Ophiocordyceps megala sp. nov (Ophiocordycepitaceae) enriches the species diversity of the O. sinensis-affined lineage

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Abstract

Ophiocordyceps is a species-rich genus in the order Hypocreales (Ascomycota) including large numbers of invertebrate-pathogen. *Ophiocordyceps sinensis* is a famous traditional Chinese medicine that adapts to the alpine environment in the Qinghai-Tibet Plateau and adjacent region. The diversity of *Ophiocordyceps sinensis* affined species could expand the traditional medicinal resources and provide insight to the adaptation to different ecological environments. In this study, a new species *O. megala* was reported from the Hengduan Mountains, one of biodiversity hotspot area. *O. megala* differed considerably from related species mainly in having massive stromata, long phialides, single big conidia and the huge-sized host. Phylogenetic analyses based on five genes of nrSSU, nrLSU, *tef*, *rpb1* and *rpb2* clarified that *O. megala* was in the *O. sinensis* Clade and closed to *O. sinensis*. The combined morphological, ecological and phylogenetic evidences supported its distinctiveness from allied *O. sinensis, O. xuefengensis* and *O. macroacicularis*. Meanwhile, the prediction of the suitable distribution of seven *O. sinensis* -affined species revealed that their potential suitable distribution extends from the southeastern QTP to the Xuefeng mountains with non-sporadically fragmented regions. The specific biodiversity corridor hypothesis was put forward in this paper, i.e., the *O. sinensis*-affined species might have an entire suitable distribution area from west to east, which could provide an excellent ecological environment for the spread and evolution of this unique group. Our results should have positive significance for the diversity and adaptive evolution of the *O. sinensis*-affined phylogenetic lineage.

Introduction

Ophiocordyceps Petch (Hypocreales, Ophiocordycipitaceae) is a large genus with 251 accepted species names (http://www.speciesfungorum.org/, 2022-9-30). The genus erected originally for species of the *Cordyceps* having asci with conspicuous apical caps and whole ascospores with distinct septation (Petch 1924, 1931).

The majority of species in *Ophiocordyceps* possess firm, darkly pigmented stromata or subiculum, especially those with *Hirsutella* Pat. asexual morphs while some species produce brightly coloured stromata with *Hymenostilbe* Petch and *Paraisaria* Samson & B.L. Brady asexual morph. The stromata are usually tough, wiry, fibrous or pliant. Perithecia are superficial to completely immersed, oblique or ordinal in arrangement. Ascospores are usually cylindrical, multiseptated, either disarticulating into part spores or remain whole after discharge (Sung et al., 2007).

Species of *Ophiocordyceps* was distributed worldwide in the forest ecosystem of the meadow, tropics and subtropics (Petch 1933, 1937, Kobayasi 1941, Tzean et al. 1997, Ban et al. 2015, Luangsa-ard et al. 2018; Wang et al. 2018; Araújo et al. 2018; 2020; Mongkolsamrit et al. 2019; Zha et al. 2021). Although tropical and subtropical forests are the regions with the richest species diversity of *Ophiocordyceps*, alpine or plateau regions cannot be ignored either. *Ophiocordyceps sinensis* (Berk.) G.H. Sung, J.M. Sung, Hywel-Jones, Spatafora, commonly known as the Chinese caterpillar fungus, is the star species as a rare transitional Chinese medicine, which is endemic to the Qinghai–Tibetan Plateau QTP) and its surroundings in high altitude cold environment (Winkler 2008; Li et al. 2011; Hu et al. 2013).

During the last two decades, we have conducted large-scale surveys of entomopathogenic fungi in the alpine area of southwestern China, one of the biodiversity hotspots in the world (Wang et al. 2020; Wang et al. 2021; Dong et al. 2022). Some specimens with huge sclerotium (parasite on the large moth) and long stromata were collected. A new taxon, closely related to *O. sinensis* was identified. As all known, *Ophiocordyceps sinensis* is a famous traditional Chinese medicine that restrict distributed to the alpine environment in the Qinghai-Tibet Plateau and adjacent region. our results may further increase the diversity of *O. sinensis* relatives, and expand the traditional medicinal resources. This study presented the morphological description and phylogenetic analyses of this fungus. The species diversity and potential distribution of the *O. sinensis*-affined phylogenetic lineage were also discussed.

Materials and Methods

Specimen collection

The specimens were collected from Lanping county, Yunnan province, China (26.46° N, 99.17° E, atitude 2500 m), in July 2015 by Hong Yu, Yong-dong Dai, Run-de Yang and Tian-Lin He, parasited on larvae cadavers of *Endoclita* sp. (Hepialidae). All specimens were deposited in the Yunnan Herbal Herbarium (YHH), China.

Fungal isolation and culture

The surface of specimen was rinsed with sterile water, followed surface sterilization with 75% ethanol for 1–3 min. The fresh tissue isolated from internal sclerotium were isolated with potato dextrose (PDA) agar. Then, the strain was cultured at 20°C under dark conditions. After purification, cultures were transplanted to PDA slant and stored at 4°C. All isolates were deposited in the Yunnan Fungal Culture Collection (YFCC), China.

Optical and scanning electron microscope observations

Specimens collected in the field were photographed and measured by stereomicroscope (Olympus SZ61). Cultures on slant were transferred to PDA plates and cultured in incubator for three weeks at 20°C. The circular agar blocks ca. 5 mm diam from a colony were digged out and placed on new PDA plates for colonial morphological observation.

For the morphological description, microscope slide cultures were prepared by placing a small piece of mycelia on 5 mm diam PDA medium block overlaid by a cover slip. Micro-morphological observations and measurements were conducted using Olympus CX40 microscope for hypha, synnema, conidial mass,

phialide and conidium.

Electron microscopy was carried out. Briefly, 1 cm wide agar blocks with hyphae of the fungus were cut from PDA cultures, and the collected samples were fixed with 4% glutaraldehyde at 4°C overnight, then washed three times with phosphate buffer solution (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) three times, 10 min/times. Fixed hyphae and conidia were dehydrated using 50%, 70%, 90% and 100% alcohol, 10 min/each level; dehydrated with supercritical carbon dioxide at last. Placed the samples to spray gold. Conidia and mucilage were examined with scanning electron microscope (SEM, S-3400N, HITACHI, Japan) and photographed.

DNA extraction, PCR amplification and sequencing

The Genomic DNA of fungus and its host were extracted with a Fungi DNA isolation Kit according to the manufacturer's instructions (Transgen Bio-Tek, USA) from the stroma and the surface of sclerotium sections respectively. The Genomic DNA was also extracted from the fungal pure cultures (0.05–0.1g axenic mycelia). The genomic DNA (> 20 ng/μL) was used as template to amplify DNA fragment.

Six nuclear loci of fungus were amplified and sequenced, including internal transcribed spacer (ITS), the small and large subunit ribosomal RNA (nrSSU, nrLSU), the transcription elongationfactor-1 alpha (*tef*), the largest and second largest subunits of RNA polymerase II (*rpb1* and *rpb2*). The mitochondria cytochrome c oxidase subunit (cox1) sequences of insect host were also amplified and sequenced. The polymerase chain reaction (PCR) assay was conducted followed by operation manual. All used primers information was supplied in Additional file 1 table S1. PCR products were sequenced on the ABI3700 automatic sequence analyzer (Sangong, Shanghai). Five species and their host insects were newly sequenced represented *Ophiocordyceps megala*, *Ophiocordyceps xuefengensis*, T.C. Wen, *Ophiocordyceps ramosissimum* T.C. Wen, J.C. Kang & K.D. Hyde and *Ophiocordyceps laojunshanensis*.

Molecular phylogeny

To construct a phylogenetic tree of *O. megala* and its related species, representative taxa with five loci (nrSSU, nrLSU, *tef*, *rpb1* and *rpb2*) were collected from the previous published phylogenetic studies in the genus *Ophiocordyceps* (Sung et al. 2007; Quandt et al. 2014; Suanjuan et al. 2014; Ban et al. 2015, Simmons et al. 2015; Mongkolsamrit et al. 2019) (Table 1). Five loci of each sample were retrieved from GenBank base on their accession numbers.

The 5- locus dataset was established combined the published data with our new sequences generated from the present study. A total of 185 taxa of 5-locus sequence data were selected to represent the diversity of *Ophiocordyceps* (Table 1). Three *Tolypocladium* species were chosen as the out groups (Kepler et al. 2014). ITS gene was used to compare the difference between *O. megala* and its relatives.

Table 1
Specimen and their 5 genes accession numbers information of nrSSU, nrLSU, *tef*, *rpb1* and *rpb2*

Species	Speciemen	accession numbers				
		nrSSU	nrLSU	tef	rpb1	rpb2
O. megala	YHH 1507001	NMDCN00011VK	NMDCN00011VM	NMDCN00011VO	NMDCN00011VQ	NMDCN00011VS
O. megala	YHH 1507002	NMDCN00011VL	NMDCN00011VN	NMDCN00011VP	NMDCN00011VR	NMDCN00011VT
Hirsutella cryptosclerotium	ARSEF_4517	KM652066	KM652109	KM651992	KM652032	
Hirsutella fusiformis	ARSEF_5474	KM652067	KM652110	KM651993	KM652033	
Hirsutella gigantea	ARSEF_30		JX566977	JX566980	KM652034	
Hirsutella guyana	ARSEF_878	KM652068	KM652111	KM651994	KM652035	
Hirsutella illustris	ARSEF_5539	KM652069	KM652112	KM651996	KM652037	
Hirsutella kirchneri	ARSEF_5551	KM652070	KM652113	KM651997		
Hirsutella lecaniicola	ARSEF_8888	KM652071	KM652114	KM651998	KM652038	
Hirsutella liboensis	ARSEF_9603	KM652072	KM652115			
Hirsutella necatrix	ARSEF_5549	KM652073	KM652116	KM651999	KM652039	
Hirsutella nodulosa	ARSEF_5473	KM652074	KM652117	KM652000	KM652040	
Hirsutella radiata	ARSEF_1369	KM652076	KM652119	KM652002	KM652042	
Hirsutella rhossiliensis	ARSEF_3207	KM652079	KM652122	KM652005	KM652044	
Hirsutella rhossiliensis	ARSEF_2931	KM652078	KM652121	KM652004	KM652043	
Hirsutella rhossiliensis	ARSEF_3751	KM652081	KM652124	KM652007	KM652046	
Hirsutella rhossiliensis	ARSEF_3747	KM652080	KM652123	KM652006	KM652045	
Hirsutella satumaensis	ARSEF_996	KM652082	KM652125	KM652008	KM652047	
Hirsutella sp1.	OSC_128575	EF469126	EF469079	EF469064	EF469093	EF469110
Hirsutella sp2.	ARSEF_2348	KM652077	KM652120	KM652003		
Hirsutella strigosa	ARSEF_2197	KM652085	KM652129	KM652012	KM652050	
Hirsutella subulata	ARSEF_2227	KM652086	KM652130	KM652013	KM652051	
Hirsutella thompsonii	ARSEF_256	KM652090	KM652135	KM652018	KM652053	
Hirsutella versicolor	ARSEF_1037	KM652102	KM652150	KM652029	KM652063	
O. acicularis	OSC_110987	EF468950	EF468805	EF468744	EF468852	
O. acicularis	OSC_110988	EF468951	EF468804	EF468745	EF468853	
O. agriota	ARSEF_5692	DQ522540	DQ518754	DQ522322	DQ522368	DQ522418
O. amazonica	Ophama2026	KJ917562	KJ917571	KM411989	KP212902	KM411982
O. aphodii	ARSEF_5498	DQ522541	DQ518755	DQ522323		DQ522419
O. appendiculata	NBRC_106959	JN941729	JN941412	AB968578	JN992463	AB968540
O. appendiculata	NBRC_106960	JN941728	JN941413	AB968577	JN992463	AB968539
O. araracuarensis	HUA 186148	KC610790	KF658679	KC610739	KF658667	KC610717
O. arborescens	NBRC_105890	AB968387	AB968415	AB968573		AB968535
O. arborescens	NBRC_105891	AB968386	AB968414	AB968572		AB968534
O. blattarioides	HUA186093	KJ917559	KJ917570	KM411992	KP212910	
O. brunneipunctata	OSC_128576	DQ522542	DQ518756	DQ522324	DQ522369	DQ522420
O. buquetii	HMAS_199617	KJ878940	KJ878905	KJ878985	KJ879020	
O. cf. acicularis	OSC_128580	DQ522543	DQ518757	DQ522326	DQ522371	DQ522423
O. clavata	NBRC_106961	JN941727	JN941414	AB968586	JN992461	AB968547
O. coccidiicola	NBRC_100682	AB968391	AB968419	AB968583		AB968545

Species	Speciemen	accession numbers				
		nrSSU	nrLSU	tef	rpb1	rpb2
O. cochlidiicola	HMAS_199612	KJ878917	KJ878884	KJ878965	KJ878998	
O. coenomyia	NBRC 108993	AB968384	AB968412	AB968570		AB968532
O. crinalis	GDGM_17327	KF226253	KF226254	KF226256	KF226255	
O. curculionum	OSC_151910	KJ878918	KJ878885		KJ878999	
O. elongata	OSC_110989		EF468808	EF468748	EF468856	
O. entomorrhiza	KEW_53484	EF468954	EF468809	EF468749	EF468857	EF468911
O. evansii	Ophsp.858	KC610796	KC610770	KC610736	KP212916	
O. formicarum	TNS_F18565			KJ878968		
O. formosana	MFLU:15-3888		KU854949			
O. formosana	NTU_00035			KT275192		
O. forquignonii	OSC_151902	KJ878912	KJ878876		KJ878991	KJ878945
O. fulgoromorphila	HUA 186139	KC610794	KC610760	KC610729	KF658676	KC610719
O. fulgoromorphila	Ophara729	KC610795	KC610761	KC610730	KF658677	AB968554
O. geometridicola	BCC35947	AB104725	KJ878877		KJ878992	
O. geometridicola	BCC79823				MH028163	MH028173
O. gracilioides	Ophuni866	KC610799		KC610742	KF658674	KC610718
O. gracilioides	Ophgrc934	KJ917556		KM411994	KP212914	
O. gracilis	EFCC_3101	EF468955	EF468810	EF468750	EF468858	EF468913
O. gracilis	EFCC_8572	EF468956	EF468811	EF468751	EF468859	EF468912
O. gracillima	Ophgrc679		KC610768	KC610744	KF658666	
O. heteropoda	NBRC 100642	JN941720	JN941721	AB968594		AB968555
O. highlandensis	HKAS83207	KM581284			KM581274	KM581278
O. hignland	YHH_OH1301	KR479869		KR479870	KR479872	KR479874
O. irangiensis	OSC_128578	DQ522556	DQ518770	DQ522345	DQ522391	DQ522445
O. karstii	MFLU:15-3884		KU854945			
O. karstii	MFLU:15-3885		KU854946			
O. konnoana	EFCC_7315	EF468959		EF468753	EF468861	EF468916
O. lanpingensis	YHOS0707	KC417459	KC417461	KC417463	KC417465	
O. lloydii	OSC_151913	KJ878924	KJ878891	KJ878970	KJ879004	KJ878948
O. longissima	TNS_F18448	KJ878925	KJ878892	KJ878971	KJ879005	
O. longissima	NBRC 106965	AB968392	AB968420	AB968584		AB968546
O. macroacicularis	NBRC_100685	AB968388	AB968416	AB968574		AB968536
O. macroacicularis	NBRC_105889	AB968390	AB968418	AB968576		AB968538
O. macroacicularis	NBRC_105888	AB968389	AB968417	AB968575		AB968537
O. melolonthae	OSC_110993	DQ522548	DQ518762	DQ522331	DQ522376	
O. myrmecophila	CEM1710	KJ878928	KJ878894	KJ878974	KJ879008	
O. myrmecophila	TNS_27120	KJ878929	KJ878895	KJ878975	KJ879009	
O. neovolkiana	OSC_151903	KJ878930	KJ878896	KJ878976		
O. nigrella	EFCC_9247	EF468963	EF468818	EF468758	EF468866	EF468920
O. nooreniae	BRIP 55363			KX673812		
O. nutans	OSC_110994	DQ522549	DQ518763	DQ522333	DQ522378	
O. nutans	NBRC_100944	JN941713	JN941428	AB968588		AB968549

Species	Speciemen	accession numbers				
		nrSSU	nrLSU	tef	rpb1	rpb2
O. pauciovoperitheciata	BCC45562		MH028162			MH028181
O. pauciovoperitheciata	BCC39781		MH028167	MH028182		
O. ponerinarum	HUA186140	KC610789	KC610767	KC610740	KF658668	
O. pseudoacicularis	BCC49256		MH028154		MH028166	MH028176
O. pseudoacicularis	BCC53843		MH028156		MH028168	MH028177
O. pulvinata	TNS-F 30044	GU904208	AB721305	GU904209	GU904210	
O. purpureostromata	TNS_F18430	KJ878931	KJ878897	KJ878977	KJ879011	
O. ramosissimum	GZUH2012HN2	KJ028013		KJ028016	KJ028018	
O. ramosissimum	GZUHHN8	KJ028012		KJ028014	KJ028017	
O. ravenelii	OSC_151914	KJ878932		KJ878978	KJ879012	KJ878950
O. rubiginosiperitheciata	NBRC_106966	JN941704	JN941437	AB968582	JN992438	AB968544
O. rubiginosiperitheciata	NBRC_100946	JN941705	JN941436	AB968581	JN992439	AB968543
O. sinensis	YN09-64	JX968028	JX968033	JX968018	JX968008	JX968013
O. sinensis	YN07-8	JX968027	JX968032	JX968017	JX968007	JX968012
O. sinensis	XZ06-44	JX968026	JX968031	JX968016	JX968006	JX968011
O. sinensis	QH06-197	JX968025	JX968030	JX968015	JX968005	JX968010
O. sinensis	QH09-201	JX968024	JX968029	JX968014	JX968004	JX968009
O. sobolifera	NBRC 106967	AB968395	AB968422	AB968590		AB968551
O. sobolifera	KEW_78842	EF468972	EF468828		EF468875	EF468925
O. spataforae	NHJ_12525	EF469125	EF469078	EF469063	EF469092	EF469111
O. sphecocephala	NBRC 101416	JN941698	JN941443		JN992432	
O. stylophora	NBRC_100948	JN941693	JN941448	AB968580	JN992427	AB968542
O. stylophora	NBRC_100949	JN941692	JN941449		JN992426	
O. stylophora	OSC_111000	DQ522552	DQ518766	DQ522337	DQ522382	DQ522433
O. stylophora	OSC_110999	EF468982	EF468837	EF468777	EF468882	EF468931
O. stylophora	NBRC_100947	JN941694	JN941447	AB968579	JN992428	AB968541
O. tettigonia	GZUHCS14062709	KT345955		KT375440	KT375441	
O. tiputini	Ophsp 1465	KC610792	KC610773	KC610745	KF658671	
O. tricentri	NBRC 106968	AB968393	AB968423	AB968593		AB968554
O. unilateralis	OSC_128574	DQ522554	DQ518768	DQ522339	DQ522385	DQ522436
O. unitubercula	YHH HU1301					
O. unitubercula	YFCC HU1302					
O. variabilis	ARSEF_5365	DQ522555	DQ518769	DQ522340	DQ522386	DQ522437
O. xuefengensis	GZUH2012HN14	KC631789		KC631793	KC631798	
O. xuefengensis	GZUH2012HN13	KC631787		KC631792	KC631797	
O. yakusimensis	HMAS_199604	KJ878938	KJ878902		KJ879018	KJ878953
Ophiocordyceps sp1	TNS_16252	KJ878941	KJ878906	KJ878986		
Ophiocordyceps sp2	NHJ_12581	EF468973	EF468831	EF468775		EF468930
Ophiocordyceps sp3	TNS_16250	KJ878942		KJ878987	KJ879021	
Ophiocordyceps sp4	OSC_110997	EF468976		EF468774	EF468879	EF468929
Ophiocordyceps sp5	NHJ_12582	EF468975	EF468830	EF468771		EF468926
Ophiocordyceps sp6	TNS_F18550	KJ878911	KJ878875	KJ878959		

Species	Speciemen	accession numbers				
		nrSSU	nrLSU	tef	rpb1	rpb2
Podonectria citrina	TNS_F18537		KJ878903	KJ878983		KJ878954
Torrubiella pruinosa	NHJ_12994	EU369106	EU369041	EU369024	EU369063	EU369084

Note: The sequences of *Ophiocordyceps megala* were submitted to NMDC (National Microbiology Data Center), and the NMDC accession numbers were list. The others sequencs accession numbers were obtained from GenBank.

Sequence alignment of nrSSU, nrLSU was conducted using MAFFT (Katoh et al. 2002) with the default settings. the codon model was set when aligned the exon regions of *tef*, *rpb1* and *rpb2*. And manual adjustments were made in MEGA5.0. Alignment lengths were total of 4515 bp, 1109 for nrSSU, 1026 for nrLSU, 931 for *tef*, 553 for *rpb1*, 935 for *rpb2*. All five loci were joined into a single dataset and 11 data partitions were defined: one each for nrSSU and nrLSU plus 9 for each of the three codon positions for the protein coding genes *tef*, *rpb1* and *rpb2*.

Best partitioning scheme and evolutionary models for 11 pre-defined partitions were selected using PartitionFinder2 (Lanfear et al. 2017), with greedy algorithm and AIC criterion. the following 5 partitions were identified: Partition. 1—nrSSU, nrLSU. Partition 2—*tef* codon1, *tef* codon2. Partition 3— *rpb1* codon1, *rpb2* codon 1. Partition 4— *rpb1* codon2, *rpb2* codon2. Partition 5—*tef* codon3, *rpb1* codon3, *rpb2* codon3. Maximum likelihood phylogenetic tree were inferred using IQ-TREE (Nguyen et al. 2015) for 2000 ultrafast (Minh et al. 2013) bootstraps, as well as the Shimodaira–Hasegawa–like approximate likelihood-ratio test (Guindon et al., 2010). The entire phylogenetic construction process is conducted in PhyloSuite (Zhang et al. 2020)

Bayesian Inference phylogenetic tree was inferred using MrBayes 3.2.6 (Ronquist et al. 2012) under partition model (2 parallel runs, 50000000 generations), in which the initial 25% of sampled data were discarded as burn-in. The operation stop rule was set when the average standard deviation of split frequencies was below 0.01. The convergence of the runs was checked using Tracer v.1.6 (Rambaut et al. 2014). Due to the huge amount of data and the time-consuming process, we used the online platform (CIPRES, https://www.phylo.org/portal2/) to complete the calculations. The contree file was visualized with FigTree v.1.6 (http://tree.bio.ed.ac.uk/software/figtree/)

In addition, the cox1 sequence was used to identify the host species of *O. megala* and its relatives. The whole process was conducted in PhyloSuite (Zhang et al. 2020)

Species distribution modelling of the O. sinensis-**affined phylogenetic lineage**

Species occurrence data was collected from ongoing field studies and published research papers. Bioclim variables were downloaded from the CliMond Archive (https://www.climond.org/) (Kriticos et al. 2012)

A total of 31 species occurrence data were used (Table 2). And a total of 35 typical climate variables at a grid resolution of 10' were obtained from CliMond the CRU CL2.0 dataset. (https://www.climond.org/). These factors contain the core set of 19 variables (temperature and precipitation), an extended set of 16 additional variables (solar radiation and soil moisture) at a global extent (Additional file 1, table S2). All climate variables data collection sources were from 1961–1990 (30 years centred on 1975).

Table 2
The species occurrence data of seven *O. sinensis* affined species

Species	Distribution		Species	Distribution	
	Longitude	**Latitude**		**Longitude**	**Latitude**
O. laojunshan-ensis	99.10°	28.93°	*O. karstii*	105.74°	28.36°
	98.95°	28.85°	*O. lanpingensis*	98.97°	26.94°
	99.52°	29.25°		99.14°	26.49°
	99.33°	27.10°		99.20°	26.46°
	98.75°	27.76°		99.26°	26.50°
	100.74°	27.27°		99.16°	26.25°
	100.12°	27.05°		99.21°	26.84°
	100.90°	26.67°		99.02°	26.95°
0.xuefenensis	110.41°	27.07°		99.51°	26.64°
	110.68°	27.19°		99.42°	26.76°
	110.53°	27.08°		99.34°	26.47°
O. liangshanensis	103.57°	28.26°		99.52°	27.33°
	104.15°	28.41°		99.57°	26.67°
	104.17°	28.46°	*O. macro-acicularis*	110.51°	26.76°
O. megala	107.03°	28.13°		106.67°	26.39°
	121.19°	24.01°			

Species distribution modelling were constructed based on species redundance with MaxEnt V3.4.1 (Phillips et al. 2006; Elith et al. 2011). randomly 25% of the data points were extracted as the test data, and “do jackknife to measure variable importance” was selected. The output grid format was set as “cloglog.” The output result of was visualized with Global mapper17.

Results

Molecular Phylogeny

Both ML and BI analyses of the combined 5-locus (nr*SSU*, nr*LSU*, *tef*, *rpb1* and *rpb2*) dataset recognized five statistically well-supported clades from a total of 185 taxa within *Ophiocordyceps*, designated here as *O. sinensis* Clade (MLBS = 94, BI = 0.999), *O. unilateralis* Clade (MLBS = 87, BPP = 0.999), *O. sphecocephala* Clade (MLBS = 100, BPP = 1.00), and *O. ravenelii* Clade (MLBS = 96, BI = 0.998) (Fig. 1). It was showed that *O. megala* clustered into the *O. sinensis* Clade. It was a sister species of *O. sinensis*, *O. macroacicularis* and *O. xuefengensis*. However, the separate clade feather with high support values also revealed the difference among *O. megala* anditsrelated species (MLBS = 99; BPP = 0.997).

Host identification

Hosts of the *O. megala* and its 5 relatives were identified based on *cox1* gene. The ML tree showed that all hosts belonged to Hepialidae (Lepidoptera), but scattered in different phylogenetic clade. The hosts of *O. megala* was identified as *Endoclita* sp. and near to *Endoclita davidi* (the host of *O. xuefengensis*). It's worth mentioning that *O. sinensis* has a very high diversity of hosts, with several clades forming in *Thitarodes* and *Ahamus*.

Taxonomy

Ophiocordyceps megala H. Yu, Y.D. Dai sp. nov. (Fig. 3).

MycoBank

MB845561

Etymology

referring to the long and large (massive) stromata and huge host.

Diagnosis

O. megala differed considerably from related species mainly in having massive stromata, long phialides, single big conidia and the huge-sized host.

Holotype: YHH 1507001, China. Yunnan Province: Lanping County, Yingpan village, at 26.46° N, 99.17° E, alt. 2800 m, on a larva of *Endoclita* sp. burried in soil, July 2015, H. Yu, R.D. Yang, Y.D. Dai (ex-holotype: YFCC15079192) (Fig. 3A).

Asexual morph

Hirsutella-like.

Colonies

on PDA reaching 18–23 mm diam after 3 weeks at 20°C, round, irregular swell, grey white to pale brown. Hyphae grew regularly after long time, forming a raised hyphae circle.

Hyphae

hyaline, septate, branched, smooth-walled, 2.6–4.5 µm wide.

Conidiogenous cells

growing from hyphae directly or laterally, monophialidic, hyaline, smooth-walled, subulate, tapering gradually into slender neck, 46.9–75.6 µm long, base (3.2–4.5 µm wide) and neck (1.0–1.5 µm wide).

Conidia

arising solitarily from the apex of conidiogenous cells, oval or orange-like shape, usually single, rare 2(-3) aggregated. 8–12 ×5–7 µm.

Host

the larvae of *Endoclita* sp. (Lepidoptera, Hepailidae). thick and solid, 19–27 × 80–130 mm.

Stromata

single, stipe clavate, solid, lignified, yellow-brown, arising from the head of host, 80–320 mm long, several small branches from tips, grayish white.

Distribution and Habitat: China, Yunnan Province: Lanping County. China, Taiwan Province. Distributed in the subtropical broadleaf forest.

Additional specimens examined: YHH 1507002 (Paratype) (Fig. 3D), China. Yunnan Province: Lanping County, Yingpan village, at 26.46° N, 99.17° E, alt. 2800 m, on a larva of *Endoclita* sp. in soil, July 2015, H. Yu, R.D. Yang, Y.D. Dai. **YHH OMLP1701-04** (Fig. 3B,C), China. Yunnan Province: Shuifu County, Taiping village, at 28.40° N, 104.1° E, alt. 2300 m, on a larva of *Endoclita* sp. in the plant root, August 2016, H. Yu, R.D. Yang, Y.D. Dai. **YHH OMM1401-05**, Myanmar. Kachin state: Muse, alt. 2650 m, July 2014, H. Yu, J.M. Xiao. **NTUH 17 – 004** (Fig. 4B-F), China. Taiwan Province. Cueifong, Nantou County (24.13° N, 121.19° E), 9 July 2017, Wei-Yu Chuang (Ariyawansa et al., 2018).

Notes

Specimens with mature sexual structure were not found among a mass specimens of *O. megala* in this study. However, a specimen numbered NTUH 17 – 004 previously identified as *O. macroacicularis* had the same ITS sequence characteristics with *O. megala* (Fig. 4A). Their 28S ribosomal RNA gene fragment (~ 800 bp) was also total the same (Ariyawansa et al., 2018). Meanwhile, NTUH 17 – 004 had significant difference in the stromata, peritheciua and asci compared with the holotype of *O. macroacicularis*, but it was highly consistent with *O. megala*, indicating that the specimen **NTUH 17 – 004** should be treated as *O. megala*. Thus, the obvious characteristics of mature ascus were supplied based on this specimen, it provided extremely important circumstantial evidence for the description of the new species *O. megala* (Fig. 4).

Based on these characteristics, we described the hypothetical graph of *O. megala* with sexual structure (Fig. 5).

The prediction of the suitable distribution area of the O. sinensis**-affined species**

The prediction result of the suitable distribution area was obtained for 7 species related to *O. sinensis* with the species distribution modeling method. Major suitable distribution areas (highlight with red colour) appear in southern and southeastern edge of the Hengduan Mountains, the Yunnan-Guizhou Plateau and local areas of the Xuefeng Mountains, and some suitable areas exist in the eastern Taiwan Island and Fujian province (Fig. 6). The main geographical distribution, especially in the southwest of China, predominantly present not sporadic but continuous large regions.

The biodiversity corridor hypothesis ( ) was deduced of the *O. sinensis*-affined species based on their potential suitable distribution prediction. The *O. sinensis*-affined species might have an entire suitable distribution area from west to east, which could provide an excellent ecological environment for the spread and evolution of this unique group, so that this group could form rich diversity and radiation adaptation characteristics. This ecological corridor mainly starts from the Qinghai-Tibet Plateau in the west and extends to the Xuefeng mountains in the east, passing through the Hengduan Mountains and the Yunnan-Guizhou plateau (Fig. 6, highlight with white circle). The discovery of *O. megala* has further enriched the species diversity associated with the phylogenetic lineages of *O. sinensis*, and might be important for the study of the origin and evolution of this group.

Discussion

Both morphological observation and phylogenetic analyses supported the distinctiveness of Ophiocordyceps megala

Ophiocordyceps megala was characterized by the long, large and lignified stromata, huge Hepialidae host, and *Hirsutella* asexual stage. In the genus *Ophiocordyceps*, species having the characters of large and lignified stromata with *Hirsutella* asexual stage, mainly includes: (1) *O. robertsii* (Cunningham, 1921), (2) *O. liangshanensis* (Zang et al.,1982; Wang et al., 2021), (3) *O. xuefengensis* (Wen et al., 2013), (4) *O. ramosissimum* (Wen et al., 2014), (5) *O. macroacicularis* (Ban et al., 2014), (6) *O. aborescens* (Ban et al., 2014) and (7) *O. karstii* (Li et al., 2016).

In this study, a detailed description of the asexual stage of *O. xuefengensis* and *O. ramosissimum* had been completed, in view of the lack of asexual stage descriptions of some related species, (Fig. 7). Thus, we could conduct a more comprehensive teleomorphic-anamorphic comparison (Table 3).

Table 3
Morphological comparison of *Ophiocordyceps megala* and its related species

Species	Host	Habitat	Stromata	Perithecium	Asci	Ascospore	Colony	Conidiogenous cells	C
O. megala	*Endoclita* sp. (Hepialidae)	in tree hole or tree root	rust, cylindrical, solitary or branched, 80–320 mm long	Superficial, long ovoid	Cylindrical	needle-shaped, multiseptate with indistict septation, 100–200 µm	18–23 mm diameter on PDA at 20°C in 3 weeks	monophialidic, clavate, swollen base and taper neck. base (3.2–4.5 µm wide) and slender neck (1.0–1.5 µm wide) (*Hirsutella*-like), 46.9–75.6 µm long.	8- µr
O. xuefengensis	*Endoclita davidi* (Hepialidae)	In tree hole or tree trunk of *Clerodendrum cyrtophyllum*	yellowish-brown, Solitary or several, 140–460×2–7 mm	Superficial, long ovoid, 20–74µm Superficial, long ovoid, 416–625 × 161–318 µm	Cylindrical, 191–392 × 4.5–8.9 µm	Thread-like, needle-shaped, multiseptate with indistinct septation, 130–380× 1.4–5.2 µm	16×20 mm diameter on PDA at 20°C in 3 week, gray-white colony with ray-like shape	swollen base (3.0–4.1 µm wide) and slender neck (1.0–1.5 µm wide) (*Hirsutella*-like) (Hirsutella-like), 31–78µm long	8- 7 co 3- ag
O. ramosissimum	*Endoclita* sp. (Hepialidae)	Living trunk or upper root near the soil in tree hole or tree trunk	One or two, 70–150×2–4 mm, branched stromata without a sterile apex	Superficial, ovoid, 340–350 × 225–255 µm	Cylindrical, 172–265 × 6.9–17.3 µm	fasciculate, thread-like, slender and long, multiseptate with indistinct septation, 130–245 × 2–3.5 µm	16×22 mm diameter on PDA at 20°C in 3 weeks	two types of conidiogenous cells (short and long types, 30–45 µm and 75–130 µm	tw of Bi × 6. sr × µr
O. aborescens	*Cossida* sp. (Hepialidae)	in the lignified roots of *Pueria lobata*	cylindrical, solitary or branched, 25-89.5 mm long	Superficial,320–570×270–350 µm	225–250 µm long	Thread-like, multiseptate with indistinct septation,120–190 × 2.3–3.75µm	15–20 mm diameter on PSA at 25°C in 2 weeks	phialidic with swollen base (3.0–4.0 µm wide) and slender neck (1.2–1.5 µm wide) (Hirsutella-like), hyaline, directly produced on prostrate or secondary branches. 30–55 µm long	7.
O. liangshanensis	*Abantiades* sp. (Hepialidae)	soil in the Qiong bamboo forest	single, or occasionally branched, cylindrical, solid, 200–300×1.5–2.5 mm long	superficial, long ovoid, 450–740 × 300–450 µm	hyaline, cylindrical, 260–480 × 8–12µm	170–240 × 3.1–4.1 µm, with many septa, 5.5–20.0 × 2.5–4.1 µm.	12–15 mm diam on PDA after 2 months, hard, round, irregular swell, brown	phialidic with swollen base (3.8–4.7 µm wide) and slender neck (2.0–3.0 µm wide) (*Hirsutella*-like), generating on hyphae laterally or terminally, hyaline, 46.9–75.6 µm long	8. 3. µr 2(ag
O. macroacicularis	Hepialidae sp.	soil near the root of *Fallopia japonica* inhabit the wood or root of Fallopia japonica	cylindrical, solitary or branched,97.2-166.1×1.3–2.4 mm	Oval, light brown, 410–760×260–420 µm	hyaline, cylindrical, 235–310 µm long	needle-shaped, multiseptate with indistinct septation, 200–300×2.3-3µm. (8-)14–16(-20) septa.	20 mm diameter on PSA at 25°C in 2 weeks	swollen base (2.9–4.1 µm wide) and slender neck (1.0–1.3. µm wide) (Hirsutella-like), 30.4–42.0 µm long	3. 5- lo

Species	Host	Habitat	Stromata	Perithecium	Asci	Ascospore	Colony	Conidiogenous cells	C
O. karstii	Hepialidae sp.	soil in the bamboo forest	cylindrical, solitary, 140-145mm long	600–765 × 247–323 μ m	186–228 × 8–12 μ m	needle-shaped, multiseptate with indistinct septation, 173–202 × 3–5 μm	not observed	not observed	n o
O. robertsii	Hepialidae sp	soil in the broad-leaf forest	cylindrical, solitary, 180–200 mm long	Perithecia dense, superficial, long ovoid, 600–680 ×500–550 μm	300–450 μm long	210–330× 3.3–4.4 μm, with many septa	not observed	not observed	n o

O. robertsii was previously known to occur only in Australia and New Zealand, far away from East Asia. The biggest difference was the broken ascospores of *O. robertsii* while the others did not break (Cunningham, 1921).

O. liangshanensis and *O. karstii* were mainly distributed in bamboo forests. And the host species and habitat ecology were obviously different from *O. megala* (Zang et al.,1982; Li et al., 2016; Wang et al., 2021). In addition, *O. liangshanensis* grew slowly with dark brown colour on PDA (Wang et al., 2021), which was also obvious different from *O. megala*.

The stromata of *O. ramosissimum* and *O. aborescens* had many branches with fertile tips. And *O. aborescens* had smaller conidia (7.5×5 μm). *O. ramosissimum* had two types of conidiogenous cells (short and long types, 30–45 μm and 75–130 μm, respecitvely)/ It was also obviously different from *O. megala*.

O. macroacicularis and *O. xuefengensis s* had highly similarity to *O. megala* in in both of sexual and asexual morphology. These 3 species also had the closest phylogenetic relationship. However, they also had significant difference in the typical microstructure. *O. macroacicularis* had shorter conidiogenous cells of 30.4–42.0 μm long, and shorter conidia 3.0–5.0 × 5–8.0 μm. *O. xuefengensis* and *O. megala* had much similarity in stromata, host type and habitat ecology, but the colony of *O. xuefengensis* was dark grayish brown, the conidia were orange -like and lotos-like shape, aggregated frequently, while the conidia of *O. megala* were more single, rare two, less aggregation. Besides, the stromata of *O. megala* was mostly smooth and brown in texture, while *O. xuefengensis* had yellow microvilli.

The new species *O. megala* had large stromata and huge host, with long phialides and big conidia, distinguishing it from the others. Both morphological and phylogenetic analyses (5-locus and ITS sequence data, respectively) showed that *O. megala* was a new species with the *Hirsutella*-like asexual stage.

We also suggested the common local name “Chongcaowang” ( ) in Lanping County as its Chinese name. the mean of “Chongcaowang” also expressed the huge morphological features.

Ophiocordyceps megala **enriches the species diversity of the** O. sinensis**-affined phylogenetic lineage**

We conducted the phylogenetic construction of *O. megala* and its relatives based on the 5-locus data. The result showed that *O. megala* had a close relationship with *O. sinensis*. Which increased the diversity of *O. sinensis* relatives. Meanwhile, the phylogenetic tree preliminarily clarified that the seven species were close related to *O. sinensis*. Except for *O. megala*, there also had *O. karstii*, *O. lanpingensis*, *O. laojunshanensis*, *O. liangshanensis*, *O. macoacicularis* and *O.xuefengensis*. Thus, we have systematically discussed and summarized the species diversity of *O. sinensis* affines. The great significance is that it could provide ideas for the research on the adaptation to different ecological environments (high cold low temperature to normal temperature) of this unique group.

Key to the species within O. sinensis**-affined phylogenetic lineage**

1a. Distribution: Hengduan Mountains and others areas……..2

1b. Distribution: restricted to Xuefengshan Mountains……*O.xuefengensis*

1c. Distribution: restricted to Oceania……*O. robertsii*

2a. Habitat: bamboo forests……..3

2b. Habitat: evergreen broad-leaf forests……..4

2c. Habitat: alpine meadow and scrub……*O. sinensis*

2d. Habitat: alpine forests……*O. laojunshanensis*

3a. ……*O. liangshanensis*

3b ……*O. karstii*

4a. Host: *Endoclita* ……*O. megala*

4b. Host: Cossida……*O. macoacicularis*

4c. Host: *Ahamus*…… *O. lanpingensis*

Species distribution modeling of the O. sinensis**-affined phylogenetic lineage supports the biodiversity corridor hypothesis**

The potential suitable distribution area was predicted to extend from the southeastern QTP to the Xuefeng mountains with non-sporadically fragmented regions. It is suggested that there might be no geographical barrier among the 7 the *O. sinensis*-affined species. Our results totally supported the biodiversity corridor hypothesis. And it also revealed that *O. sinensis* and its relatives have gradually developed radiatively adapted evolutionary features from west to north towards different altitude gradients. The results of this paper present positive significance for the study of the diversity of *O. sinensis* affined species.

Conclusions

In this study, we report the new species *O. megala* from the biodiversity hotspot area, Lanping County, Chins. Its taxonomic position and phylogenetic status were described and illustrated in detail. The 5-gene phylogenetic result shows that *O. megala* had a close relationship with *O. sinensis*. Thus, our discovery of *O. megala* enriches the species diversity of the *O. sinensis*-affined phylogenetic lineage. The prediction of potential suitable distribution of the 7 *O. sinensis*-affined species totally supports the biodiversity corridor hypothesis. On one hand, the great significance is that it could expand the traditional medical resources. On the others, it could provide ideas for the study of the adaptation to different ecological environments of this unique group.

Abbreviations

MLBS: Maximium likelihood bootstrap support. BPP: Bayesian inference posterior probability

Declarations

Ethics approval and consent to participate

Not applicable.

Adherence to national and international regulations

Not applicable.

Consent for publication

All authors agreed to publishing the manuscript in IMA Fungus.

Availability of data and material

All specimens were deposited in the Yunnan Herbal Herbarium (YHH), all isolations were deposited in the Yunnan Fungal Culture Collection (YFCC). All sequences were submitted to GenBank and NMDC.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YD and SC Conceived and designed experiments: YD, YBW, YW. analyzed the data. All authors analyzed them and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

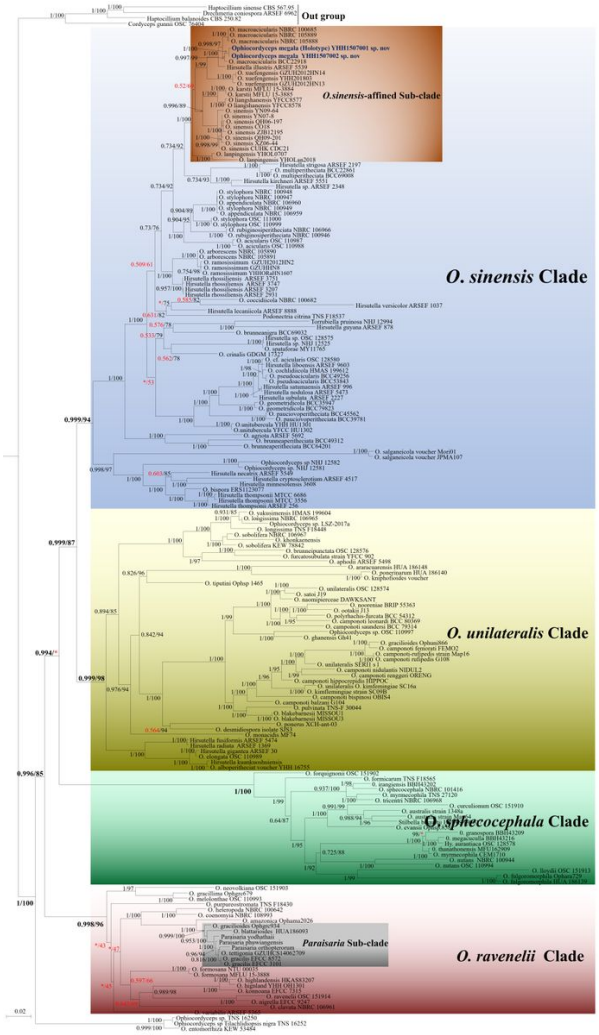

Figure 1

The phylogeny of *Ophiocordyceps* with emphasized on *O. megala* and its related species based on 5-locus datasets

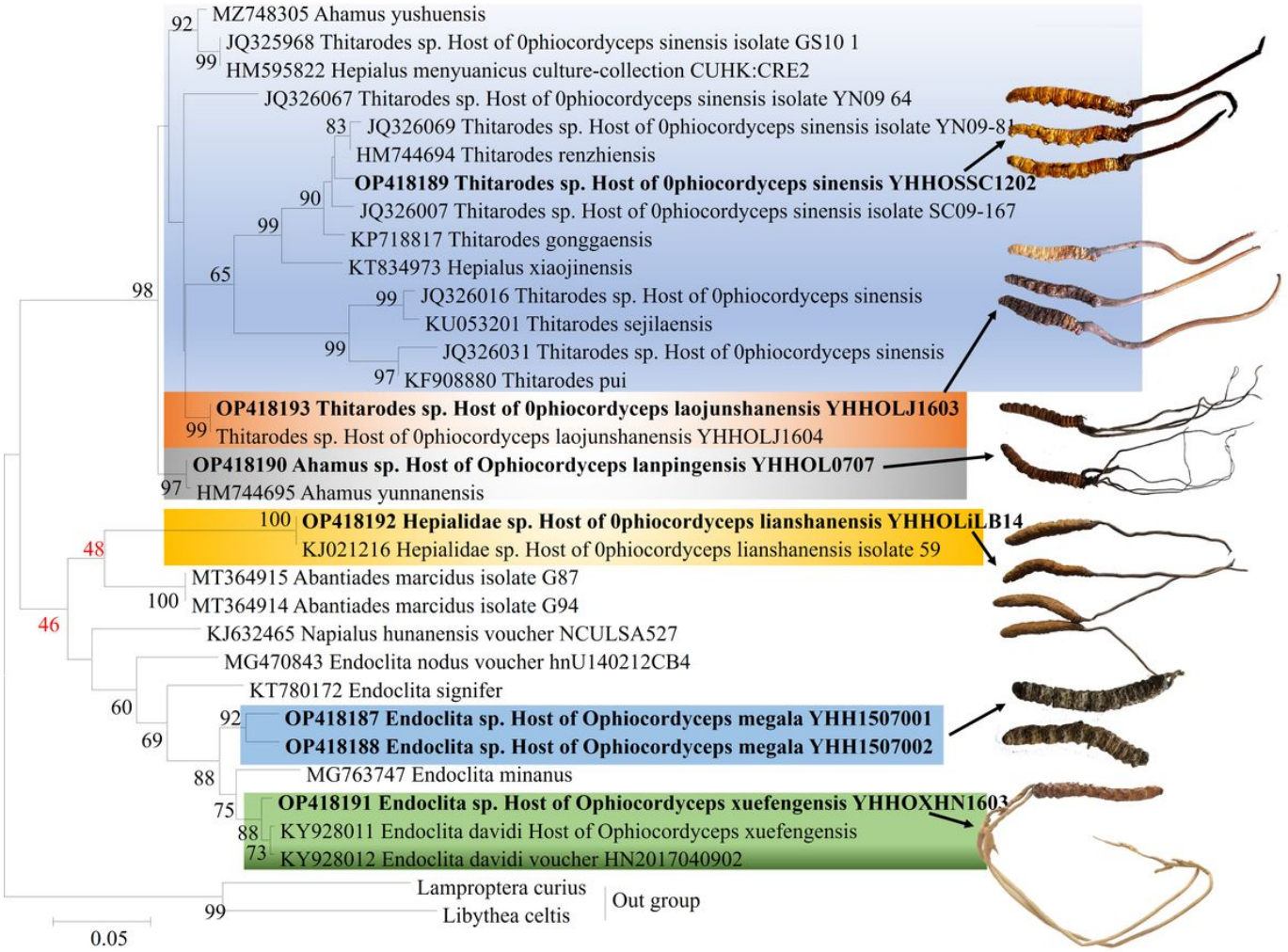

Figure 2

The host identification of *O. megala* and its related species based on cox1 sequence

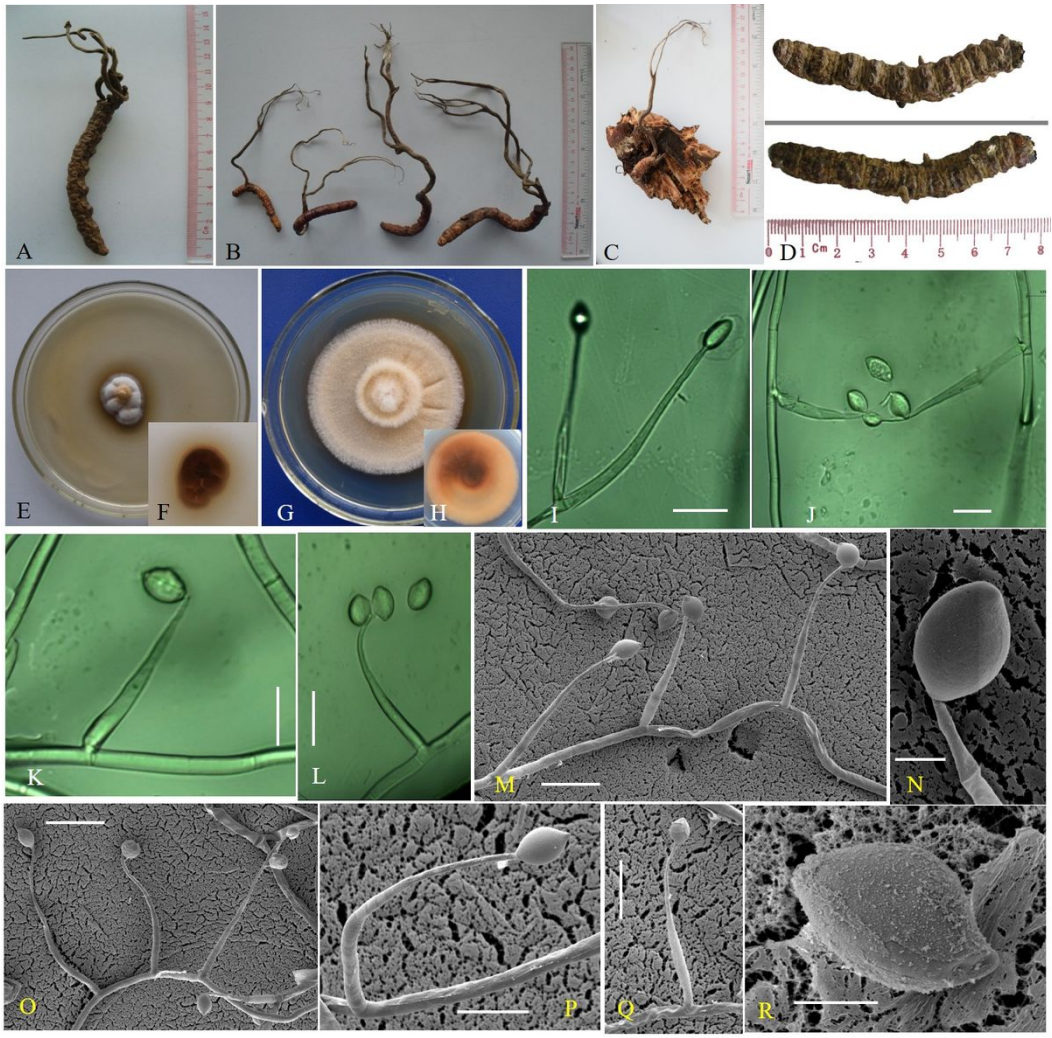

Figure 3

The morphiological and micromorphological characterastics of *Ophiocordyceps megala*

A-C. Wild morph (A. **Holotype** YHH 1507001), D. Host morph; E-H. Colony (YFCC15079192); I-L. Phialide and conidium; M-R. Phialide and conidium under electron microscope. I-Q, bar=10 μm, R, bar=5 μm.

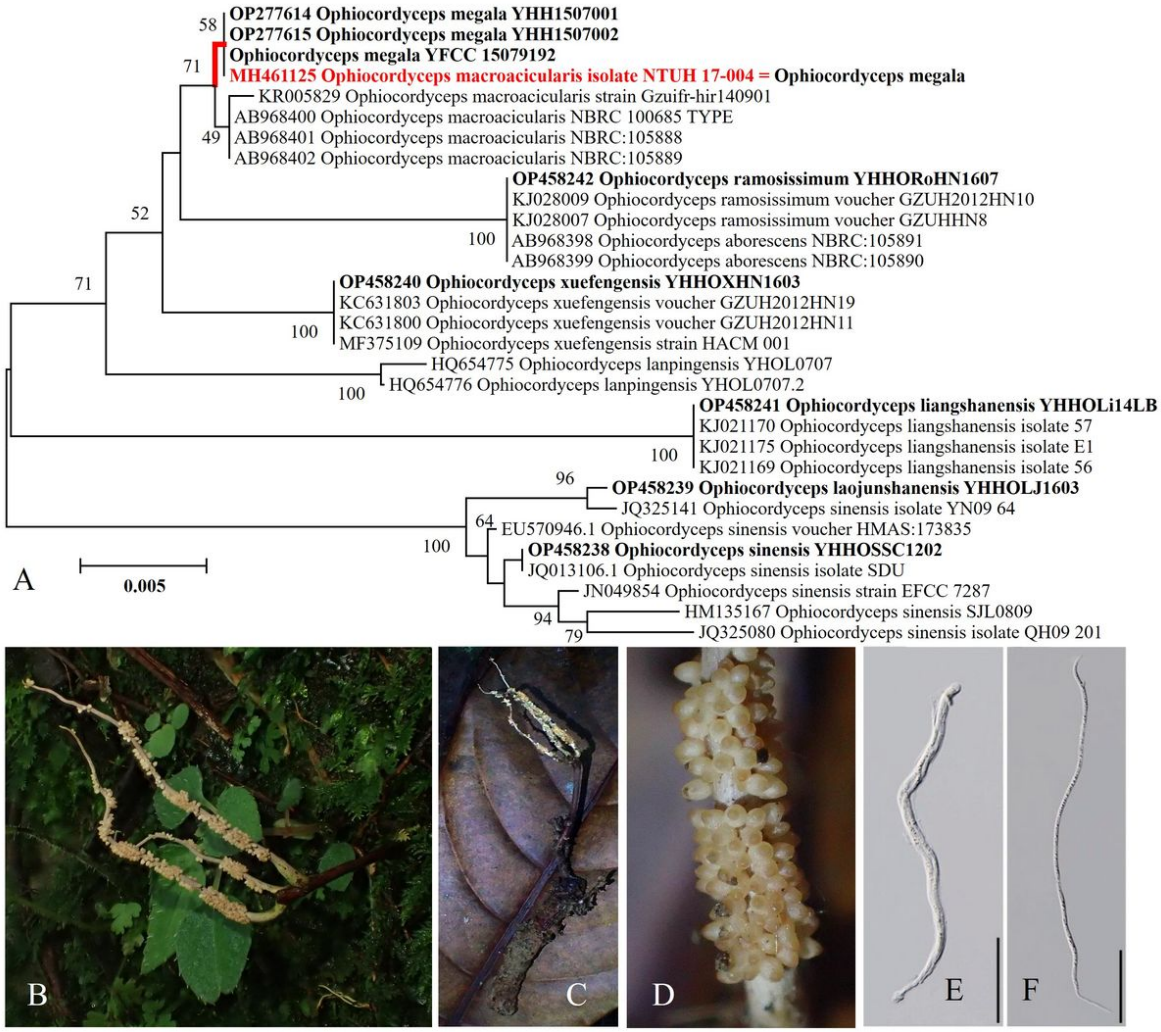

Figure 4

The ITS sequence comparison and morphological characters of the specimen NTUH 17-004 (B-F were referred from Ariyawansa et al. 2018))

A. phylogenetic identification; B-C. Stromata arising from the host in the field; perithecia; E-F. ascus and ascospore

Figure 5

The hypothetical graph of *O. megala* with sexual structure (drawing by Zeng Xiaolian)

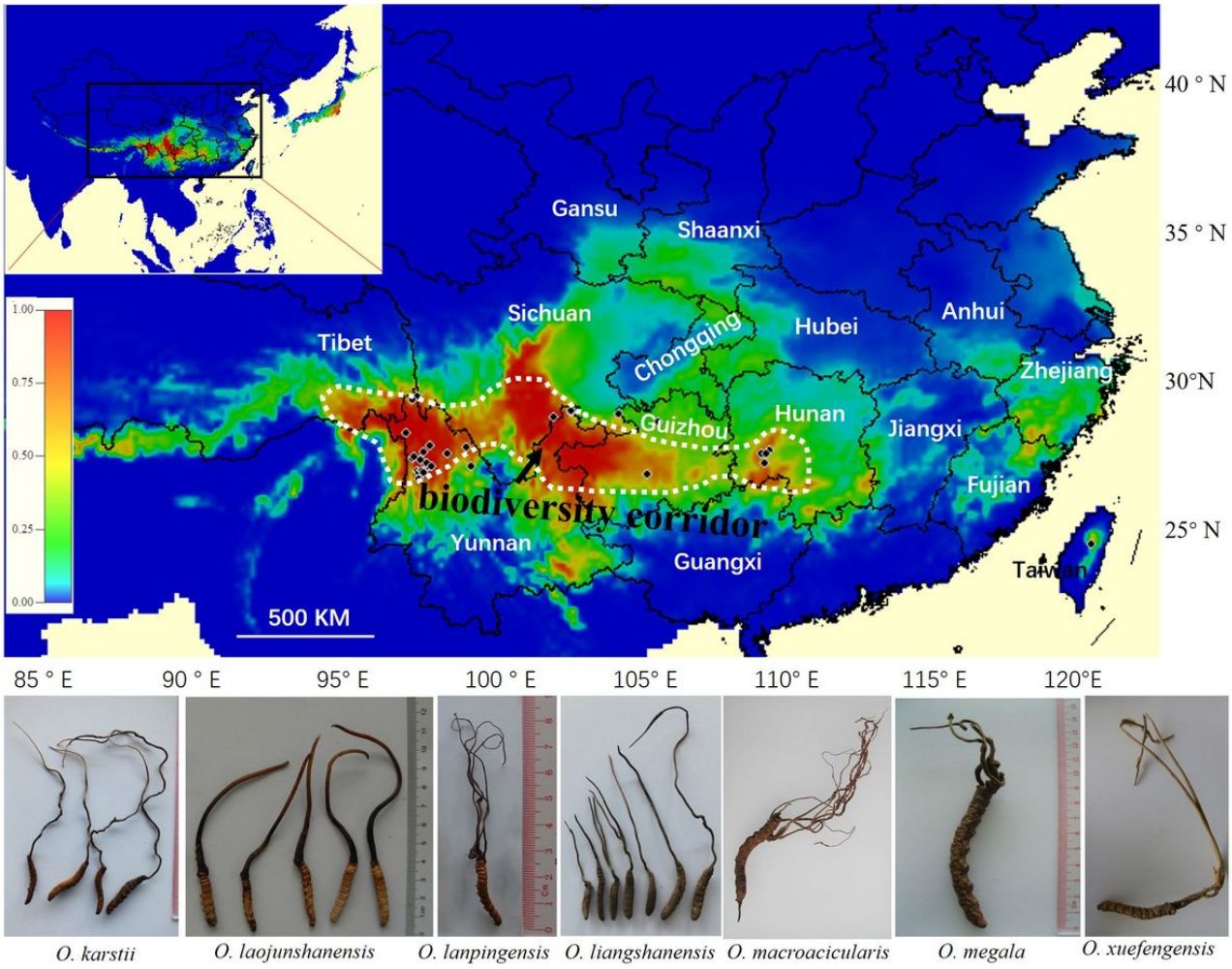

Figure 6

The prediction of the suitable distribution area of *O.sinensis*-affined phylogenetic lineage

Note: Species occurrence were marked with black dots of the 7 species

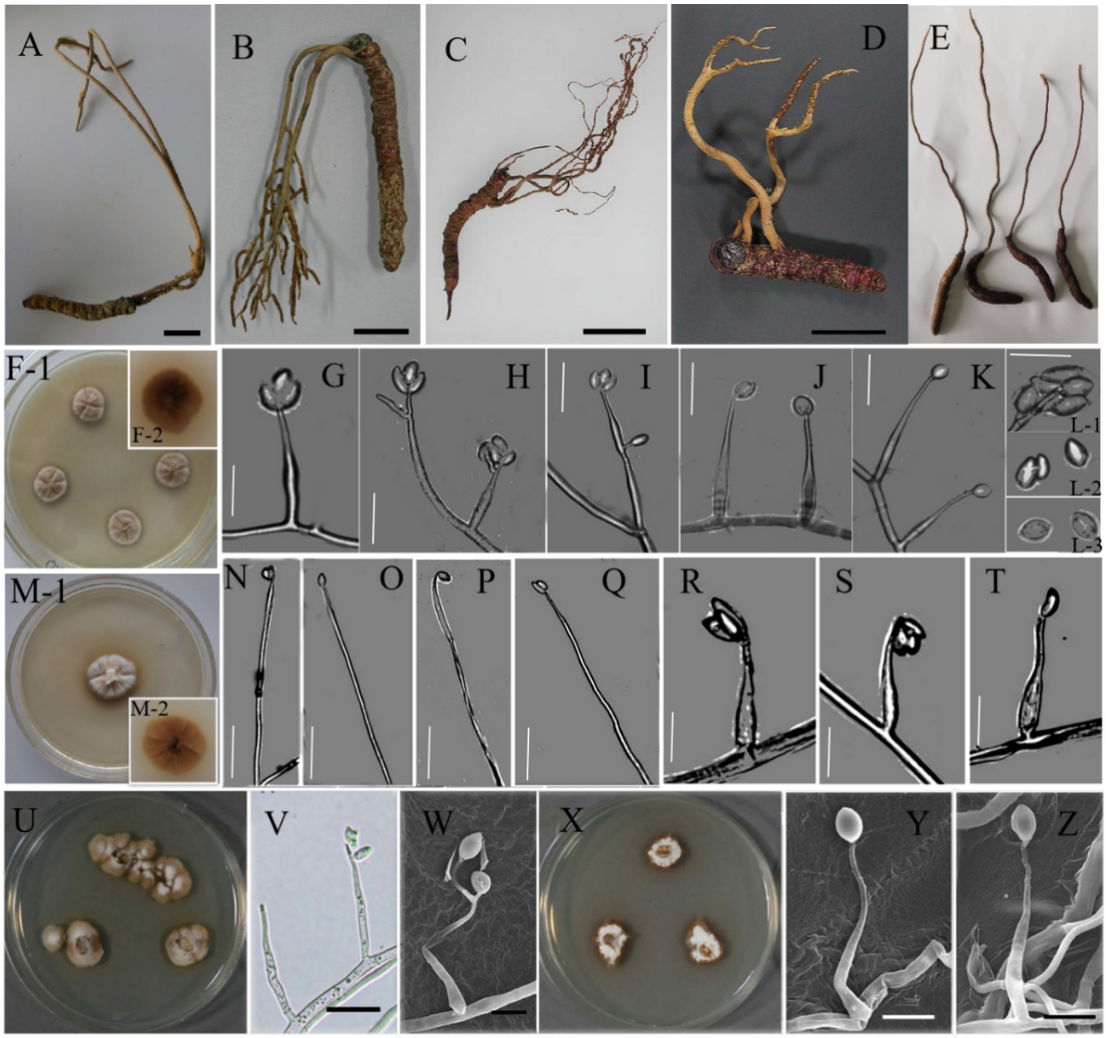

Figure 7

The morphological and micromorphological characteristics of 5 *Ophiocordyceps* species (Fig.C-D, U-Z were cited from Ban et al., 2014)**)**

A, F—I. *O. xuefengensis*; B, M—T. *O. ramosissimum*; C, U-W. *O. macroacicularis*; D, X-Z:*O. aborescens*; E. *O.liangshanensis* Bar=2μm

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- additionalfile1.xlsx