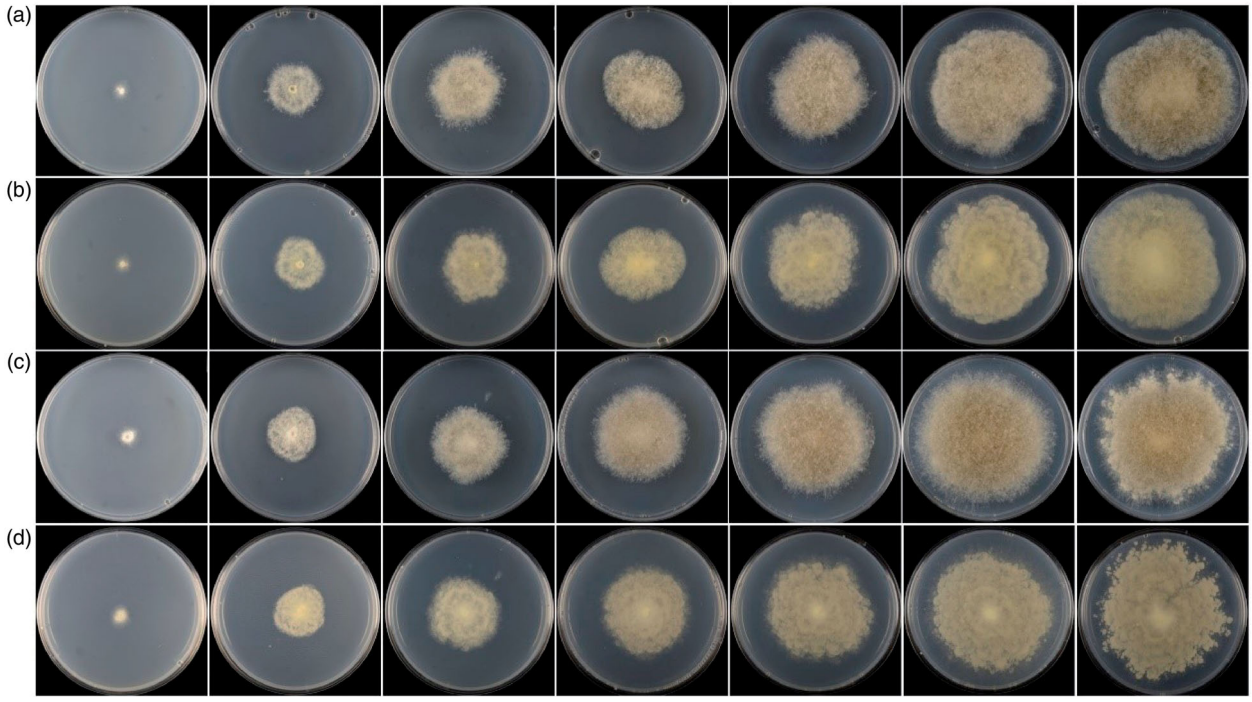

Figure 1. Colonies of *Absidia ovalispora* sp. nov. on SMA at 28 °C in 1–7 d (from left to right). (a) Obverse of CGMCC 3.16018 (ex-holotype strain); (b) Reverse of CGMCC 3.16018 (ex-holotype strain); (c) Obverse of CGMCC3.16019; (d) Reverse of CGMCC3.16019.

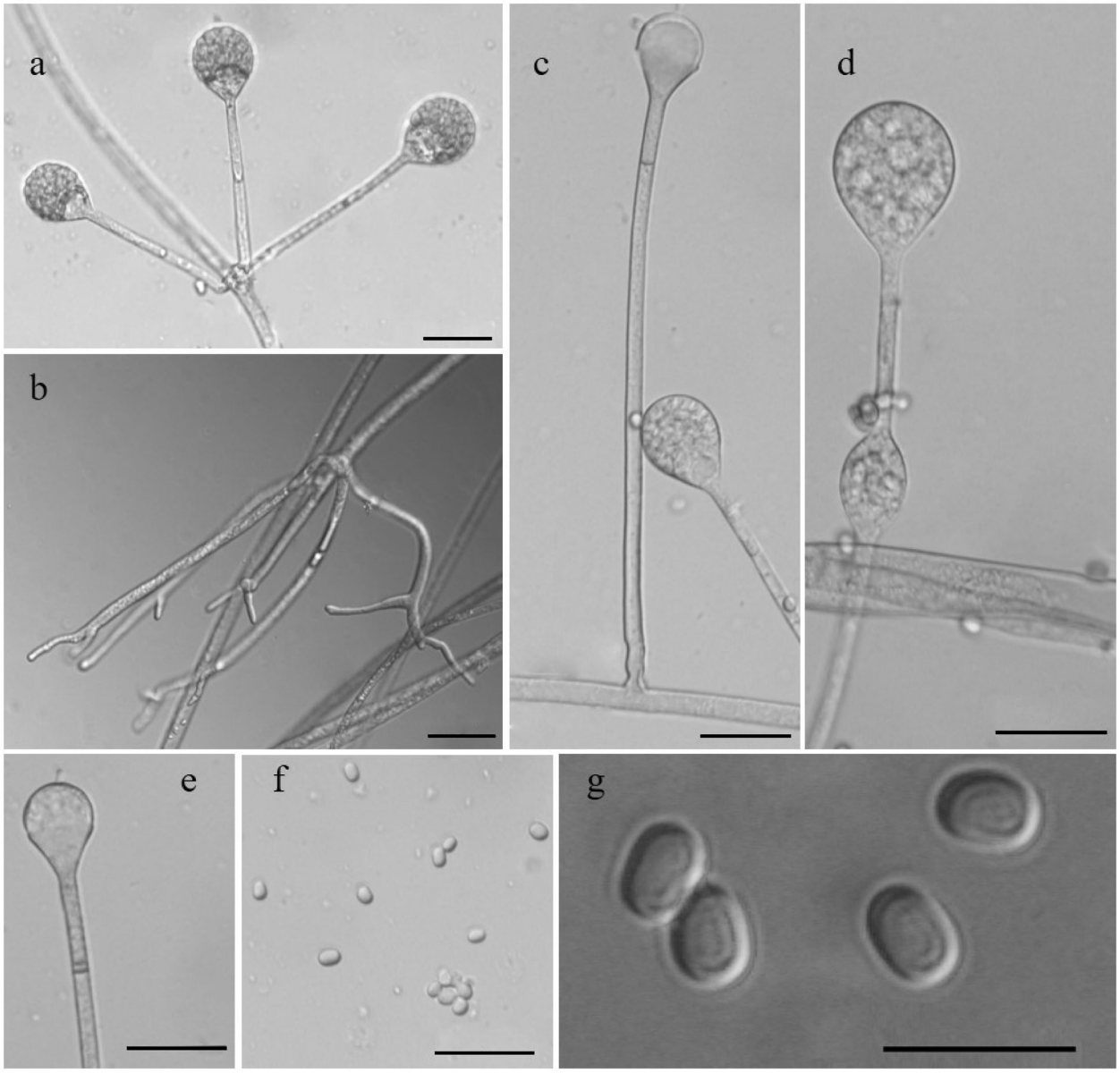

Figure 2. Morphology of *Absidia ovalispora* sp. nov. CGMCC 3.16018 (ex-holotype strain). (a) Verticillately branched sporangiophores; (b) Rhizoids; (c) Single sporangiospore; (d) Swelling on sporangiosphores; (e) Columella with a septum and an apical projection; (f, g) Sporangiospores. Scale bars: (a, c–f) = 20 μm; (b) = 50 μm; (g) = 10 μm.

Maximum growth temperature: 37 °C.

Note: On the medium SMA, *Absidia ovalispora* is distinguished from its allied species *Absidia koreana* H.B. Lee, H.W. Lee & T.T.T. Nguyen 2015 in sporangiospores, sporangia, sporangiophores, columellae, collars, and rhizoids. Sporangiospores are ovoidal to ellipsoidal in *A. ovalispora*, while short-cylindrical or cylindrical in *A. koreana* [40]. Sporangia/sporangiophores/columellae in *A. ovalispora* (12.54–40.80 μm × 12.47–38.54 μm/2.14–6.39 μm wide/7.99–20.80 μm × 7.01–23.88 μm) are all bigger than those in *A. koreana* (19.33–23.64 μm × 21.06–26.35 μm/3.84–4.60 μm wide/10.90–16.96 μm × 11.46–18.89 μm) [40]. Collars are absent in *A. ovalispora* but present in *A. koreana* [40]. Rhizoids are always observed in *A. ovalispora* but were not described in *A. koreana* [40]. Apart from these differences, they are similar in the branching manner, that is, single or up to 6 sporangiophores are arising from the same point on the stolons. This paper reported two strains in the *A. ovalispora*. It is worth noting the difference in colonies between these two strains, that is, the strain CGMCC 3.16019 is lighter, more vigorous than the ex-holotype strain CGMCC 3.16018. Moreover, CGMCC 3.16019 extends some small satellite colonies around the main colony (Figure 1).

***Cunninghamella globospora* H. Zhao & X.Y. Liu sp. nov.**

MycoBank No.: 838023

Figures 3 and 4

Typification: CHINA, XINJIANG: Manas County, 44°43′10″N, 86°17′03″E, from the soil sample in salt marshes, July 1, 2015, Jing Zhu, (holotype

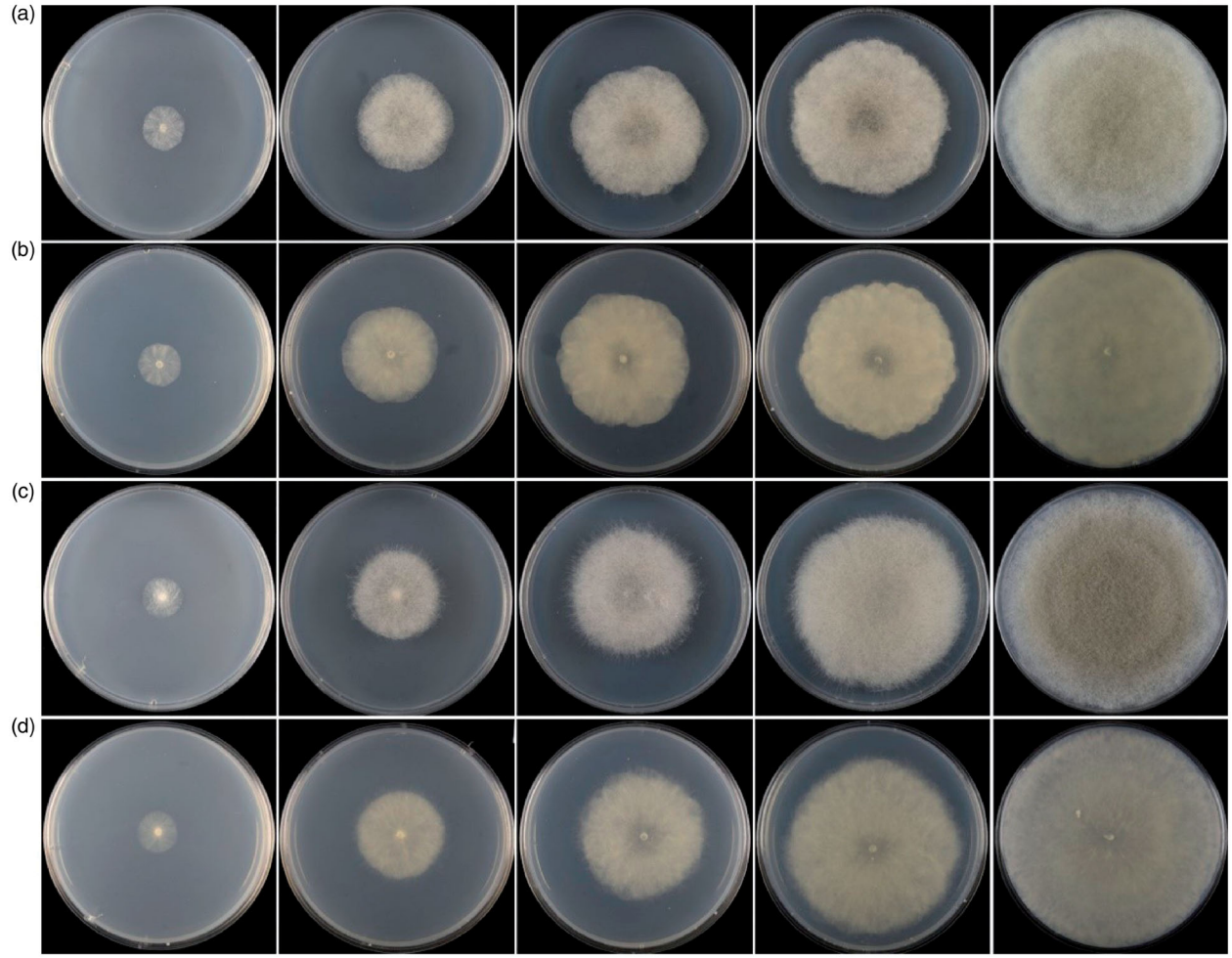

Figure 3. Colonies of *Cunninghamella intermedia* CBS 347.69 and *C. globospora* sp. nov. CGMCC 3.16020 (ex-holotype strain) on SMA at 28 °C in 1–5 d. (a) Obverse of CBS 347.69; (b) Reverse of CBS 347.69; (c) Obverse of CGMCC 3.16020; (d) Reverse of CGMCC 3.16020.

HMAS 249159, living ex-holotype culture CGMCC 3.16020. GenBank: ITS = MW264132; LSU = MW264073.)

Additional culture examined: CHINA, BEIJING: Yanqing County, 40°33′50″N, 116°26′26″E, from a soil sample, November 13 2020, Tong-Kai Zong, (HMAS 249160, living culture CGMCC 3.16021. GenBank: ITS = MW264133; LSU = MW264074).

Etymology: *globospora* (Lat.), referring to the shape of the sporangiospores.

Ecology and distribution: Soil in Xinjiang and Beijing, China.

Morphological characteristics: Colonies on SMA floccose, reaching 9 cm in 5 d at 28 °C, initially white, soon gray, finally gray-brownish, and gray reversely. *Hyphae* branched, 4.34–18.00 µm diameter, and septate when old. *Stolons* present. *Rhizoids* simple, and fingerlike. *Sporophores* erect or recumbent, arising from stolons and aerial hyphae, main axes usually aequilate and 4.79–24.85 µm diameter, rarely gradually thicken upwards, primary branches 37.85–459.72 µm long and 2.86–13.74 µm wide, rebranching or third-branching into branchlets. *Vesicles* hyaline to brownish, globose or subglobose, sometimes expanded at the bottom, bearing lots of pedicels which are left after sporangiola fall off, axial vesicles 19.04–43.62 µm diameter, and lateral vesicles 13.74–27.46 µm diameter. *Pedicels* 1.00–3.07 µm long. *Sporangiola* globose, 9.09–14.73 µm diameter, and growing on vesicles with pedicels. *Chlamydospores* absent. *Zygospores* absent.

Maximum growth temperature: 46 °C.

Notes: On the medium SMA at 28 °C, the *Cunninghamella globospora* is distinguished from its closely related species *Cunninghamella intermedia* K.B. Deshp. & Mantri 1966 by sporangiola and colonies. The species *C. intermedia* was nominated with the sporangiola being intermediate among four species [41]. The sizes of sporangiola in the new species *C. globospora* (9.09–14.73 µm diameter) are smaller and more uniform than those in *C. intermedia* (8.00–23.00 µm diameter) [22]. And, colonies are gray-brownish in *C. globospora* but "Hair Brown" in *C. intermedia* [22]. Except for these discrepancies, they are similar in other characteristics, such as branched and septate hyphae, fingerlike rhizoids, and long primary branches [22].

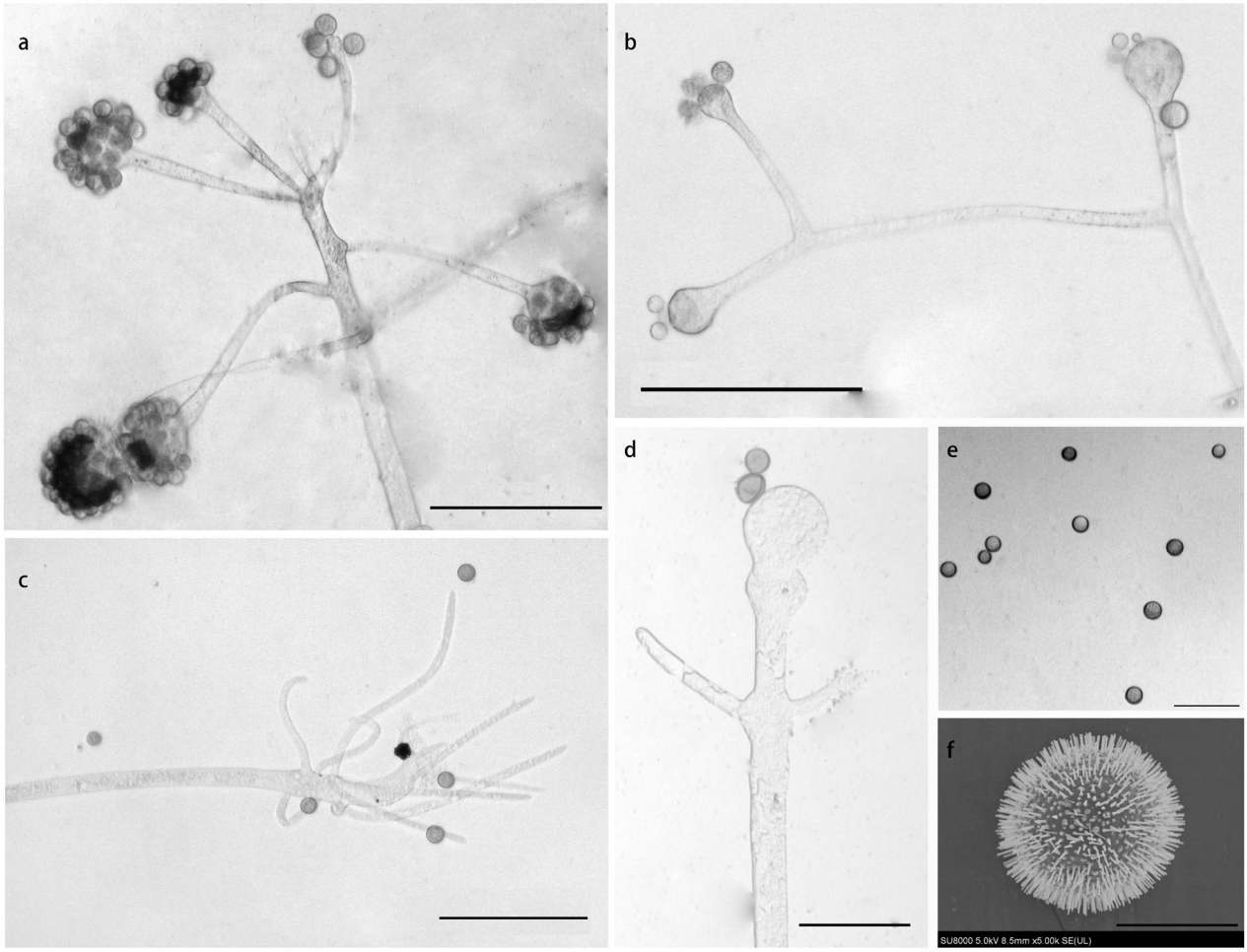

Figure 4. Morphology of *Cunninghamella globospora* sp. nov. CGMCC 3.16020 (ex-holotype strain). (a, b) Sporophores showing characteristic branching patterns; (c) Rhizoids; (d–f) Sporangiola. Scale bars: (a–c) = 100 μm; (d, e) = 50 μm; (f) = 10 μm.

3.2. Phylogenetic analyses

The combined ITS and LSU rDNA dataset consisted of 42 taxa representing 26 species, and 2108 characters including 811 constant, 303 parsimony-uninformative, and 994 parsimony-informative. The generated MP tree length (TL), consistency index (CI), homoplasy index (HI), retention index (RI) and rescaled consistency index (RC) were 5227, 0.4534, 0.5466, 0.6629 and 0.3006, respectively. The optimal model for the BI inference was GTR + I + G, and the final optimization likelihood value is −22601. When convergent, and the average standard deviation of split frequencies was 0.008871. The topologies of the three phylogenetic trees were similar. Hence, the ML tree was selected to represent the evolutionary history, being supported with ML bootstrap, MP bootstrap, and BI posterior probability values (Figure 5). *Absidia ovalispora* was closely related to *A. koreana* (ML = 89, MP = 36, BI = 1.00); and *Cunninghamella globospora* was closely related to *C. intermedia* (ML = 100, MP = 100, BI = 1.00).

4. Discussion

The genus *Absidia* is characterized by sporangiophores originating from stolons, apophysate sporangia with deliquescent walls, septa usually seen below sporangia, and heterothallic zygospores with appendages [9,17,40]. The species of *Absidia* were always isolated from soils and animal dung [42]. In this study the new species of *Absidia ovalispra* also possesses these morphological features and was isolated from soil in Xinjiang, China.

In the genus *Cunninghamella*, stable traits used for identification are sporangia, zygospores, maximum growth temperatures, mating compatibilities, and ITS rDNA sequences [22]. According to this criterion, *Cunninghamella bigelovii*, *Cunninghamella gigacellularis*, and *Cunninghamella guizhouensis* were recently proposed [23,27,43]. In this paper, *C. globospora* was proposed from China based on molecular data and morphological observation.

The phylogenetic analyses on ITS and LSU rDNA (Figure 1) showed that *A. ovalispora* and *C. globospora* were closely related to *A. koreana* (ML = 89, MP = 36, BI = 1.00) and *C. intermedia* (ML = 100,

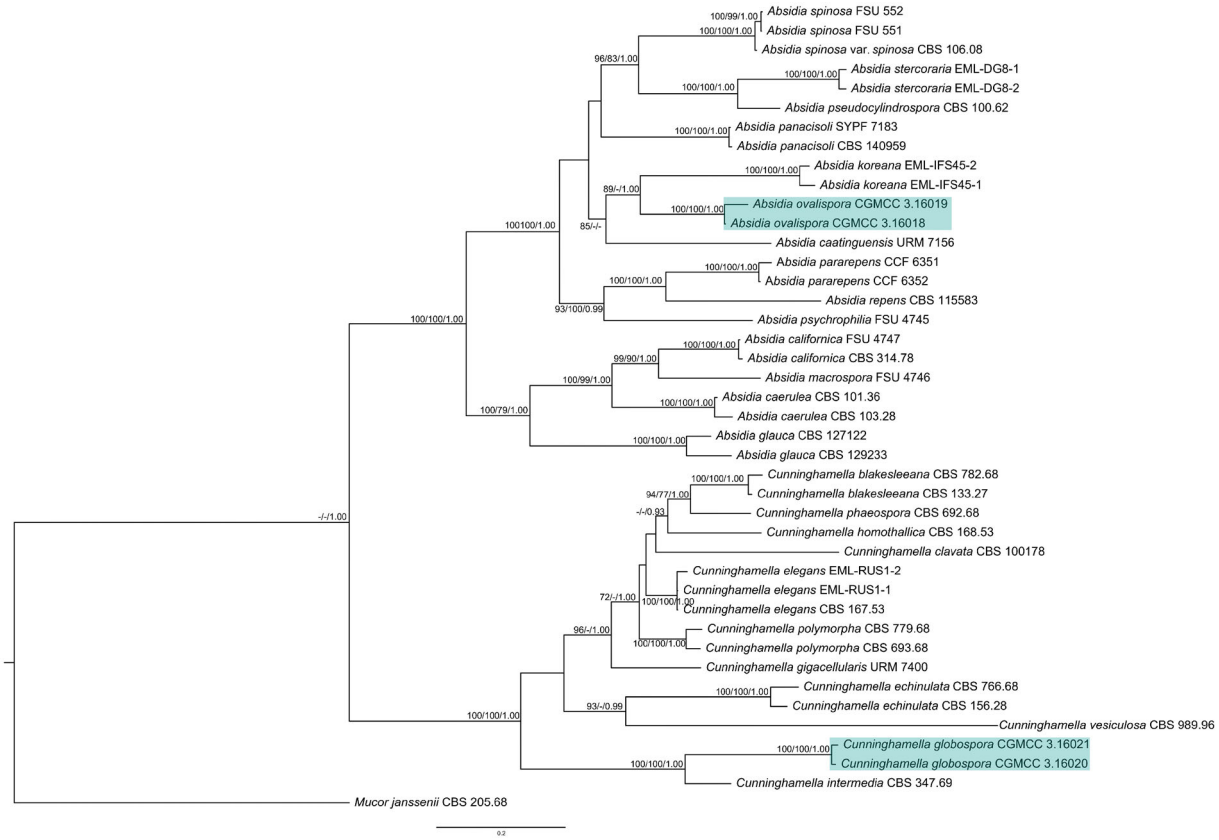

Figure 5. Maximum-likelihood phylogenetic tree of *Absidia* and *Cunninghamella* based on ITS and LSU rDNA sequences, with *Mucor janssenii* as outgroup. Two new species *A. ovalispora* sp. nov. and *C. globospora* sp. nov. are in shade. Maximum-parsimony (MP) bootstrap values (≥70%)/maximum-likelihood (ML) bootstrap values (≥70%)/Bayesian Inference (BI) posterior probabilities (≥0.95) of each clade are indicated along branches. Scale bar indicates substitutions per site. "T" indicates the ex-holotype cultures.

MP = 100, BI = 1.00), respectively. Their differences in morphology were compared in the respective Notes part. Apart from these, the two new species were both distinct from their allies in the shape of sporangiospores, and thus they were nominated. The maximum growth temperature of *C. intermedia* was 43 °C in a previous study [22], but 46 °C in this study, same as in the new species *C. glaobospora*. It is maybe because that the strains are different, IMI 200623 in Zheng and Chen [22] and CBS 347.69 in this study.

With the proposal of these two species of Cunninghamellaceae, the species and variety number of *Absidia* and *Cunninghamella* are now 32 and 18, respectively.

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Author contributions

Heng zhao, Jing Zhu, Zhi-Dong Zhang, and Xiao-Yong Liu conceived and designed this research. Heng Zhao analyzed data. Xiao-Yong Liu improved the manuscript. Tong-Kai Zong, Qing Lin, and Min Qiao collected and isolated samples. Other authors conducted part experiments and advised on analyses.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The datasets generated during the current study are available in the TreeBASE repository at https://treebase.org/.

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