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RESEARCH ARTICLE

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Morphological and Molecular Phylogenetic Data of the Chinese Medicinal Fungus *Cordyceps liangshanensis* Reveal Its New Systematic Position in the Family Ophiocordycipitaceae

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ABSTRACT

A cordycipitoid fungus infecting Hepialidae sp. in Nepal was supposed to be identical to *Cordyceps liangshanensis*, originally described from southwestern China, and thus, transferred to the genus *Metacordyceps* or *Papiliomyces* in previous studies. However, our multi-gene (nrSSU-nrLSU-*tef-1α*-*rpb1*-*rpb2*) phylogenetic and morphological studies based on the type specimen and additional collections of *C. liangshanensis* revealed that the fungus belongs to the genus *Ophiocordyceps* (Ophiocordycipitaceae). Therefore, a new combination *O. liangshanensis* was made, and a detailed description of this species was provided.

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1. Introduction

Cordyceps liangshanensis M. Zang, D.Q. Liu & R.Y. Hu is well-known in southwestern China and has been used as a Traditional Chinese Medicine (TCM) named "Mai-Gan-Chong-Cao" for a long time. Like *Ophiocordyceps sinensis* (Berk.) G.H. Sung et al., *C. liangshanensis* parasitizes soil-borne larvae of Hepialidae sp., and has a limited distribution in China [1]. The caterpillar-fungus resulting from fungal parasitism has been frequently used for the treatment of chronic cough, hemoptysis, asthma, lumbago, impotence and seminal emissions, and other diseases in Yi Nationality areas as it has special effects on cough expectorant, reinforcing kidney, nourishing lung, etc. [1,2]. The main ingredients of the natural *C. liangshanensis* are similar to the those of the natural *O. sinensis*, which contains amino acids, mannitol, adenosine, ergosterol, stearic acid, alkaloids, and organic acids [1,3]. The contents of polysaccharides, flavonoids, and nucleosides of *C. liangshanensis* were a little bit lower than those of *O. sinensis*, while the mannitol and saponins were higher than those of *O. sinensis* [3]. It has been long recognized as a prized medicinal fungus and a desirable alternative for natural *O. sinensis* by local people. This medicinal fungus was firstly recorded in "Sichuan Tongzhi," and it was treated as a new species and named *Cordyceps liangshanensis* by Zang et al. [4]. The medicinal virtue of *C. liangshanensis* is also highly valued by herbalists. This fungus was included in the standardized herbal medicines of Sichuan (enlarged edition) in 1987.

With the rapid development of molecular phylogenetic techniques, considerable changes to the taxonomy of *Cordyceps* s. l. have occurred. Sung et al. [5] proposed the genus *Metacordyceps* to accommodate some species of *Cordyceps* s. l., which was characterized by solitary or grouped stromata which are simple or branched, with a fleshy or tough whitish stipe, a greenish yellow to greenish cylindrical to enlarged fertile part, and perithecia partially or completely immersed in stromata.

Specimens collected from Nepal were once regarded as *Cordyceps liangshanensis* and, subsequently this species was moved from *Cordyceps* to the genus *Metacordyceps* based on multi-gene phylogenetic evidence [5]. Based on the DNA sequences generated by Sung et al. [6], *C. liangshanensis* was transferred to *Papiliomyces* in the family Clavicipitaceae. However, the type or authentic materials of *C. liangshanensis* were not examined in the aforementioned studies. There is a need to

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reinvestigate the type and additional collections of *C. liangshanensis*.

In this study, the type and other specimens from type locality of *C. liangshanensis* were collected and examined. The redescription was carried out on the basis of five-gene (nrSSU, nrLSU, *tef-1α*, *rpb1*, and *rpb2*) molecular phylogenetic analysis and morphological observations. Our data indicated that collections of *C. liangshanensis* differ from those from Nepal generated by Sung et al. [5], and the species transferred to *Metacordyceps* or *Papiliomyces* was not justified.

2. Materials and methods

2.1. Specimens

The type specimen of *Cordyceps liangshanensis* (KUN-HKAS 7723) was borrowed from Kunming Institute of Botany, Chinese Academy of Sciences. Additional collections were made in two locations in southwestern China, one location in Leibo County, Sichuan Province, and another in Shuifu County, Yunnan Province. Specimens were stored in plastic containers at low temperature and transported to the laboratory for identification and isolation. Afterward, they were deposited at Yunnan Herbal Herbarium (YHH), Yunnan University.

2.2. Fungal isolation and culture

Specimens were rinsed with tap water, and then washed with sterile distilled water. For the purpose of obtaining pure cultures, stromata were immersed in 30% H_2O_2 for 5 min, rinsed with sterile water, and then dried on sterilized filter paper. Stromata were then cut off and a small piece of tissue was inoculated onto potato dextrose agar (PDA: fresh diced potato 200 g, dextrose 20 g, agar 18 g, in 1000 ml distilled water) plates. The purified fungal strains were maintained in a culture room at 25 °C or transferred to PDA slants and stored at 4 °C, and were deposited to the Yunnan Fungal Culture Collection (YFCC) at the Institute of Herb Biotic Resources of Yunnan University.

2.3. Morphological observations

Specimens were examined in the laboratory using the Canon 750 D camera (Canon Inc., Tokyo, Japan) and Olympus SZ60 stereo dissecting microscope (Olympus Corporation, Tokyo, Japan). Cultures on PDA slants were transferred to PDA plates and incubated at 25 °C for 2 months. The colors of fresh specimens and cultures were characterized by the color standard [7]. Frozen sections and glass slides with lactic acid phenol cotton blue solution were prepared for morphological observation and measurement of sexual morph under a light microscope (BX53, Olympus Corporation, Tokyo, Japan). Morphological description of asexual morph was conducted as the method described by Wang et al. [8]. Micro-morphological observations and measurements were performed using the Olympus BX53 stereomicroscope and a scanning electron microscope (Quanta 200 FEG, FEI Company, Hillsboro, USA).

2.4. DNA extraction, PCR, and sequencing

Specimens and live axenic cultures were prepared for DNA extraction. Genomic DNA was extracted using a Genomic DNA Purification Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The primers used for PCR amplification of nrSSU, nrLSU, *tef-1α*, *rpb1*, and *rpb2* are listed in Table 1. All PCR reactions were performed in a final volume of 50 μl containing 25 μl 2 × Taq PCR Master Mix (Tiangen Biotech Co., Ltd, Beijing, China), 0.5 μl of each primer (10 μM), 1 μl of genomic DNA, and 23 μl of RNase-free water. Target gene amplification and sequencing were performed according to the methods described in our previous publication [9].

2.5. Phylogenetic analysis

Five gene sequences were retrieved from GenBank, and combined with those generated in our study (Table 2). The sequences were aligned using the programs Clustal X 2.0 and MEGA v6.06 [13,14]. After sequence alignment, the aligned sequences of five genes were concatenated. Partition homogeneity test was conducted using PAUP* 4.0b10 [15], and the result revealed that there was no significant conflict among different data partitions. Program PartitionFinder V1.1.1 identified eleven data partitions, one each for nrSSU and nrLSU, and nine for each of the three codon positions for the protein coding genes *tef-1α*, *rpb1*, and *rpb2* [16,17]. The results showed that the phylogenetic signals for the five genes were congruent ($p = 0.02$). Phylogenetic analysis of the five-gene dataset was conducted

Table 1. PCR primers used in this study.

Gene	Primer	5′-Sequence-3′	Reference
nrSSU	nrSSU-CoF	TCTCAAAGATTAAGCCATGC	[9]
	nrSSU-CoR	TCACCAACGGAGACCTTG	
nrLSU	LR5	ATCCTGAGGGAAACTTC	[10,11]
	LR0R	GTACCCGCTGAACTTAAGC	
tef-1α	EF1α-EF	GCTCCYGGHCAYCGTGAYTTYAT	[5,12]
	EF1α-ER	ATGACACCRACRGCRACRGTYTG	
rpb1	RPB1-5′F	CAYCCWGGYTTYATCAAGAA	[5,12]
	RPB1-5′R	CCNGCDATNTCRTTRTCCATRTA	
rpb2	RPB2-5′F	CCCATRGCTTGTYYRCCCAT	[5,12]
	RPB2-5′R	GAYGAYMGWGATCAYTTYGG	

Table 2. Specimen information for the materials used in this study.

Taxon	Host/substrate	Voucher information	GenBank accession number				
			nrSSU	nrLSU	*tef1-α*	*rpb1*	*rpb2*
Akanthomyces coccidioperitheciatus	Araneae	NHJ 6709	EU369110	EU369042	EU369025	EU369067	EU369086
Akanthomyces muscarius	Hemiptera: Aleyrodidae	CBS 143.62^{T}	KM283774	KM283798	KM283821	KM283841	KM283863
Akanthomyces tuberculatus	Lepidopteran adult	BCC 16819	MF416600	MF416546	MF416490	MF416647	MF416444
Balansia henningsiana	Plant (*Panicum* sp.)	AEG 96-27a	AY545723	AY545727	AY489610	AY489643	DQ522413
Balansia pilulaeformis	Plant (Poaceae sp.)	AEG 94-2	AF543764	AF543788	DQ522319	DQ522365	DQ522414
Beauveria bassiana	Lepidoptera: Cossidae	YFCC 3369	MN576768	MN576824	MN576994	MN576884	MN576938
Beauveria brongniartii	Coleoptera: Scarabaeidae	ARSEF 617^{T}	AB027335	AB027381	HQ880991	HQ880854	HQ880926
Beauveria scarabaeidicola	Coleoptera: Scarabaeidae	ARSEF 5689	AF339574	AF339524	DQ522335	DQ522380	DQ522431
Blackwellomyces cardinalis	Lepidoptera: Tineidae	OSC 93609^{T}	AY184973	AY184962	DQ522325	DQ522370	DQ522422
Blackwellomyces pseudomilitaris	Lepidopteran larva	BCC 2091	MF416589	MF416535	MF416479		MF416441
Claviceps paspali	Plant (Poaceae sp.)	ATCC 13892	U32401	U47826	DQ522321	DQ522367	DQ522416
Claviceps purpurea	Plant (Poaceae sp.)	S.A. cp11	EF469122	EF469075	EF469058	EF469087	EF469105
Conoideocrella luteorostrata	Hemiptera: Coccoidea (scale insect)	NHJ 12516	EF468994	EF468849	EF468800	EF468905	EF468946
Conoideocrella tenuis	Hemiptera: Coccoidea (scale insect)	NHJ 6293	EU369112	EU369044	EU369029	EU369068	EU369087
Cordyceps kyusyuensis	Lepidopteran pupa	EFCC 5886	EF468960	EF468813	EF468754	EF468863	EF468917
Cordyceps militaris	Lepidopteran pupa	YFCC 6587	MN576762	MN576818	MN576988	MN576878	MN576932
Cordyceps pruinosa	Lepidoptera: Limacodidae	ARSEF 5413	AY184979	AY184968	DQ522351	DQ522397	DQ522451
Cordyceps tenuipes	Lepidopteran pupa	ARSEF 5135^{T}	MF416612	JF415980	JF416020	JN049896	JF416000
Drechmeria balanoides	Nematode	CBS 250.82^{T}	AF339588	AF339539	DQ522342	DQ522388	DQ522442
Drechmeria gunnii	Lepidoptera (Hepialidae larva)	OSC 76404	AF339572	AF339522	AY489616	AY489650	DQ522426
Gibellula pulchra	Araneae	NHJ 10808	EU369099	EU369035	EU369018	EU369056	EU369076
Gibellula ratticaudata	Araneae	ARSEF 1915	DQ522562	DQ518777	DQ522360	DQ522408	DQ522467
Gliocephalotrichum bulbilium	Soil	ATCC 22228^{T}	AY489700	AY489732	AY489627	AY489664	EF469114
Harposporium harposporiferum	Arthropod	ARSEF 5472^{T}	AF339569	AF339519	DQ118747	DQ127238	
Harposporium helicoides	Nematode	ARSEF 5354	AF339577	AF339527			
Hevansia arachnophilus	Araneae	NHJ 10469	EU369090	EU369031	EU369008	EU369047	
Hevansia novoguineensis	Araneae	NHJ 11923	EU369095	EU369032	EU369013	EU369052	EU369072
Hirsutella citriformis	Hemiptera: Cixiidae	ARSEF 1446	KM652065	KM652106	KM651990	KM652031	
Hirsutella fusiformis	Coleoptera: Curculionidae (*Brachyderes incanus*)	ARSEF 5474	KM652067	KM652110	KM651993	KM652033	
Hirsutella gigantea	Hymenoptera: Pamphiliidae	ARSEF 30		JX566977	JX566980	KM652034	
Hirsutella guyana	Hemiptera: Cicadellidae (*Empoasca kraemeri*)	ARSEF 878	KM652068	KM652111	KM651994	KM652035	
Hirsutella illustris	Hemiptera: Aphididae (*Eriosoma lanigerum*)	ARSEF 5539	KM652069	KM652112	KM651996	KM652037	
Hirsutella kirchneri	Acari: Eriophyidae (*Abacarus hystrix*)	ARSEF 5551	KM652070	KM652113	KM651997		
Hirsutella lecaniicola	Hemiptera: Coccidae (*Parthenolecanium corni*)	ARSEF 8888	KM652071	KM652114	KM651998	KM652038	
Hirsutella liboensis	Lepidoptera: Cossidae (larva)	ARSEF 9603^{T}	KM652072	KM652115	KY415588	KY945367	
Hirsutella minnesotensis	Tyle nchida (*Heterodera glycin*)	3608	JPUM01000376	JPUM01000376	JPUM01000211	JPUM01000139	JPUM01000138
Hirsutella necatrix	Acari	ARSEF 5549	KM652073	KM652116	KM651999	KM652039	
Hirsutella nodulosa	Lepidoptera: Pyralidae (*Dioryctria zimmermani*)	ARSEF 5473	KM652074	KM652117	KM652000	KM652040	
Hirsutella radiata	Diptera	ARSEF 1369	KM652076	KM652119	KM652002	KM652042	
Hirsutella rhossiliensis	Tylenchida: Criconematidae (*Mesocriconema xenoplax*)	ARSEF 3747	KM652080	KM652123	KM652006	KM652045	
Hirsutella strigosa	Hemiptera: Cicadellidae (*Nephotettix virescens*)	ARSEF 2197	KM652085	KM652129	KM652012	KM652050	
Hirsutella subulata	Lepidoptera: Microlepidoptea	ARSEF 2227	KM652086	KM652130	KM652013	KM652051	
Hirsutella thompsonii	Acari	MTCC 3556	APKB01000383	APKB01000383	APKB01000061	APKB01000125	APKB01000164
Hirsutella thompsonii var. *synnematosa*	Acari: Eriophyidae (*Aceria sheldoni*)	ARSEF 2459	KM652099	KM652147	KM652027	KM652061	
Hirsutella thompsonii var. *thompsonii*	Acari: Eriophyidae (*Phyllocoptruta oleivora*)	ARSEF 137	KM652087	KM652131	KM652014	KM652052	
Hirsutella thompsonii var. *vinacea*	Acari: Eriophyidae (*Acalitus vaccinii*)	ARSEF 254	KM652101	KM652149	KM652028	KM652062	
Hypocrella schizostachyi	Hemiptera: Coccoidea (scale insect)	BCC 14123	DQ522557	DQ518771	DQ522346	DQ522392	DQ522447

(*continued*)

Table 2. Continued.

Taxon	Host/substrate	Voucher information	GenBank accession number				
			nrSSU	nrLSU	*tef1-α*	*rpb1*	*rpb2*
Hypocrella siamensis	Hemiptera: Coccoidea (scale insect)	BCC 8105	DQ522537	DQ518752	DQ522317	DQ522363	DQ522411
"*Metacordyceps liangshanensis*"	**Lepidoptera (pupa)**	**EFCC 1452**	**EF468962**	**EF468815**	**EF468756**		
"*Metacordyceps liangshanensis*"	**Lepidoptera (pupa)**	**EFCC 1523**	**EF468961**	**EF468814**	**EF468755**		**EF468918**
Metacordyceps shibinensis	Lepidoptera (pupa)	GZUHSB13050311^T	KR153588		KR153589	KR153590	
Metacordyceps neogunnii	Lepidoptera (pupa)	GZUHSB13050302^T	KU729722		KU729727	KU729732	
Metacordyceps neogunnii	Lepidoptera (pupa)	GZUHHS14061253	KU729725		KU729730	KU729735	
Metapochonia goniodes	Fungi	CBS 891.72^T	AF339599	AF339550	DQ522354	DQ522401	DQ522458
Metapochonia microbactrospora	Rotifera: Bdelloidea (bdelloid rotifer)	CBS 101433^T	AF339587	AF339538	KJ398794	KJ398605	KJ398701
Metapochonia rubescens	Nematode eggs	CBS 464.88^T	AF339615	AF339566	EF468797	EF468903	EF468944
Metarhizium album	Hemiptera	ARSEF 2082	DQ522560	DQ518775	DQ522352	KJ398617	KJ398715
Metarhizium anisopliae	Soil	BUM 1900	MH143837	MH143820	MH143854	MH143869	MH143884
Metarhizium carneum	Dune sand	CBS 239.32^T	EF468988	EF468843	EF468789	EF468894	EF468938
Metarhizium flavoviride	Coleoptera: Curculionidae (*Ceutorrhynchus macula-alba*)	CBS 218.56^T		MH869139	KJ398787	KJ398598	KJ398694
Metarhizium indigoticum	Lepidoptera (Hepialidae larva)	TNS-F 18553	JF415952	JF415968	JF416010	JN049886	JF415992
Metarhizium khaoyaiense	Lepidoptera (larva)	BCC 14290	KX983469	KX983463	KJ398797	JN049888	KJ398704
Metarhizium kusanagiense	Lepidoptera	NBRC 109322	JF415954	JF415972	JF416014	JN049890	
Metarhizium majus	Coleoptera	ARSEF 3145	AF339579	AF339530	AF543774	DQ522399	DQ522453
Metarhizium marquandii	Soil	CBS 182.27^T	EF468990	EF468845	EF468793	EF468899	EF468942
Metarhizium minus	Hemiptera	ARSEF 2037^T	AF339580	AF339531	DQ522353	DQ522400	DQ522454
Metarhizium owariense	Hemiptera	NBRC 33258	HQ165669	HQ165730	JF416017	KJ398596	JF415996
Metarhizium samlanense	Hemiptera: Cicadellidae (leafhopper)	BCC 17093	HQ165666	HQ165728	HQ165687	HQ165746	HQ165647
Metarhizium takense	Hemiptera: Cicadidae (cicada nymph)	BCC 30934	HQ165658	HQ165720	HQ165679	HQ165740	HQ165639
Metarhizium yongmunense	Lepidoptera (pupa)	EFCC 2131	EF468977	EF468833	EF468770	EF468876	
Nectria cinnabarina	Plant (*Betula* sp.)	CBS 114055	U32412	U00748	AF543785	AY489666	DQ522456
Ophiocordyceps acicularis	Coleoptera (larva)	OSC 110987	EF468950	EF468805	EF468744	EF468852	
Ophiocordyceps acicularis	Coleoptera (larva)	OSC 110988	EF468951	EF468804	EF468745	EF468853	
Ophiocordyceps agriotidis	Coleoptera (larva)	ARSEF 5692	DQ522540	DQ518754	DQ522322	DQ522368	DQ522418
Ophiocordyceps amazonica	Orthoptera (Acrididae adult)	HUA 186143^T	KJ917562	KJ917571	KM411989	KP212902	KM411982
Ophiocordyceps aphodii	Coleoptera (Scarabaeidae larva)	ARSEF 5498	DQ522541	DQ518755	DQ522323		DQ522419
Ophiocordyceps appendiculata	Coleoptera (larva)	NBRC 106960	JN941728	JN941413	AB968577	JN992462	AB968539
Ophiocordyceps arborescens	Lepidoptera (*Pueraria lobata* larva)	NBRC 105891^T	AB968386	AB968414	AB968572		AB968534
Ophiocordyceps bispora	Isoptera (adult termite)	ERS1123077	FKNF01000183	FKNF01000183	FKNF01000002	FKNF01000038	FKNF01000031
Ophiocordyceps brunneanigra	Hemiptera: Cicadellidae	TBRC 8093^T		MF614654	MF614638	MF614668	MF614681
Ophiocordyceps brunneaperitheciata	Lepidoptera (larva)	TBRC 8100^T		MF614658	MF614643		MF614685
Ophiocordyceps brunneipunctata	Coleoptera (Elateridae larva)	OSC 128576	DQ522542	DQ518756	DQ522324	DQ522369	DQ522420
Ophiocordyceps citrina	Hemiptera	TNS F18537		KJ878903	KJ878983		KJ878954
Ophiocordyceps cochlidiicola	Lepidoptera (Cochlididae pupa)	HMAS 199612	KJ878917	KJ878884	KJ878965	KJ878998	
Ophiocordyceps coenomyia	Diptera: Coenomyiidae (*Coenomyia* sp. larva)	NBRC 106964	AB968385	AB968413	AB968571		AB968533
Ophiocordyceps crinalis	Lepidoptera (larva)	GDGM 17327	KF226253	KF226254	KF226256	KF226255	
Ophiocordyceps evansii	Hymenoptera (*Pachycondyla harpax* adult ant)	HUA 186159^T	KC610796	KC610770	KC610736	KP212916	
Ophiocordyceps formicarum	Hymenoptera: Formicidae	TNS F18565	KJ878921	KJ878888	KJ878968	KJ879002	KJ878946
Ophiocordyceps forquignonii	Diptera (adult fly)	OSC 151902	KJ878912	KJ878876		KJ878991	KJ878945
Ophiocordyceps fulgoromorphila	Hemiptera (Fulgoridae adult)	HUA 186139^T	KC610794	KC610760	KC610729	KF658676	KC610719
Ophiocordyceps geometridicola	Lepidoptera (Geometridae larva)	TBRC 8095^T		MF614648	MF614632	MF614663	MF614679
Ophiocordyceps gracilis	Lepidoptera (larva)	EFCC 8572	EF468956	EF468811	EF468751	EF468859	EF468912
Ophiocordyceps heteropoda	Hemiptera (cicada nymph)	NBRC 100644	JN941718	JN941423	AB968596	JN992452	AB968557
Ophiocordyceps karstii	Lepidoptera (*Hepialus jianchuanensis*)	MFLU:15-3884^T	KU854952		KU854945	KU854943	

(*continued*)

Table 2. Continued.

Taxon	Host/substrate	Voucher information	GenBank accession number				
			nrSSU	nrLSU	*tef1-α*	*rpb1*	*rpb2*
Ophiocordyceps kimflemingiae	Hymenoptera (*Camponotus castaneus/americanus*)	SC09B	KX713631	KX713620	KX713698	KX713724	
Ophiocordyceps kniphofioides	Hymenoptera (*Cephalotes atratus* adult ant)	HUA 186148	KC610790	KF658679	KC610739	KF658667	KC610717
Ophiocordyceps konnoana	Coleoptera (larva)	EFCC 7315	EF468959		EF468753	EF468861	EF468916
Ophiocordyceps lanpingensis	Lepidoptera (Hepialidae larva)	YHOS0705	KC417458	KC417460	KC417462	KC417464	KC456333
Ophiocordyceps liangshanensis	**Lepidoptera (Hepialidae larva)**	**KUN-HKAS7723**				**MW168192**	
Ophiocordyceps liangshanensis	**Lepidoptera (Hepialidae larva)**	**YFCC 8577**	**MT774218**	**MT774225**	**MT774246**	**MT774232**	**MT774239**
Ophiocordyceps liangshanensis	**Lepidoptera (Hepialidae larva)**	**YFCC 8578**	**MT774219**	**MT774226**	**MT774247**	**MT774233**	**MT774240**
Ophiocordyceps liangshanensis	**Lepidoptera (Hepialidae larva)**	**YHH 17007**	**MT774220**	**MT774227**	**MT774248**	**MT774234**	**MT774241**
Ophiocordyceps longissima	Hemiptera: Cicadidae (cicada nymph)	NBRC 106965	AB968392	AB968420	AB968584		AB968546
Ophiocordyceps macroacicularis	Lepidoptera (Cossidae larva)	NBRC 100685^{T}	AB968388	AB968416	AB968574		AB968536
Ophiocordyceps multiperitheciata	Lepidoptera (larva)	BCC 69008^{T}		MF614657	MF614641		MF614682
Ophiocordyceps myrmicarum	Hymenoptera: Formicidae	HIRS 45	KJ680150	JX566965	JX566973	KJ680151	
Ophiocordyceps nigrella	Lepidoptera (larva)	EFCC 9247	EF468963	EF468818	EF468758	EF468866	EF468920
Ophiocordyceps pseudoacicularis	Lepidoptera (larva)	TBRC 8102^{T}		MF614646	MF614630	MF614661	MF614677
Ophiocordyceps pruinosa	Hemiptera	NHJ 12994	EU369106	EU369041	EU369024	EU369063	EU369084
Ophiocordyceps pulvinata	Hymenoptera (*Camponotus* adult ant)	TNS-F 30044	GU904208	AB721305	GU904209	GU904210	
Ophiocordyceps ramosissimum	Lepidoptera (*Phassus nodus* larva)	GZUHHN8	KJ028012		KJ028014	KJ028017	
Ophiocordyceps ravenelii	Coleoptera (beetle larva)	OSC 110995	DQ522550	DQ518764	DQ522334	DQ522379	DQ522430
Ophiocordyceps robertsii	Lepidoptera (Hepialidae larva)	KEW 27083		EF468826	EF468766		
Ophiocordyceps rubiginosiperitheciata	Coleoptera (larva)	NBRC 106966	JN941704	JN941437	AB968582	JN992438	AB968544
Ophiocordyceps satoi	Hymenoptera (*Polyrhachis lamellidens*)	J19	KX713650	KX713601	KX713684	KX713710	
Ophiocordyceps sinensis	Lepidoptera (Hepialidae larva)	EFCC 7287	EF468971	EF468827	EF468767	EF468874	EF468924
Ophiocordyceps sinensis	Lepidoptera (Hepialidae larva)	YHH 1805	MK984568	MK984580	MK984572	MK984587	MK984576
Ophiocordyceps sobolifera	Hemiptera: Cicadidae (cicada nymph)	KEW 78842	EF468972	EF468828		EF468875	EF468925
Ophiocordyceps sobolifera	Hemiptera: Cicadidae (cicada nymph)	TNS F18521	KJ878933	KJ878898	KJ878979	KJ879013	
Ophiocordyceps spataforae	Hemiptera (adult)	NHJ 12525	EF469125	EF469078	EF469063	EF469092	EF469111
Ophiocordyceps sphecocephala	Hymenoptera (adult wasp)	NBRC 101753	JN941695	JN941446	AB968592	JN992429	AB968553
Ophiocordyceps stylophora	Coleoptera (Elateridae larva)	OSC 110999	EF468982	EF468837	EF468777	EF468882	EF468931
Ophiocordyceps tiputinii	Megaloptera (larva)	QCNE 186287^{T}	KC610792	KC610773	KC610745	KF658671	
Ophiocordyceps thanathonensis	Hymenotera (adult ant)	MFLU 16-2910	MF882926	MF850377	MF872614	MF872616	
Ophiocordyceps tricentri	Hemiptera (Cercopoidea adult)	NBRC 106968	AB968393	AB968423	AB968593		AB968554
Ophiocordyceps unilateralis	Hymenoptera (*Camponotus* adult ant)	OSC 128574	DQ522554	DQ518768	DQ522339	DQ522385	DQ522436
Ophiocordyceps unituberculata	Lepidoptera (larva)	YFCC HU1301^{T}	KY923214	KY923212	KY923216	KY923218	KY923220
Ophiocordyceps xuefengensis	Lepidoptera (*Phassus nodus* larva)	GZUH2012HN14^{T}	KC631789		KC631793	KC631798	
Ophiocordyceps yakusimensis	Hemiptera: Cicadidae (cicada nymph)	HMAS 199604	KJ878938	KJ878902		KJ879018	KJ878953
Orbiocrella petchii	Hemiptera: Coccoidea (scale insect)	NHJ 6209	EU369104	EU369039	EU369023	EU369061	EU369081
Orbiocrella petchii	Hemiptera: Coccoidea (scale insect)	NHJ 6240	EU369103	EU369038	EU369022	EU369060	EU369082
Pochonia chlamydosporia	Mollusca	CBS 101244	DQ522544	DQ518758	DQ522327	DQ522372	DQ522424
Pochonia chlamydosporia var. *catenulata*	Soil	CBS 504.66^{T}	AF339593	AF339544	EF469069	EF469098	EF469120
Pochonia chlamydosporia var. *chlamydosporia*	Soil under *Brassica napus*	CBS 103.65^{T}			KJ398786	KJ398597	KJ398693
Pochonia boninensis	Soil	JCM 18597	AB758255	AB709831	AB758463	AB758666	AB758693
Purpureocillium atypicolum	Araneae	CBS 744.73	EF468987	EF468841	EF468786	EF468892	
Purpureocillium lilacinum	Soil	CBS 284.36^{T}	AY526475	FR775484	EF468792	EF468898	EF468941
Rotiferophthora angustispora	Rotifera: Bdelloidea (bdelloid rotifer)	CBS 101437	AF339584	AF339535	AF543776	DQ522402	DQ522460
Samsoniella alboaurantium	Soil	CBS 262.58^{T}	AB023943	AB080087	MF416497	MF416654	MF416448
Samsoniella inthanonensis	Lepidopteran pupa	TBRC 7915^{T}		MF140725	MF140849	MF140790	MF140815
Shimizuomyces paradoxus	Plant (*Smilax sieboldii*)	EFCC 6279	EF469131	EF469084	EF469071	EF469100	EF469117

(*continued*)

Table 2. Continued.

Taxon	Host/substrate	Voucher information	GenBank accession number				
			nrSSU	nrLSU	*tef1-α*	*rpb1*	*rpb2*
Shimizuomyces paradoxus	Plant (*Smilax sieboldii*)	EFCC 6564	EF469130	EF469083	EF469072	EF469101	EF469118
Simplicillium lamellicola	Fungi (*Agaricus bisporus*)	CBS 116.25^T	AF339601	AF339552	DQ522356	DQ522404	DQ522462
Simplicillium lanosoniveum	Fungi (*Hemileia vastatrix*)	CBS 704.86	AF339602	AF339553	DQ522358	DQ522406	DQ522464
Sphaerostilbella berkeleyana	Fungi (Polyporaceae sp.)	CBS 102308	AF543770	U00756	AF543783	AY489671	DQ522465
Tolypocladium capitatum	Fungi (*Elaphomyces* sp.)	NBRC 100997	JN941740	JN941401	AB968597	JN992474	AB968558
Tolypocladium inflatum	Soil	OSC 71235	EF469124	EF469077	EF469061	EF469090	EF469108
Tolypocladium paradoxum	Hemiptera: Cicadidae (larva)	NBRC 100945	JN941731	JN941410	AB968599	JN992465	AB968560
Trichoderma deliquescens	On decorticated conifer wood	ATCC 208838	AF543768	AF543791	AF543781	AY489662	DQ522446
Trichoderma stercorarium	Cow dung	ATCC 62321	AF543769	AF543792	AF543782	AY489633	EF469103

Notes: ^{T}ex-type culture.
Boldface: *Ophiocordyceps lianshanensis* and "*Metacordyceps liangshensis*" were treated as two different species here.

using maximum-likelihood (ML) methods. The ML analysis was run on RaxML v7.9.1 using the optimal model GTR + I with 1000 rapid bootstrap replicates [18]. The reliability of nodes was assessed 1000 replicates of non-parametric bootstrap proportions on the combined 5-gene dataset.

3. Results

3.1. Phylogenetic analysis

The 149 taxa were used for phylogenetic analysis from four families (Ophicordycipitaceae, Clavicipitaceae, Cordycipitaceae, and Hypocreaceae), with *Gliocephalotrichum bulbilium* and *Nectria cinnabarina* included as outgroups. The concatenated sequence dataset of five genes was composed of 5567 bp sequence data (1690 bp for nrSSU, 972 bp for nrLSU, 1035 bp for *tef-1α*, 781 bp for *rpb1*, and 1089 bp for *rpb2*). Phylogenetic tree inferred from ML analysis recognized four statistically well-supported clades in *Ophiocordyceps*, designated as *Hirsutella* Pat., *O. sobolifera* (Hill ex Watson) G.H. Sung et al., *O. sphecocephala* (Klotzsch ex Berk.) G.H. Sung et al. and *O. ravenelii* (Berk. & M.A. Curtis) G.H. Sung et al. clades (Figure 1). The *Hirsutella* clade included six distinct subclades, namely, *H. citriformis* Speare, *H. thompsonii* F.E. Fisher, *H. nodulosa* Petch, *H. Guyana* Minter & B.L. Brady, *H. sinensis* X.J. Liu et al., and the *Hirsutella* ant pathogen subclades. Phylogenetic analysis of combined dataset placed four samples of *C. liangshanensis* in the *H. sinensis* subclade. *Cordyceps liangshanensis* was closely clustered with *O. karstii* T.C. Wen, Y.P. Xiao & K.D. Hyde and well-supported by ML bootstrap proportions (ML-BP = 100%). However, three samples of *C. liangshanensis* clustered together and formed a separate clade from *O. karstii* with 100% statistical support.

3.2. Morphological features

The morphological characteristics of various specimens of *C. liangshanensis* are shown, and the photomicrographs of morphological structures are shown in Figure 2. The detailed fungal morphological descriptions are in the Taxonomy section. Distinct morphological features between *C. liangshanensis* and its related species are summarized in Table 3.

3.3. Taxonomy

Ophiocordyceps liangshanensis (M. Zang, D.Q. Liu & R.Y. Hu) H. Yu, Y. Wang, Y.D. Dai, Zhu L. Yang & Y.B. Wang, comb. nov. (Figure 2).

MycoBank MB837859

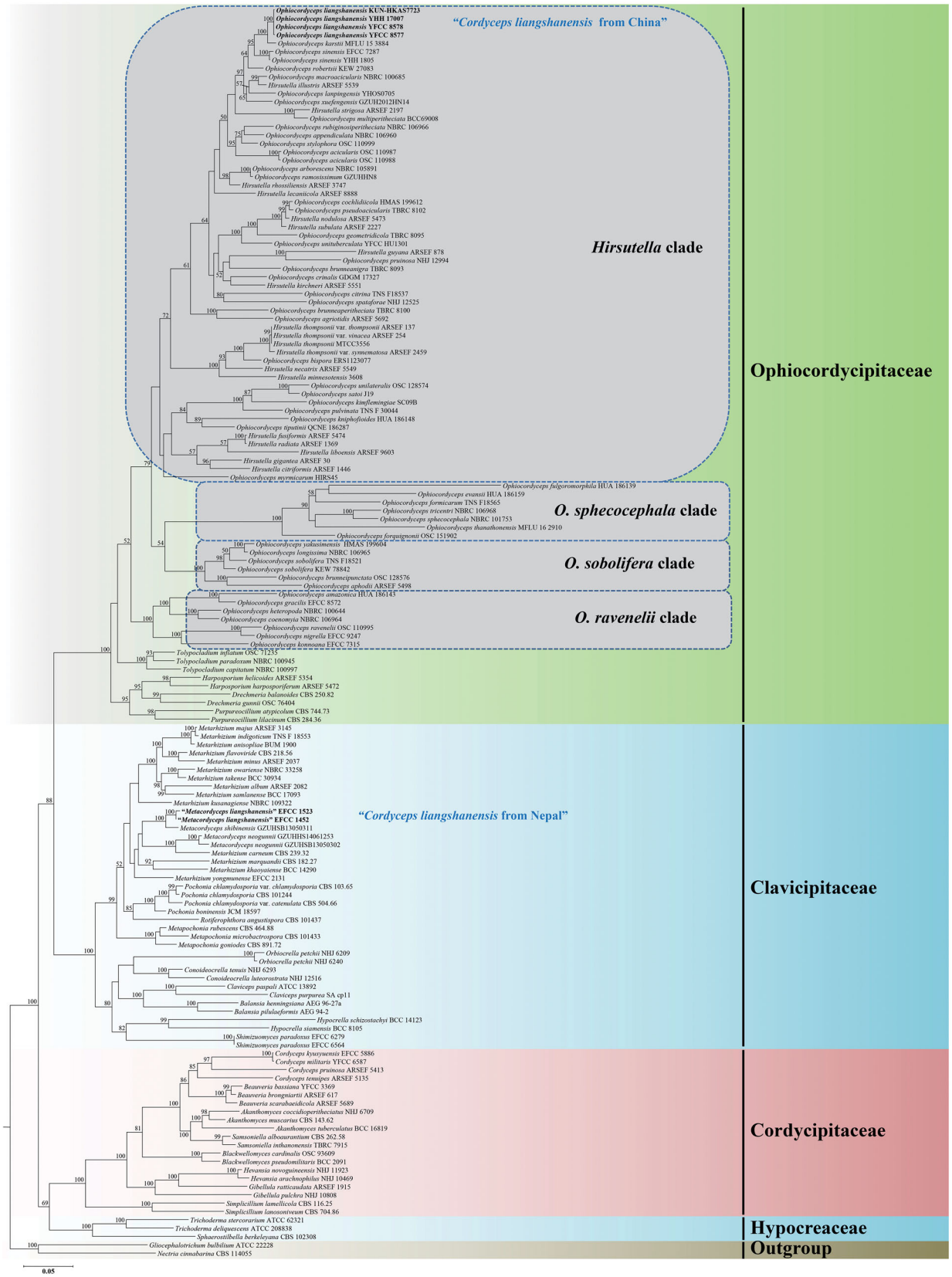

Figure 1. Phylogenetic tree of *Ophiocordyceps liangshanensis* with related species based on maximum likelihood analysis from a five-locus (nrLSU, nrSSU, *tef-1α*, *rpb1*, and *rpb2*) dataset. Statistical support values (50%) are shown at the nodes for ML bootstrap support.

≡ *Cordyceps liangshanensis* M. Zang, D.Q. Liu & R.Y. Hu, Acta Botonic Yunnanica 4(2): 174 (1982).

= *Metacordyceps liangshanensis* (M. Zang, D. Liu & R.Y. Hu) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora, Studies in Mycology 57: 35 (2007, misinterpretation).

= *Papiliomyces liangshanensis* (M. Zang, D. Liu & R.Y. Hu) Luangsa-ard, Samson &

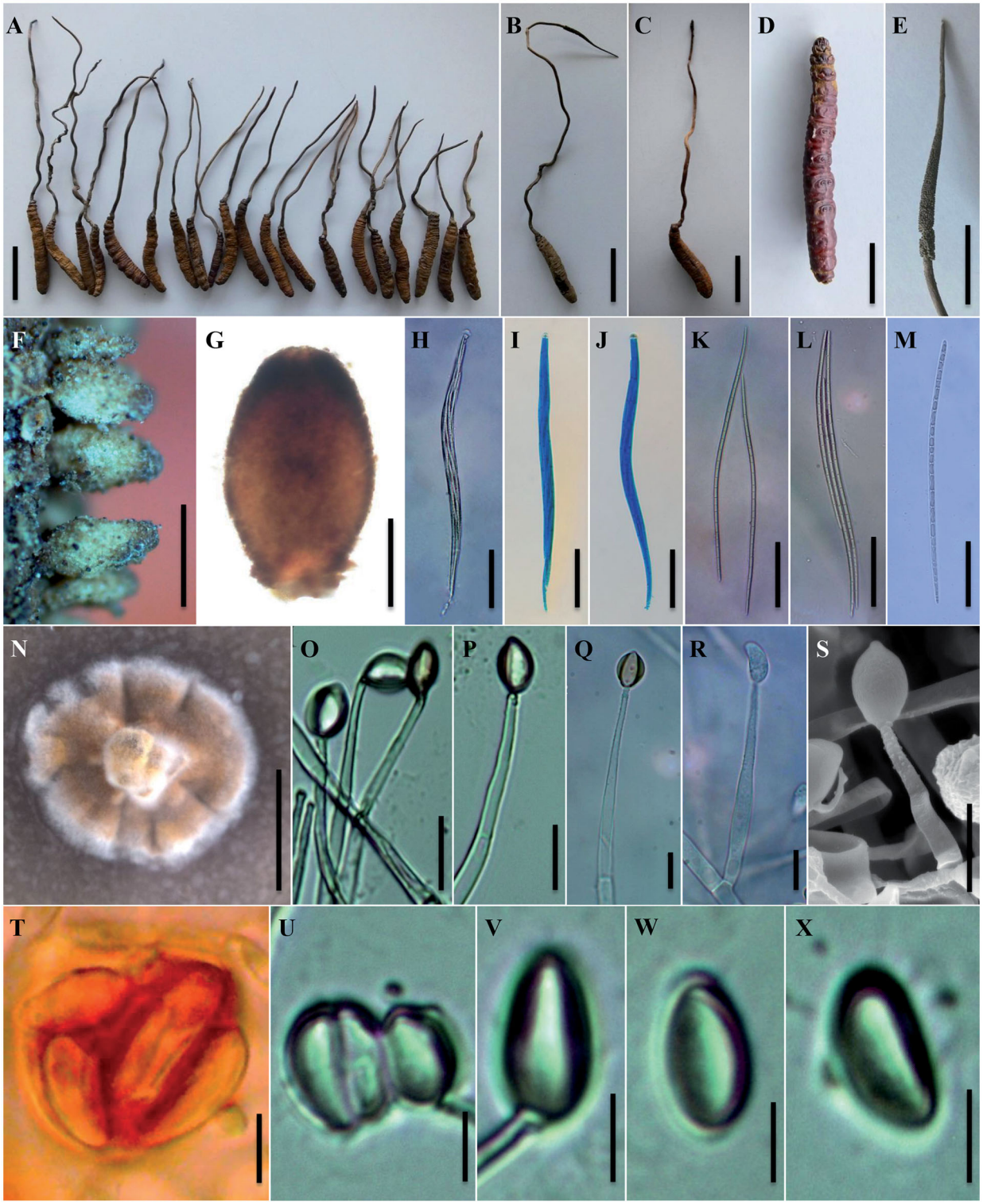

Figure 2. Morphology of *Ophiocordyceps liangshanensis*. (A) Slender stromata arising from Hepialidae larvae; (B) Mature stroma arising from the larva of Hepialidae. (C) The type specimen of *O. liangshanensis* (KUN-HKAS7723). (D) The reddish dark brown host of *O. liangshanensis*. (E) Fertile part; (F–G) Perithecia. (H–J) Asci. (K–M) Ascospores; (N) Colony on PDA; (O–S) Conidiogenous cells; (T–X) Conidia embedded in mucous sheaths. Scale bars: A–C = 3 cm; D, E, N = 1 cm; F = 500 μm; G = 200 μm; H–M = 50 μm; O–S = 10 μm; T–X = 5 μm.

Thanakitpipattana, Studies in Mycology 95: 240 (2020, misinterpretation).

Holotype: CHINA. Sichuan Province: Liangshan Yi Autonomous Prefecture, Leibo County, alt. 1500 m, on the larva of Hepialidae sp. living in *Qiongzhuea tumidinoda* forest, 25 July 1980, Jiyuan Li (KUN-HKAS7723, holotype).

Sexual morph: Stromata cylindrical, solid, yellow-brown to brown, 1–2 arising mainly from the head of host, 200–300 mm long, 1.5–2.5 mm wide. Stipes

Table 3. Morphological comparisons of *Ophiocordyceps liangshanensis* with its related species.

Species	Host	Stromata	Perithecia	Asci	Ascospores	Reference
Ophiocordyceps liangshanensis	Hepialidae larva	Single or occasionally branched, cylindrical, 200–300 × 1.5–2.5 mm	Superficial, long ovoid, 450–740 × 300–450 μm	Cylindrical, 260–480 × 8–12 μm	170–240 × 2.5–4.1 μm. Septa, 5.5–19.8 × 2.5–4.1 μm	This study
Ophiocordyceps robertsii	Hepialidae larva	Single, cylindrical, 100–380 × 3–4 mm	Superficial, elongate-obvate or elliptical, 600–880 × 300–400 μm	Narrowly cylindrical, 280–400 × 9–10 μm	280 × 3 μm. Secondly ascospores, 5.0–6.0 × 3.0 μm	[19]
Ophiocordyceps xuefenensis	Hepialidae larva	Single or occasionally branched, cylindrical, 140–460 × 2–7 mm	Superficial, long ovoid, 416–625 × 161–318 μm	Cylindrical, 191–392 × 4.5–8.9 μm	130–380 × 1.4–5.2 μm. Septa not measured	[20]
Ophiocordyceps ramosissimum	Hepialidae larva	One or two, 70–150 × 2–4 mm	Superficial, ovoid, 340–350 × 225–255 μm	Cylindrical, 172–265 × 6.9–17.3 μm	130–245 × 2.0–3.5 μm. Septa not measured.	[21]
Ophiocordyceps karstii	Hepialidae larva	Mostly single, 140–145 × 2–4 mm	Superficial, flask-shaped, thick-walled, 600–765 × 247–323 μm	Narrowly cylindrical, with a thickened apex. 186–228 × 8–12 μm	173–202 × 3.0–5.0 μm. Septa not measured	[22]
Ophiocordyceps lanpingensis	Hepialidae larva	Single to several, or fascioled, 50–160 × 0.2–1.3 mm	Superficial, ovoid, 310–370 × 200–240 μm	Cylindrical, 240–300 × 5.1–6.5 μm	240–300 × 1.4 μm. Septa, 3.3–4.9 × 1.1–1.4 μm	[23]
Ophiocordyceps emeiensis	Hepialidae larva	Single, occasionally 2, 100–160 × 1.5–3.0 mm	Superficial, ellipsoidal to ovoid, 320–460 × 220–320 μm	Cylindrical, 170–215 × 7.5–8μm	Whole ascospore was not measured. Septa, 9.8–16.0 × 1.0–1.5 μm	[24]
Ophiocordyceps larvarum	Lepidoptera larva	100–380 × 2.0–2.5 mm	Superficial, ovoid, 600–700 × 330–370 μm	Narrowly cylindrical, 180–200 × 8.5 μm	Whole ascospore was not measured. Septa, 4.0–9.0 × 2.0–2.5 μm	[2,25]
Ophiocordyceps sinensis	Hepialidae larva	Single, occasionally 2–3, 40–110 × 1.5–4.0 mm	Nearly superficial, ellipsoidal to ovoid, 380–550 × 140–240 μm	Slender, long, 240–485 × 12–16 μm	160–470 × 5.0–6.0 μm. Septa, 5.0–6.3 × 4.6–6.0 μm	[2]

subcylindrical, slender, and long. Fertile parts cylindrical or clavate, yellow-brown to dark brown, covering apex to middle part of stromata, 30–60 mm long, 2–2.5 mm diam., often with a 3–5 mm long sterile apex ($n = 5$). Perithecia dense, superficial, long ovoid, with a basal stipe connected to the stromata, becoming yellowish brown to black brown when mature, 450–740 × 300–450 µm ($n = 10$). Asci hyaline, cylindrical, 8-spored, 260–480 × 8–12 µm ($n = 10$). Apical caps conspicuous and thick, hemiglobose to taper, 7.2–10.0 µm wide, 4.4–6.4 µm high ($n = 10$). Ascospores hyaline, fasciculate, thread-like, slender and long, 170–240 × 2.5–4.1 µm ($n = 10$), with many septa, not breaking into secondary ascospores. Septa, 5.5–19.8 × 2.5–4.1 µm ($n = 10$).

Asexual morph: *Hirsutella*. Colonies on PDA growing very slowly, reaching 12–15 mm diam after 2 months at 25 °C, hard, round, irregular swell, brown, and radial growth of white. Cell secretoried dark brown pigment material. Hyphae hyaline, septate, branched, smooth-walled, 3.2–5.4 µm wide ($n = 10$). Conidiogenous cells monophialidic, sometimes polyphialidic with swollen base and slender neck, generating on hyphae laterally or terminally, hyaline, 46.9–75.6 µm long ($n = 20$), smooth and subcylindrical in the basal region, reaching 3.8–4.7 µm wide ($n = 20$), tapering gradually or abruptly to a straight neck, minutely warty, 2.0–3.0 µm wide at the tip ($n = 20$). Conidia hyaline, aseptate, smooth-walled, arising in groups at the apex of the neck, ellipsoid, citriform or shape of an orange segment, 8.0–12.6 × 3.6–5.0 µm ($n = 25$), single or 2–4 aggregated, embedded in a pigmented mucous sheath.

Host: Larvae of Hepialidae sp., reddish dark brown, 31–55 mm long, 6–10 mm wide ($n = 10$).

Other materials examined: CHINA. Sichuan Province: Liangshan Yi Autonomous Prefecture, Leibo County, Xining Town (N 28.26°, E 103.57°), alt. 1540 m, on larvae of Hepialidae sp. living in *Qiongzhuea tumidinoda* forests, 12 July 2011, Hong Yu (YHH 16800, epitype, designated here; YFCC 8577, ex-epitype living culture); Ibid., 5 August 2016, Lei Ding (YHH 17007–YHH 17050). CHINA. Yunnan Province: Zhaotong City, Shuifu County, Taiping Town, Tongluoba National Forest Park (N 28.41°, E 104.15°), alt. 1750 m, on larvae of Hepialidae sp. living in *Q. tumidinoda* forests, 20 June 2015, Yong-Dong Dai (YHH 16861–YHH 16900; YFCC 8578, living culture).

Known distribution: this species is distributed in Sichuan, Yunnan, and Guizhou, southwestern China.

4. Discussion

Ophiocordyceps liangshanensis and *O. robertsii* share similar morphological characteristics by producing long stromata with a sterile apex, wide, and brown perithecia, long and cylindrical asci, except that *O. robertsii* produces secondary ascospores [19]. There are more than 270 known species of *Ophiocordyceps* but only a few species (i.e., *O. liangshanensis*, *O. xuefenensis* T.C. Wen, R.C. Zhu, J.C. Kang & K.D. Hyde, *O. ramosissimum* T.C. Wen, J.C. Kang & K.D. Hyde, *O. karstii*, *O. lanpingensis* H. Yu & Z. H. Chen, *O. emeiensis* (A.Y. Liu & Z.Q. Liang) G.H. Sung *et al.*, *O. larvarum* (Westwood) G.H. Sung et al., and *O. sinensis*) have long stromata, superficial perithecia and ascospores not breaking into secondary ascospores. *Ophiocordyceps liangshanensis* differs from the other species mentioned above in having relatively wide perithecia (300–450 µm) (Table 3). Its asexual state has long conidiogenous cells, and is similar to that of *H. illustris* Minter & B.L. Brady and *H. strigosa* Petch. However, *O. liangshanensis* differs from *H. illustris* by its smaller size in conidia (8.0–12.6 × 3.6–5.0 µm). The conidiogenous cells of *O. liangshanensis* generate on hyaline hyphae laterally or terminally, whereas those of *H. strigosa* arise at right angle from brown hyphae. To fix the species concept, a recently collected specimen (YHH 16800), is designated here as the epitype of *O. liangshanensis*.

No serious comparison was made for the materials of *C. liangshanensis* from China and those from Nepal previously [4,5]. Based on multi-gene phylogeny, "*C. liangshanensis*" from Nepal reported by Sung et al. [5] was identified as a member in Clavicipitaceae, whereas "*C. liangshanensis*" from Sichuan (type locality) and Yunnan, China, belongs to Ophiocordycipitaceae in the present study. Thus, its new combination is proposed as *Ophiocordyceps liangshanensis* instead of the previous names, "*Metacordyceps liangshanensis*" and "*Papiliomyces liangshanensis*."

The genus *Papiliomyces*, consisting of two species, was proposed for the type species "*P. liangshanensis*" by Mongkolsamrit et al. [6] based on its phylogenetic placement. However, it is clear that the Nepalese collections differ from the Chinese collections, and should be restudied and described in detail, as well as other nomenclatural and taxonomic confusions should be verified in the future.

Disclosure statement

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

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