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# DNA barcoding the commercial Chinese caterpillar fungus

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## Abstract

Chinese caterpillar fungus (*Ophiocordyceps sinensis*) has been widely used as tonic in Asian medicine. Considering its curative effect and high cost, various counterfeit versions of *O. sinensis* have been introduced and are commercially available. These counterfeits have morphological characteristics that are difficult to distinguish based on morphology alone, thereby causing confusion and threatening its safe use. In this study, internal transcribed spacer (ITS) sequences as a DNA barcode were analyzed and assessed for rapid and accurate identification of 131 *O. sinensis* samples and 12 common counterfeits and closely related species. Results showed that sufficient ITS sequence differences, also known as 'barcode gaps', existed to distinguish between *O. sinensis* and counterfeit species. ITS sequence correctly identified 100% of the samples at the species and genus level using the Basic Local Alignment Search Tool 1 and the nearest distance method. Furthermore, *O. sinensis*, counterfeits, and closely related species can be successfully identified using tree-based methods including maximum parsimony, neighbor-joining, and maximum likelihood analysis. These results indicated that DNA barcoding could be used as a fast and accurate identification method to distinguish *O. sinensis* from counterfeits and closely related species to ensure its safe use.

## Introduction

*Ophiocordyceps sinensis* (anamorph: *Hirsutella sinensis*), which is synonymous to *Cordyceps sinensis* in the historical classification system (Sung *et al.*, 2007), is one of the most common medicinal entomogenous fungi used as tonic and health-promoting food in China (Shrestha *et al.*, 2010). The combination of a mummified caterpillar larva and the fungal stroma resulting from parasitism is widely used to treat asthma, bronchitis, lung inflammation, nocturnal emissions, and night sweats (Wang *et al.*, 2009). *Ophiocordyceps sinensis* and its host are geographically distributed in Alpine regions between 3000 and 5000 m elevation on the Tibetan Plateau, including some parts of China, Bhutan, and Nepal (Shrestha *et al.*, 2010; Li *et al.*, 2011). *Ophiocordyceps sinensis* and its host are distributed in China, particularly in eastern Tibet, northeastern Qinghai, northern Yunnan, and northwestern Sichuan (Zhang *et al.*, 2010; Li *et al.*, 2011). Given its curative effect and limited supply, *O. sinensis* costs \$53.7 $g^{-1}$ and is commonly known as the 'golden' Chinese medicine (Guo *et al.*, 2012).

Considering its high cost, *O. sinensis* has been produced using various counterfeit versions with very similar morphology, thereby causing confusion in market management and challenging the safe use of *O. sinensis*. These counterfeits are in the form of natural counterfeits, such as *Ophiocordyceps robertsii*, *Cordyceps gunnii*, *Cordyceps militaris*, and *Cordyceps cicadae* (Liu *et al.*, 2011; Au *et al.*, 2012), and artificial complexes, which can cause illness or even death when improperly used (Ji *et al.*, 2008; Huang *et al.*, 2011). Studies have shown that *O. sinensis* can be identified from its counterfeits or closely related species based on its macroscopic and microscopic properties (Liu *et al.*, 2011; Au *et al.*, 2012). However, various counterfeits have indistinct morphological characters that are difficult to distinguish based on morphology alone. Therefore, a quick, inexpensive, and







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reliable assay to discriminate *O. sinensis* from its counterfeits is clearly needed for its safe use.

As a rapid and accurate identification method, DNA barcoding technology has been widely applied in animals, plants, and fungi for species identification (Seifert, 2009). Prior to DNA barcoding, few species-level markers were used, including nuclear ribosomal internal transcribed spacers (ITS), small or large ribosomal subunits, ribosomal polymerase B1 and B2, β-tubulin, and calmodulin (Hinrikson *et al.*, 2005; Seifert, 2009). At present, the ITS region of a nuclear ribosomal repeat unit is considered by most mycologists as a universal DNA barcode candidate for identifying fungi (Nilsson *et al.*, 2008; Schoch *et al.*, 2012). This barcode is also supported by the Consortium of the Barcode of Life (Seifert, 2009). The ITS region of this barcode comprises ITS1 (rapidly evolving), 5.8S (very conserved), and ITS2 (moderately rapid) domains and has been widely used as a DNA barcode to identify fungal species (Nilsson *et al.*, 2008).

Liu *et al.* (2002) and Chen *et al.* (2001, 2004) used nrDNA ITS sequences to determine the relationship between anamorph and teleomorph portions of several *Cordyceps* species. Zhang *et al.* (2009, 2010) and Zhu *et al.* (2010) reported the genetic diversity and genotypes of ITS sequence in *O. sinensis*. However, molecular identification of *O. sinensis* and counterfeits by ITS sequence has not been reported. In this study, DNA barcoding technology was applied for the first time to distinguish *O. sinensis* from its counterfeits. To verify the applicability of DNA barcoding, ITS sequences of 131 test organisms of *O. sinensis* and 12 common counterfeits and closely related species were used. The application of DNA barcoding in trade and market management could ensure the safe use of *O. sinensis*.

## Materials and methods

### Materials

A total of 53 authentic *O. sinensis* samples and six common counterfeit species were collected, including five natural counterfeits *C. gunnii* (10 samples), *C. cicadae* (19 samples), *C. militaris* (3 cultured products), *Ophiocordyceps nutans* (5 samples), *O. robertsii* (4 samples), and one artificial counterfeit concoction of the dried caterpillar and plant stem of *Ligularia hodgsonii* (10 samples; Fig. 1 and Table S1, Supporting information). All of the samples were collected from herbal medicine or rural markets in China except *O. robertsii*, which was collected from New Zealand in 2012 (Table S1). Four fresh *H. sinensis* (anamorph of *O. sinensis*) samples were incubated at 16 °C for 30 days on potato dextrose agar medium. The first three samples were isolated from Sichuan and

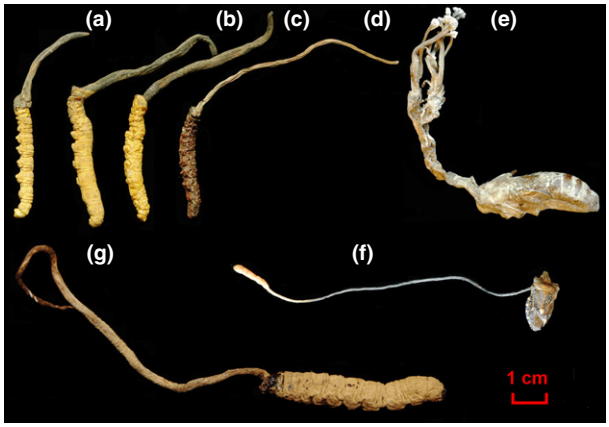


**Fig. 1.** *Ophiocordyceps sinensis* and the common counterfeits appearing on the market: (a) authentic *O. sinensis*; (b) natural counterfeit *Cordyceps gunnii*; (c) artificial counterfeit concoction of dried caterpillar and plant stem of *Ligularia hodgsonii*; (d) natural counterfeit *Matacordyceps liangshanensis*, which was collected but not used in this study because its genomic DNA was degraded; (e) natural counterfeit *Cordyceps cicadae*; (f) natural counterfeit *Ophiocordyceps nutans*; (g) natural counterfeit *Ophiocordyceps robertsii* collected from New Zealand.

Qinghai in 2010. The fourth sample (code GCMCC3.14243) was collected from China General Microbiological Culture Collection Center. Cultured *C. militaris* samples were used in this study because of their rare nature. All the samples used in this study were morphologically identified, and corresponding voucher samples are curated in the Herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medicinal Sciences. In addition, 78 published sequences isolated from *O. sinensis* and 9 sequences isolated from counterfeit *C. militaris*, and six closely related species (*Ophiocordyceps emeiensis*, *Ophiocordyceps sobolifera*, *Cordyceps brongniartii*, *Matacordyceps brittlebankisoides*, *Paecilomyces sinensis*, and *Tolypocladium caledonica*) were downloaded from GenBank for completeness (Table S1).

### DNA extraction, amplification, and sequencing

Specimens were divided into stromata and sclerotia, but only stromata were used. In brief, single dry stroma (20–30 mg) and fresh mycelia of *H. sinensis* (40–50 mg) were homogenized in liquid nitrogen. nrDNA was subsequently isolated using a plant genomic DNA kit (Tiangen Biotech Co., Ltd., Beijing, China). The ITS regions of nrDNA were amplified using an LA *Taq* polymerase chain reaction (PCR) kit (Takara Biotech Inc.) with a forward primer of ITS5F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and a reverse primer of ITS4R (5′-TCCTCCGCTTA TTGATATGC-3′; Liu *et al.*, 2002). The PCR mixture contained 0.1 μL of LA *Taq* (5 U $\mu L^{-1}$), 2.5 μL of 10× LA

*Taq* PCR buffer II ($Mg^{2+}$ Plus), 1 μL of dNTP mixture (2.5 mM each), 0.6 μL of each primer (10 μM), and 1 μL of genomic DNA in a total volume of 25 μL. The samples were amplified using a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA) under the following conditions: initial denaturation at 97 °C for 1 min, followed by 30 cycles of denaturation at 97 °C for 1 min, annealing at 48 °C for 1 min, extension at 72 °C for 3 min, and a final elongation step at 72 °C for 7 min (Liu *et al.*, 2002). After the PCR products were confirmed by 1.2% agarose gel electrophoresis in 1× TBE buffer, the fragments were purified using a gel band purification kit (Tiangen Biotech Co., Ltd.) and then sequenced bidirectionally using an ABI 3730 XL sequencer (Applied Biosystems).

### Sequence analysis

A CodonCode ALIGNER v 3.5.4 (CodonCode Co.) was used to edit the sequences and assemble the contigs. HMMer software was used to annotate the ITS2 region (Eddy, 1998; Keller *et al.*, 2009; Koetschan *et al.*, 2012). ITS1 and 5.8S sequences were retrieved according to their annotations in GenBank. The partial 18S and 28S regions were excluded from both sides of the sequences. The alignment was performed with MUSCLE in MEGA 5.0 (Tamura *et al.*, 2011). The Kimura 2-parameter (K2P) distances were computed with PAUP4b10 (Florida State University) to quantify sequence divergence. The presence of a 'barcode gap' (interspecific divergences that are clearly greater than intraspecific variation) was verified (Blagoev *et al.*, 2009). Species determination was based on the best hit and the smallest genetic distance of the query sequence in the Basic Local Alignment Search Tool 1 (BLAST1) and the nearest distance method, respectively (Ross *et al.*, 2008). The tree-based methods used for species identification analyses included the neighbor-joining (NJ) and maximum parsimony (MP) methods from the PAUP4b10 software with 1000 bootstrap replicates (Florida State University), and the maximum likelihood (ML; RAXML with PROTGAMMAWAG model) prepared for 100 bootstraps using RAXML version 7.0.4 (Stamatakis, 2006). Two ITS haplotypes of the artificial counterfeit *L. hodgsonii* were designated as outgroup for rooting the trees.

## Results

### Universality

DNA was successfully extracted from 104 samples of *O. sinensis* and six counterfeits. Gel electrophoresis revealed that the DNA of *O. sinensis* and five natural counterfeits had an optical density ($OD_{260:280\ nm}$) ratio of 1.75–2.02, concentrations of 64.9–156.3 ng $\mu L^{-1}$, and a single band for each sample. However, high-quality genomic DNA from the artificial counterfeit *L. hodgsonii* was difficult to extract, and a DNA smear was observed through gel electrophoresis.

ITS regions were successfully amplified from *O. sinensis* and five natural counterfeit samples using one pair of universal primers, ITS4 and ITS5, and a clear single target band was observed. Unfortunately, only three ITS regions were amplified from 10 *L. hodgsonii* samples, and nonspecific bands were observed. High-quality bidirectional sequences were obtained from the ITS regions after sequence quality and coverage were assessed.

### Genetic divergence analysis

Sequence length, GC content, and variable sites of ITS regions of *O. sinensis* and six counterfeit samples were analyzed and summarized (Table 1). Among the 131 ITS sequences of *O. sinensis*, the sequence length varied between 485 and 493 bp, and the average GC content was 63.97%. A total of 53 nucleotide variation sites were detected in the ITS region, including 39 informative sites and 17 singleton variable sites (Fig. S1). The informative sites in ITS1, 5.8S, and ITS2 sequences were 15, 5, and

**Table 1.** ITS sequence characteristics and K2P distances of *Ophiocordyceps sinensis* and six counterfeits used in this article

| | | Sequence length (average GC content) | | | | Variable site | | |
|---|---|---|---|---|---|---|---|---|
| Species | Sample number | ITS1 (bp/%) | 5.8S (bp/%) | ITS2 (bp/%) | Haplotype | Informative site | Singleton variable site | Intraspecific K2P distance (mean) |
| *Ophiocordyceps sinensis* | 131 | 152–160 (66.1) | 153 (50.4) | 176–180 (75.4) | 33 | 39 | 17 | 0–0.056 (0.011) |
| *Ophiocordyceps nutans* | 5 | 138–141 (31.1) | 157 (42.7) | 158–160 (32.2) | 4 | 11 | 3 | 0–0.029 (0.018) |
| *Ophiocordyceps robertsii* | 4 | 155 (56.6) | 166 (47.6) | 189 (73.0) | 2 | 0 | 1 | 0–0.002 (0.001) |
| *Cordyceps militaris* | 6 | 150–151 (59.9) | 165 (46.7) | 165 (67.0) | 4 | 1 | 2 | 0–0.004 (0.002) |
| *Cordyceps cicadae* | 19 | 182 (62.6) | 155 (49.0) | 163 (69.3) | 1 | 0 | 0 | 0 |
| *Cordyceps gunnii* | 10 | 207 (52.2) | 153 (48.4) | 209–210 (67.1) | 3 | 1 | 1 | 0–0.002 (0.001) |
| *Ligularia hodgsonii* | 3 | 256 (53.9) | 164 (49.4) | 225 (53.8) | 2 | 0 | 2 | 0–0.003 (0.002) |

19, respectively, whereas the singleton variable sites for these sequences were 9, 5, and 3, respectively. Among the 39 informative sites, the following contents were detected (Fig. S1): 27 T/C (14) and A/G (13) transitions; 8 G/C (2), A/C (2), T/G (1), and T/A (3) transversions; and 4 T/C/A (2) and G/A/C (2) substitutions. In addition, the following deletions were detected: 8 bp within the ITS1 region of KF003038 (YC0170MT03) at site 59; 4 bp within the ITS2 region of FJ654148 and FJ654149 at site 485; and 1 bp deletion within the ITS regions of 12 samples at sites 59, 341, 351, 462, and 469. For the 131 *O. sinensis* samples, 33 ITS haplotypes were detected (Fig. S1 and Table S1).

A total of 97 ITS sequences of *O. sinensis* and counterfeits generated in this study were submitted to GenBank (Table S1). All the ITS haplotypes of *O. sinensis* and counterfeits have been listed (Table S1). Among the 53 ITS sequences of *O. sinensis*, 8 ITS haplotypes were detected, five of which were reported for the first time. The GenBank accession numbers are JX402624 (YC017 0MT05), JX402625 (YC0170MT08), JX402626 (YC0 170MT09), JX402627 (YC0170MT47), and JX402628 (YC0170MT26). The intraspecific divergence calculated using the K2P model indicated that the ITS sequences of *O. nutans* had the highest average intraspecific distance 0.018, and *O. sinensis* had the highest maximum intraspecific distance 0.056 (Table 1). No variable sites were detected among the ITS regions of the 19 *C. cicadae* samples collected from Sichuan, Anhui, and Jiangsu (Table S1).

In an ideal DNA barcode, genetic variation should demonstrate separate, nonoverlapping distributions between intraspecific and interspecific samples. A barcode gap exists if the minimum interspecific variation is larger than the maximum intraspecific variation. The species can then be given reliable identification. Our results demonstrated that the distribution of the intraspecific and interspecific variations of ITS sequences exhibited distinct gaps at a scale of 0.001 distance units in *O. sinensis* and five natural counterfeits species, including *C. gunnii*, *C. cicadae*, *C. militaris*, *O. nutans*, and *O. robertsii* (Fig. 2). All these six species fall above the 1 : 1 line, indicating the presence of a 'barcode gap.' These DNA barcode gaps clearly identified the authentic *O. sinensis* and the counterfeit species.

### Efficiency of species authentication

BLAST1, nearest genetic distance, and tree-based methods were used to determine whether *O. sinensis* and counterfeits could be identified by ITS regions.

We initially constructed a library containing 184 ITS sequences from *O. sinensis*, counterfeits, and closely related species (Table S1). We then searched the database with the 'test' sequences generated in this study from samples with confirmed taxonomic identity. The species identities of these 'test' sequences were then determined using the BLAST1 or nearest genetic distance method. In the BLAST1 method, species determination was based on the best hit of the query sequence and an *E*-value for the match less than a cutoff value. In comparison, the nearest genetic distance method determines the identity of a sample based on the sequence in the database that has the smallest genetic distance from the query sequence; this distance must be less than the distance threshold (Chen *et al.*, 2010). The results indicated that ITS correctly identified 100% of the samples at both the species and genus level using the BLAST1 method. Moreover, the nearest genetic distance method also had 100% success rate of identification at the species and the genus level.

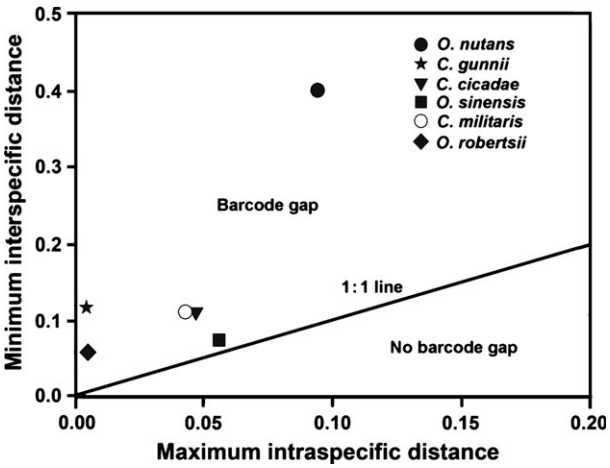


**Fig. 2.** Presence of barcode gap between inter- versus intraspecific divergences in *Ophiocordyceps sinensis* and counterfeit species.

Among the 184 ITS sequences, 53 haplotypes from *O. sinensis*, counterfeits, and closely related species were used for species identification on the basis of the tree-based methods. No significant differences in the tree topologies were observed among the NJ, MP, and ML trees (Fig. 3 and Figs S2 and S3). The ITS sequences in each of these polytypic species were separated into pairs of divergent clusters. Therefore, *O. sinensis* can be successfully distinguished from counterfeits using the tree-based methods.

## Discussion

Chinese caterpillar fungus, along with ginseng and deer antler, has been considered as one of the 'treasure of tonics.' However, various counterfeit versions of *O. sinensis*, including natural counterfeits and artificial complexes,

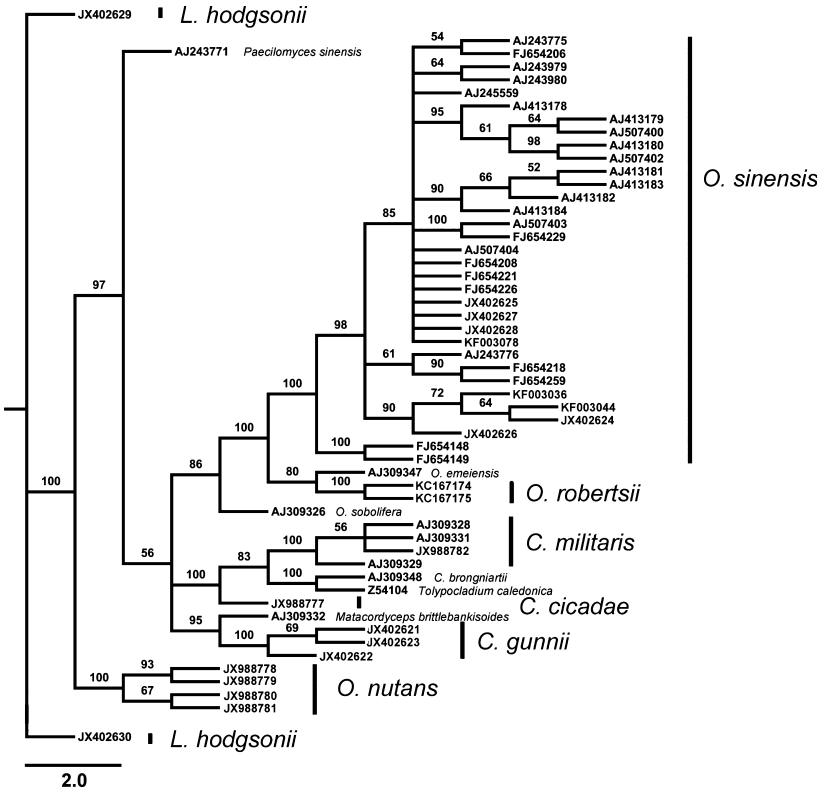


**Fig. 3.** NJ tree based on the haplotype ITS sequences of *Ophiocordyceps sinensis* and counterfeits/closely related species by implementing NJ tree in the PAUP4b10 software with 1000 bootstrap replicates and bootstrap values more than 50%. Two ITS haplotypes of artificial counterfeit of plant stem of *Ligularia hodgsonii* were designated as outgroup for rooting tree.

have been introduced to the market because *O. sinensis* is expensive (Liu *et al.*, 2011; Au *et al.*, 2012). With the improvement of the herb market, most counterfeits that are easily distinguished have disappeared. For example, we collected only several aged samples of *Matacordyceps liangshanensis* with degraded genomic DNA. However, counterfeits with very similar morphology are common in the herb market and are difficult to distinguish. *Cordyceps gunnii*, a synonym of *Cordyceps hawkesii* that parasitizes the larvae of *Napialus hunanensis*, is a most similar natural counterfeit (Liang, 1985). Both *O. sinensis* and *C. gunnii* are insect pathogenic fungi, and their host larvae belong to the same family, Hepialidae (Liang, 1985). These organisms exhibit similar lifestyles, which result in morphologies that are difficult to distinguish via macroscopic and microscopic methods (Liu *et al.*, 2011). Another most common counterfeit introduced to the market is an artificial complex consisting of caterpillars and the plant stem of *L. hodgsonii* as the stromata. The roots of *L. hodgsonii* contain sesquiterpenes and pyrrolizidine alkaloids (Huang *et al.*, 2011). However, the pyrrolizidine alkaloid clivorine isolated from *L. hodgsonii* is toxic to the human liver (Ji *et al.*, 2008) and can cause illness or even death when used improperly. Both *C. cicadae* and *C. militaris* are traditional Chinese medicinal fungi; however, they are commercially available as counterfeits of *O. sinensis* in informal markets. *Ophiocordyceps nutans* and *O. robertsii* are unusual counterfeit species, but they are also introduced to the market sometimes. Therefore, a quick and accurate molecular identification method is needed to discriminate *O. sinensis* from its counterfeits.

*Ophiocordyceps sinensis* parasitizes approximately 57 different species of caterpillar larvae including *Hepialus* and *Thitarodes* distributed in the Tibetan Plateau (Wang & Yao, 2011; Zhang *et al.*, 2012). *Ophiocordyceps sinensis* with any of the 57 caterpillar species are considered authentic as the medicinal product. The authentic *O. sinensis* is unrelated to the host species. Therefore, only the stromata were used in this study for species identification. The host caterpillar larvae of *O. sinensis* can also be parasitized with other entomogenous fungi, such as *C. brongniartii* (anamorph: *Beauveria brongniartii*), which was used as closely related species in this manuscript (Table S1). Some mummified caterpillar larvae of other moth species parasitized with other entomogenous fungi without stromata were also used as counterfeits in the past. However, those mummified caterpillar larvae counterfeits are easily distinguished by morphological characteristics and are rare in the market at present.

ITS1, ITS2, and ITS1 + ITS2 were researched as the DNA barcode for *O. sinensis* and its counterfeits. Both ITS1 and ITS1 + ITS2 can be used to distinguish the authentic *O. sinensis* from counterfeits. The ITS2 region

has been proposed as a universal DNA barcode for plants and animals identification in our published studies (Chen *et al.*, 2010; Yao *et al.*, 2010). However, the authenticity of *O. sinensis* cannot be verified using ITS2 sequences on the basis of tree-based methods. Furthermore, low PCR amplification and sequencing success eliminated both ITS1 and ITS2 as universal barcode for the identification of *O. sinensis*. 5.8S was contained in the ITS region and was not deleted deliberately in this manuscript. Therefore, only the ITS (ITS1 + 5.8S + ITS2) region was used as DNA barcode for the identification of *O. sinensis* from counterfeits in this article.

In an ideal DNA barcode, PCR fragments should be easily amplified, and sequence-based identification should be accurate, rapid, cost effective, universally accessible, and useful for nonexperts (Seifert, 2009; Schoch *et al.*, 2012). Our study suggests that the ITS region is a valuable DNA barcode to authenticate *O. sinensis* from its counterfeits and closely related species. ITS fragments can be easily amplified from *O. sinensis* and from natural counterfeit samples (*O. robertsii*, *C. gunnii*, *C. cicadae*, and *C. militaris*). A total of 53 *O. sinensis* samples with different states (e.g. fresh, dry, or aged) were collected from different markets. High-quality DNA was easily extracted from these samples, and the ITS regions were successfully amplified. Identification based on the ITS sequence was made accurate using the barcode gap, BLAST1, nearest genetic distance, and tree-based methods. The distinct barcode gaps found in the six species were given reliable identifications from *O. sinensis* and its natural counterfeits. On the basis of the ITS sequence, the samples can be correctly identified at both the species and genus levels using the BLAST1 and nearest genetic distance methods. The tree-based methods were also utilized for species identification. Species could be distinguished from other species on the basis of the ITS haplotypes in the MP, ML, and NJ trees. Therefore, *O. sinensis* could be identified from its counterfeits or closely related species using these ITS sequences. DNA extraction, PCR amplification, and sequencing are rapid and inexpensive methods. According to the protocols in this study, DNA extraction and PCR amplification can be completed in 1 d by experts or nonexperts. The sequencing cost for one ITS sequence is approximately USD 3.00, and this cost would become cheaper with the development of sequencing technology. Therefore, DNA barcoding may be used as an effective tool to identify *O. sinensis* from its counterfeits and closely related species for its safe use.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** The intraspecific variable sites in the ITS sequence of *Ophiocordyceps sinensis*.

**Fig. S2.** MP tree based on the haplotype ITS sequences of *Ophiocordyceps sinensis* and counterfeits/closely related species by implementing MP trees in the PAUP4b10 software with 1000 bootstrap replicates and bootstrap values more than 50%.

**Fig. S3.** ML tree based on the haplotype ITS sequences of *Ophiocordyceps sinensis* and counterfeits/closely related species by implementing ML tree (RAXML with PROTGAMMAWAG model) prepared for 100 bootstrap values using RAXML version 7.0.4.

**Table S1.** The species information of *Ophiocordyceps sinensis* and counterfeits used in this study.