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# Molecular Analyses of *Cordyceps gunnii* in China

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## ABSTRACT

*Cordyceps gunnii* Berk., originally named for a specimen discovered in Tasmania, Australia, is commonly reported in China as an adulterant of the valuable Chinese medicinal fungus, *Cordyceps sinensis*. Correct identification of this adulterant species bears close relevance to herb control, legal disputes and forensic measures. Analyses of the internal transcribed spacers of the nuclear ribosomal DNA repeats (ITS) and three loci [nuclear ribosomal large subunit (nrLSU), elongation factor 1α (EF-1α) and the largest subunit of RNA polymerase II (rpb1)] of *C. gunnii* from China and from Tasmania revealed that they are not conspecific. The *Cordyceps* species recognized as *C. gunnii* in China is clustered with those species that are regarded as belonging to the genus *Metacordyceps* and placed in the *Clavicipitaceae* family. Tasmanian samples, however, are closer to *C. sinensis* and belong to the genus *Ophiocordyceps* in the *Ophiocordycipitaceae* family. Re-investigation of the taxonomic status of this adulterant fungus is urgently needed to avoid unnecessary dispute. At this stage, reference to the Chinese samples of *Cordyceps gunnii* can be tentatively labeled as *Cordyceps gunnii* auctorum non Berkeley (*Cordyceps gunnii* auct. non Berk.). The tested sequences can also be useful for differentiating the adulterant '*Cordyceps gunnii*' from genuine samples of *Cordyceps sinensis*.

Key words: *Cordyceps gunnii*, *Cordyceps sinensis*, *Metacordyceps*, *Ophiocordyceps*, adulterants

## INTRODUCTION

Cordyceps (Dongchong Xiacao) is a treasured herb in traditional Chinese medicine for tonifying 'lungs', replenishing 'kidneys', stopping bleeding and resolving phlegm. It is commonly used for treating chronic coughs, debilitating asthma, consumptive coughs with hematemesis, impotence, spermaturia, and lumbar and knee pains[1]. According to the Pharmacopoeia of the People's Republic of China, Cordyceps is the fruiting body of *Cordyceps sinensis* (Berk.) Sacc. [syn. *Ophiocordyceps sinensis* (Berk.) G. H. Sung, J. M. Sung, Hywel-Jones & Spatafora] growing on ghost moth caterpillars belonging to the family *Hepialidae*[1]. Market demands for this herb keep rising in recent years, and quality items are being sold for US$40,000 per kilogram[2], which is a price higher than gold. It is not surprising that adulteration and substitution of this herb are very frequent on the market[3-5]. One of the commonly reported adulterants is the fruiting bodies of *Cordyceps gunnii* Berk., known as Guni Chongcao in China, which is also a parasite fungus of ghost moth caterpillars[6-8]. Another adulterant called Yaxiangbang Chongcao has been identified as *C. hawkesii* Gray, but it is now regarded as a synonym of *C. gunnii* based on molecular findings[9]. Both *C. gunnii* and *C. hawkesii* were first named after collections made in Tasmania, Australia.

The Chinese herbal community considers *C. gunnii* an adulterant and also harmful to health. However, a few reports suggested they have beneficial health effects[10-13]. Correct identification and proper understanding of this species, thus, bears close relevance to herb control, legal disputes and forensic measures. This study applies DNA barcoding techniques of nucleotide sequences from multiple nuclear loci with an aim to clarify the identity of Chinese samples of *C. gunnii*.

## MATERIALS AND METHODS

### I. *Source of Samples*

Live cultures of *Cordyceps gunnii* (CG), *Cordyceps militaris* (CM) and *Hirsutella sinensis* (HS) were

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kindly provided by the U. S. Department of Agriculture, Agricultural Research Service (USDA-ARSEF). Five samples of Cordyceps (CS1-5) retailed as substitute of *C. sinensis* and confirmed as *C. gunnii* by morphological comparison and three authentic *C. sinensis* samples (CS6-8) were examined in this study. Downloaded from NCBI GenBank and applied in cluster analyses in this study were 29 ITS sequences of *Cordyceps* including 7 of *C. gunnii* from Chinese sources and 42 sequences of three loci (nrLSU, EF-1α and rpb1). As AT-biased genotypes are known in *Cordyceps sinensis*[14], ITS sequences of AT-biased genotypes (AB067740 and AB067744) of *C. sinensis* were also included in the MP tree of ITS region. Details of the samples are listed in Tables 1 and 2.

II. *DNA Extraction, Polymerase Chain Reaction and DNA Sequencing*

Approximately 0.1 g of sample materials from the fruiting body was mixed with 500 μL of extraction buffer [200 mM Tris-HCl (pH 8.0), 25 mM EDTA, 200 mM NaCl, 0.5% SDS] at 37°C for 1 h[15]. The sample solution was then mixed with 400 μL of CTAB [2% CTAB (w/v), 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH. 8.0), 1.4 M NaCl, 1% PVP (polyvinylpyrrolidone)] and 800 μL of phenol : chloroform : isoamyl alcohol (25 : 24 : 1)]. The two phases were separated by centrifugation at 13,000 rpm for 15 min. Then the upper phase was transferred to a new Eppendorf tube. The extraction was repeated with an equal volume of chloroform : isoamyl alcohol (24 : 1). DNA was precipitated by adding 500 μL of isopropanol and standing on an ice bath for 30 min. Crude DNA pellet was harvested by centrifugation at 13,000 rpm for 15 min. The DNA pellet was then washed using 70% ethanol and resuspended in 30 μL of double deionized water and stored at -20°C.

Polymerase chain reactions of the genomic ITS, nrLSU, EF-1α and rpb1 were performed in a 25 μL reaction mixture containing 1 μL of DNA, 17.3 μL of double deionized water, 2.5 μL of 10X buffer, 2 μL of 2.5 μM dNTP, 0.2 μL of *Taq* polymerase and 1 μL of 10 μM primers[16]. The primers sets and annealing temperatures of the fungal-specific gene regions used are listed in Table 3. The mixtures were initially subjected to 95°C for 5 min, and then 35 thermal cycles of 1 min at 95°C, 1 min at a region-specific annealing temperature (Table 2), 1 min at 72°C, and finally with an extension of 5 min at 72°C. DNA fragments were visualized on 1% agarose gels using Tris-acetate-EDTA (TAE) and stained with ethidium bromide[16].

DNA fragments were recovered from the agarose gel using the manufacturer's protocol (Gel-M Extraction System, Viogene, Taiwan). The amplified ITS region fragments were cloned with pGEM®-T Easy Vector System (Promega, USA), and three plasmids were randomly picked and purified according to the manufacturer's protocol (Mini *Plus*™ Plasmid DNA Extraction System, Viogene, Taiwan). Plasmids were purified with protocols outlined in the Mini *Plus*™ Plasmid DNA Extraction System (Viogene, Taiwan). Primer T7P (5′ TAA TAC GAC TCA CTA TAG GG 3′) was used for sequencing the cloned products of ITS fragment in the recombinant plasmid. For the amplicons of other gene regions, both strands were sequenced using the forward or reverse

**Table 1.** Live cultures and Cordyceps samples used in the analysis and the NCBI accession numbers assigned to their sequences

| Sample name | Source location | Code | GenBank accession number | | | |
|---|---|---|---|---|---|---|
| | | | ITS | nrLSU | EF-1α | rpb1 |
| *Cordyceps gunnii* (ARSEF 6828) | Tasmania, Australia | CG | HM140630 | HM140633 | HM140636 | HM140639 |
| *Cordyceps militaris* (ARSEF 6248) | Anhui, China | CM | HM140632 | HM140635 | HM140638 | HM140641 |
| *Hirsutella sinensis* (ARSEF 6282) | Qinghan, China | HS | HM140631 | HM140634 | HM140637 | HM140640 |
| Cordyceps | / | CS1 | HM149352 | HM149357 | HM149362 | HM149367 |
| Cordyceps | / | CS2 | HM149353 | HM149358 | HM149363 | HM149368 |
| Cordyceps | / | CS3 | HM149354 | HM149359 | HM149364 | HM149369 |
| Cordyceps | / | CS4 | HM149355 | HM149360 | HM149365 | HM149370 |
| Cordyceps | / | CS5 | HM149356 | HM149361 | HM149366 | HM149371 |
| Cordyceps | / | CS6 | HM595996 | HM595901 | HM595935 | HM595967 |
| Cordyceps | / | CS7 | HM595999 | HM595902 | HM595936 | HM595968 |
| Cordyceps | / | CS8 | HM595981 | HM595885 | HM595918 | HM595952 |

**Table 2.** Sequences of *Cordyceps* and related species and their corresponding NCBI accession numbers downloaded in this study

| Species | Source location / Voucher Info.[a] | NCBI accession number | | | |
|---|---|---|---|---|---|
| | | ITS | nrLSU | EF1 | rpb1 |
| *Cordyceps gunnii* | Guangdong, China | AJ243772 | – | – | – |
| | Guangdong, China | AJ243773 | – | – | – |
| | Yunnan, China | AJ309340 | – | – | – |
| | Tasmania, Australia | AJ309344 | – | – | – |
| | OSC 76404, Tasmania, Australia | – | AF339522 | AY489616 | AY489650 |
| *Cordyceps hawkesii* | Hunan, China | AJ309341 | – | – | – |
| *Cordyceps militaris* | Guizhou, China | AJ309329 | – | – | – |
| | OSC 93623 | – | AY184966 | DQ522332 | DQ522377 |
| *Cordyceps sinensis* | EFCC 7287 | – | EF468827 | EF468767 | EF468874 |
| | / | AB067740 | – | – | – |
| | / | AB067744 | – | – | – |
| | / | AJ309354 | – | – | – |
| | / | AJ309357 | – | – | – |
| | / | AJ309361 | – | – | – |
| *Cordyceps taii* | HMIGD 20925 | EF495099 | – | – | – |
| | ARSEF 5714 | – | AF543787 | AF543775 | DQ522383 |
| *Haptocillium balanoides* | ARSEF 3350 | EU086434 | – | – | – |
| | CBS 250.82 | AJ292414 | AF339539 | DQ522342 | DQ522388 |
| *Haptocillium sinense* | CBS 282.90 | AJ292415 | – | – | – |
| | CBS 567.95 | AJ292417 | AF339545 | DQ522343 | DQ522389 |
| *Hirsutella sinensis* | HMAS 55469 | AJ243980 | – | – | – |
| *Metarhizium anisopliae* | British Columbia | EU307926 | – | – | – |
| | ARSEF 3145 | – | AF339530 | AF543774 | DQ522399 |
| *Nectria cinnabarina* | NBRC 30679 | AB237663 | – | – | – |
| | CBS 713.97 | – | AF193237 | – | – |
| | CBS 114055 | – | – | AF543785 | AY489666 |
| *Nomuraea rileyi* | NBRC 8560 | – | AB047211 | – | – |
| | CBS 806.71 | AY624205 | – | EF468787 | EF468893 |
| *Paecilomyces carneus* | CBS239.32 | AY624171 | EF468843 | EF468789 | EF468894 |
| | CBS399.59 | AY624170 | EF468842 | EF468788 | EF468895 |
| *Paecilomyces gunnii* | / | AJ309339 | – | – | – |
| | / | AJ309343 | – | – | – |
| *Paecilomyces hawkesii* | / | AJ309342 | – | – | – |
| *Paecilomyces lilacinus* | CBS 431.87 | AY624188 | EF468844 | EF468791 | EF468897 |
| *Paecilomyces marquandii* | CBS 182.27 | AY624193 | EF468845 | EF468793 | EF468899 |
| *Pochonia bulbillosa* | CBS 145.70 | AJ292410 | AF339542 | EF468796 | EF468902 |
| | RCEF 3428 | EU000247 | – | – | – |

[a]ARSEF: USDA-ARS Collection of Entomopathogenic Fungal cultures, Ithaca, NY; CBS: Centraalbureau voor Schimmelcultures, Itrecht, the Netherlands; CG: Embrapa Collection, Brasília, Brazil; EFCC: Entomopathogenic Fungal Culture Collection, Chuncheon, Korea; HMAS: Herbarium of the Institute of Microbiology, Chinese Academia of Sciences, China; HMIGD: Herbarium of Guangdong Institute of Microbiology, Guangzhou, China; JCM: Japan Collection of Microorganisms, Japan; NBRC: NITE Biological Resource Center; OSC: Oregon State University Herbarium, Corvallis, OR; RCEF: Research Centre Entomogenous Fungi of Anhui Agricultural University, China.

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**Table 3.** Forward (F) and reverse (R) PCR primers and annealing temperatures used

| Name | Sequences (5′ – 3′)[b] | Annealing temperature | References |
|---|---|---|---|
| **ITS** | | 53°C | |
| ITS5 (F) | GGA AGT AAA AGT CGT AAC AAG G | | (40) |
| ITS4 (R) | TCC TCC GCT TAT TGA TAT GC | | (40) |
| **nrLSU** | | 50°C | |
| LROR (F) | GTA CCC GCT GAA CTT AAG C | | (41) |
| LR5 (R) | ATC CTG AGG GAA ACT TC | | (41) |
| **EF-1α** | | 55°C | |
| EF-983F (F) | GCY CCY GGH CAY GGT GAY TTY AT | | (42) |
| EF-2218R (R) | GAC TTG ACT TCR GTV GTG AC | | (42) |
| **rpb1** | | 53°C | |
| CRPB1 (F) | CCW GGY TTY ATC AAG AAR GT | | (43) |
| RPB1Cr (R) | CCN GCD ATN TCR TTR TCC ATR TA | | (43) |

[b]H = (A/C/T), N = (A/G/C/T), R = (A/G), V = (A/G/C), W = (A/T), Y = (C/T)

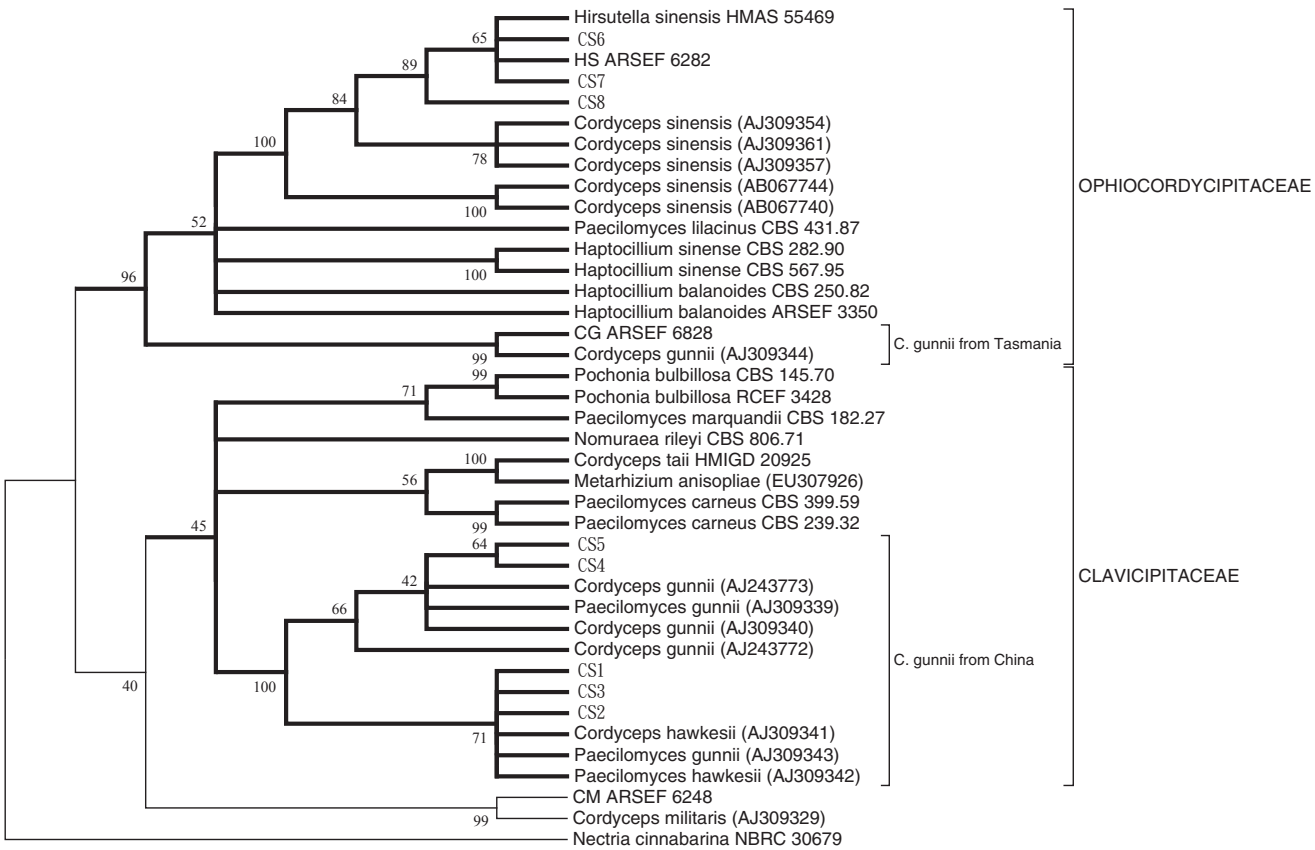


**Figure 1.** Consensus Maximum Parsimony tree of ITS region of Cordyceps samples and related sequences assessed with 1000 bootstrap replicates was constructed by Bootstrap analyses with bootstrap values indicated at branches (branches corresponding to partitions reproduced in less than 40% were collapsed).

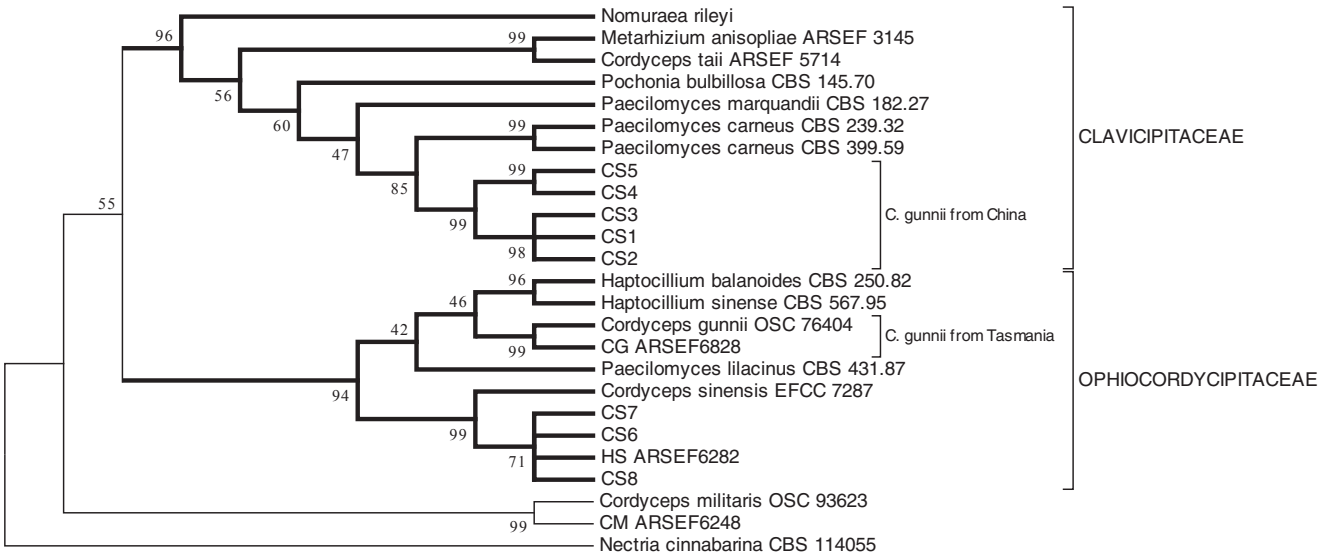


**Figure 2.** Consensus Maximum Parsimony tree of combined data set of three genes (nrLSU, EF-1α and rpb1) from Cordyceps samples and related sequences assessed with 1000 bootstrap replicates was constructed by Bootstrap analyses with bootstrap values indicated at branches (branches corresponding to partitions reproduced in less than 40% were collapsed).

primer alone and the PCR Product Pre-sequence Kit (USE Co., Cleveland, OH, USA). Cycle sequencing was performed using ABI PRISM$^{TM}$ BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster, CA, USA).

III. *Data Analysis*

DNA sequences generated in this study are deposited in GenBank nucleotide sequence database in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/), and their corresponding accession numbers are listed in Table 1. Additional sequences of corresponding regions of closely-related species were downloaded from NCBI and integrated for present analyses (Table 2). DNA sequences were assembled and edited in the program BioEdit 7.0.5.3[17]. Multiple alignments were performed in program Clustal W[18]. Maximum parsimony (MP) tress based on ITS and combined data set of three genes (nrLSU, EF-1α and rpb1) were constructed with Close-Neighbor-Interchange algorithm[19] using program MEGA 4[20]. Bootstrap analyses[21] for 1000 replicates were performed to provide confidence estimates for tree topologies. The trees were rooted with *Nectria cinnabarina*, which belongs to a sister order of *Cordyceps*.

## RESULTS

DNA sequences obtained in this analysis, together with sequences from related species from NCBI GenBank were aligned for FINS (forensically informative nucleotide sequencing) analyses. The aligned 40-taxa ITS region data set consisted of 639 base pairs (bp). As a result of complete deletion of all positions containing gaps and missing data, there were a total of 377 positions in the final data set, out of which 129 were parsimony informative. Maximum parsimony (MP) analyses resulted in 49 equally parsimonious trees. The strict consensus tree is shown in Figure 1.

The combined 25-taxa 3-gene data set consisted of 1,852 bp (nrLSU 795 bp, EF-1α 539 bp, and rpb1 518 bp). After eliminating positions with gaps and missing data, there was a total of 1,305 positions in the final data set, out of which 361 are parsimony informative. MP analyses resulted in 17 equally parsimonious trees and the strict consensus tree is shown in Figure 2.

As revealed in the consensus MP tree based on ITS region (Figure 1), adulterant Cordyceps samples (CS1-CS5) and sequences of *Cordyceps gunnii* samples from China clustered in a group, distinctly separate from the samples of *C. gunnii* from Tasmania. As AT-biased genotypes are known in *Cordyceps sinensis*[14], ITS sequences of AT-biased genotypes (AB067740 and AB067744) of *C. sinensis* were also included in the MP tree of ITS region (Figure 1). The clusters were not affected by the AT-biased genotypes of *C. sinensis*[14]. Similar discrepancy of sequences of *C. gunnii* from China and Tasmania was found in the consensus MP tree of combined data set of the three genes (nrLSU, EF-1α and rpb1) (Figure 2). Again, adulterant Cordyceps samples (CS1-CS5), generally regarded as *C. gunnii* in China, also clustered in a clade, and excluded *C. gunnii* samples from Tasmania.

The molecular results clearly demonstrate that Cordyceps samples generally identified as *C. gunnii* in China is definitely not conspecific with Tasmanian samples. If the new classification based on cladistic conclusions[22-24] is followed, the Chinese '*C. gunnii*' samples, which are closer

to *C. taii* and related species (Figure 1-2), would have to be removed to the new genus *Metacordyceps* G. H. Sung, J. M. Sung, Hywel-Jones & Spatafora and placed in the *Clavicipitaceae* family. On the other hand, Tasmanian samples are closer to *C. sinensis*, and would belong to the genus *Ophiocordyceps* and the *Ophiocorydycipitaceae* family.

As a side issue, the two trees (Figures 1 and 2) also reveal that *Paecilomyces lilacinus* appears in the *Ophiocorydycipitaceae* clade while the other *Paecilomyces* species in the *Clavicipitaceae* clade. This result confirms the previous observation that the genus *Paecilomyces* is polyphyletic and a thorough taxonomic review is urgently required[25-27].

## DISCUSSION

In the pharmaceutical and herbal industry, correct identification of source material is fundamental. Improper labeling of an organism with a scientific name can lead to serious consequences, including legal disputes and ineffective conservation, as well as misleading information filing and retrieval.

*Cordyceps* Link (sensu lato) is a diversified genus with more than 400 species and a wide host range[28-32]. There are recent suggestions to divide this genus into four genera: (1) *Cordyceps* sensu stricto, with *C. militaris* (L.) Link as the type species, (2) *Metacordyceps* G. H. Sung, J. M. Sung, Hywel-Jones & Spatafora, (3) *Elaphocordyceps* G. H. Sung & Spatafora, and (4) *Ophiocordyceps* Petch to which genus *C. sinensis* would belong[22-24]. Before their relationship is better clarified, we choose to consider *Cordyceps* in a broad sense.

*Cordyceps gunnii* was first reported by Berkeley[33] as *Sphaeria gunnii* Berk. growing on caterpillars of *Cossus* (*Cossideae* family) or *Hepialus* (*Hapialidae* family). It was first recorded in Franklin Village, Tasmania[33], but was later also found in other parts of southern Australia[34]. According to the descriptions of Mr. Gunn, the collector of the type specimen of this species, the fungus was abundant in sandy locations, and the caterpillar and fruiting bodies varied from 5 - 18 inches long, depending on the depth of the burrow. The fruiting body underground was white in color, while the exposed portion was about 2 - 4 inches long and dark olive black in color[33]. Another closely-related species, *Cordyceps hawkesii*, was reported by M.C. Cooke in 1891 and named after the collector, Mr. Hawkes[35]. The specimen was first reported in Tasmania[35]. Its host was suggested to be a *Pielus* species (*Limnephilinae* family) or some closely allied genera[34], while Lloyd[36] suggested that although this fungal species which shared the same host with *C. gunnii*, *C. hawkesii* resembles *C. gunnii*. Its fruiting body is comparatively more slender, irregular, contorted and knotted besides being woolly[35].

Both *C. gunnii* and *C. hawkesii* have been reported in China. Li *et al.*[37] discovered *C. hawkesii* in Hunan, Anhui and Fujian Provinces. Liang[6,7], on the other hand, claimed that *C. gunnii* also grows in Guizhou. A variant of this species, *C. gunnii* var. *minor*, was also reported[38]. Their hosts were suggested to be some species from the *Hapialidae* family[6,7,38,39]. Both species shared similar morphological characters, and Liang[7] differentiated *C. gunnii* from *C. hawkesii* by the size of asci, ascospores and part-spores. *C. gunnii* contained larger asci, ascospores and part-spores than *C. hawkesii*.

Liu *et al.*[9] first suggested the two species available in China are conspecific, based on DNA sequences of ITS region. They regarded *C. hawkesii* as synonymous with *C. gunnii*. Various molecular studies have looked into the phylogeny of *Cordyceps*. However, there is no thorough comparison between samples of *C. gunnii* in China and Tasmania[22-24,32]. Our results conclusively demonstrate that the Chinese samples identified as *C. gunnii* is very likely a misidentification. Re-investigation into the taxonomic status of this common fungus is urgently needed. At this stage, reference to the Chinese samples of *C. gunnii* can be tentatively labeled as *Cordyceps gunnii* auctorum non Berkeley (*Cordyceps gunnii* auct. non Berk.). Moreover, our results also show that the internal transcribed spacers of the nuclear ribosomal DNA repeats (ITS) and three loci [nuclear ribosomal large subunit (nrLSU), elongation factor 1α (EF-1α) and the largest subunit of RNA polymerase II (rpb1)] can be used for differentiating the adulterant '*C. gunnii*' from genuine samples of *C. sinensis*.

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