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Quality Control of *Cordyceps sinensis* Teleomorph, Anamorph, and Its Products

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1. Introduction

1.1 *Cordyceps sinensis* general description and habitat

Cordyceps sinensis (Berk.) Sacc. is a caterpillar fungus of the genus *Cordyceps* (Clavicipitaceae, Hypocreales, Pyrenomycetes, Ascomycota, Eukaryota) (Fig. 1).

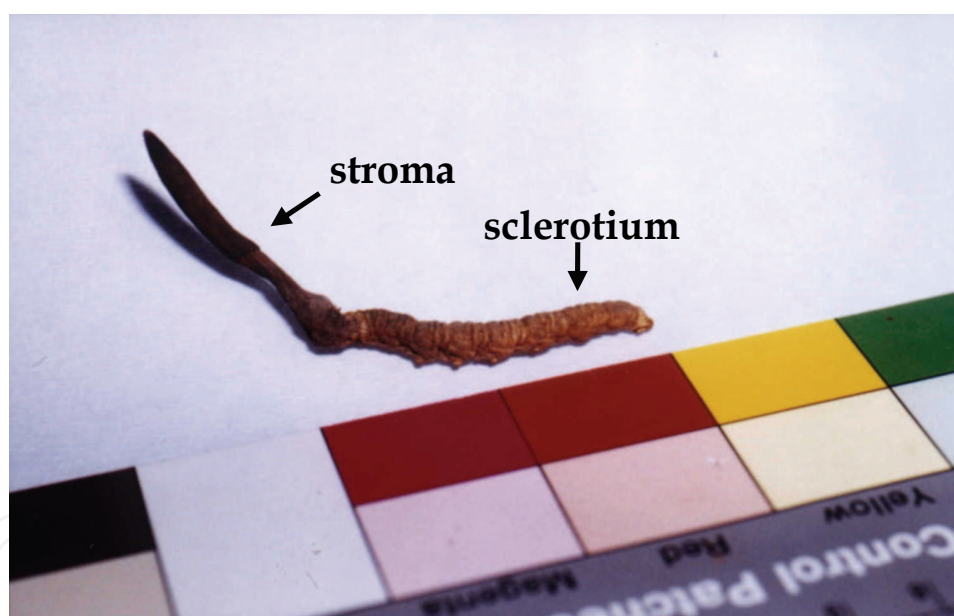


Fig. 1. The stroma and sclerotium of a *Cordyceps sinensis* specimen (image with back shadow)

The fungus is endemic mostly to the alpine shrub-meadow zone of high mountains (3000–5000 m altitude) or highlands of the south western of China (the Tibet Autonomous Region and the Qinghai, Sichuan, Yunnan and Gansu provinces) and to some countries of the Himalayan south slope (Nepal, Bhutan, and North East India) (Fig. 2). It grows parasitically on Lepidoptera larvae, particularly those belonging to the genus *Hepialus* (bat moths), which are found in cool weather conditions (Kinjo & Zang, 2001; Pegler et al., 1994).

After host larval infection with either meiotic or mitotic spores, the fungus multiplies in the host by yeast-like budding, eventually killing the host. The fungus then grows in the form of

thread-like hyphae. Following overwintering, the fungus ruptures the host body, forming a sexual sporulating structure (a perithecial stroma) that is connected to the dead larva below ground and grows upward to emerge above the soil surface. It is this stroma, either with or without the host larva, that is traditionally used for medicinal purposes (Buenz et al., 2005).



Fig. 2. Distribution of *Cordyceps sinensis* (Buenz et al., 2005)

1.2 Medicinal effects of *C. sinensis*

In Asia, *C. sinensis* is considered a valuable traditional herb that has several medicinal effects. It has been reported that *C. sinensis* nourishes the lungs and kidneys (Pegler et al., 1994), strengthens the immune system (Kuo et al., 1996), revitalizes the body after serious illness, acts as an aphrodisiac (Bhattarai, 1993), is a free-radical scavenger (Yamaguchi et al., 2000), exerts an anti-tumour effect (Chen et al., 1997b), and possesses the ability to alter apoptotic homeostasis (Buenz et al., 2005).

1.3 Morphology of the *C. sinensis* teleomorph

C. sinensis has a sexual stage (teleomorph) and an asexual stage (anamorph). It is generally seen in the sexual stage with a stalked fruit body. Recently, an inadequate teleomorph supply has raised its price to around 30,000–50,000 USD per kilogram (depending on size and quality). This high price results in many fake products on the market, e.g. other *Cordyceps* spp. (e.g. *C. cicadicola*, *C. liangshanensis*, *C. hawkesii*, *C. militaris*, *C. ophioglossoides*, *C. shanxiensis*, *C. sobolifera*, etc.) or some plant root and stem products; therefore, accurate *C. sinensis* teleomorph identification is very important (Chen et al., 2009).

Fungus identification is traditionally via morphological observation. The fungus holotype (K. H0221) kept in the Herbarium of the Royal Botanic Gardens, Kew, was labelled as *Sphaeria sinensis* Berk., Stroma, China. Morphological characteristics according to holotype were described as 'the stroma single, cylindrical 1.7 - 2.2 cm, 2.4 - 4 mm diameter, with sterile acuminate apex. Ascogenous portion glabrous or punctate with ostioles of the perithecia. Stipes 2-2.6 cm, 2-2.6 mm diameter with silky surface. Perithecia globoid or vase-shaped, 150-380 (-550) × 110-240 µm. The cortex usually consisting of one layer of closely interwoven hyphae, pseudoparenchymatous 80-170-µm thick. Asci cylindrical, narrowing below, 160-240 (-400) × 5.2-6.5 (-12) µm with a hemispherical thickening of the wall at the apex, asci not fully mature and hence thinner. Ascospores hyaline, filiform, smooth, mutiseptate, 120-190 × 0.6-1.3 µm, never breaking up into secondary spores; faintly tapered to both ends' (Zang & Kinjo, 1996).

All of these characteristics should be observed using mature and complete tissues (stroma, ascus, ascospore, etc.), but most *C. sinensis* teleomorphs are collected before maturation and sold as desiccated specimens. Nothing about the powdered products has been said. Therefore, it is important that molecular methods to accurately identify *C. sinensis* be developed.

1.4 *C. sinensis* anamorph

Due to the serious decline of teleomorph resources, cultured *C. sinensis* anamorph mycelia have been used as a substitute. However, anamorph culture did not produce a uniform mycelium, and several species have subsequently been reported as possible *C. sinensis* anamorphs, including *Stachybotrys* sp. (Kobayasi, 1982), *Paecilomyces sinensis* (Chen et al., 1984), *Scytalidium hepiali* (Li & Sun, 1988), *Tolypocladium sinensis* (Li, 1988), *Chrysosporium sinensis* (Liang, 1991a), *Hirsutella sinensis* (Liu et al., 1989), *Synnematium sinensis* (Yin & Shen, 1990), *Cephalosporium* sp. (Shen, 1983), *Paecilomyces hepiali*, *Mortierella hepiali*, and *Scytalidium hepiali* (Chen et al., 2009). Many mycelia products made by these species are marketed as '*C. sinensis* mycelia products', which confuses consumers.

H. sinensis has been confirmed as a *C. sinensis* anamorph based on microcyclic conidiation (short life cycle) observation (Liang, 1991b; Liu et al., 2001). However, the short life cycle is difficult to observe, and its use is very limited for other species. Similar problems have been encountered with regard to the morphological identification of the desiccated and powdered mycelia products. Thus, development of molecular biological techniques is important in order to extract and analyse fungal DNA even from dead fungi and to infer simple, rapid, and reliable anamorph-teleomorph connections (Egger & Sigler, 1992).

1.5 *C. sinensis* identification using molecular biology methods

Although *C. sinensis* has different phenotypes during its life cycle, its genotype during the different stages is unique. Genetic analyses of *C. sinensis* examined the patterns of genetic variability exhibited by randomly amplified polymorphic DNA (RAPD) markers and nuclear ribosomal DNA (nrDNA) sequence diversity. However, most DNA-based studies have examined genetic differentiation at the population rather than the species level (Buenz et al., 2005).

RAPD-polymerase chain reaction (RAPD-PCR) techniques have been used to study the relationship between *H. sinensis* and *C. sinensis* (Chen et al., 1999; Li et al., 2000) and those among the geographical populations of *C. sinensis* (Chen et al., 1999; Chen et al., 1997a). Twenty-nine *C. sinensis* samples were divided into 3 clusters, i.e. the north population (NP),

middle population (MP), and south population (SP) and were considered as different subspecies rather than as different species (Chen et al., 1999; Chen et al., 1997a). The unstable RAPD patterns and large time commitment restricted the application of RAPD-PCR.

Using phylogenetic trees and probes based on ITS-region nrDNA sequences (ITS 1, 5.8S, and ITS 2 nrDNA sequences), Chen et al. (2001b) designed 2 *C. sinensis*-specific probes for species-level identification. A PCR single-stranded conformation polymorphism (PCR-SSCP)-based method was developed in Taiwan to identify *C. sinensis* and its fermented products (Kuo et al., 2006; Kuo et al., 2005).

Kinjo and Zang (2001) suggest that the 17 collections of *C. sinensis* isolates from 11 southwestern localities in China could be divided into 2 subgroups based on their ITS region sequences. Stensrud et al. (2007) analysed ITS-region nrDNA variations among 71 sequences of *C. sinensis* made available by the EMBL/GenBank databases. These authors suggested that *C. sinensis* isolates can be divided into 3 sub-species groups; however, the 2 *C. sinensis*-specific probes (Chen et al., 2001b), as well as the PCR-SSCP-based method (Kuo et al., 2006; Kuo et al., 2005), could only detect group 2 of Kinjo and Zang (2001) and group A of Stensrud et al. (2007).

The objective of this study was to develop an innovative and direct method that can detect all 3 subspecies groups of *C. sinensis* teleomorph, anamorph, and its derivative products.

2. Materials and methods

2.1 Fungal specimens and strains

The specimens and strains used in this study are listed in Table 1. The specimens were washed with sterile water and divided into 3 parts: the stroma (fruiting body), upper part of the sclerotium (head, h), and lower part of the sclerotium (tail, t). Some of the smaller sclerotia (body, b) were used for subsequent experiments.

The strains were cultured in 250 mL of potato dextrose broth (PDB; DIFCO, Detroit, MI, USA) in 500-mL flasks and agitated at 100 rpm at 14 °C. The mycelia were harvested after 8 weeks and washed with sterile water. All the specimens and mycelia were then lyophilized and stored at -20°C for subsequent analysis.

2.2 DNA preparation

DNA was isolated as described by Moncalvo et al. (1995). In brief, the ground sample (60 mg) was transferred to a 1.5-mL microcentrifuge tube containing 600 µL of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% SDS, and 1% 2-mercaptoethanol; pH 7.2). The tube was incubated in a water bath at 65 °C for 1 h, and the aqueous phase was then extracted twice using 600 µL of PCI (phenol:chloroform:isoamyl alcohol = 25:24:1; Sigma Co., St. Louis, MO, USA). After extraction, the aqueous phase was transferred to a new tube and the precipitated DNA was mixed with 0.1 volumes of 3 M sodium acetate and 0.6 volumes of isopropanol. The DNA was pelleted by centrifugation at $15,000 \times g$ for 5 min, washed twice with cold 70% ethanol, and dried for 30 min in a vacuum oven at 37 °C.

The DNA was resuspended in 100 µL of TE buffer (10 mM Tris-HCl and 1 mM EDTA; pH 8.0) containing 2 µL of RNase (500 µg/mL; Roche Applied Science Co., Mannheim, Germany) and incubated in a water bath at 37 °C for 1 h. After addition of 100 µL of chloroform, the aqueous phase was transferred directly into a new tube. DNA was

precipitated with 0.1 volumes of 3 M sodium acetate and 0.6 volumes of isopropanol and then pelleted by centrifugation at $15,000 \times g$ for 5 min. The DNA was resuspended in 100 μL of sterile water and stored at -20°C .

2.3 PCR amplification

Partial 18S nrDNA were amplified using the primer pairs NS5 (5'-AACTTAAAGGAATTGACGGAAG-3')/NS6 (5'-GCATCACAGACCTGTTATTGCCTC-3'). The 18S (partial), ITS 1, 5.8S, ITS 2, and 28S (partial) regions were amplified using the following primer pairs (Fig. 3) (White et al., 1990):

ITS1 (5'-TCCGTAGGTGAACCTGCGG-3')/ITS4, (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3')/ITS4

The amplifications were carried out in a Perkin-Elmer Thermocycler 480 (Applied Biosystems, Foster City, CA, USA) in 25 μL of the reaction mixture containing 50 ng template DNA, 2.5 μL of 10 \times PCR buffer (ProTech Professional Technical Services, Inc., Pittsburgh, PA, USA), 0.2 mM of each dNTP, 0.5 μM of each primer, and 0.625 U of Super Taq DNA polymerase (ProTech Professional Technical Services, Inc.).

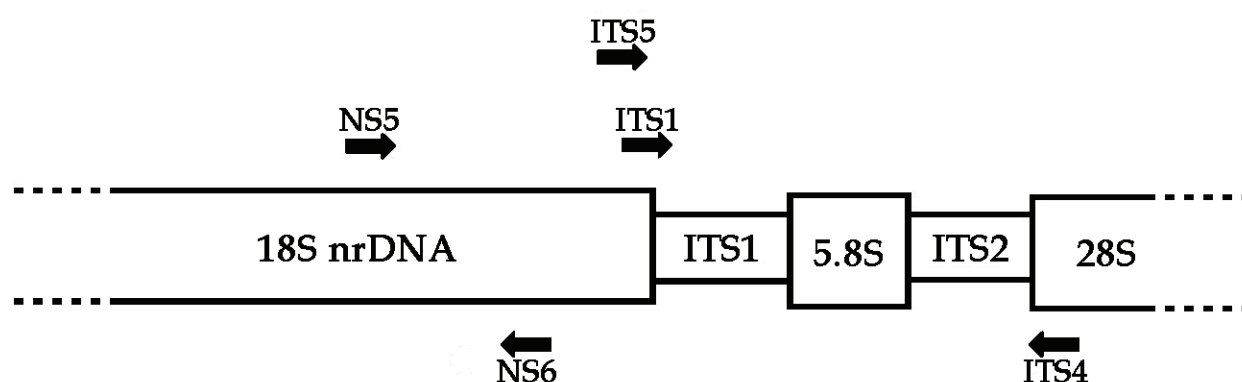


Fig. 3. PCR primer locations on the nrDNA. The arrowheads represent the 3' end of each primer (White et al., 1990)

Amplifications were performed using the following PCR program: initial denaturation at 96°C for 2 min followed by 35 cycles at 96°C for 45 s, 52°C for 45 s, 72°C for 2 min, and a final extension step at 72°C for 10 min. A negative control (dsH_2O) was included. Gel electrophoresis of the PCR products was performed on a 1.2% agarose gel. The gels were photographed under a UV transilluminator.

2.4 DNA sequencing and analyses

The PCR-amplified products were sequenced by the Mission Biotech Company (Taipei, Taiwan) (Chen & Hseu, 2002). The sequences were analyzed by an autosequencer (Applied Biosystems) using a Terminator Cyclor Sequencing Ready Reaction Kit (Applied Biosystems). The sequences were imported into the BioEdit Sequence Alignment Editor version 7.0.9.0 (Hall, 1999) and aligned using the CLUSTAL W (Thompson et al., 1994) option.

2.5 Restriction fragment length polymorphisms (RFLPs)

For RFLP analysis, 20 μL of the PCR products was digested with 1 μL each of the restriction enzyme *Cfo*I (GCG↓C; 10 U/ μL ; Roche Applied Science Co.) and *Rsa*I (GT↓AC; 10 U/ μL ;

Roche Applied Science Co.), 5 µL of 10× SuRE/Cut buffer L (Roche Applied Science Co.), and distilled water to a final volume of 50 µL. Tubes were incubated at 37 °C for at least 4 h before separation on a 2.0% agarose gel and visualization by staining in ethidium bromide and UV transillumination (Chen & Hseu, 1999).

Species	Isolate No.	Tissue	Locality/Source	Collection date	ITS	18S
<i>Cordyceps sinensis</i>	Cs824a	Stroma	Sichuan, China	Oct. 1995	AJ488272	-
		Sclerotium (body)	Sichuan, China	Oct. 1995	-	AJ274527
	Cs824b	Sclerotium (body)	Sichuan, China	Oct. 1995	AJ488267	AJ274528
	W1023	Stroma	Sichuan, China	Sep. 1996	AJ488260	AJ274531
		Sclerotium (tail)	Sichuan, China	Sep. 1996	AJ488259	-
	Cs1014C	Sclerotium (body)	Tibet, China	May 1997	AJ488268	-
	Cs7528A	Stroma	Tibet, China	May 1997	AJ488253	AJ007567
		Sclerotium	Tibet, China	May 1997	AJ488252	AJ007566
	Cs7528J	Stroma	China	May 1997	AJ488278	AJ007568
		Sclerotium (tail)	China	May 1997	AJ488239	AJ007569
	Cs7528H	Stroma	China	May 1997	AJ488245	-
		Sclerotium (head)	China	May 1997	AJ488251	-
		Sclerotium (tail)	China	May 1997	AJ488250	AJ274529
	RSCsI	Stroma	-	May 1997	AJ488236	-
		Sclerotium (head)	-	May 1997	AJ488237	-
		Sclerotium (tail)	-	May 1997	AJ488238	-
	RSCsK	Stroma	-	May 1997	AJ488249	-
		Sclerotium (head)	-	May 1997	AJ488248	-
		Sclerotium (tail)	-	May 1997	AJ488247	-
	RSCsL	Stroma	-	May 1997	AJ488240	-
		Sclerotium (head)	-	May 1997	AJ488246	-
		Sclerotium (tail)	-	May 1997	AJ488241	-
	RSCsM	Stroma	-	May 1997	AJ488242	-

Species	Isolate No.	Tissue	Locality/Source	Collection date	ITS	18S
<i>Hirsutella sinensis</i>	Csd	Sclerotium (head)	-	May 1997	AJ488243	-
		Sclerotium (tail)	-	May 1997	AJ488244	-
		Stroma	-	May 1997	AJ488269	-
		Sclerotium (body)	-	May 1997	AJ488270	-
		Stroma	-	May 1997	AJ488271	-
	S1023	Stroma	Tibet, China	Oct. 1998	AJ488257	AJ274534
		Sclerotium (head)	Tibet, China	Oct. 1998	AJ488256	-
		Sclerotium (tail)	Tibet, China	Oct. 1998	AJ488258	-
	T1023	Stroma	Qinghai, China	Oct. 1998	AJ488263	AJ274532
		Sclerotium (head)	Qinghai, China	Oct. 1998	AJ488261	-
		Sclerotium (tail)	Qinghai, China	Oct. 1998	AJ488262	-
	H1023	Stroma	Qinghai, China	Oct. 1998	AJ488254	AJ274535
		Sclerotium (tail)	Qinghai, China	Oct. 1998	AJ488255	-
	K1023	Stroma	-	Oct. 1998	AJ488264	AJ274536
		Sclerotium (head)	-	Oct. 1998	AJ488265	-
		Sclerotium (tail)	-	Oct. 1998	AJ488266	-
	RS2	Mycelium	Tibet, China	May 1997	AJ488275	AJ274540
	RS3	Mycelium	Tibet, China	May 1997	AJ488274	AJ274537
<i>C. liangshanensis</i>	CC1014a	Stroma	China	-	-	AJ239070
<i>Cordyceps</i> sp.	ATCC 36337	Mycelium	ATCC	-	-	AJ239076
<i>C. memorabilis</i>	ATCC 36743	Mycelium	ATCC	-	-	AJ242432
<i>C. militaris</i>	ATCC 26848	Mycelium	ATCC	-	-	AJ239074
<i>C. ophioglossoides</i>	CMC2	Stroma	China	-	AJ488277	-
	ATCC 36865	Mycelium	ATCC	-	-	AJ239075

Table 1. Examined species and isolates and their GenBank nrDNA sequences

3. Results

3.1 PCR amplification and DNA sequencing

We amplified and sequenced the NS5/NS6 region and ITS region nrDNA sequences of the *Cordyceps* species collected in our laboratory. Their GenBank Accession Numbers (<http://www.ncbi.nlm.nih.gov/>) are provided in Table 1. The ITS region yielded products of approximately 550 bp, while those of the NS5/NS6 region were approximately 310 bp.

In the present study, the 18S or ITS nrDNA sequences of the stroma and sclerotium (head, tail, and whole body) from each isolate were identical [Cs7528A(b) vs. Cs7528A(f); W1023(f) vs. W1023(t); Cs824a(f) vs. Cs824a(b); Cs7528H(f) vs. Cs7528H(h) vs. Cs7528H(t); Cs7528J(f) vs. Cs7528J(t); K1023(f) vs. K1023(h) vs. K1023(t); H1023(f) vs. H1023(t); T1023(f) vs. T1023(h) vs. T1023(t); S1023(f) vs. S1023(h) vs. S1023(t); Csd(f) vs. Csd(b); RSCsM(f) vs. RSCsM(h) vs. RSCsM(t); RSCsL(f) vs. RSCsL(h) vs. RSCsL(t); RSCsK(f) vs. RSCsK(h) vs. RSCsK(t); and RSCsI(f) vs. RSCsI(h) vs. RSCsI(t)] (Table 1). This result suggested that the stroma and sclerotium from each isolate originated from the same species.

3.2 Phylogenetic analysis

The phylogenetic tree based on the ITS region sequences is illustrated in Fig. 4. It was constructed using the sequencing data collected in this study as well as from GenBank. The *C. memorabilis* strain ATCC 36743 was set as the out-group. The 3 *C. militaris* sequences – GenBank Accession Nos. AJ242923, AJ243774, and CMC2 – were grouped.

The *C. sinensis* specimens could be divided into 5 subgroups. Group A included the following isolates: (1) Tibet isolates Cs824d(f), K1023(f), RS2, Csd(b), and Cs7528A(f) as well as those with GenBank Accession Nos. AJ243775, AJ243776, AJ243778, AJ243979, AJ243980, AJ245559, AJ413178, AJ413179, AJ413180, AJ507399, AJ507400, AJ507401, AJ507402, and AB067713; (2) Sichuan isolates Cs824a(f) and Cs824b(b) as well as those with GenBank Accession Nos. AJ413183, AJ413184, AB067715, and AB067717; (3) Qinghai isolates T1023(t) and those with GenBank Accession Nos. AJ413181, AJ413182, and AJ507404; (4) Yunnan isolates with GenBank Accession Nos. AJ309354, AJ309361, and AJ507403; (5) Gansu isolates with GenBank Accession Nos. AJ507405 and AB067716; and (6) uncertain locality isolates with GenBank Accession Nos. AB067718, AB067721, and AJ309357. This group is similar to subgroup 2 of Kinjo and Zang (2001), and the bootstrap level was 994.

Group B included the following isolates: (1) Tibet isolates with GenBank Accession Nos. AB067739 (isolate Nyaramu-1), AB067740 (isolate Nyaramu-2), AB067743 (isolate Yagyusan), and AB067744 (isolate Satoku); (2) Sichuan isolates with GenBank Accession Nos. AB067742 (isolate Ritan), AB067745 (isolate Kandin Clone A1), AB067746 (isolate Kandin Clone A2), AB067747 (isolate Kandin Clone A4), AB067748 (isolate Kandin Clone B3), and AB067749 (isolate Kandin Clone B6); and (3) Qinghai isolate with GenBank Accession No. AB067741 (isolate Rusyasya). This group is close to subgroup 1 of Kinjo and Zang (2001), and the bootstrap level was 1000.

Group C, which we discovered earlier (Chen et al., 2004), included the following: (1) Tibet isolate Cs1014C(b) and (2) Sichuan isolate W1023(f). The bootstrap level of Group C was 1000. Although this group was remote from *C. sinensis* Groups A and B, the 2 isolates were identified to be *C. sinensis* based on morphological observations.

Group D included the following isolates/strains: GenBank Accession Nos. AF122030 (strain BCRC 36421), AF291749 (strain MPNU 8002), and AB067720 (isolate SHANGHAI). The bootstrap level of Group D was 1000. Although these isolates were scientifically named *C.*

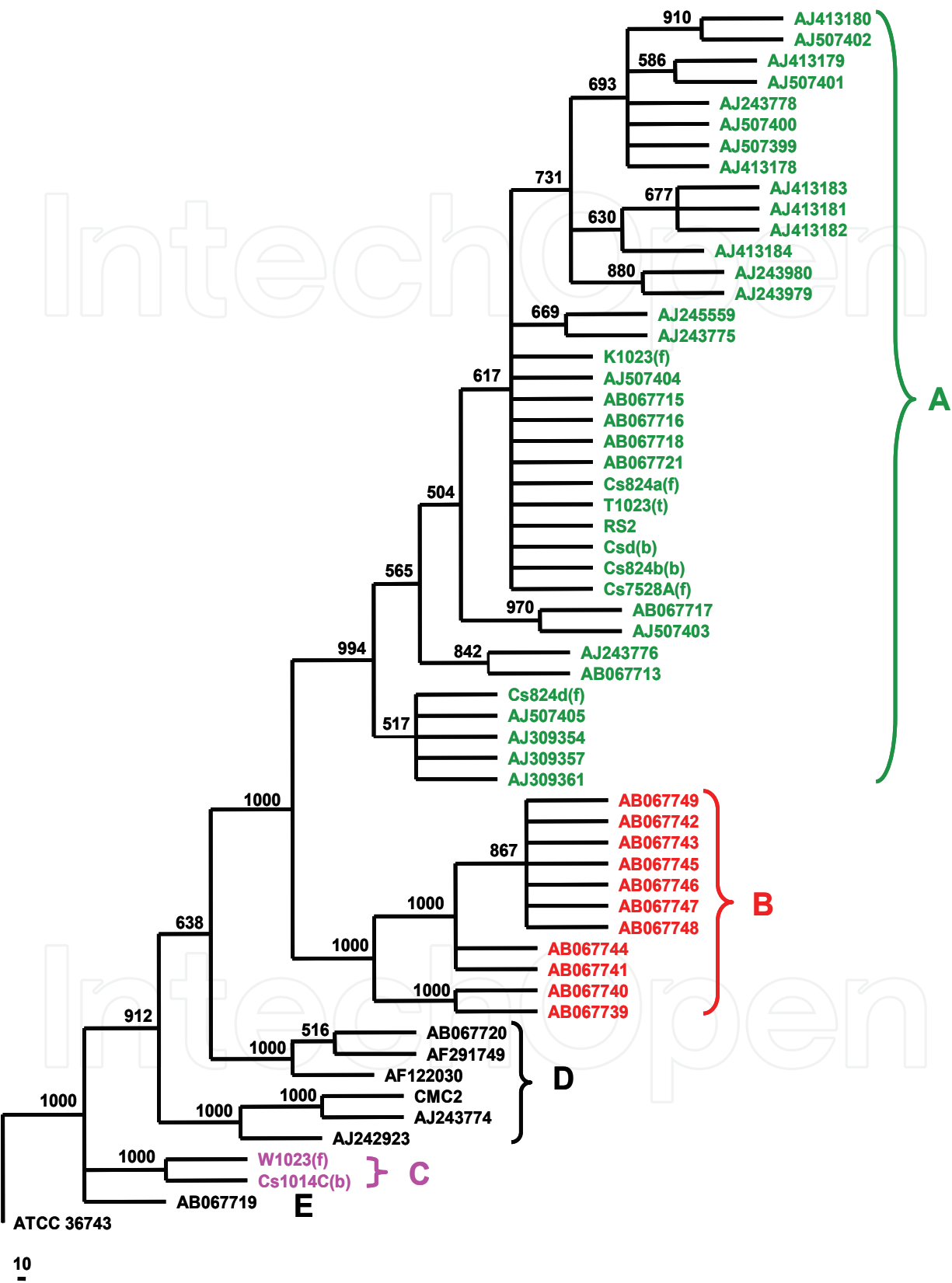


Fig. 4. Phylogenetic tree resulting from the Neighbour-Joining Method (NJ) of the nrDNA ITS1, 5.8S, and ITS2 region sequences of *Cordyceps sinensis* and other related fungi. NJ bootstrap percentage values are shown at each branch

sinensis, the 3 strains shall not be considered *C. sinensis*. They might belong to the anamorphic species *Tolypocladium*, a relative of *C. subsessilis* (Hodge et al., 1996). Another possibility was that the 3 strains might be contaminants or associated fungi of *C. sinensis* because many contaminant anamorphic fungi were associated with *Cordyceps* species (Kinjo & Zang, 2001).

Group E included only 1 isolate that had the GenBank Accession No. AB067719 (isolate SANMEI). It was not accurately represented in the GenBank format by Kinjo. The isolate was not considered a *C. sinensis* anamorph strain because of the large identity difference in its 18S nrDNA sequences and those of *C. sinensis* specimens (discussed below).

According to the above-mentioned findings, Groups A, B, and C were considered the real *C. sinensis*. Group A included *C. sinensis* isolates from 5 sources (Tibet, Sichuan, Qinghai, Yunnan, and Gansu); Group B, 3 sources (Tibet, Sichuan, and Qinghai); and Group C, 2 sources (Tibet and Sichuan). This indicated that the intra-group isolates from different geographic regions were identical subspecies. However, the inter-group isolates were different subspecies. The identity between Groups A and B was 86.0%–89.4% as compared to 63.2%–66.0% between Groups A and C and 56.6%–57.8% between Groups B and C. Only Group A members could be detected using the 2 probes developed by Chen et al. (2001a).

The ITS region and NS5/NS6 region sequences of *C. sinensis* in the present study and GenBank (Table 1) were aligned and analysed. The identity between each ITS region sequence was 56.6%–100%; however, the NS5/NS6 region sequences of the isolates were almost completely identical. Therefore, the NS5/NS6 region sequence of *C. sinensis* was compared to those of other *Cordyceps* spp. to determine the diversity therein.

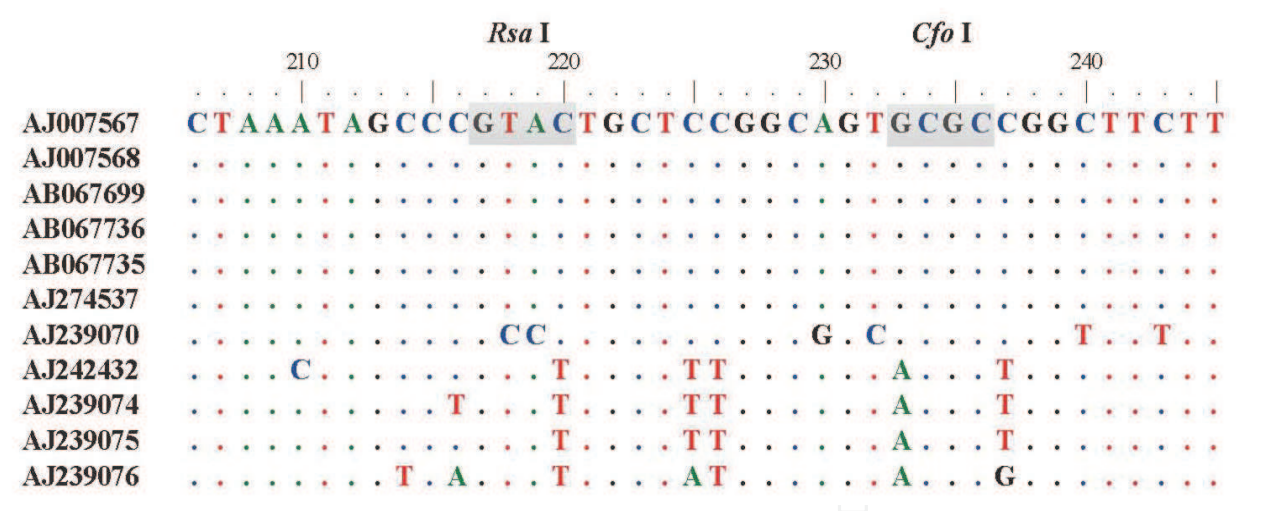


Fig. 5. Sequence alignment of 18S nrDNA NS5/NS6 region sequences of *C. sinensis* Cs7528A(f) (Accession No. AJ007567), *C. sinensis* Cs7528J(f) (Accession No. AJ007568), *C. sinensis* MARCH (Accession No. AB067699), *C. sinensis* Kandin (Accession No. AB067736), *C. sinensis* Nyaramu-1 (Accession No. AB067735), *H. sinensis* (Accession No. AJ274537), other *C. liangshanensis* CC1014a (Accession No. AJ239070), *C. memorabilis* ATCC 36743 (Accession No. AJ242432), *C. militaris* ATCC 26848 (Accession No. AJ239074), *C. ophioglossoides* ATCC 36865 (Accession No. AJ239075), and *Cordyceps* sp. ATCC 36337 (Accession No. AJ239076) in the present study. The shades are the restriction sites of *Cfo*I (GCG↓C) and *Rsa*I (GT↓AC)

Obviously, *C. sinensis* had 2 restriction sites—*Cfo*I and *Rsa*I (the signature sequence)—that could be used to differentiate *C. sinensis* from other *Cordyceps* spp. (Fig. 5). To ensure specificity of the signature sequence, the GenBank database was searched using the search phrase '*Cordyceps* 18S rRNA gene'. Simultaneously, the NS5/NS6 region sequence was uploaded to GenBank and the database was searched for the sequences that were mostly closely related using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al., 1990). None of the sequences contained both restriction sites (data not shown); thus, *C. sinensis* could be definitively identified based on the 2 restriction sites. The PCR-RFLP method based on the signature sequence was developed and used to characterize *C. sinensis* fermented products in Taiwan (Table 3).

Marketing Sample ^a	Sample Origin ^b	Collection Date
<i>C. sinensis</i> mycelium	A	June 2004
<i>C. sinensis</i> mycelium	AV	June 2004
<i>C. sinensis</i> mycelium	B	June 2004
<i>C. sinensis</i> mycelium	C	June 2004
<i>C. sinensis</i> and <i>Ganoderma lucidum</i> mycelia	DP	June 2004
<i>C. sinensis</i> mycelium	G1	June 2004
<i>C. sinensis</i> mycelium	Gen	June 2004
<i>C. sinensis</i> mycelium	L	June 2004
<i>C. sinensis</i> mycelium	NT	June 2004
<i>C. sinensis</i> mycelium	P	June 2004
<i>C. sinensis</i> mycelium	PH	June 2004
<i>C. sinensis</i> mycelium	4B	June 2004

^aThe marketing sample content descriptions were provided by the respective suppliers.

^bFull company names related to the marketing samples are not shown here.

Table 3. *Cordyceps sinensis* mycelium fermented products collected in the present study

Among the 12 fermented products, only AV and L matched the signature sequence and were, therefore, considered genuine *C. sinensis* mycelium products (Fig. 6). Another 8 products—A, B, C, G1, Gen, NT, PH, and 4B, none of which were digested by *Cfo*I and *Rsa*I—were considered fake products. The product DP, whose content was labeled *C. sinensis* and *Ganoderma lucidum* mycelia, had 2 corresponding polymorphism patterns; however, the product P, whose content was labeled only as *C. sinensis* mycelium, must not exclusively comprise pure *C. sinensis* mycelium.

4. Discussion

ITS-region nrDNA, which consists of the 2 variable non-coding regions ITS 1 and ITS 2, was more broadly used in phylogenetic analysis than 18S nrDNA. However, the 2 *C. sinensis*-

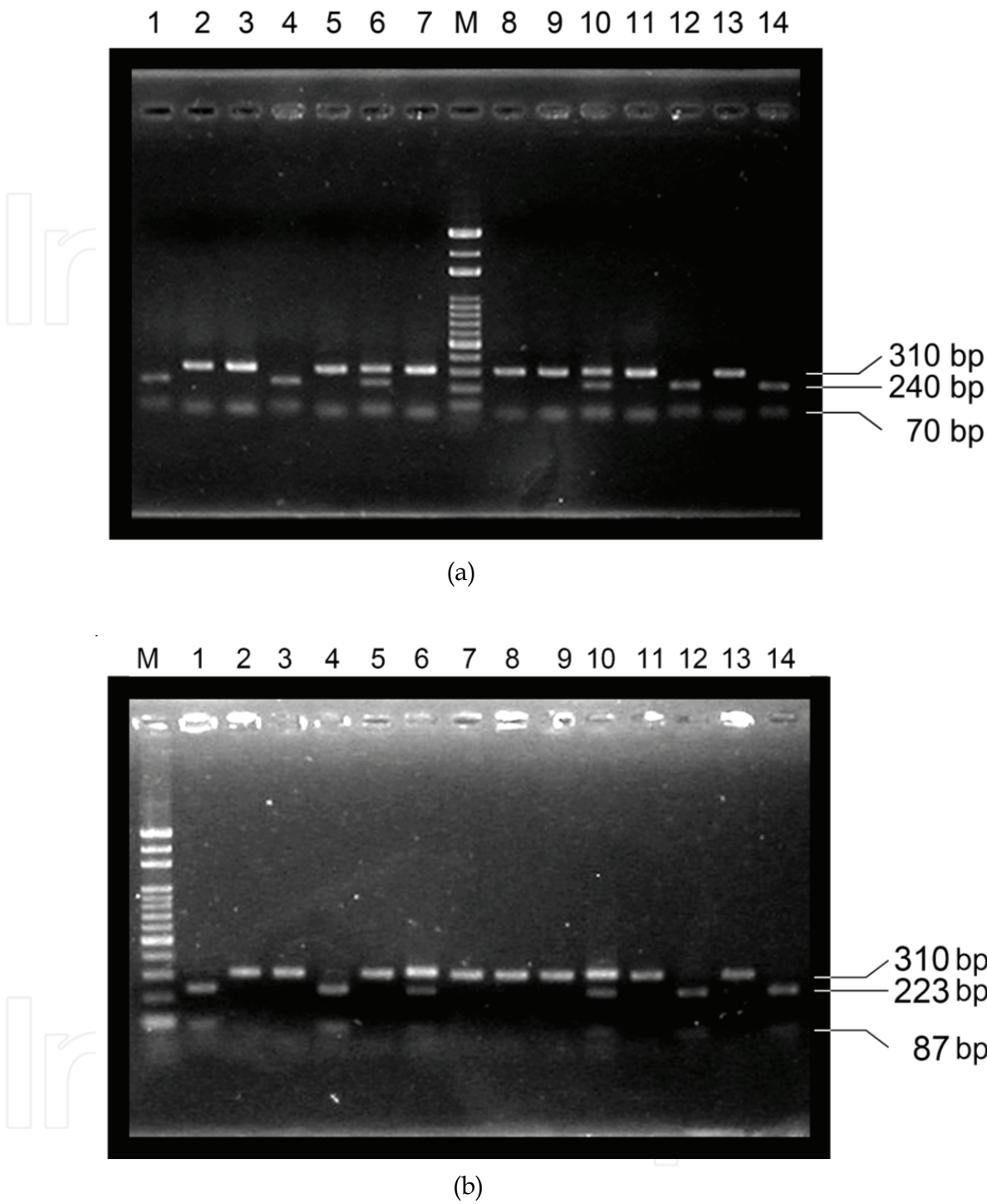


Fig. 6. Restriction maps of the 18S nrDNA PCR products of *C. sinensis* and various mycelium fermented products. Amplification of 18S nrDNA by PCR using primer pair NS5/NS6. The products were digested using restriction enzyme (A) *CfoI* (GCG↓C) and (B) *RsaI* (GT↓AC) and then visualized in 2.0% agarose gel stained with ethidium bromide. Lane M, 100-bp DNA marker; lane 1, *C. sinensis* Cs7528A; lane 2, product A; lane 3, product B; lane 4, product AV; lane 5, product C; lane 6, product DP; lane 7, product G1; lane 8, product Gen; lane 9, product NT; lane 10, product P; lane 11, product PH; lane 12, product L; lane 13, product 4B; and lane 14, *C. sinensis* Cs7528J

specific probes (Chen et al., 2001b) and the PCR-SSCP method (Kuo et al., 2006; Kuo et al., 2005) based on the ITS region nrDNA sequence could detect only group A in Fig. 4. Thus, the 18S nrDNA was used in the present study instead of the ITS region to develop a method by which to differentiate *C. sinensis*.

The signature sequence based on the *Cfo*I and *Rsa*I restriction sites of the NS5/NS6 region was an innovative and species-level genetic marker of *C. sinensis*. It could be broadly used to determine *C. sinensis* teleomorph, anamorph, and the identification and differentiation of derivative products. In addition, the *C. sinensis* was further divided into 3 intra-species groups, based on the ITS-region sequences. These molecular systematic indicators could serve as the foundation for further research and applications.

Many teleomorph and anamorph fermented products in the market do not comprise *C. sinensis* (i.e. fake products), and some of them are not made exclusively of pure *C. sinensis* (Fig. 6). They might be made of or mixed with other *Cordyceps* spp. – *C. sinensis*-related anamorph strains, plant powders, or other materials. Although many of these ingredients were reported as having various biological functions, the fake or impure products, labelled as '*C. sinensis* mycelium products', might cause serious problems with regards to food safety. Thus, the signature sequence will be a powerful tool in assaying *C. sinensis* fermented products and performing quality control measures.

On the basis of morphological observation and signature sequence confirmation, strain RS3 was identified as *C. sinensis* anamorph, i.e. *H. sinensis*. According to the ITS-region nrDNA sequence, it belongs to group A of Fig. 4. However, the genomic statuses of the anamorph strains of groups B and C remain unclear. Further work should be undertaken to collect live *C. sinensis* specimens for group B and group C anamorph strain isolation and for research into the morphological characteristics and medicinal effects of each group isolate.

5. Conclusion

In the present study, we successfully developed a molecular method that can detect all 3 sub-species groups of *C. sinensis*. This innovative method can be applied to *C. sinensis* teleomorph and anamorph identification and can be used to improve quality control.

6. Acknowledgment

This study was supported by a grant (Grant No. DOH-91-TD-1163) from the Department of Health, Executive Yuan, Taiwan, Republic of China.

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Quality Control of Herbal Medicines and Related Areas

Edited by Prof. Yukihiro Shoyama

ISBN 978-953-307-682-9

Hard cover, 282 pages

Publisher InTech

Published online 07, July, 2011

Published in print edition July, 2011

The authors of this thematic issue provide a comprehensive summary of most recent knowledge and references on quality control in wide fields. Quality control is essential for natural products like natural medicine and related food products. In this issue fifteen chapters have been included, discussing in detail various aspects of quality control. It will certainly prove useful not only for phytochemical researchers, but also many scientists working in numerous fields. Much effort has been invested by the contributors to share current information. Without their efforts and input 'Quality Control of Herbal Medicine and Related Areas' could not exist.

How to reference

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Chih-Sheng Chen, Ruey-Shyang Hseu and Ching-Tsan Huang (2011). Quality Control of Cordyceps sinensis Teleomorph, Anamorph, and Its Products, Quality Control of Herbal Medicines and Related Areas, Prof. Yukihiro Shoyama (Ed.), ISBN: 978-953-307-682-9, InTech, Available from:
<http://www.intechopen.com/books/quality-control-of-herbal-medicines-and-related-areas/quality-control-of-cordyceps-sinensis-teleomorph-anamorph-and-its-products>

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