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Identification of Chinese Medicinal Fungus *Cordyceps sinensis* by PCR-Single-Stranded Conformation Polymorphism and Phylogenetic Relationship

HSIAO-CHE KUO,[†,‡] YONG-LIN SU,[†] HUEY-LANG YANG,[†,‡,§] AND TZONG-YUEH CHEN*,[†,‡,§]

Institute of Biotechnology, Agriculture Biotechnology Research Center, and Center for Bioscience and Biotechnology, National Cheng Kung University, Tainan 701, Taiwan

Fungi belonging to the *Cordyceps* species have long been used as food and herbal medicines in Asia and are especially popular as commercially available powdered supplements. Despite this acceptance and use, little is known of the phylogenetic relationships of the genus. Presently, the neighbor-joining method based on the ITS1, 5.8S rRNA, and ITS2 regions was used to construct a phylogenetic tree of 17 *Cordyceps* isolates. Five major groups were evident. *Cordyceps sinensis* was less closely related to 15 *Cordyceps* species but shared a closer relationship with *Cordyceps agriota.* PCR-single-stranded conformational polymorphism was applied to differentiate seven *Cordyceps* isolates: five were different from those used to construct the phylogenetic tree, based on differences in the internal spacer 2 (ITS2). The length of ITS2, amplified by primers 5.8SR and ITS4, vary between 334 and 400 bp. This segment could be used for intraspecies classification or detection of mutations and represents potential novel means of identification of this fungal genus in herbal medicines and in quality control applications in the fermentation industry.

KEYWORDS: ***Cordyceps*; identification; PCR−SSCP; phylogenetic analysis**

INTRODUCTION

The ascomycetous genus *Cordyceps* is an entomopathogenic fungus. Many fungi belonging to *Cordyceps* have long been used as food and herbal medicines in Asia (*1*, *2*). Pharmacologically, the immunomodulation and antioxidant activities of *Cordyceps cicadae* (*C. cicadae*) (*2*) have been exploited in addressing hyperglycemia, respiratory disease, liver disease, and renal dysfunction. Both *C. cicadae* and *C. ophioglossoides* exhibit antitumor activity (*2*, *3*). *C. sinensis* possesses a polysaccharide that protects against free-radical-induced neuronal cell toxicity (*4*). Finally, *C. pruinosa* has been used to treat stomach disorders, inflammatory disease, and endotoxin shock or sepsis (*5*). Approximately 400 species of *Cordyceps* are known. They are distinguished from one another and classified according to the color and shape of their fruiting bodies (*6*, *7*), possession of spores, ascus shape, and host insect species (*7*) and by other morphological characteristics (*8*).

In the past few years, molecular biology techniques have been applied to classify fungal species (*9*). In particular, methods that utilize the polymerase chain reaction (PCR) have significantly increased the sophistication of fungal systematic investigation. The simplicity and speed of PCR techniques, coupled with the use of particular regions of the fungal genome, has increased the understanding of fungal taxonomic groupings, evolutionary relationships, and functional properties. PCR-based techniques developed from the characteristics of DNA encoding for ribosomal RNA (rDNA) and PCR-based DNA fingerprinting have in particular been successful at discriminating fungi at the inter- and intraspecific levels (*10*, *11*).

Sequence differences can be demonstrated by direct sequencing or by PCR-based single-stranded conformational polymorphism (PCR−SSCP) (*12*). In the latter, nucleotide differences between homologous sequence strands are detected by electrophoresis of single-stranded DNA under nondenaturing conditions (*12*). SSCP analyses have been used for viroid characterization (*13*), elucidating DNA sequence variation (*14*), detecting genetic mutations in humans (*15*, *16*) classification of fish species (*17*), and the identification of bacteria, spirochetes, and fungi (*18*−*21*). A comparative study of spirochete identification involving restriction fragment length polymorphism (RFLP) and PCR−SSCP (*22*) found the latter technique to be a better tool for rapid identification for spirochetes at the intraspecific level, particularly when faced with large numbers of samples.

Whether SSU (small subunit) rDNA provides sufficient variation to separate closely related species has been questioned. We have successfully used SSU rDNA for speciation of *Cordyceps* fungi following the identification of single-stranded conformation polymorphisms of the ITS2 region by PCR−SSCP analysis (unpublished data). In the present paper, we have

* To whom correspondence should be addressed. Phone: 886-6-2757575 ext 65622. Fax: 886-6-2766505. E-mail address: ibcty@mail.ncku.edu.tw.
[†] Institute of Biotechnology.
[‡] Agriculture Biotechnology Research Center.
[§] Center for Bioscience and Biotechnology.

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Table 1. List of Fungal Isolates for Construction of the Phylogenetic Tree and PCR-Product Sequence Length and Accession Numbers

species	origin[b]	NCBI GenBan accession no.	length (bp) of ITS1, 5.8S, and ITS2 regions[c]
Cordyceps agriota	ARSEF 5692	AY245626	586
Cordycesp bifusispora	ARSEF 5690	AY245627	587
Cordyceps brongniartii	ATCC 66779	AY245628	569
Cordyceps dipterigena	CCRC 35725	AY245629	601
Cordyceps heteropoda	IFO 33060	AY245630	569
Cordyceps japonica	IFO 9647	AY245645	557
Cordyceps memorabilis	CCRC 32218	AY245632	583
Cordyceps militaris[a]	CBS 178.59	AY245634	567
Cordyceps militaris	CCRC 32219	AY245633	568
Cordyceps myrmecophila	CCRC 35726	AY245635	583
Cordyceps ochraceostromata	ARSEF 5691	AY245646	587
Cordyceps ophioglossoides	CCRC 32220	AY245636	564
Cordyceps scarabaeicola	ARSEF 5689	AY245639	572
Cordyceps sinensis	CCRC 36421	AY245638	556
Cordyceps sphingum	CBS 114.22	AY245641	588
Phytocordyceps ninchukispora	CCRC 31900	AY245642	586
Podostroma cordyceps	IFO 9019	AY245647	610

[a] This species of *Cordyceps militaris* was collected from the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. [b] ARSEF: Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, U.S. ATCC: American Type Culture Collection Center. CBS: Centraalbureau voor Schimmelcultures Utrecht, The Netherlands. CCRC: Bioresources Collection and Research Center in Taiwan. IFO: Institute for Fermentation, Osaka, Japan. [c] The DNA segment was amplified by the primers ITS1 and ITS4. For the DNA sequence; see the section of Material and Methods.

constructed a phylogenetic tree based on the ITS1, 5.8S rRNA, and ITS2 regions to understand the phylogenetic relationships between 17 isolates collected from culture collection centers. Herein, we report on the use of PCR−SSCP to differentiate between species and isolates of *Cordyceps* based on sequence differences in the gene coding for the ITS2 region in rDNA. Using this method provides a simple, rapid, and accurate means of identification for the Chinese medicinal fungus *Cordyceps*.

MATERIALS AND METHODS

Fungal Isolates for Construction of Phylogenetic Tree. Fungal materials in this study were collected from five culture collection centers around the world. The names and origins of the 17 isolates are listed in Table 1.

DNA Extraction. Fungal isolates from the culture collection centers were cultivated in PDA (potato dextrose agar: 15 g of potato, 15 g of glucose, and 20 g of agar for 1 L of water) plates and subjected to DNA extraction. The total genomic DNA extraction method was modified from the description of Lu et al., 2002 (*23*). Hyphae of fungal isolates was ground in liquid nitrogen and transferred into a 1.5 mL microfuge tube. Lyses buffer (100 mM Tris-HCl, pH 8.0, 1% mercaptoethanol, 10 mM EDTA, 2% sodium dodecyl sulfate (SDS), and 100 μg/mL proteinase K) was then added, and the mixture was placed in a water bath at 65 °C for 3 h. The solution was extracted at least twice with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1 (v/v)), then centrifuged at 13 000 rpm for 10 min, and followed by extraction with chloroform: isoamyl alcohol (24:1) until the top phase was clear. The upper phase was transferred to a new tube, and DNA was precipitated with 0.7 volume 2-propanol, washed with 70% ethanol, dried, and resuspended in 100−200 mL of doubly distilled H_2O. Genomic DNA was visualized in 0.7% agarose gel. The concentration and purity of the DNA were determined using a BioDoc-It system (Ultra-Violet Products Ltd., Cambridge, U.K.).

PCR, Cloning, and Sequencing. From the total genomic DNA, a DNA segment containing the 3′ end of the nuclear 18S rDNA, ITS1, 5.8S rDNA, and ITS2 and the 5′ end of 28S rDNA was amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (*24*). The temperature profiles for the PCR cycles were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles at 94 °C for 2 min, 50 °C for 1 min, and 72 °C for 2 min, and a final extension reaction at 72 °C for 10 min. The internal transcribed spacer 2 (ITS 2) region segments were amplified by PCR using primers 5.8SR (5′-TCGATGAAGAACG-CAGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (*24*). PCR was under the temperature profiles of 94 °C for 5 min, followed by 35 cycles at 98 °C for 1 min, 52.4 °C for 1 min, and 68 °C for 2 min, and final extension at 60 °C for 10 min. Another DNA segment comprising the partial length of 18S rDNA was amplified by PCR using primers NS7 (5′-GAGGCAATAACAGGTCTGTGATGC-3′) and NS8 (5′-TCCGCAGGTTCACCTACGGA-3′) (*24*) and followed the temperature profiles of 94 °C for 5 min, 35 cycles at 98 °C for 1 min, 50 °C for 1 min, and 68 °C for 2 min, and final extension at 60 °C for 10 min. The amplification was carried out in a T1 Thermocycler (Biometra, Inc., Goettingen, Germany) in a 50 μL reaction mixture containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each dNTP, 0.4 mM of each primer, 50 ng of template DNA, and 1.5 U *Taq* DNA polymerase (Promega Corp., Wisconsin). Amplified products were purified using a PCR-M clean up system (Viogene Biotek Corp., Illinois) and cloned into a pGEM-T easy vector system (Promega) and transformed into *Escherichia coli* strain RR1 competent cells. Plasmids containing the PCR products were isolated using the Mini-M plasmid DNA extraction system (Viogene Biotek) and then sequenced directly with a BigDye primer cycle sequencing kit (Applied Biosystems and Hitachi, Ltd., California) on a ABI PRISM 3100 genetic analyzer automated DNA sequencer (Applied Biosystems and Hitachi, Ltd.). The nucleotide sequences determined were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases (for accession numbers, see Table 1). The positions of primers, which were used for amplified the ribosomal DNA, are showed in Figure 1.

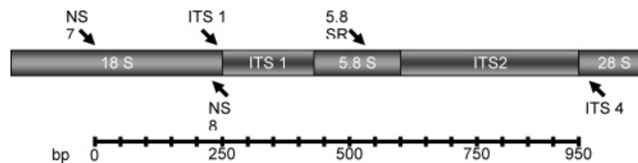

Figure 1. Primers used in this study and their positions on rDNA. Three (NS7, ITS1, and 5.8SR) forward primers and two (NS8 and ITS4) reverse primers were illustrated, and a scale of nucleotide length was drawn below.

Data Analyses. Sequences were aligned using the multiple alignment program CLUSTAL W (*25*). A neighbor-joining (NJ) tree (*26*) based on ITS regions and 5.8S rDNA sequences was constructed with Kimura's (*27*) two-parameter distance using the program PAUP*, version 4.0b2 (*28*). Heuristic tree searches were executed utilizing the tree-bisection-reconnection (TBR) branch-swapping algorithm with random sequence analysis. The bootstrap values were obtained from 1000 replications of NJ analyses. The pair distances of DNA sequences (Table 2) were calculated by the computer program CLUSTAL W (*25*) and the similarity of DNA sequences shown by percentage.

Sampling for SSCP Analyses. Fungal materials for SSCP analyses were collected from three culture collection centers around the world. The names and origins of the 7 isolates are listed in Table 3.

Screening of SSCP−PCR Products. PCR products were run on 2% agarose gels and examined for the presence of bands after ethidium bromide staining. The SSCP of PCR products were analyzed by electrophoresis with 15% MDE (mutation detection enhancement) gels (0.5× MDE gel: MDE gel, 16.0 mL; ddH_2O, 44.2 mL; 10XTBE (Tris-borate-EDTA), 3.84 mL; 10% APS (ammonium persulfate), 256 μL; TEMED (N,N,N′,N′-Tetramethyl-ethylenediamine), 25.6 μL). Gel will polymerize in about 1 h. In brief, 25 μL of the amplified product was diluted with 100 μL of buffer (0.1% sodium dodecyl sulfate; ethylenediaminetetraacetic acid (EDTA), 10 mM); 1 μL of this dilution was mixed with 9 μL of loading buffer (95% formamide, 10 mM NaOH, and 0.025% of both bromophenol blue and xylene cyanol). The mixtures were boiled (heat-denatured samples at 95 °C) for 10 min, cooled in ice for 5 min, and then loaded on the gel at 150 V for 1 h and 10 min at room temperature. Gels were run in 0.5× TBE buffer.

Table 2. Pair Distances of 17 *Cordyceps* Fungi Examined in This Study Based on ITS Regions and 5.8S RDNA Sequences[a]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. *C.–agriota*	-	80.2	81.5	75.8	81.4	78.8	81.5	83.1	82.6	78.6	80.4	75.7	87.9	81.1	80.0	82.8	80.5
2. *C. bifusispora*		–	91.7	76.5	91.4	82.0	79.2	92.9	92.8	84.4	99.1	76.2	84.4	92.1	95.7	82.5	91.5
3. *C. brongniartii*			–	77.5	97.0	82.4	84.2	93.1	92.8	90.0	91.2	76.1	85.3	97.5	93.5	85.1	91.2
4. *C. dipterigena*				–	78.4	77.7	78.6	79.4	78.9	76.2	76.1	77.3	82.4	79.0	77.0	77.4	76.8
5. *C. heteropoda*					–	82.2	82.4	91.9	91.5	91.2	90.7	75.4	85.4	94.9	93.3	84.9	91.2
6. *C. japonica*						–	82.0	80.8	80.6	92.6	80.4	77.9	83.6	81.5	81.5	82.4	80.8
7. *C. memorabilis*							–	81.8	81.5	81.3	78.6	76.4	87.2	82.0	79.1	85.2	79.2
8. *C. militaris*[b]								–	99.8	86.6	91.5	75.4	85.1	92.9	93.7	84.0	92.2
9. *C. militaris*									–	85.9	91.7	75.0	85.6	92.4	93.5	83.8	91.9
10. *C. myrmecophila*										–	83.7	77.5	84.7	87.8	85.6	81.6	84.6
11. *C. ochraceostromata*											–	75.7	84.4	92.0	95.6	82.3	91.1
12. *C. ophioglossoides*												–	76.8	74.8	75.0	76.1	74.5
13. *C. sinensis*													–	84.4	83.5	88.5	84.4
14. *C. scarabaeicola*														–	92.8	85.1	90.4
15. *C. sphingum*															–	82.0	91.8
16. *Po. cordyceps*																–	85.0
17. *Ph. ninchukispora*																	–

[a] Pair distances of 17 Cordyceps fungi calculated by the computer program ClustalW. The percent similarities are shown in the triangular data sheet. The names of the isolates are shown on the left; the numbers shown in front of the species correlate to the numbers list on the first line of this table. [b] This species of *Cordyceps militaris* was collected from the Centraalbureau voor Schimmelcultures Utrecht, The Netherlands.

Table 3. Fungal Samples Used in This Study for Examination of the SSCP Test

fungal isolate	origin[a]
Cordyceps pseudomilitaris	BCC 1386
Cordyceps roseostromata	ARSEF 4870
Cordyceps scarabaeicola	ARSEF 5689
Cordyceps sinensis	CCRC 36421
Cordyceps sp.	BCC 1519
Cordyceps sp.	CCRC 32221
Cordyceps sp.	BCC 1443

[a] ARSEF: Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, U.S. CBS: Centraalbureau voor Schimmelcultures Utrecht, The Netherlands. CCRC: Bioresources Collection and Research Center in Taiwan.

The gels were silver stained using the Pharmacia Biotech DNA silver staining kit (Amersham Biosciences Corp., New Jersey) and by following the standard staining procedure.

RESULTS

Phylogenetic Analyses Based on ITS1, 5.8S rDNA, and ITS2 Regions. The DNA fragments amplified using PCR by primers ITS1 and ITS4 vary between 556 and 610 bp in length. The fragment contained the 3′ end of 18S rDNA, ITS1, 5.8S rDNA, and ITS2 and the 5′ end of 28S rDNA. The presence and similarity of the sequence in 17 *Cordyceps* isolates was ascertained using the CLUSTAL W computer program (*25*). The similarity based on the ITS region (ITS1, 5.8S rDNA, and ITS2 regions) between these isolates varied from 75 to 99.8% (Table 2). Figure 2 displays the NJ tree constructed by comparing the sequence identities of the ITS regions in different *Cordyceps* taxa. In our experience, about one in 3000 nucleotides will mutate after PCR with *Taq* DNA polymerase, a rate that seems higher than others. To avoid introduction of mutations by *Taq* DNA polymerase, each sequence that was amplified by PCR for sequencing was obtained from seven clones of the same fungal isolate. At least five of the seven clones sequenced were the same and could be used for constructing the phylogenetic tree.

The 17 *Cordyceps* isolates could be divided into five major groups based on the phylogenetic analyses. *C. sinensis*, the traditional species used in Chinese medicinal preparations, was less closely related to the other 15 isolates, but shared a closer relationship with *C. agriota.* On the other hand, *C. japonica* and *C. myrmecophila* formed a group with a bootstrap value of 99%. *C. dipterigena* and *C. ophioglossoides* comprised a group

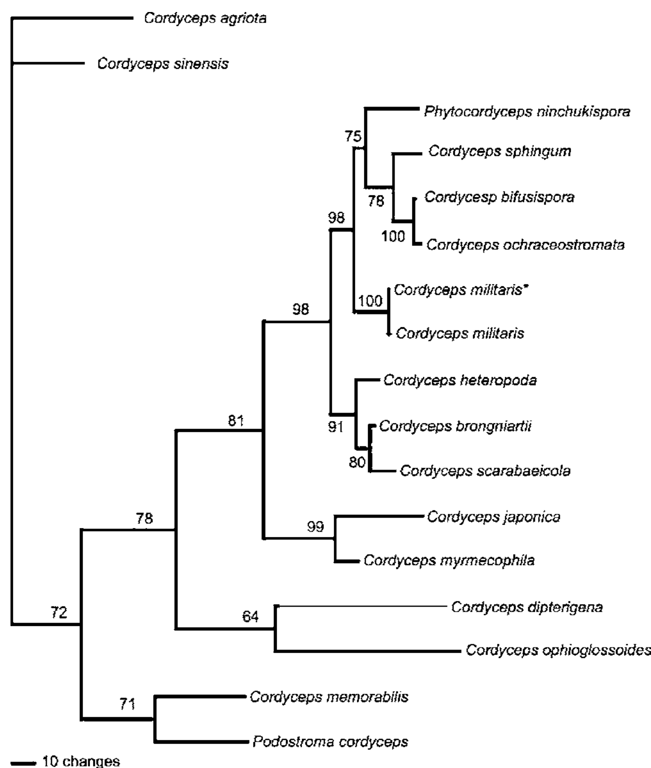

Figure 2. Phylogenetic relationship of the 17 isolates of *Cordyceps* fungi based on ITS1, 5.8S rDNA, and ITS2 regions of DNA sequences. The branch-swapping algorithm used tree-bisection-reconnection (TBR) and bootstrap method with heuristic search. Tree description: Optimality criterion = parsimony. Character-status summary: there are a total of 667 characters, 342 characters are constant; 119 variable characters are parsimony-uninformative; the number of parsimony-informative characters = 206. A total of 667 unambiguously aligned nucleotide sites were subjected to neighbor-joining (NJ) analyses. Bootstrap values were obtained with 1000 replications Bootstrap values are shown at the nodes. Tree length = 743; consistency index (CI) = 0.650; homoplasy index (HI) = 0.350; retention index (RI) = 0.587; rescaled consistency index (RC) = 0.381. Numbers on tree branches indicate the percentage of bootstrap replications derived from 1000 replications of NJ analysis and supporting the internal branches by ≥50%.

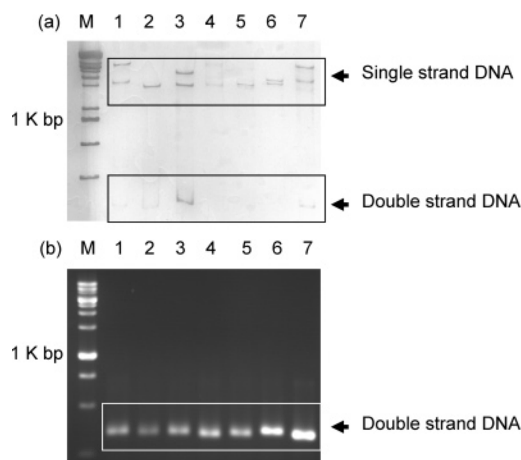

Figure 3. PCR product and PCR–SSCP analyses based on the ITS2 region of entomopathogentic fungi: (**a**) SSCP profiles on the MDE gel; (**b**) PCR products on the agarose gel. M, 1 Kb DNA ladder; lane 1, *C. pseudomilitaris*; lane 2, *C. roseostromata*; lane 3, *C. scarabaeicola*,; lane 4, *C. sinensis*; lane 5, *C. sp.*; lane 6, *C. sp.*; lane 7, *C. sp*.

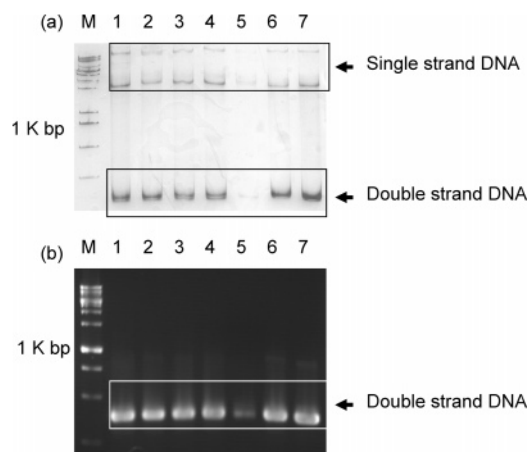

Figure 4. PCR product and PCR–SSCP analysis based on the 3′ end of the 18S rDNA region of entomopathogenic fungi: (**a**) SSCP profiles on the MDE gel; (**b**) PCR products on the agarose gel. M, 1 Kb DNA ladder; lane 1, *C. pseudomilitaris*; lane 2, *C. roseostromata*; lane 3, *C. scarabaeicola*; lane 4, *C. sinensis*; lane 5, *C. sp.*; lane 6, *C. sp.*; lane 7, *C. sp*.

with a bootstrap value of 64%. *C. memorabilis* and *Podostroma cordyceps* (*Po. cordyceps*) formed a group with a bootstrap value of 71%. The largest group, representing a bootstrap value of 98%, was comprised of *C. bifusispora*, *C. brongniartii*, *C. heteropoda*, *C. militaris*, *C. militaris*, *C. ochraceostromata*, *C. scarabaeicola*, *C. sphingum*, and *Phytocordyceps ninchukispora (Ph. ninchukispora)*.

PCR Products and PCR−SSCP Analyses of the ITS2 Region and the 3′ End of the 18S rRNA Gene. Using the foregoing phylogenetic tree, we aimed to design a reliable, inexpensive, and rapid *Cordyceps* identification and speciation method. Our previous experience using PCR−SCCP made this approach feasible. The primers used for amplifying the DNA fragment for SSCP examination (5.8SR and ITS4) varied between 334 and 400 bp in length, respectively. These sizes produce readily identifiable SSCP patterns in MDE gels. Primers NS7 and NS8 were used to PCR amplify an approximately 250 bp DNA fragment representing the 18S rRNA gene. Figure 1 shows the positions of primers used in this study on rDNA.

PCR−SSCP assays were repeated at least three times for all isolates, and reproducibility was 100%. On the basis of the SSCP results, the optimal conditions were 25 °C, a MDE (15% polyarceramide) gel, 150 V, and 70 min. The combination of the double strand size, agarose gel electrophoresis, and SSCP patterns of DNA proved useful for the differentiation of *Cordyceps* fungi. As shown in Figure 3, all seven isolates listed in Table 3 could be differentiated by their SSCP patterns and PCR. A summary of the seven isolates is presented in Table 3. The other segments from 18S rDNA were tested by SSCP. Identical patterns of the seven isolates were obtained on the basis of their 18S rDNA (Figure 4).

DISCUSSION

The phylogenetic relationships between the 17 *Cordyceps* isolates we used were closely related to each other on the basis of the ITS region and still could be divided into five major groups. *C. sinensis*, the traditional species used in Chinese medicinal preparations, was less closely related to the other 15 isolates, but shared a closer relationship with *C. agriota.* The origin of the geographical location of *C. sinensis* is China, Tibet, where the fungus has an isolated environment. This isolated environment might have caused *C. sinensis* to evolve away from the core species of *Cordyceps* as found on the phylogenetic tree we constructed from 17 *Cordyceps* fungi. The phylogenetic tree only could show the relationships between the species but still provided much useful information.

Our previous and present studies have demonstrated that the ITS2 region and 18S of rDNA are useful for the distinction and identification of *Cordyceps* species. It indicates that they have great potential in a novel SSCP-based test which could be easily, accurately, and inexpensively used to identify the Chinese medicinal fungus *Cordyceps*. This represents a significant improvement from the traditional morphological and biochemical classification criteria. Our other study has set up a SSCP pattern database from 49 different *Cordyceps* isolates. To identify optimal primer pairs, amplified DNA fragment, and universal PCR conditions, we tested 22 primer pairs (unpublished data). Some of the primers were from White et al., 1990 (*24*) but most designed by ourselves. Nevertheless, most of these primer pairs showed nonspecific binding to genomic DNA, and the PCR products showed a complex pattern, not just a single band. Finally, the primer pairs of 5.8SR/ITS4 and NS7/NS8 were the most suitable of the 22 primer pairs.

Our earlier result was focused on searching suitable primer pairs and setting up a database of SSCP pattern for *Cordyceps* fungi. In this paper we sequenced the 17 fungal isolates and constructed a phylogenetic tree to find the position of *C. sinensis* and its phylogentic relationship to the 17 fungal isolates. The *C. sinensis* obtained from CCRC should be the same as the *C. sinensis* that is used in medicine. However, our study has shown the *C. sinensis* from CCRC might have been incorrectly named. CCRC 36421 is another *Cordyceps* fungus but not *C. sinensis* (data not shown). On the basis of the phylogenetic tree we constructed, *C. pseudomilitaris*, which was close to *C. cardinalis* (*29*), was chosen for application of the SSCP test. *C. cardinalis* (AY184976) shares 97.6% similarities to *C. agriota* (AY245651) on the SSU rDNA and is most similar to *C. pseudomilitaris* in the microscopic characters of nondisarticulating ascospore morphology and its host affinity for lepidopteran larvae (*29*). On the other hand, *C. sinensis* was closely related to *C. agriota* as shown on the phylogenetic tree (Figure 2). According to the above information, *C. sinensis* and *C. pseudomilitaris* were put in the SSCP gel for testing. Therefore, we used SSCP to know what *Cordyceps sinensis* is and also to try to identify the three

unknown *Cordyceps* species, which were shown on lanes 4−6 of Figure 3. Although the above information was considered, *C. pseudomilitaris* and *C. sinensis* could be the same species. Indeed, given the similar habitats of the two species, it is entirely conceivable that they might in fact represent a single species. But, using SSCP, we found different patterns between *C. pseudomilitaris* and *C. sinensis*. They might have a very close relationship but are not the same species. When the other 18S rRNA segments were tested by SSCP, the results were consistent with the seven isolates being a single species (Figure 4). This contrasts markedly with the normal differentiation of isolates that typically results from use of 18S rRNA. The high degree of similarity of the sequence analyses based on the ITS1, 5.8S rDNA, and ITS2 regions from 17 isolates of *Cordyceps* (Table 2) reinforces this close association. The confidence in the validity of SCCP result is high, given the relative ease of the technique, as compared to DNA extraction, sequencing, and sequence analysis.

In conventional DNA-based classification methods, the genetic variation of individuals can be detected by RFLP analysis (*30*). The basis of RFLP, namely, the discrimination of differently sized DNA fragments, cannot distinguish DNA sequence variations in fragments of the same size. Presently, restriction cutting sites based on the rDNA of the 17 *Cordyceps* isolates were examined and computationally predicted (data not show). These results failed to identify unique sites that could be exploited to differentiate between species using restriction enzymes. Direct sequencing is an exquisitely accurate means of species differentiation. But this accuracy comes with the price of time-consuming sample preparation and analysis. When a large number of samples are being examined, this task becomes exceedingly onerous. Other methods, including the random amplification of polymorphic DNA (RAPD) (*31*) and amplified fragment length polymorphism (AFLP) (*32*, *33*), could be used as identification methods. RAPD is a very fast method that provides a great number of polymorphisms, but its major drawback is poor reproducibility (*17*). This was confirmed presently by the use of more than five different 10-mer primers on the 17 *Cordyceps* isolates (data not shown).

The ITS region consists of two variable noncoding regions, ITS1 and ITS2, nested within the rDNA repeat among the highly conserved 18S, 5.8S, and 28S (*34*). ITS regions are commonly used to examine phylogenetic positions or relationships at a species or interspecies level (*11*, *35*). Information gained in an examination of individuals of a given species has not been as encouraging. The present success incorporating ITS2 in a SSCP-based test indicates that differences between individuals of a species (such as mutants) may be possible to identify with this technique. Conceivably, SSCP could be exploited in culture quality control protocols.

In conclusion, PCR−SSCP provides a reliable method to distinguish between species of *Cordyceps* fungi, in particular those species that are used as Chinese medicine. PCR−SSCP is fast, simple, and clearly differentiates *Cordyceps* species. The incorporation of ITS as an analytical probe enables the dissection of differences between individuals of a single species, allowing mutations to be revealed. SSCP profiles of the ITS region may offer a powerful means of identifying the *Cordyceps* species in powdered preparations of Chinese medicine, and as a quality control tool during the fermentation procedure in the preparation of the medicine.

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