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Conlarium sichuanense sp. nov., on *Ficus virens* from Sichuan Province, China

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Abstract

A new hyphomycetous species, *Conlarium sichuanense* was found on dead branches of *Ficus virens* (*Moraceae*) from a terrestrial habitat in Sichuan Province, China. The new species has sporodochial colonies on natural substate, with micronematous conidiophores and muriform, brown and subglobose to irregular-shaped conidia. The phylogenetic analysis based on combined LSU, ITS and SSU sequence data showed that *Conlarium sichuanense* clustered together with *C. aquaticum* and *C. thailandense* and presented as a distinct lineage. A detailed, illustrated description and comparison with related *Conlarium* species are provided.

Keywords: 1 new species, Asexual morph, Multi-gene, Phylogeny, Taxonomy

Introduction

The genus *Conlarium* was introduced by Liu *et al.* (2012) with *C. dupliciascosporum* as type species. Sexual morph of *C. dupliciascosporum* was reported from submerged wood in a freshwater stream, which has dark brown to black ascomata with a long neck, cylindrical asci with a large apical ring and fusiform ascospores with or without papillary appendages, while asexual morph was reproduced from culture, characterized by micronematous or semi-micronematous conidiophores, monoblastic, doliiform, cylindrical conidiogenous cells and mostly irregular, brown, muriform conidia (Liu *et al.* 2012). *Conlarium* was originally assigned in *Sordariomycetidae* or *Sordariomycetes* genera *incertae sedis* (Liu *et al.* 2012, Maharachchikumbura *et al.* 2015). Zhang *et al.* (2017) introduced the first asexual morph of *Conlarium*, namely *C. aquaticum* on natural substrate and established *Conlariaceae* to accommodate *Conlarium*. Based on the phylogenetic analysis (LSU, ITS and SSU and *rpb2* sequence data), a new order *Atractosporales* was introduced by Zhang *et al*. (2017) to include *Atractosporaceae*, *Conlariaceae* and *Pseudoproboscisporaceae.* Luo *et al.* (2019) treated *Conlariaceae* as *Diaporthomycetidae* family *incertae sedis* due to its uncertain phylogenic affinity with the type family *Atractosporaceae* of *Atractosporales*. Hyde *et al.* (2021) raised *Conlariaceae* to *Conlariales* since the stem age of *Conlariaceae* (138 MYA) fall within the order range suggested by Hyde *et al.* (2017) based on the divergence time establishment and we follow their treatment in this study. Up to date, eight species are accepted in *Conlarium,* of which *C. aquaticum*, *C. dupliciascosporum* and *C. subglobosum* were reported from freshwater habitats (Liu *et al*. 2012, Zhang *et al*. 2017, Dong *et al*. 2021), and *C. indicum* and *C. thailandense* were known from terrestrial habitats (Phookamsak *et al*. 2019, Dubey & Manikpuri 2021). Moreover, *Conlarium baiseense*, *C. nanningense* and *C. sacchari* were reported as endophytes isolated from sugarcane rhizosphere (Xie *et al.* 2019).

In this study, we introduce a new species collected on dead branches of *Ficus virens* from terrestrial habitats in Sichuan Province, China. Based on morphological characteristics coupled with phylogenetic analysis of combined LSU, ITS and SSU sequence data, it belongs to *Conlarium* and can be recognized as a new species *C. sichuanense*. Detailed description and illustration are provided, as well as the comparison among *Conlarium* species.

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Materials and methods

Isolation and morphology

Samples were collected from decaying woods in Sichuan Province, China in November 2020. The specimens were taken to the laboratory in paper envelopes. Fungal fruiting bodies were observed using a Motic SMZ (Stereoscopic Zoom Microscope) 168 series stereomicroscope and photographed by a Nikon E80i microscope-camera system. Measurements were taken using Tarosoft Image Frame Work program v. 0.9.7 (Liu *et al.* 2010) and images used for photo-plate were processed with Adobe Photoshop CS6. Single conidium isolation was made following the method described in Senanayake *et al.* (2020). Germinated conidia were individually transferred to potato dextrose agar (PDA) media plates and incubated at 25 ˚C.

Herbarium specimens were deposited at the Herbarium of Cryptogams, Kunming Institute of Botany Academia Sinica (HKAS), Kunming, China, and Herbarium, University of Electronic Science and Technology (HUEST), Chengdu, China. The living cultures were deposited in the China General Microbiological Culture Collection Center (CGMCC) in Beijing, China, and University of Electronic Science and Technology Culture Collection (UESTCC) in Chengdu, China.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from fresh fungal mycelia grown on PDA at 25 ˚C after three months by using Tsingke Fungus Genomic DNA Extraction Kit (TSINGKE Biotech, Shanghai, P.R. China) according to the manufacturer's instructions. DNA amplification was performed by Polymerase Chain Reaction (PCR) for three gene regions, namely the large subunits of the nuclear ribosomal RNA genes (LSU), the internal transcribed spacers (ITS) and the small subunits of the nuclear ribosomal RNA (SSU). The primers used were LR0R/LR5 (Vilgalys & Hester 1990) for LSU, ITS5/ITS4 (White *et al.* 1990) for ITS and NS1/NS4 (White *et al.* 1990) for SSU. PCR was carried out in 25 μL reaction volume containing 12.5 μL PCR Master Mix (Sangon Biotech, Shanghai, P.R. China), 9.5 μL ddH_2O, 1 μL of DNA template and 1 μL of each primer. The amplification condition for all three genes consisted of initial denaturation at 94 °C for 3 min; followed by 40 cycles of 45 s at 94 °C, 50 s at 56 °C and 1 min at 72 °C, and a final extension period of 10 min at 72 °C. PCR products were sequenced using the same set of primers used in PCR in Beijing Tsingke Biological Engineering Technology and Services Co. Ltd. (Beijing, P.R. China).

Phylogenetic analyses

Sequences generated from different primers were analyzed with other sequences obtained from GenBank. The related sequences were determined by using a BLAST search to reveal the closest matches with taxa in *Conlarium* (TABLE 1). Individual loci were aligned using MAFFTv.7 (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013) and manually improved when necessary with Aliview (Larsson & Anders 2014), and then were concatenated with Mesquite v. 3.01 (Maddison & Maddison 2014).

Phylogenetic analysis was performed by maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) as detailed in Dissanayake *et al*. (2020). A maximum likelihood analysis was performed using raxmlGUI v. 1.3 (Silvestro & Michalak 2011) with 1,000 rapid bootstrap replicates. The final tree was selected among suboptimal trees from each run by comparing likelihood scores under the GTR+GAMMA substitution model. Maximum parsimony analyses were performed using PAUP v. 4.0b10 (Swofford 2002) with the heuristic search option with 1,000 random taxa addition and tree bisection and reconnection (TBR) as the branch-swapping algorithm. The branches of zero length were collapsed and all equally parsimonious trees were saved. Tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC], homoplasy index [HI] were calculated. Bayesian analyses was performed with MrBayes v.3.2.2 (Ronquist *et al*. 2012) and posterior probabilities (PP) were determined by Markov Chain Monte Carlo sampling (MCMC). The best-fit model of evolution was determined with MrModeltest v. 2.2 (Nylander 2004), SYM+I+G substitution model was decided for ITS, GTR+I for LSU and GTR for SSU. Bayesian analyses of four simultaneous Markov chains were run for 10,000,000 generations with trees sampled every 1,000th generations. The first 25% of trees, representing the burn-in phase of the analyses, were discarded and the remaining trees were used for calculating posterior probabilities in the majority rule consensus tree.

Phylogenetic tree was visualized by FigTree v.1.4.0 (Rambaut 2006) and further edited using Adobe Illustrator (Adobe Systems, USA).

TABLE 1. Taxa used in this study and GenBank accession numbers.

Species	Voucher/Culture	GenBank accession numbers		
		LSU	ITS	SSU
Conlarium aquaticum	**MFLUCC 15–0992**	**MF374363**	**MF374354**	**MF374372**
Conlarium aquaticum	MFLUCC 18–0338	MK849850	MK828698	N/A
Conlarium aquaticum	MFLUCC 18–1417	MW287759	MW286486	N/A
Conlarium baiseense	HMAS 247986	MK164655	MK164653	MK164657
Conlarium baiseense	**HMAS 247298**	**MF083158**	**MF083157**	**MF083159**
Conlarium dupliciascosporum	**CGMCC 3.14938**	JN936991	JN936995	**JN936987**
Conlarium dupliciascosporum	CGMCC 3.14939	JN936992	JN936996	JN936988
Conlarium dupliciascosporum	CGMCC 3.14940	JN936993	JN936997	JN936989
Conlarium indicum	**NFCCI 4841**	**MT586317**	**MT586318**	**N/A**
Conlarium nanningense	**HMAS 247075**	**KX886202**	**KX886204**	**KX886203**
Conlarium nanningense	HMAS 247985	MK164656	MK164654	MK164658
Conlarium sacchar	**HMAS 247299**	**MF083167**	**MF083166**	**MF083168**
Conlarium sacchari	HMAS 247300	MF083164	MF083163	MF083165
Conlarium sacchari	**HMAS 247301**	**MF083161**	**MF083160**	**MF083162**
Conlarium sichuanense	**CGMCC 3.20406**	**MZ519553**	**MZ519551**	**MZ519549**
Conlarium sichuanense	UESTCC 21.0015	MZ519554	MZ519552	MZ519550
Conlarium subglobosum	**MFLU 17–1728**	**MW287768**	**MW286494**	**N/A**
Conlarium thailandense	**MFLUCC 1–2349**	**MH624127**	**MH624129**	**MH624128**
Riomyces rotundus	**AF303–1 (ILL)**	**JF775589**	**N/A**	**N/A**
Atractospora aquatica	S–1297	MK849849	MK828697	MK828308
Atractospora aquatica	MFLU 18–2322	MK849848	MK828696	MK828307

Ex-type isolates are in bold, newly generated sequences are in red. "N/A" sequence is unavailable

Abbreviation: **CGMCC:** China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China; **HMAS:** Mycological Herbarium, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China. **ILL:** Fungarium of the University of Illinois at Urbana-Champaign, Illinois, USA. **MFLU:** Mae Fah Luang University Herbarium Collection, Chiang Rai, Thailand; **MFLUCC:** Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; **NFCCI:** National Fungal Culture Collection of India, Agarkar Research Institute, Pune, Maharashtra, India; **UESTCC:** University of Electronic Science and Technology Culture Collection.

Results

Phylogenetic analyses

The combined LSU, ITS and SSU sequence matrix comprised 21 taxa including the new collections and comprised 2,269 characters (LSU: 1–772; ITS: 773–1,281; SSU: 1,282–2,269) including gaps. The maximum parsimonious dataset consists of 2,269 characters, of which 2,023 characters were constant, and 13 variable characters were parsimony-uninformative, and number of parsimony-informative characters was 233. Two equally most parsimonious trees (TL = 330, CI = 0.848, RI = 0.887, RC = 0.753, HI = 0.152) were yielded from the heuristic search. The RAxML analysis resulted in a best scoring likelihood tree (FIG. 1) with a final value lnL = -4932.948862. Estimated base frequencies were as follows: A = 0.253993, C = 0.223201, G = 0.274418, T = 0.248388; substitution rates AC = 1.752154, AG = 2.555021, AT = 1.824612, CG = 0.686188, CT = 8.293199, GT = 1.000000. Maximum likelihood, maximum parsimony and Bayesian analyses of the combined dataset inferred in phylogenetic reconstructions with largely similar topologies, and the RAxML tree is shown in FIGURE 1.

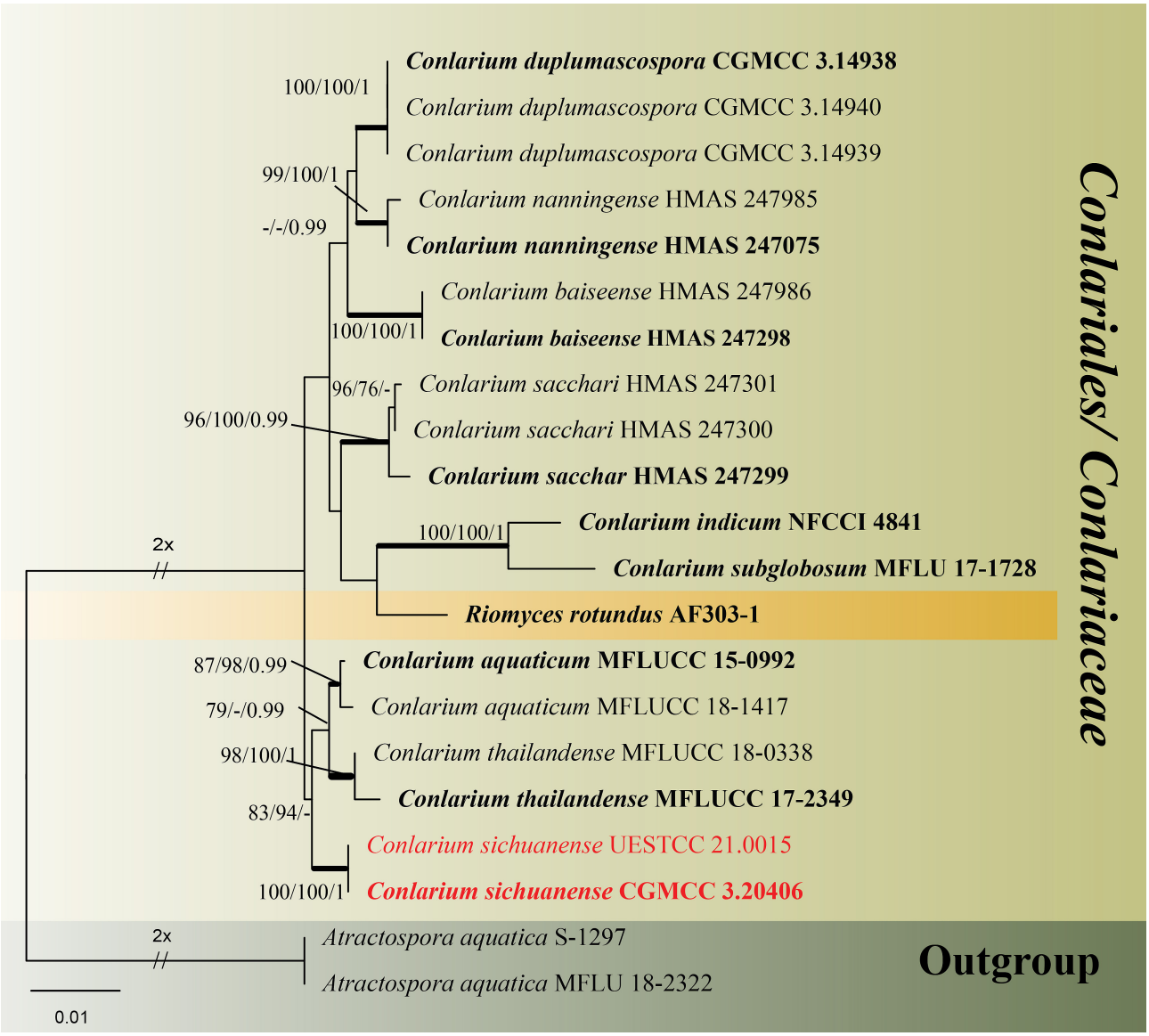

FIGURE 1. Phylogenetic tree based on RAxML analyses of a combined LSU, ITS and SSU dataset. Bootstrap support values for ML and MP equal to or greater than 75% and PP value greater than 0.95 are in thickened. Ex-type isolates are in bold, and new taxa are indicated in red. The tree is rooted with *Atractospora aquatica* (S-1297) and *A. aquatica* (MFLU 18–2322).

Our collections clustered together with *Conlarium aquaticum* (ITS gene) and *C. thailandense* (LSU gene) as a sister clade respectively in the individual loci analyses (data not shown). The multi-gene analyses (FIG. 1) showed that two isolates of *Conlarium sichuanense* formed a distinct lineage and close to *C. aquaticum* and *C. thailandense* which indicated its stable phylogenetic placement. *Riomyces rotundus* (AF303-1(ILL)) formed a distinct lineage within species of *Conlarium* which agrees with the previous studies (Luo *et al*. 2019, Dong *et al*. 2021, Hyde *et al*. 2021).

Taxonomy

Conlarium sichuanense Tian Zhang & Jian K. Liu, ***sp. nov.*** (FIG. 2)

MycoBank number: MB 840499

Etymology: Name refers the location where the fungus was collected, Sichuan Province, China,

Saprobic on dead branches of *Ficus virens* in terrestrial habitat. **Sexual morph**: Undetermined. **Asexual morph**: Hyphomycetous. *Colonies* on natural substrate superficial, sporodochial, punctiform, gregarious, brown to black. *Mycelium* mostly immersed on substratum, consisting of septate, thin-walled, hyaline hyphae. *Conidiophores* absent or

reduced to conidiogenous cells. *Conidiogenous cells* up to 2.3 µm wide, monoblastic, integrated, hyaline, subcylindrical, smooth. *Conidia* 15.5–19.5× 13.5–17 µm ($\bar{x}$ = 17.5 × 15 µm, n = 30), acrogenous, solitary, dry, mostly subglobose, or irregular in shape, muriform, 0–2-transversely septate, 1–3-longitudinal septa, constricted at septa, median brown, smooth, thin-walled, sometimes with a hyaline or pale brown, protruding basal cell.

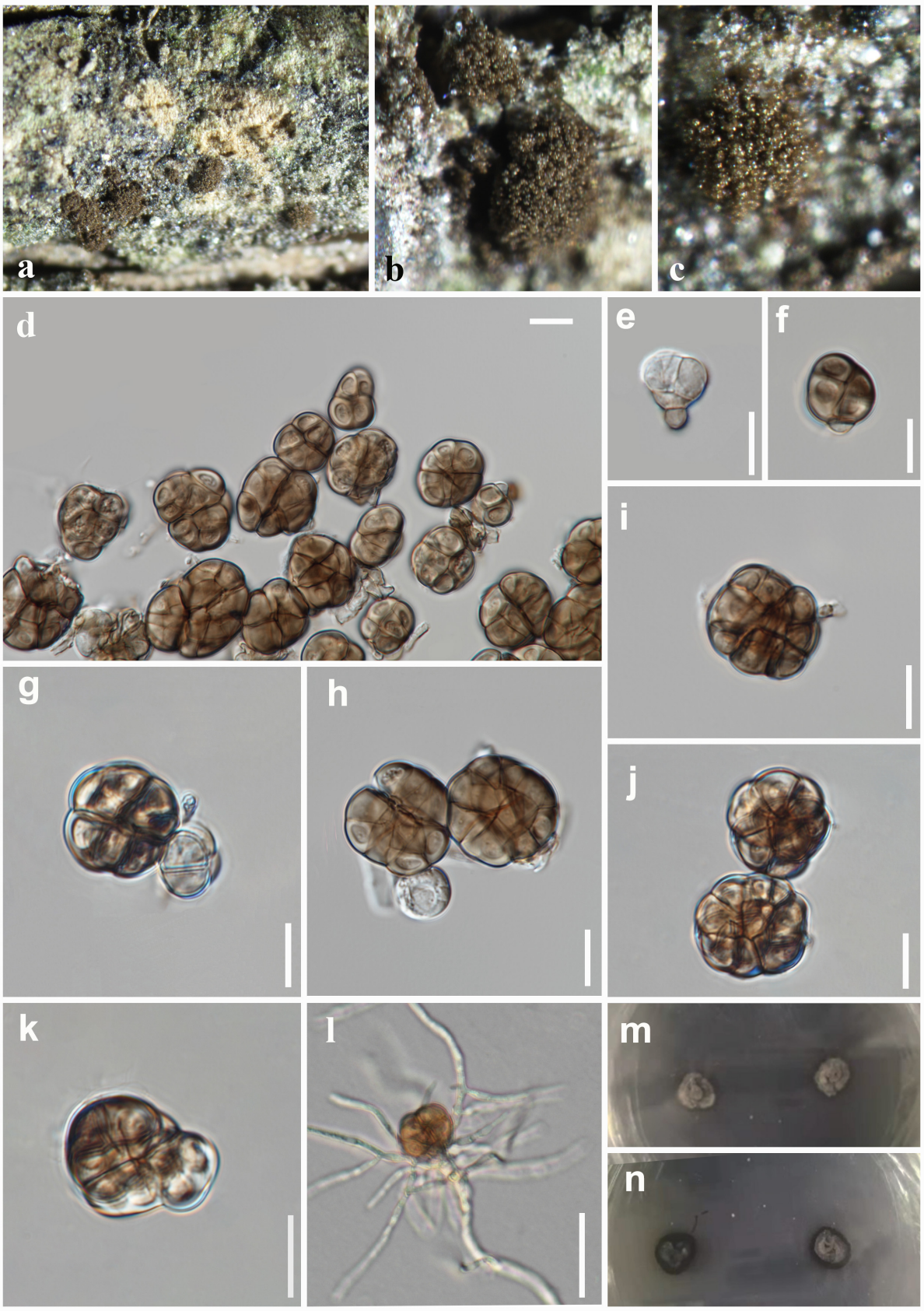

FIGURE 2. ***Conlarium sichuanense*** (HKAS 113024, holotype) **a–c** Colonies on dead branches. **d–k** Conidiogenous cells and conidia. **l** Germinated conidium. **m, n** Colony on PDA from surface and reverse. Scale bars: d-k = 10 µm, l = 20 µm.

TABLE2. Asexual morphology comparison and habitats among *Conlarium* species.

Species	Conidia				Conidiogenous cells			Host/Habitat	References
	Color	Shape	Septa	Size/μm	Color	Shape	Size/μm		
C. aquaticum	dark brown to black	subglobose, ellipsoidal, oblong or irregular	6–12-transverse, 4–10-longitudinal	45–70 × 20–57	hyaline to pale brown	cylindrical	up to 18 μm long	decaying wood/ freshwater	Zhang *et al*. (2017)
C. baiseense	yellow-brown to brown	irregularly globose or subglobose	0–1-transverse, 0–4-longitudinal; or only transverse septa 2–5-	15–25 × 12–19 (muriform);21–35 × 7–12(columnar)	yellow-brown to brown	doliiform	3–8 × 5–12	sugarcane rhizosphere/ terrestrial	Xie *et al*. (2019)
C. dupliciascosporum	brown	irregularly globose or subglobose	0–2-transverse, 0–1-longitudinal	15.5–35.0 × 11–26.5	–	doliiform, cylindrical	5.0–10.0 × 3.0–6.0	decaying wood/ freshwater	Liu *et al*. (2012)
C. indicum	yellow-brown to brown	irregularly globose or subglobose	1–3-transverse, 1–3-longitudinal	60–103 × 39–88	yellow brown to brown	doliiform	3–8 × 5–12	bamboo/ terrestrial	Dubey *et al*. (2021)
C. nanningense	brown	irregularly globose or subglobose	0–1-transverse, 0–4-longitudinal	11–21 × 9–21	–	doliiform, cylindrical	4–13 × 5–10	sugarcane rhizosphere/ terrestrial	Xie *et al*. (2019)
C. sacchari	yellow-brown to brown	irregularly globose or subglobose	0–1-transverse, 0–4-longitudinal	14–19 × 13–22	yellow-brown to brown	doliiform	4–12 × 2–7	sugarcane rhizosphere/ terrestrial	Xie *et al*. (2019)
C. sichuanensis	brown to dark brown	subglobose, or irregular	0–2-transverse, 1–3-longitudinal	15.5–19.5× 13.5–17	hyaline	subcylindrical	up to 2.3 μm wide	decaying wood/ terrestrial	This study
C. subglobosum	brown to dark brown	subglobose	1–2-transverse,1–2-longitudinal	14.5–24	–	–	–	decaying wood/ freshwater	Dong *et al*. (2021)
C. thailandense	brown to dark brown	irregular, subglobose to ellipsoidal	4–8-transverse, 4–6-longitudinal	25–45 × 17–33	hyaline	cylindrical	up to 5.5 μm long	decaying wood/ terrestrial	Phookamsak *et al*. (2019)

Culture characteristics: Colonies on PDA reaching 10 mm in 3 months at 25 °C, surface rough, dry, gray, umbonate, dense, raised, reverse black to grey.

Material examined: CHINA. Sichuan Province, Chengdu city, University of Electronic Science and Technology of China (UESTC) (Qingshuihe campus), on dead branches of *Ficus virens*, 13 November 2020, Wen-Li Li, HUEST 21.0010 (HKAS 113024, holotype; ex-type living culture: CGMCC 3.20406 = UESTCC 21.0014; *Ibid*., on dead branches of *Ficus virens*, 06 December 2020, Tian Zhang, HUEST 21.0011 (paratype), living culture UESTCC 21.0015.

Notes: *Conlarium sichuanense* resembles to *C. dupliciascosporum*, *C. indicum* and *C. subglobosum* in having subglobose or irregular, brown, muriform conidia. In addition, the morphological comparison of *Conlarium* species is shown in TABLE 2. The phylogenetic results (FIG. 1) showed that *Conlarium sichuanense* formed a distinct lineage and differed with the closely related species *C. aquaticum* and *C. thailandense.* Moreover, a comparison of ITS sequence showed that *C. sichuanense* differs from *C. aquaticum* and *C. thailandense* in having 18 bp (base pair) and 20 bp differences (without gaps) respectively. Therefore, the new species establishment of *C. sichuanense* is justified with morphological and phylogenetic evidence.

Discussion

Conlarium species are found from different habitats, such as freshwater and terrestrial, and three species are reported as endophytes (Liu *et al*. 2012, Zhang *et al*. 2017, Phookamsak *et al*. 2019, Xie *et al.* 2019, Dong *et al*. 2021, Dubey & Manikpuri 2021). As known currently, members of *Conlarium* have only been reported from Asia (China, India and Thailand). However, considering their diverse lifestyles and adaption to different environments, *Conlarium* species may have a worldwide distribution which indicates that there are many species remaining to be discovered.

Riomyces is a monotypic sexual morph genus introduced by Ferrer *et al.* (2012). Although *Riomyces* has close phylogenetic relationship with *Conlarium* (Luo *et al*. 2019, Dong *et al*. 2021, Hyde *et al*. 2021), they can be easily distinguishable by morphology. *Conlarium* has a long neck of ascomata, cylindrical asci with a large bipartite apical ring and fusiform ascospores with or without papillary appendages at each end, while *Riomyces* has a short neck of ascomata, asci lacking an apical pore and other apical structures, and ellipsoidal-fusiform ascospores surrounded by a gelatinous sheath (Ferrer *et al*. 2012, Liu *et al*. 2012). Since *Riomyces* is only represented by a single species with one isolate, more fresh collections of *Riomyces* are required to confirm its phylogenetic placement. Further discovery of asexual morph of *Riomyces* is also very important to clarify the relationship between *Conlarium* and *Riomyces*. In addition, it is worthy to mention that *Conlarium* is morphologically similar to the genus *Pseudoconlarium,* which recently was described in *Diaporthomycetidae*, genus *incertae sedis* by Hyde *et al.* (2020), but they are phylogenetically distinct, thus we speculate that *Conlarium* may be polyphyletic and remains further studies.

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