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Multigene phylogeny and HPLC analysis reveal fake *Ophiocordyceps sinensis* in markets

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Abstract

Ophiocordyceps sinensis (*Cordyceps sinensis*) has long been a Chinese Traditional Medicine and functional food in China. Because of its valued medicinal effect and improvement in the Chinese economy, market demand for *O. sinensis* has significantly increased in recent years. Here, we use multigene and High Performance Liquid Chromatography (HPLC) analysis of specimens bought from markets to reveal the sale of fake *O. sinensis* in Traditional Chinese Medicine markets. The insect larvae (belonging to Lepidopteran) was a different species to that normally infected by *O. sinensis*. Combined sequence analysis of ITS, nrSSU, EF-1α and RPB1 gene markers also revealed the putative *O. sinensis* to be *Metacordyceps taii*. Producers had gone to great lengths to produce remarkably similar fake *O. sinensis* specimens. The insect body was infected with *M. taii*, but the fungus stromata were made from a *Ligularia hodgsonii* stem that had been molded into stromata and stuck into the insect body. We analyzed the nucleoside constitutes of the insect bodies and fake *Cordyceps* stromata which were very different to those of authentic wild *O. sinensis* samples from Tibet. It is not clear what the consumers of these products are actually ingesting and whether it may be harmful. In the future, sequence data should be used to test the authenticity of *O. sinensis*, and the development a real-time PCR assay for species-specific diagnosis is needed. The use of multi-gene phylogeny could have a wide application in verification of other Traditional Chinese Medicined and fungal biotechnology products.

Key words – Chemical analysis – Combined sequence analysis – fake *Cordyceps* – *Ligularia hodgsonii* – *Metacordyceps taii*

Introduction

Ophiocordyceps sinensis (syn. *Cordyceps sinensis*) has long been used as a Traditional Chinese Medicine (TCM) and a tonic in China (Zhou et al. 2009). Because of its revered medicinal effect, and economic improvements in China, the market demand for *O. sinensis* has increased significantly. The price of *O. sinensis* per gram is now higher than that of gold, with best quality samples being 100-130 USD/g in 2012 (Shrestha & Bawa 2013). This is the result of increased

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demand and over gathering, the latter which has caused substantial reductions in populations (Mortimer et al. 2012).

Ophiocordyceps sinensis, which is gathered from the wild, is a rich source in bioactive compounds (De Silva et al. 2013), such as polysaccharides (Li et al. 2006), adenosine and other nucleotide/nucleotides derivatives (Li et al. 2004a, 2006), ergosterol (Li et al. 2004a, 2006), δ-3-ergosterol (Zhu et al. 1998), cordycepic acid (mannitol) (Li et al. 2006), crude protein, amino acids, and metal elements (Zhu et al. 1998). They manifest a wide range of pharmacological functions (Li et al. 2002, De Silva et al. 2013), and its immunity regulation function plays an important role in antitumor and anticancer activity (De Silva et al. 2012a), prevention and control of diabetes (De Silva et al. 2012b), organ transplants and therapy for some diseases of the kidney, liver and heart (Kuo et al. 1996, Zhou et al. 2009).

Ophiocordyceps sinensis is restricted to the high altitudes of the Himalayas and Tibetan Plateau (Shrestha & Bawa 2013). It has been collected at altitudes of about 3200–4200 m in India (Singh et al. 2010), 3540–5050 m in Nepal (Devkota 2000), 4200-5200 m in Bhutan (Cannon et al. 2009), and 3000-5000 m in Tibet, Qinghai, Sichuan, Yunnan and Gansu Provinces in China (Zhou et al. 2009). Winkler (2009) analyzed available production data and estimated that the total annual production was in the range of 85 to 185 tons for all production areas in the Himalayas and Tibetan plateau. This yields a huge total estimated global market value of US $5–11 billion (Shrestha 2012). The market price climbed more than nine times in Tibet from 1997 to 2008 (Winkler 2009), and as a result collectors are extending their collecting areas year by year. This has resulted in over exploitation of the pristine landscapes with significant ecosystem degradation (Shrestha 2012).

Methods for the artificial cultivation of *O. sinensis* have not yet been successfully developed, despite considerable funds being committed for this by the Chinese government over the past 30 years (Zhou et al. 2009). Some fermentation products have been produced using the anamorph state of *O. sinensis* (Li et al. 1999, 2006, Jiang & Yao 2003). With high increase in the price of wild *O. sinensis* and a decrease in the annual collection, fake *O. sinensis* have started to emerge in TCM markets. This is because it is difficult for the average customer to identify fake *O. sinensis*. An x-ray machine was developed to reveal the fraudulent practice of placing copper and other metals in the *O. sinensis* insect host which increased the sale value though increased weight (Tuli et al. 2013). However, this cannot detect fake *O. sinensis*, comprising other species of *Cordyceps sensu lato*, sold in the market.

The purpose of this study was to establish weather fake *O. sinensis* specimens were being sold in TCM markets. In this study, we used analysis of combined ITS, nrSSU, EF-1α, RPB1 sequenced data and HPLC detection of nucleosides to establish weather the *O. sinensis* samples were authentic.

Materials & Methods

Specimens and host

Ophiocordyceps sinensis specimens were collected in Linzhi and Basu County, Tibet, China in May 2010. Specimens of *O. sinensis* were also bought from a Traditional Chinese Medicine market in October 2012. Specimens were stored in plastic containers and transported to the laboratory for identification and analysis.

Morphological characterization

Material was examined under an Optec SZ660 stereo microscope (Chongqing Optec Instrument Co., Ltd, Chongqing, China) where photographs were taken.

DNA extraction, PCR amplification and determination of DNA sequences

Total genomic DNA of fungal was extracted from dried specimens using an E.Z.N.A.™ Fungal DNA MiniKit (Omega Biotech Inc., CA, USA) and Plant DNA was extracted from dried specimens using an EZgene™ Plant gDNA Kit (Biomega Inc., CA, USA) according to manufacturers' protocols and the extracted DNA was stored at -20 C. Two nuclear (ITS, nrSSU)

and two protein gene (EF-1α, RPB1) loci were amplified and sequenced (Sung et al. 2007), for the undetermined fruiting body of the specimens from market, ITS loci was amplified and sequenced.

The PCR amplification and sequencing of ITS1-5.8S-ITS2 rDNA (ITS) were conducted as described in White *et al.*(1990), The ITS was amplified and sequenced with the primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al.* 1990), while primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used when amplifying the undetermined fruiting body (White *et al.* 1990). The PCR amplification and sequencing of nrSSU were conducted as described in Sung *et al.* (2007). The nrSSU was amplified and sequenced with the primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') (White *et al.* 1990). In the amplification of EF-1α and RPB1, we followed Sung *et al.* (2007) and Castlebury *et al.* (2004). For the amplification of EF-1α, the primers 983F (5'-GCYCCYGGHCAYCGTGAYTTYAT-3') and 2218R (5'- ATGACACCRACRGCRACRGTYTG-3') were used (Sung *et al.* 2007). Sequencing of RPB1 was performed with the same primers used in the amplification. For RPB1, the primers CRPB1A (5'-CAYCCWGGYTTYATCAAGAA-3') and RPB1Cr (5'-CCNGCDATNTCRTTRTCCATRTA-3') were used in PCR amplification and sequencing (Castlebury *et al.* 2004).

All PCR products were sequenced by GenScript Biotechnology Co. Ltd. (Nanjing, China).

Sequence alignment and phylogenetic analysis

The taxon information and GenBank accession numbers used in the molecular analysis are listed in Table 1, the plant information are listed in Table 2. The four gene datasets (ITS, nrSSU, EF-1α, RPB1) from the undetermined specimens and natural *Ophiocordyceps sinensis* from Tibet, plus datasets obtained from GenBank, were aligned using MEGA5.05 Tamura *et al.* (2011). Alignments were manually adjusted to allow maximum sequence similarity. Gaps were treated as missing data. Unweighted maximum Parsimony (MP) analysis were performed using PAUP* 4.0b10 (Swofford 2002). Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Maxtrees were 5000, branches of zero length were collapsed and all multiple parsimonious trees were saved. Clade stability of the trees resulting from the parsimony analyses were assessed by bootstrap analysis with 1000 replicates, each with 10 replicates of random stepwise addition of taxa (Felsenstein 1985). Trees were viewed in Treeview and exported to graphics programs (Page 1996).

Nucleosides Analytical methods

The samples were dried overnight to a constant weight at 55ºC. Cordycepin, adenosine and other nucleosides in fruiting bodies and the host insect was analyzed by HPLC (1200 series, Agilent Technology, U.S.) with a C18 reverse phase column (5 μm, 4.6 ×150 mm, Upelco, Bellefonte, PA, USA). Standard cordycepin, adenosine and other nucleoside samples from Sigma were dissolved in distilled water for calibration. The mobile phase was 10mM KH_2PO_4, which was dissolved in methanol/distilled water (6:94). Elution was performed at a flow rate of 1 ml/min with column temperature at 45ºC and at a UV wavelength of 259 nm.

Results

The partition homogeneity test ($P = 0.01$) suggested that the individual gene partitions were not highly incongruent (Farris et al. 1994, Cunningham 1997). Blast searches were made and recent publications studied to reveal the closest matches in GenBank for phylogenetic analysis (Table 1). The combined datasets comprised 3456 characters after alignment, of which 914 characters are parsimony-informative, 2089 constant, and 453 parsimony-uninformative. Parsimony analysis generated 5000 trees; SH test verified that they were similar, one of which (tree length = 3907 steps, CI = 0.530, RI = 0.777, RC = 0.412, HI = 0.470) and the most parsimonies tree is shown in Fig. 2.

Table 1 Taxa used in molecular phylogenetic analyses in fungals.

Species	Voucher Info. [1]	Host/Substratum	GenBank Accession Number				References
			ITS	nrSSU	EF-1α	RPB1	
Metacordyceps taii (host)	GZUH 2012HK2	Lepidopteran pupa	KJ364485	KR153587	KJ364493		In this study
Metacordyceps taii (host)	GZUH 2012HK6	Lepidopteran pupa	KJ364486		KJ364494		In this study
Ophiocordyceps sinensis	GZUH 2010LL4	*Hepialus armoricanus*	KJ364487	KJ364490	KJ364495	KJ364498	In this study
Ophiocordyceps sinensis	GZUH 2010RW4	*Hepialus armoricanus*	KJ364488	KJ364491	KJ364496	KJ364499	In this study
Ophiocordyceps sinensis	GZUH 2010RW5	*Hepialus armoricanus*	KJ364489	KJ364492	KJ364497		In this study
Cordyceps brongniartii	NBRC 101395		JN943298	JN941759		JN992493	Schoch et al. 2012
Cordyceps brongniartii	BCC 16585		JN049867	JF415951	JF416009	JN049885	Kepler et al. 2012
Cordyceps cardinalis	CBS 113411*	Lepidopteran larva		AY184973	DQ522325	DQ522370	Sung et al. 2007
Cordyceps cardinalis	OSC 93610		JN049843	AY184974	EF469059	EF469088	Kepler et al. 2012
Cordyceps militaris	OSC 93623	Lepidopteran pupa	JN049825	AY184977	DQ522332	DQ522377	Sung et al. 2007
Cordyceps militaris	NBRC 100741		JN943437	JN941755		JN992489	Schoch et al. 2012
Elaphocordyceps japonica	OSC 110991	*Elaphomyces* sp. (Eurotiomycetes)	JN049824	DQ522547	DQ522330	DQ522375	Sung et al. 2007
Elaphocordyceps japonica	IFO 9647		AB027366	AB027320			Nikoh & Fukatsu 2000
Elaphocordyceps ophioglossoides	OSC 106405	*Elaphomyces* sp. (Eurotiomycetes)		AY489691	AY489618	AY489652	Sung et al. 2007
Elaphocordyceps ophioglossoides	NBRC 106331		JN943320	JN941733		JN992467	Schoch et al. 2012
Elaphocordyceps subsessilis	OSC 71235	Scarabaeid larva (Coleoptera)	JN049844	EF469124	EF469061	EF469090	Sung et al. 2007
Metacordyceps atrovirens	TNM-F 10184	Coleoptera	JN049882	JF415950		JN049884	Kepler et al. 2012
Metacordyceps brittlebankisoides	G97025*		AJ309332				Liu et al. 2002
Metacordyceps campsosterni	HMIGD 20885*		DQ150247				Zhang 2005
Metacordyceps campsosterni	HMIGD 20884		DQ150246				Zhang 2005
Metacordyceps chlamydosporia	CBS 101244		JN049821	DQ522544	DQ522327	DQ522372	Kepler et al. 2012
Metacordyceps chlamydosporia	CBS 504.66	Nematode	AJ292398	AF339593	EF469069	EF469098	Sung et al. 2012
Metacordyceps guniujiangensis	GNJ020527-04*		AY913757				Li et al. 2010
Metacordyceps indigotica	TNS-F18553	Lepidoptera	JN049874	JF415952	JF416010	JN049886	Kepler et al. 2012
Metacordyceps indigotica	TNS-F18554	Lepidoptera	JN049875	JF415953	JF416011	JN049887	Kepler et al. 2012
Metacordyceps khaoyaiensis	BCC 12687	Lepidoptera	JN049868		JF416013	JN049889	Kepler et al. 2012
Metacordyceps khaoyaiensis	BCC 14290	Lepidoptera	JN049869		JF416012	JN049888	Kepler et al. 2012
Metacordyceps kusanagiensis	TNS F18494	Coleoptera	JN049873	JF415954	JF416014	JN049890	Kepler et al. 2012

Metacordyceps liangshanensis	EFCC 1452	Lepidopteran pupa		EF468962	EF468756		Sung et al. 2007
Metacordyceps liangshanensis	EFCC 1523	Lepidopteran pupa		EF468961	EF468755		Sung et al. 2007
Metacordyceps martialis	TTZ070716-04	Lepidoptera	JN049871	JF415955		JN049891	Kepler et al. 2012
Metacordyceps martialis	HMAS 197472(S)	Lepidoptera	JN049881	JF415956	JF416016	JN049892	Kepler et al. 2012
Metacordyceps owariensis	NBRC 33258	Hemiptera	JN049883		JF416017		Kepler et al. 2012
Metacordyceps pseudoatrovirens	TNSF 16380	Coleoptera	JN049870			JN049893	Kepler et al. 2012
Metacordyceps taii	ARSEF 5714	Lepidoptera	JN049829	AF543763	AF543775	DQ522383	Sung et al. 2007
Metacordyceps yongmunensis	EFCC 2131	Lepidoptera	JN049856	EF468977	EF468770	EF468876	Kepler et al. 2012
Metacordyceps yongmunensis	EFCC 2135	Lepidoptera		EF468979	EF468769	EF468877	Kepler et al. 2012
Metarhizium acridum	ARSEF 324	Orthoptera	HQ331457		EU248844	EU248896	Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium acridum	ARSEF 7486*	Orthoptera	HQ331458		EU248845	EU248897	Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium anisopliae	ARSEF 7450	Coleoptera	HQ331464		EU248852	EU248904	Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium anisopliae	ARSEF 7487*	Orthoptera	HQ331446		DQ463996	DQ468355	Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium brunneum	ARSEF 2107{	Coleoptera	KC178691		EU248855	EU248907	Kepler et al. 2013 & Bischoff et al. 2009
Metarhizium brunneum	ARSEF 4152	Soil	HQ331452		EU248853	EU248905	Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium flavoviride	ARSEF 2133*	Coleoptera			DQ463999	DQ468358	Bischoff et al. 2009
Metarhizium frigidum	ARSEF 4124*	Coleoptera			DQ464002	DQ468361	Bischoff et al. 2009
Metarhizium globosum	ARSEF 2596*	Lepidoptera	HQ331459		EU248846	EU248898	Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium guizhouense	CBS 258.90*	Lepidoptera	HQ331448		EU248862	EU248914	Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium guizhouense	ARSEF 6238	Lepidoptera	HQ331447		EU248857	EU248909	Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium lepidiotae	ARSEF 7412	Coleoptera	HQ331455		EU248864	EU248916	Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium lepidiotae	ARSEF 7488*	Coleoptera	HQ331456		EU248865	EU248917	Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium majus	ARSEF 1015	Lepidoptera	HQ331444		EU248866	EU248918	Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium majus	ARSEF 1914{	Coleoptera	HQ331445		EU248868	EU248920	Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium pingshaense	ARSEF 3210	Isoptera	HQ331449		DQ463995	DQ468354	Schneider et al. 2011 & Bischoff

Metarhizium pingshaense	CBS 257.90*	Coleoptera	HQ331450		EU248850	EU248902	et al. 2009 Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium robertsii	ARSEF 727	Orthoptera	HQ331453		DQ463994	DQ468353	Schneider et al. 2011 & Bischoff et al. 2009
Metarhizium robertsii	ARSEF 7501				EU248849	EU248901	Bischoff et al. 2009
Ophiocordyceps rhizoidea	N.H.J. 12522	Termite (Isoptera)	JN049857	EF468970	EF468764	EF468873	Sung et al. 2007
Ophiocordyceps rhizoidea	N.H.J. 12529	Termite (Isoptera)		EF468969	EF468765	EF468872	Sung et al. 2007
Ophiocordyceps sinensis	EFCC 7287	Lepidopteran pupa	JN049854	EF468971	EF468767	EF468874	Sung et al. 2007
Ophiocordyceps sinensis	ARSEF 6282		HM595981		HM595918	HM595952	Chan et al. 2011
Ophiocordyceps stylophora	OSC 111000	Elaterid larva (Coleoptera)	JN049828	DQ522552	DQ522337	DQ522382	Sung et al. 2007
Ophiocordyceps stylophora	OSC 110999	Coleopteran larva		EF468982	EF468777	EF468882	Sung et al. 2007
Ophiocordyceps yakusimensis		Nymph of cicada (Hemiptera)	AB044643	AB044632			Nikoh & Fukatsu 2000
Tyrannicordyceps fratricida	TNS 19011*	Fungi	JQ349068	JQ257022	JQ257028	JQ257016	Kepler et al. 2012
Glomerella cingulata	CBS 114054	*Fragaria* sp. (Rosaceae)	DQ286202	AF543762		AY489659	Sung et al. 2007

1 A.E.G., A. E. Glenn personal collection; ARSEF, USDA-ARS Collection of Entomopathogenic Fungal cultures, Ithaca, NY; ATCC, American Type Culture Collections, Manassas, VA; BCC, BIOTEC Culture Collection, Klong Luang, Thailand; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; EFCC, Entomopathogenic Fungal Culture Collection, Chuncheon, Korea; F.A.U., F. A. Uecker personal collection; E.G.S., E, G. Simmons personal collection; GAM, Julian H. Miller Mycological Herbarium Athens, GA; G.J.S., G. J. Samuels personal collection; GZUH, Guizhou University Herbarium, Guiyang, Guizhou, China; KEW, mycology collection of Royal Botanical Garden, KEW, Surrey, UK; MICH, University of Michigan Herbarium, Ann Arbor, MI; N.H.J., Nigel Hywel-Jones personal collection; OSC, Oregon State University Herbarium, Corvallis, OR; S.A., S. Alderman personal collection.

* Denotes an ex-type isolate. { Denotes an ex-epitype isolate.

The data set comprises 36 species including three *Cordyceps*, three *Ophiocordyceps*, three *Elaphocordyceps*, and 27 *Metacordyceps/Metarhizium* species. The samples sold in a TCM market as *Ophiocordyceps sinensis* clustered with *M. taii* in the *Metacordyceps-Metarhizium* clade with relatively high bootstrap support (97%). The *Ophiocordyceps sinensis* samples from Tibet clustered with *O. sinensis* in the *Ophiocordyceps* clade with high bootstrap support (100%).

The host insect of the fake specimens is not *Hepialus armoricanus*, and the fungus is not *Ophiocordyceps sinensis*, but is *Metacordyceps taii* (asexual state = *Metarhizium guizhouense*). In the fake samples the insect was identified as a lepidopteran larvae and these were infected by *Metacordyceps taii*. However, the fungus stromata was in fact false and was the stem of a plant (Fig. 1). Phylogenetic analysis of ITS gene of the genus *Ligularia* has proved by 100% bootstrap that this stem belongs to the clade of *Ligularia hodgsonii* (Fig. 4). This had been stuck to the insect body and its resemblance to authentic *Ophiocordyceps sinensis* specimens was remarkable. When chemically tested, the false fungal stromata contained only nine nucleosides, and lacked uracil, adenine, and guanosine which are found in natural *O. sinensis* stromata. We also analyzed the nucleosides of the insect bodies from the fake ones which were different from the wild *O. sinensis* from Tibet (Fig. 3). The insect body of wild *O. sinensis* contains 13 nucleosides, while the fake ones contained 12 nucleosides.

Discussion

Ophiocordyceps sinensis is one of three *Cordyceps sensu lato* species listed in Chinese pharmacopoeia (The State Pharmacopoeia Committee of People Republic of China 2010) and is highly valued in TCM attracting a price of 100-130 USD/g per gram, which is higher than that of gold (Shrestha & Bawa 2013). Thus the practice of producing fake *Ophiocordyceps sinensis* is likely to become more common and elaborate methods are likely to be developed to produce specimens to sell in the TCM markets. Previous studies have found that nucleosides are the most important bioactive constituents in *O. sinensis* (Tuli et al. 2013, Yue et al. 2013) and thus the determination of nucleosides in *O. sinensis* is very important for its quality control (Zhu et al. 1998). Winkler (2009) estimated that the total annual production of *O. sinensis* was in the range of 85 to 185 tons for all production areas in the Himalayas and Tibetan plateau, but the total amount sold in markets is over 200 tons per year all over the world. One fraudulent practice of placing copper, mercury and other materials in the *O. sinensis* insect host body was developed to increase the weight to increase profits (Wu et al. 1996). This creates a health risk for patients who take the medicine, and there have been several reported clinical poisoning cases (Wu et al. 1996).

Table 2 Taxa used in molecular phylogenetic analyses in fake fruiting bodies.

Species	GenBank Accession No. (ITS)	References
Ligularia hodgsonii (GZUH 2012HK2)	KR153586	In this study
Ligularia anoleuca	AB557884	Nagano et al. 2010
Ligularia brassicoides	AY723266	Liu et al. 2006
Ligularia cymbulifera	JF976813	Li et al. 2011
Ligularia dentata	AY723256	Li et al. 2011
Ligularia dictyoneura	JF976815	Li et al. 2011
Ligularia fischeri	AB369647	Nomura et al. 2010
Ligularia lamarum	AB426703	Saito et al. 2011
Ligularia lankongensis	AB684267	Hirota et al. 2012
Ligularia liatroides	AY723268	Liu et al. 2006
Ligularia lingiana	JF767246	Yu et al. 2011
Ligularia nelumbifolia	JF976821	Li et al. 2011
Ligularia pleurocaulis	JF976823	Li et al. 2011
Ligularia przewalskii	AY723263	Liu et al. 2006
Ligularia pubifolia	GU444022	Lei et al. 2011
Ligularia purdomii	AY723257	Liu et al. 2006
Ligularia sagitta	AY723265	Liu et al. 2006
Ligularia stenocephala	AB369649	Nomura et al. 2010
Ligularia subspicata	JF976829	Li et al. 2011
Ligularia tongolensis	JF976833	Li et al. 2011
Ligularia tsangschanensis	AY723264	Liu et al. 2006
Ligularia veitchiana	AB557886	Nagano et al. 2010
Ligularia vellerea	JF976836	Li et al. 2011
Ligularia virgaurea	JF976840	Li et al. 2011
Tussilago farfara	EU785941	GenBank
Ligularia hodgsonii	FJ980336	GenBank
Ligularia hodgsonii	JX402629	Xiang et al. 2013
Ligularia hodgsonii	KF003090	Xiang et al. 2013

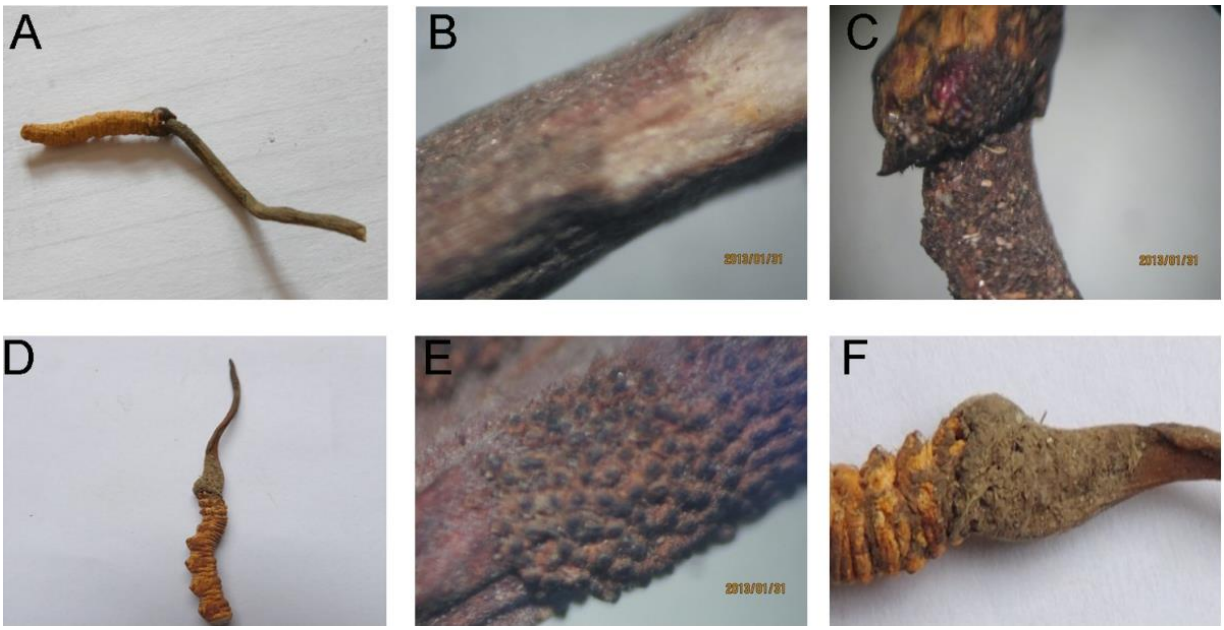
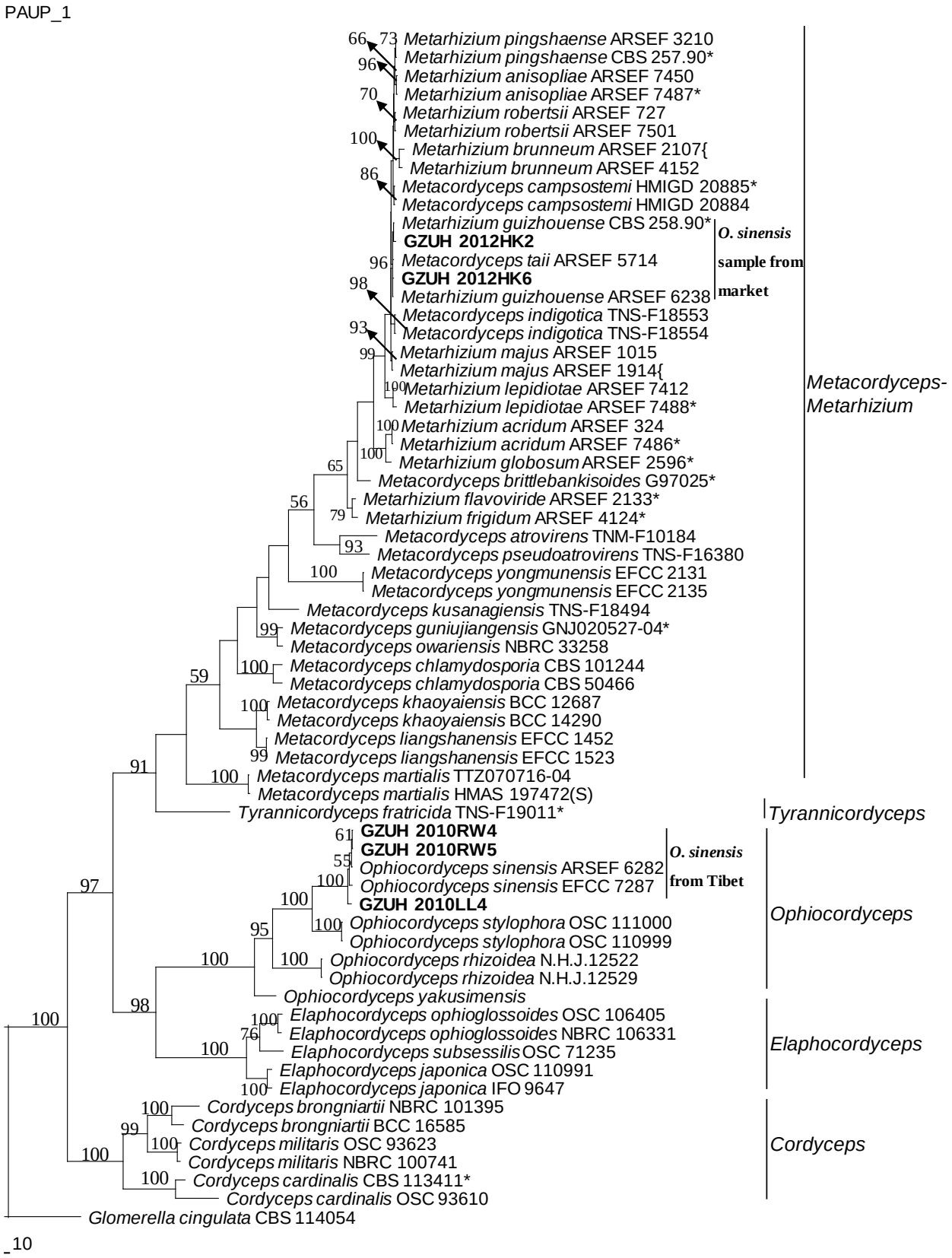


Fig. 1 – Fake (A-C) and wild (D-F) *Ophiocordyceps sinensis.* A. Fake *Ophiocordyceps sinensis* showing dead insect and false stroma. Note how the fungal stroma appears to be stuck in the insect. B. Fake stromata with surface sliced (white area) to reveal inner part. There are no ascomata. C. Join between fake stromata and insect larvae. It appears to have been stuck inside the insect. D. Wild *Ophiocordyceps sinensis* with insect and fungus stroma. E. Dark brown, nearly superficial ascomata on stromata of wild *Ophiocordyceps sinensis*. F. Area where fungus emerges from insect larvae.

The need to develop methods to detect fake *O. sinensis* is paramount. In China, adenosine is presently the only chemical constituent tested to authenticate and control the quality of *O. sinensis* (The State Pharmacopoeia Committee of People Republic of China 2010). X-ray machines however, were developed to reveal the fraudulent practice of placing copper and other metals in the *O. sinensis* insect host (Tuli et al. 2013). Au et al. (2012) used light and polarized microscopy to compare the cell tissues of the insect and stromata to authenticate *O. sinensis* artificial counterfeits and some fermented *Cordyceps* products as well as *Cordyceps* capsules available in markets. They showed that microscopy is a reliable method that needs only few samples for the authentication of *Ophiocordyceps sinensis* and its related products. Markers and distinct fingerprints from different constituents from sources of *Cordyceps* have also been reported (Gong et al. 2004, Li et al. 2004b). Several analytical techniques are available to detect of the main nucleosides in *Ophiocordyceps sinensis*, such as thin layer chromatography (TLC) (Hu et al. 2008, Ma et al. 2008), and capillary electrophoresis (Li et al. 2008, Sun et al. 2008). However, these methods can only determine one or several nucleosides with relatively high content in *O. sinensis*, and trace amounts of several other nucleosides or its derivatives cannot be detected by the above two methods. This is not accurate for distinguishing the more than 400 species of the *Cordyceps sensu lato* (Wen et al. 2013) and counterfeit production of *Ophiocordyceps sinensis* and may be why the counterfeiters added nucleotides to the false stromata. In this study, 13 nucleosides and derivatives were detected from natural *O. sinensis* in a single experimental run with high performance liquid chromatography (HPLC). This method can therefore be used for effective quality control of *O. sinensis*.

Some authors have reported that a single gene marker can be used for the identification of *O. sinensis* and its related products (Hseu & Chen 2001, Weng & Li 2008). Unfortunately, these methods are not thoroughly optimized, and extensive work is still needed to accurately identify different *Cordyceps sensu lato* species and products in the market with high efficiency. No effective molecular diagnosis protocol provides a specific and sensitive probe for this important and expensive TCM. It is necessary to identify a SNP - rich region to enable rapid species-specific



Figs 2 – Phylogenetic relationships among fake *Ophiocordyceps sinensis* and other related species based on combination of four genes (ITS, nrSSU, EF-1α, RPB1). Bootstraps values (1000 replicates) are indicated above the nodes. The tree is routed to *Glomerella cingulata*.
* Denotes an ex-type isolate. { Denotes an ex-epitype isolate.

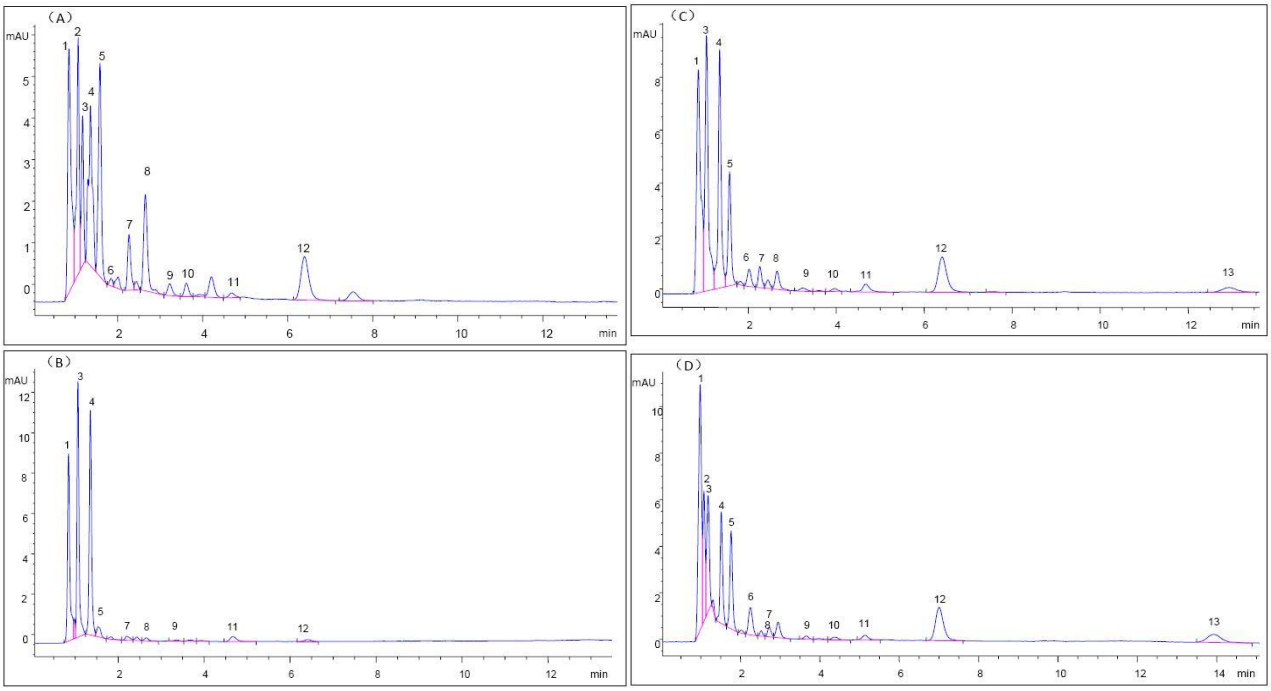


Fig. 3 – HPLC chromatograms of different simples. (A) Host of fake *Ophiocordyceps sinensis*; (B) Fruiting-body of fake *O. sinensis*; (C) Host of wild *O. sinensis*; (D) Fruiting-body of wild *O. sinensis*. 1: cytosine; 2: uracil; 3: guanine; 4: cytidine; 5: hypoxanthin; 6: adenine; 7: uridine; 8: thymine; 9: inosine; 10: guanosine; 11: thymidine; 12: adenosine; 13: undetermined.

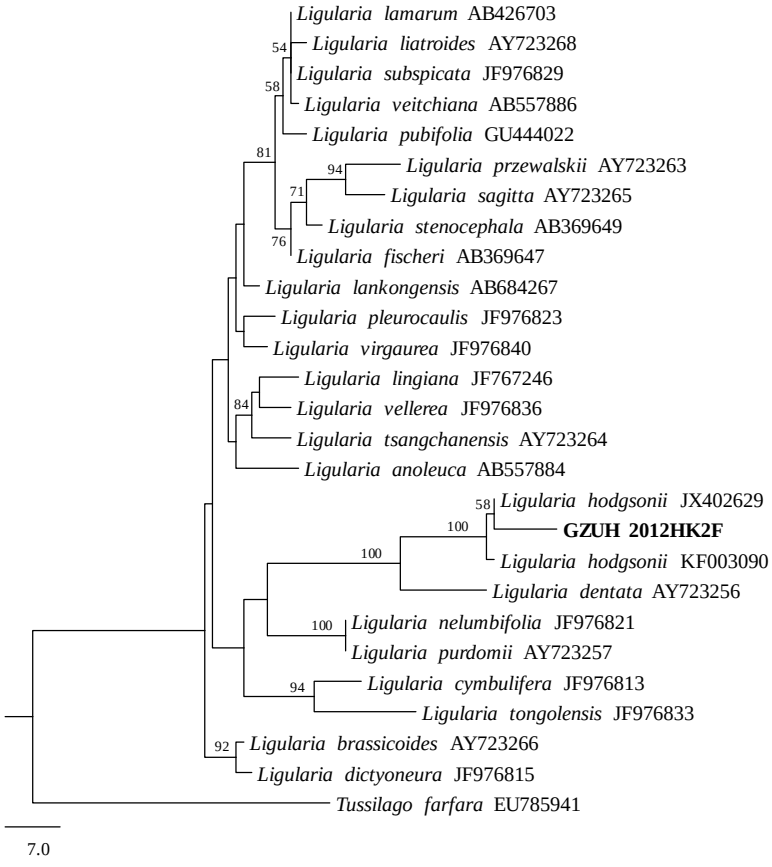


Fig. 4 – Phylogram generated from parsimony analysis based on ITS sequenced data of *Ligularia*. Parsimony bootstrap support values greater than 50% are indicated above the nodes. The sample from fake ones in this study is given in bold. The tree is rooted with *Tussilago farfara*.

primer and probe designs for *Ophiocordyceps sinensis* in the future like as has been developed for some plant pathogenic fungi (Tao et al. 2013). As previous studies have indicated, an appropriate DNA barcode marker for fungal species identification is judged by two important criteria including the suitable intra- and interspecific variations and the high success rate of PCR amplification and sequencing (Hollingsworth 2009, Peng et al. 2011). In this study, we confirmed that the partial EF-1α and RPB1 are good DNA markers for *Cordyceps sensu lato* species as compared with ITS and nrSSU. ITS has been routinely used to study the phylogenetic relationships among different species of varies fungal groups and has also been regarded as the universal DNA barcode for the kingdom Fungi (Schoch et al. 2012, Nilsson et al. 2014), but does not work in many fungal groups (Maharachchikumbura et al. 2012, Hyde et al. 2014, Udayanga et al. 2014). In this study, we found that ITS and nrSSU have a high similarity among the tested species in *Cordyceps sensu lato* with some intra - and interspecific overlapping between species in the single gene tree.

The method for quality control is important to ensure authenticity and quality of *Cordyceps* species and products (Li et al. 2006). In this study, chemical and multi-gene loci phylogeny are simultaneously used for identification and quality control of *Ophiocordyceps sinensis*; the methodology is quick and highly efficient. This is the first time to report fake *O. sinensis* made from *Metacordyceps taii*, however this method may become common for counterfeiting *Ophiocordyceps sinensis* in the future because of its remarkable similarity.

The strategies used for identification *O. sinensis* using combined sequenced data from the multi-gene loci could have a wide application in other TCM and in fungal biotechnology.

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References

Au D, Wang L, Yang D, Mok DK, Chan AS, Xu H. 2012 – Application of microscopy in authentication of valuable Chinese medicine I - *Cordyceps sinensis*, its counterfeits, and related products. Microscopy Research and Technique 75, 54–64.

Bischoff JF, Rehner SA, Humber RA. 2009 – A multilocus phylogeny of the *Metarhizium* anisopliae lineage. Mycologia 101, 512-530.

Cannon PF, Hywel-Jones NL, Maczey N, Norbu L, Tshitila, Samdup T, Lhendup P. 2009 – Steps towards sustainable harvest of *Ophiocordyceps sinensis* in Bhutan. Biodiversity & Conservation 18, 2263–2281.

Castlebury LA, Rossman AY, Sung GH, Hyten AS, Spatafora JW. 2004 – Multigene phylogeny reveals new lineage for *Stachybotrys chartarum*, the indoor air fungus. Mycological Research 108, 864–872.

Chan WH, Ling KH, Chiu SW, Shaw PC, But PPH. 2011 – Molecular analyses of *Cordyceps gunnii* in China. Journal of Food and Drug Analysis 19, 18-25.

Cunningham CW. 1997 – Can three incongruence tests predict when data should be combined? Molecular Biology and Evolution 14, 733–740.

De Silva DD, Rapior S, Fons F, Bahkali AH, Hyde KD. 2012a – Medicinal mushrooms in supportive cancer therapies: an approach to anti-cancer effects and putative mechanisms of action. Fungal Diversity 55, 1–35.

De Silva DD, Rapior S, Hyde KD, Bahkali AH. 2012b – Medicinal mushrooms in prevention and control of diabetes mellitus. Fungal Diversity 56, 1–29.

De Silva DD, Rapior S, Sudarman E, Stadler M, Xu J, Alias SA, Hyde KD. 2013 – Bioactive metabolites from macrofungi: ethnopharmacology, biological activities and chemistry. Fungal Diversity 62, 1–40.

Devkota S. 2000 – *Ophicordyceps sinensis* (Yarsagumba) from Nepal Himalaya: status, threats and management strategies. In: Zhang P, Hao W (Ed.), *Cordyceps* Resources and Environment. Grassland Supervision Center by the Ministry of Agriculture, People's Republic of China. 91–108.

Farris JS, Källersjö M, Kluge AG, Bult C. 1994 – Testing significance of incongruence. Cladistics 10, 315–319.

Felsenstein J. 1985 – Confidence Limitson Phylogenies: An Approach using the Bootstrap. Evolution 39, 783–791.

Gong YX, Li SP, Li P, Liu JJ, Wang YT. 2004 – Simultaneous determination of six main nucleosides and bases in natural and cultured *Cordyceps* by capillary electrophoresis. Journal Of Chromatography A 1055, 215–221.

Hirota H, Horiguchi Y, Kawaii S, Kuroda C, Hanai R, Gong X. 2012 – Four new bisabolane-type sesquiterpenes from *Ligularia lankongensis*. Natural Product Communications 7, 451-454.

Hollingsworth PM, Forrest LL, Spouge JL, Hajibabaei M, Ratnasingham S, van der Bank M, Chase MW, Cowan RS, Erickson DL, Fazekas AJ, Graham SW, James KE, Kim KJ, Kress WJ, Schneider H, van AlphenStahl J, Barrett SC, van den Berg C, Bogarin D, Burgess KS, Cameron KM, Carine M, Chacón J, Clark A, Clarkson JJ, Conrad F, Devey DS, Ford CS, Hedderson TA, Hollingsworth ML, Husband BC, Kelly LJ, Kesanakurti PR, Kim JS, Kim YD, Lahaye R, Lee HL, Long DG, Madriñán S, Maurin O, Meusnier I, Newmaster SG, Park CW, Percy DM, Petersen G, Richardson JE, Salazar GA, Savolainen V, Seberg O, Wilkinson MJ, Yi DK, Little DP. 2009 – A DNA barcode for land plants. Proceedings of the National Academy of Sciences 106, 12794–12797.

Hseu RS, Chen CS. Gene sequence and method for distinguishing *Cordyceps sinensis*. US Patent 6271003, 2001.

Hu K, Fang DS, Tang YP, Jia JJ, Li M. 2008 – Comparison of *Cordyceps Sinensis* and solid fermentation of *Cordyceps Militaris* by TLC. China Pharmacy 19, 1180–1182.

Hyde KD, Nilsson RH, Alias SA, Ariyawansa HA, Blair JE, Cai L, de Cock AWAM, Dissanayake AJ, Glockling SL, Goonasekara ID, Gorczak M, Hahn M, Jayawardena RS, van Kan JAL, Laurence MH, Lévesque CA, Li X, Liu JK, Maharachchikumbura SSN, Manamgoda DS, Martin FN, McKenzie EHC, McTaggart AR, Mortimer PE, Nair PVR, Pawlowska J, Rintoul TL, Shivas RG, Spies CFF, Summerell BA, Taylor PWJ, Terhem RB, Udayanga H, Vaghefi N, Walther G, Wilk M, Wrzosek M, Xu JC, Yan JY, Zhou N. 2014 – One stop shop: backbones trees for important phytopathogenic genera: I (2014). Fungal Diversity 67, 21–125.

Jiang Y, Yao Y. 2003 – Anamorphic fungi related to *Cordyceps sinensis*. Mycosystema 22, 161–176.

Kepler RM, Sung GH, Ban S, Nakagiri A, Chen MJ, Huang B, Li ZZ, Spatafora JW. 2012 – New teleomorph combinations in the entomopathogenic genus *Metacordyceps*. Mycologia 104, 182–197.

Kuo YC, Tsai WJ, Shiao MS, Chen CF, Lin CY. 1996 – *Cordyceps sinensis* as an immunomodulatory agent. American Journal of Chinese Medicine 24, 111–125.

Lei W, Chen H, Zhang GR, Li SS, Peng QY, Zhong X and Liu X. 2011 – Molecular identification and food source inference of constructive plants, native to the *Ophiocordyceps sinensis* habitat. African Journal of Biotechnology 10, 159-167.

Li CR, Huang B, Fan MZ, Lin YR, Li ZZ. 2010 – *Metacordyceps guniujiangensis* and its *Metarhizium* anamorph: a new pathogen on cicada nymphs. Mycotaxon 111, 221–231.

Li DS, Liu ZX, Zhang Y, Zhang CK. 2002 – Contrast analysis of mainly chemical ingredients of *Cordyceps menshanensis* and *Cordyceps sinensis*. Edible Fungi of China 21, 35–37.

Li DZ, Gao LM, Li HT, Wang H, Ge XJ, Liu JQ, Chen ZD, Zhou SL, Chen SL, Yang JB, Fu CX, Zeng CX, Yan HF, Zhu YJ, Sun YS, Chen SY, Zhao L, Wang K, Yang T, Duan GW. 2011 – Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should

be incorporated into the core barcode for seed plants. Proceedings of the National Academy of Sciences of the United States of America 108, 19641-19646.
Li J, Feng CQ, NI XM, Zhang WS. 2008 – Determination of nucleosides of natural *Cordyceps sinensis* in Qinghai province by capillary electrophoresis. Chinese Pharmaceutical Journal 43, 1105–1107.
Li S, Yang F, Tsim KWK. 2006 – Quality control of *Cordyceps sinensis*, a valued traditional Chinese medicine. Journal of Pharmaceutical and Biomedical Analysis 41, 1571–1584.
Li SP, Li P, Lai C, Gong YX, Kan KK, Dong TT, Tsim KW , Wang YT. 2004a – Simultaneous determination of ergosterol, nucleosides and their bases from natural and cultured *Cordyceps* by pressurised liquid extraction and high-performance liquid chromatography. Journal of Chromatography A 1036, 239–243.
Li SP, Song ZH, Dong TTX, Ji ZN, Lo CK, Zhu SQ, Tsim KWK. 2004b – Distinction of water-soluble constituents between natural and cultured *Cordyceps* by capillary electrophoresis. Phytomedicine 11, 684–690.
Li XQ, Wang Y, Bao TT. 1999 – RP-HPLC determination of adenosine in fermented *Cordyceps*. China journal of Chinese materia medica 24, 12–14, 62.
Liu JQ, Wang YJ, Wang AL, Hideaki O, Abbott RJ. 2006 – Radiation and diversification within the *Ligularia-Cremanthodium-Parasenecio* complex (asteraceae) triggered by uplift of the Qinghai-Tibetan plateau. Molecular Phylogenetics and Evolution 38, 31–49.
Liu ZY, Liang ZQ, Liu AY, Yao YJ, Hyde KD, Yu ZN. 2002 – Molecular evidence for teleomorph-anamorph connections in *Cordyceps* based on ITS-5.8S rDNA sequences. Mycological Research 106, 1100–1108.
Ma Y, Wang Y, Yang GZ, Yu RM. 2008 – Determination of nucleosides in *Cordyceps sinensis* preparation by dual-wavelength TLC-scanning. China Pharmacy 19, 2375–2377.
Maharachchikumbura SSN, Guo LD, Cai L, Chukeatirote E, Wu WP, Sun X, Crous PW, Bhat DJ, McKenzie EHC, Bahkali AH, Hyde KD. 2012 – A multi-locus backbone tree for *Pestalotiopsis*, with a polyphasic characterization of 14 new species. Fungal Diversity 56, 95–129.
Mortimer PE, Karunarathna SC, Li Q, Gui H, Yang X, Yang X, He J, Ye L, Guo J, Li H. 2012 – Prized edible Asian mushrooms: ecology, conservation and sustainability. Fungal Diversity 56, 31–47.
Nagano H, Kanda M, Yamada H, Hanai R, Gong X, Kuroda C. 2010 – Chemical and genetic study of *Ligularia anoleuca* and *L.veitchiana* in Yunnan and Sichuan provinces of China. Helvetica Chimica Acta 93, 1945–1952.
Nikoh N, Fukatsu T. 2000 – Interkingdom host jumping underground: phylogenetic analysis of entomoparasitic fungi of the genus *Cordyceps*. Molecular Biology and Evolution 17, 629–638.
Nilsson RH, Hyde KD, Pawłowska J, Ryberg M, Tedersoo L, Aas AB, Alias SA, Alves A, Anderson CL, Antonelli A, Arnold AE, Bahnmann B, Bahram M, Bengtsson-Palme J, Berlin A, Branco S, Chomnunti P, Dissanayake A, Drenkhan R, Friberg H, Frøslev TG, Halwachs B, Hartmann M, Henricot B, Jayawardena R, Jumpponen A, Kauserud H, Koskela S, Kulik T, Liimatainen K, Lindahl BD, Lindner D, Liu JK, Maharachchikumbura S, Manamgoda D, Martinsson S, Neves MA, Niskanen T, Nylinder S, Pereira OL, Pinho DB, Porter TM, Queloz V, Riit T, Sánchez-García M, de Sousa F, Stefańczyk E, Tadych M, Takamatsu S, Tian Q, Udayanga D, Unterseher M, Wang Z, Wikee S, Yan JY, Larsson E, Larsson KH, Kõljalg U, Abarenkov K. 2014 – Improving ITS sequence data for identification of plant pathogenic fungi. Fungal Diversity 67, 11–19.
Nomura N, Takaso T, Peng C, Kono Y, Oginuma K, Mitsui Y, Setoguchi H. 2010 – Molecular phylogeny and habitat diversification of the genus *Farfugium* (asteraceae) based on nuclear rDNA and plastid DNA. Annals of Botany 106, 467–482.
Page RDM. 1996 – TreeView: an application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences Cabios 12, 357–358.

Peng Z, Jing L, Zhuang WY, Liu XZ, Bing W. 2011 – Erratum to:DNA barcoding of the fungal genus *Neonectria* and the discovery of two new species. Science China Life Sciences 54, 664–674.

Saito Y, Hattori M, Iwamoto Y, Takashima Y, Mihara K, Sasaki Y, Fujiwara M, Sakaoku M, Shimizu A, Chao X, Kuroda C, Gong X, Hanai R, Tori M. 2011 – Overlapping chemical and genetic diversity in *Ligularia lamarum* and *Ligularia subspicata*. isolation of ten new eremophilanes and a new seco-bakkane compound. Tetrahedron 67, 2220-2231.

Schneider S, Rehner SA, Widmer F, Enkerli J. 2011 – A PCR-based tool for cultivation-independent detection and quantification of *Metarhizium* clade 1. Journal of Invertebrate Pathology 108, 106-114.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Fungal Barcoding Consortium. 2012 – Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Science 109, 6241–6246.

Shrestha UB, Bawa KS. 2013 – Trade, harvest, and conservation of caterpillar fungus (*Ophiocordyceps sinensis*) in the Himalayas. Biological Conservation 159, 514–520.

Shrestha UB. 2012 – Asian medicine: A fungus in decline. Nature 482 (7383):35.

Singh N, Rakshita P, Kathait AS, Rautela D, Dubey A. 2010 – Collection of *Cordyceps sinensis* (Berk.) Sacc. in the interior villages of Chamoli District in Garhwal Himalaya (Uttarakhand) and its social impacts. Journal of American Science 6, 5–9.

Sun YQ, Sun GX, Jin Y. 2008 – Quality control of traditional Chinese medicines by the capillary electrophoresis fingerprint and capillary electrophoresis-mass spectrometry. Chinese Journal of Chromatography 26, 160–165.

Sung GH, Sung JM, Hywel-Jones NL, Spatafora JW. 2007 – A multi-gene phylogeny of Clavicipitaceae (Ascomycota, Fungi): Identification of localized incongruence using a combinational bootstrap approach. Molecular Phylogenetics and Evolution 44, 1204–1223.

Swofford DL. 2002 – PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sinauer Associates, Sunderland.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011 – MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28, 2731–2739.

Tao G, Hyde KD, Cai L. 2013 – Species-specific real-time PCR detection of *Colletotrichum kahawae*. Journal of Applied Microbiology 114, 828–835.

The State Pharmacopoeia Committee of People Republic of China. 2010 – Pharmacopoeia of the People's Republic of China. Part I. Beijing: Chemical Industry Publishing House, 106, 352.

Tuli HS, Sandhu SS, Sharma AK. 2013 – Pharmacological and therapeutic potential of *Cordyceps* with special reference to Cordycepin. 3 Biotech 4, 1–12.

Udayanga D, Castlebury LA, Rossman AY, Chukeatirote E, Hyde KD. 2014 – Insights into the genus *Diaporthe*: phylogenetic species delimitation in the *D. eres* species complex. Fungal Diversity 67, 203-229.

Wen TC, Zhu RC, Kang JC, Huang MH, Tan DB, Ariyawansha H, Hyde KD, Liu H. 2013 – *Ophiocordyceps xuefengensis* sp. nov. from larvae of *Phassus nodus* (Hepialidae) in Hunan Province, southern China. Phytotaxa 123, 41–50.

Weng RA, Li SH. 2008 – Identification of *Cordyceps* and its adulterants *Cordyceps militaris* using ITS sequencing. Journal of Hunan Normal University (Medical Science) 5, 42-45..

White TJ, Bruns T, Lee S, Taylor JW. 1990 – Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols A Guide to Methods and Applications, 315–322.

Winkler D. 2009 – Caterpillar fungus (*Ophiocordyceps sinensis*) production and sustainability on the Tibetan Plateau and in the Himalayas. Asian Medicine 5, 291–316.

Wu TN, Yang KC, Wang CM, Lai JS, Ko KN, Chang PY, Liou SH. 1996 – Lead poisoning caused by contaminated *Cordyceps*, a Chinese herbal medicine: two case reports. Science of the Total Environment 182, 193–195.

Xiang L, Song JY, Xin TY, Zhu YJ, Shi LC, Xu XL, Pang XH, Yao H, Li WJ, Chen SL. 2013 – DNA barcoding the commercial Chinese caterpillar fungus. FEMS Microbiology Letters 347, 156–162.

Yu JJ, Kuroda C, Gong X. 2011 – Natural hybridization and introgression in sympatric *Ligularia* species (asteraceae,senecioneae). Journal of Systematics and Evolution 49, 438–448.

Yue K, Ye M, Zhou Z, Sun W, Lin X. 2013 – The genus *Cordyceps*: a chemical and pharmacological review. Journal of Pharmacy and Pharmacology 65, 474–493.

Zhang WM, Hu B, Zhong H, Chen YQ, Tao MH. 2005 – Study on the anamorph of *Cordyceps campsosterna*. Proceedings of the First Symposium on Development of China's Medicinal Fungi Industry 81-82.

Zhou XW, Gong ZH, Su Y, Lin J, Tang KX. 2009 – *Cordyceps* fungi: natural products, pharmacological functions and developmental products. Journal of Pharmacy and Pharmacology 61, 279–291.

Zhu JS, Halpern GM, Jones K. 1998 – The scientific rediscovery of a precious ancient Chinese herbal regimen: *Cordyceps sinensis*: part II. Journal of Alternative and Complementary Medicine 4, 429–457.