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Research Article

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Determination of the Ribosomal DNA Internal Transcribed Spacers and 5.8S rDNA Sequences of *Cordyceps* Species

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The sequences of the internal transcribed spacers (ITS1 and ITS2) and 5.8S ribosomal DNA gene from five *Cordyceps* species and one *Paecilomyces japonica* were determined. The total length of the ITS1, 5.8S and ITS2 regions ranged from 528 to 549 bp. When the *C. militaris* collected from Korea was used as a standard genotype, the sequence showed 88.4%, 88.6%, 91.1% and 86.8% identity to *C. pruinosa*, *C. sphecocephala*, *C. scarabaeucika* and *P. japonica*, respectively, while the lowest identity was found with *C. sinensis* (75.4%). Interestingly, *C. sinensis* was phylogenetically distant from the other *Cordyceps* species. To test geographic variation, furthermore, sequences of the ITS regions in the 8 samples of *C. militaris* collected from two localities in Korea and China analyzed and compared with the GenBank-searched sequences from Japan and China. The total length of the ITS regions of *C. militaris* from Korea, Japan and China was completely identical to each other with 528 bp, and the sequence divergence among three localities in pairwise comparisons ranged from 0.2% (1 bp) to 0.4% (2 bp).

Key words: Fungi, *Cordyceps*, ITS, 5.8S rDNA, Phylogeny

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Introduction

Cordyceps species, which are belonged to the Hypocreals of the ascomycetes, are fungal parasites of insects. They infect the larva or imago of insects, kill them, and then form a fruit body on the insect.

In the Orient, some *Cordyceps* species are used in traditional medicine. It has been reported that *Cordyceps* species can produce many kinds of bioactive compounds and the medicinal benefits (Fujita *et al.*, 1994; Furuya *et al.*, 1983; Kneifel *et al.*, 1977; Zhu *et al.*, 1998a, b). Along with an extensive medicinal interest, *C. militaris* and *Paecilomyces japonica* are mass-produced in the silkworm larva and pupa, *Bombyx mori* (Cho, 1999; Lee *et al.*, 2001a,b).

Approximately 400 species of *Cordyceps* are known, and they are classified by color and shape of fruit body or spore, shape of ascus, and kind of host insect (Shimizu, 1994). For *Cordyceps* the application of molecular methods to phylogenetic analysis has been enhanced ability to understand the genetic diversity (Chen *et al.*, 2001; Ito and Horano, 1997; Nikoh and Fukatsu, 2000). Internal transcribed spacer (ITS) regions are rapidly evolving regions of the nuclear ribosomal DNA, which have been widely used for species or population analysis of various organisms (Gonzalez *et al.*, 1990; Grutell, 1993; Lee and Taylor, 1992; Morales *et al.*, 1993; Wilmotte *et al.*, 1993). These sequences from some *Cordyceps* were determined to infer their phylogenetic relationships (Chen *et al.*, 2001; Ito and Hirano, 1997; Nikoh and Fukatsu, 2000).

In this study, we determined the sequences of the internal transcribed spacers (ITS1 and ITS2) and 5.8S ribosomal RNA gene from five *Cordyceps* species and analyzed their phylogenetic relationships. Additionally, we

Table 1. A brief information of strains of the entomopathogenic fungi sequenced in this study and GenBank-searched *C. militaris* sequences

Species	Sample number	Host	Host stage	Origin	GenBank accession number
Sequences obtained from this study					
Cordyceps militaris	E11	Sphinx moth	Pupa	Changnyeong-gun, Gyeongsangnam-do, Korea	
	E12	Sphinx moth	Pupa	Changnyeong-gun, Gyeongsangnam-do, Korea	
	E13	Sphinx moth	Pupa	Changnyeong-gun, Gyeongsangnam-do, Korea	
	E14	Sphinx moth	Pupa	Changnyeong-gun, Gyeongsangnam-do, Korea	
	E15	Sphinx moth	Pupa	Changnyeong-gun, Gyeongsangnam-do, Korea	
	E16	Sphinx moth	Pupa	Gangwon University, Korea	
	E21	–	Larva	Mt. Jangbag, China	
	E22	–	Pupa	Mt. Jangbag, China	
Cordyceps pruinosa	E7	–	–	Changnyeong-gun, Gyeongsangnam-do, Korea	
	E8	–	–	Changnyeong-gun, Gyeongsangnam-do, Korea	
	E9	–	–	Changnyeong-gun, Gyeongsangnam-do, Korea	
	E10	–	–	Changnyeong-gun, Gyeongsangnam-do, Korea	
Cordyceps sphecocephala	E6	Bee	Adult	Millyang-si, Gyeongsangnam-do, Korea	
	E18	–	Pupa	Changnyeong-gun, Gyeongsangnam-do, Korea	
Cordyceps scarabaeucika	E5	Scarab beetle	Adult	Gangwon University, Korea	
Cordyceps sinensis	E20	–	–	Tibet, China	
Paecilomyces japonica	E1	Silkworm	Pupa	NIAST, RDA, Suwon-si, Gyeonggi-do, Korea	
	E2	Silkworm	Pupa	Gangwon University, Korea	
	E3	Silkworm	Pupa	Gangwon University, Korea	
Sequences obtained through GenBank search					
Cordyceps militaris	–	Moth	Pupa	Tsuruoka, Yamagata, Japan	AB027379
	–	–	–	Beijing, China	AJ243774

–, Not determined.

determined the sequences from the *C. militaris* collected from two localities in Korea and China to test if any geographic variation exists among them and compared with the GenBank-searched sequences from Japan and China.

Materials and Methods

Fungal strains

Information on fungal strains used in this study is presented in Table 1. Most of the *Cordyceps* species were collected in Korea and China. *Cordyceps militaris* was collected from Korea (sample number E11-16) in 2000 to 2001 and from Mt. Jangbag, China (sample number E21-22) in 2001.

DNA extraction and PCR amplification

DNA was isolated from the fruit body of the fungi. Fruit bodies of the fungal strains were surface-sterilized in 70% ethanol and cut by a clean razor to obtain uncontaminated fungal tissue from the core region. The tissue was ground well into powder in a mortar in the presence of liquid nitrogen, from which DNA was isolated with the Wizard™ Genomic DNA Purification Kit, according to the manufacturers instructions (Promega). The primers used for amplification of the sequences of the internal tran-

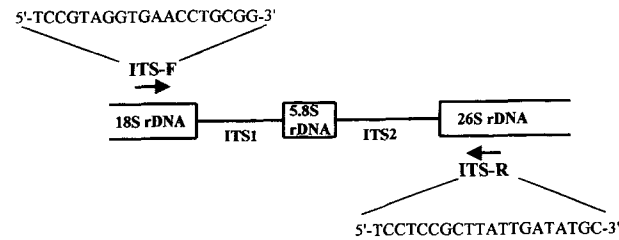

Fig. 1. Localization of the primers used for the amplification and sequencing reaction of internal transcribed spacers (ITS1 and ITS2) and 5.8S rDNA from *Cordyceps* and *Paecilomyces*.

scribed spacers (ITS1 and ITS2) and 5.8S ribosomal RNA gene were primer ITS-F (forward), 5-TCCGTAGGTGAACCTGCGG-3, and primer ITS-R (reverse), 5-TCCTCCGCTTATTGATATGC-3 (Bachellerie and Qu, 1993; White *et al.*, 1990; see Fig. 1). The PCR amplication condition consisted of an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 40 s and 72°C for 2 min, and a final extension step of 72°C for 10 min. The amplified products were purified with Qiaquick PCR Purification kit (QIAGEN), and then used directly for sequencing.

DNA sequencing and data analysis

DNA sequencing was performed using an automatic sequencer (model 310 Genetic Analyzer; Perkin-Elmer Applied Biosystems, Foster City, CA). Including the sequences of the internal transcribed spacers (ITS1 and ITS2) and 5.8S ribosomal DNA gene sequenced in this study and obtained through GenBank search, phyloge-

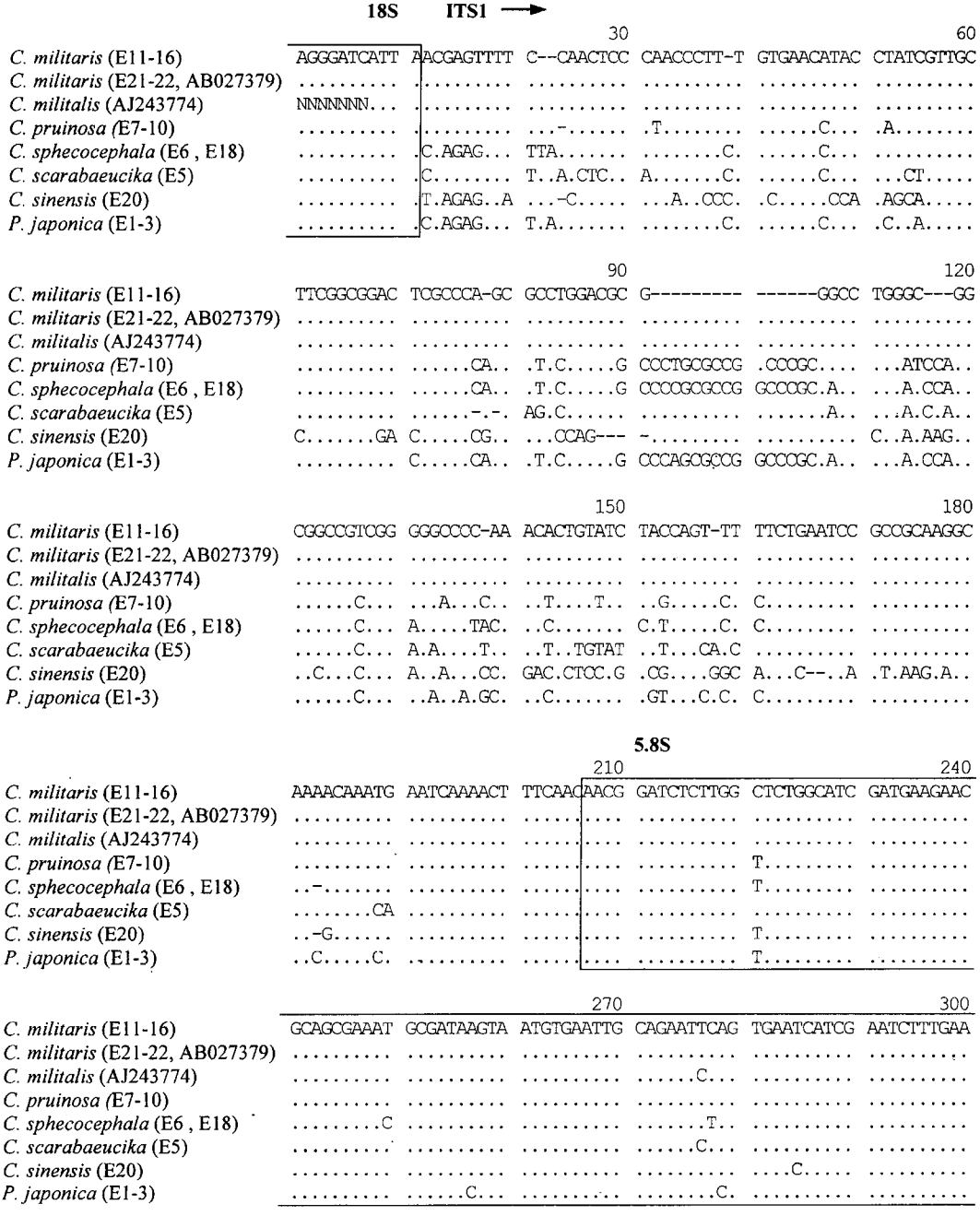

Fig. 2. Sequence alignment of ITS regions and 5.8S rDNA gene from five *Cordyceps* species and one *Paecilomyces* species. Only nucleotides that differ from *C. militaris* (E11-16) are indicated.

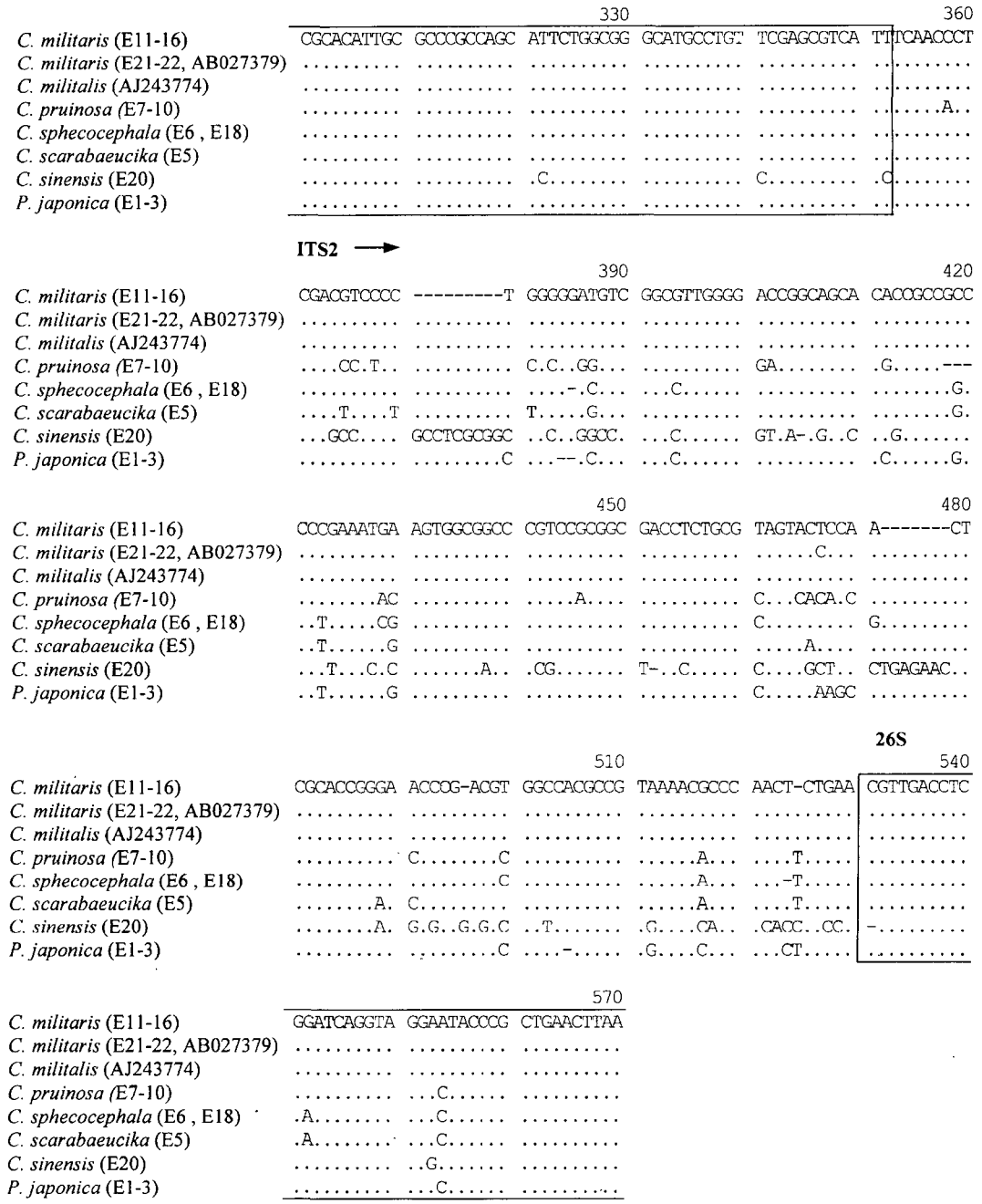

Fig. 2. Continued

netic analysis among *Cordyceps* species was performed using PAUP (Phylogenetic Analysis using Parsimony) version 3.1 (Swofford, 1990). The accession numbers of the sequences in the GenBank are as follows: *Cordyceps militaris* E11-E16 (this study) and E21-E22 (this study), *Cordyceps pruinosa* E7-E10 (this study), *Cordyceps sphecocephala* E6 and E18 (this study), *Cordyceps scarabaeucika* E5 (this study), *Cordyceps sinensis* E20 (this study), *Paecilomyces japonica* E1-E3 (this study), and *Cordyceps militaris* (AB027378; AJ243774).

Results and Discussion

Sequence alignment of the ITS1, 5.8S and ITS2 regions of five *Cordyceps* species and one *P. japonica* is presented in Fig. 2. The alignment of each other nucleotide sequences

Table 2. Length (in nucleotides) and GC ratio (%) of sequences of the ITS regions and 5.8S rDNA gene from the entomopathogenic fungi in this study

Species	18S (partial)	ITS1	5.8S	ITS2	26S (partial)	total
C. militaris (E11-16)	11 (36.4)	171 (55.6)	146 (49.3)	160 (67.5)	40 (50.0)	528 (56.63)
C. militaris (E21-22)	11 (36.4)	171 (55.6)	146 (49.3)	160 (68.1)	40 (50.0)	528 (56.82)
C. pruinosa (E7-10)	11 (36.4)	189 (59.3)	146 (48.6)	158 (69.0)	40 (52.5)	544 (58.27)
C. sphecocephala (E6, E18)	11 (36.4)	193 (60.1)	146 (48.6)	159 (69.2)	40 (50.0)	549 (58.47)
C. scaravaeucika (E5)	11 (36.4)	174 (53.4)	146 (50.0)	161 (64.6)	40 (50.0)	532 (55.26)
C. sinensis (E20)	11 (36.4)	170 (62.9)	146 (51.4)	176 (74.4)	39 (51.3)	542 (62.18)
P. japonica (E1)	11 (36.4)	193 (62.2)	146 (50.0)	158 (71.5)	40 (52.5)	548 (60.40)

Table 3. Pairwise comparisons among ITS regions and 5.8S rDNA sequences from the entomopathogenic fungi in this study and internet down-loaded, homologous sequences of two *C. militaris* (AB027379, AJ243774)

	1	2	3	4	5	6	7	8
1. *C. militaris* (E11-16)	–	0.002	0.002	0.116	0.114	0.089	0.246	0.132
2. *C. militaris* (E21-22, AB027379)	1	–	0.004	0.114	0.116	0.091	0.244	0.132
3. *C. militalis* (AJ243774)	1	2	–	0.118	0.116	0.088	0.247	0.133
4. *C. pruinosa* (E7-10)	66	65	67	–	0.1	0.14	0.263	0.109
5. *C. sphecocephala* (E6 , E18)	65	66	66	57	–	0.128	0.27	0.058
6. *C. scarabaeucika* (E5)	51	52	50	80	73	–	0.267	0.142
7. *C. sinensis* (E20)	140	139	141	150	154	152	–	0.27
8. *P. japonica* (E1-3)	75	75	76	62	33	81	154	–

Numbers above the diagonal are mean distance values; numbers below the diagonal are absolute distance values.

revealed significant differences and also there was considerable sequence variations in the ITS1 and ITS2, but was little in the regions of the 5.8S rDNA.

Table 2 showed the total length of the ITS1, 5.8S and ITS2 regions. The total length of the ITS1, 5.8S and ITS2 regions of five *Cordyceps* species and one *P. japonica* ranged from 528 to 549 bp (Table 2). The shortest size of the ITS1-5.8S-ITS2 was 528 nucleotides of *C. militaris*, and the longest size of the ITS regions was 549 nucleotides of *C. sphecocephala*. There was considerable length variation within the *Cordyceps* for the ITS1 and ITS2 regions. However, the conserved 5.8S rDNA from the six species showed identical nucleotide length to each other. ITS1 in most species except *C. sinensis* was longer than ITS2. It has been reported that ITS2 in *C. sinenesis* was longer than ITS1 (Chen *et al.*, 2001). In the G+C content of ITS1, 5.8S and ITS2 regions, the highest content of the ITS1-5.8S-ITS2 was 62.18% of *C. sinensis*, and the lowest content of the ITS regions was 55.26% of *C. scaravaeucika*. In all species determined in this study, G+C content in ITS2 was higher than ITS1. Torres *et al.* (1990) found a phenomenon in the G + C content of ITS regions in a wide range of organisms and called it "GC balance". Further study is needed to determine to effect of G + C content to ecological and physiological characteristics on *Cordyceps* species.

Additionally, sequences of the ITS1, 5.8S and ITS2 regions in the 8 samples of *C. militaris* collected from two localities in Korea and China to test if any geographic variation exists among them were obtained and compared with the GenBank-searched sequences from Japan and China. The total length of the ITS1, 5.8S and ITS2 regions of *C. militaris* from Korea, Japan, and China was completely identical to each other with 528 bp (Table 2 and Fig. 2). The sequence divergence among three localities in pairwise comparisons ranged from 0.2% (1 bp) to 0.4% (2 bp), indicating relatively lower genetic diversity (Table 3).

When the *C. militaris* (E11-E16) from Korea was used as a standard genotype in this study, the sequence showed 88.4%, 88.6%, 91.1% and 86.8% identity to *C. pruinosa*, *C. sphecocephala*, *C. scarabaeucika* and *P. japonica*, respectively, while the lowest identity was found with *C. sinensis* (75.4%) (Table 3). PAUP analysis was performed to investigate phylogenetic relationships among 16 isolates of five *Cordyceps* species and 3 isolates of *P. japonica* as an outgroup (Fig. 3). However, a phylogenetic analysis using the ITS1, 5.8S and ITS2 sequences revealed that the sequences of the *C. sinensis* formed a subgroup with *C. scaravaeucika*. The sequence identity was not reflected to the phylogenetic analysis. This result

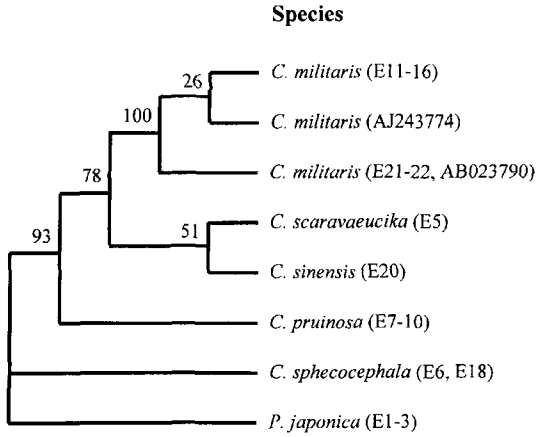

Fig. 3. PAUP analysis of ITS regions and 5.8S rDNA sequences. The tree shown is a single-most parsimonious tree from the heuristic search using *P. japonica* (E1-3) as an outgroup. The numbers shown on the branches represent bootstrap values for 1,000 replicates. Tree length is 292 steps, Consistency Index is 0.836, and Retention Index is 0.669.

is not consistent with the current phylogenetic hypothesis among *Cordyceps* species reported by ITS sequences (Nam *et al.*, 1999). Interestingly, Ito and Hirano (1997) found that *C. sinensis* was phylogenetically distant from the other *Cordyceps* species. Also, the previous report (Ito and Hirano, 1997) proposed that a new classification of *Cordyceps* species could be constructed according to accumulated phylogenetic information obtained from rRNA sequences. Thus, our results of molecular phylogenetic analysis based on ITS sequences will expand information on *Cordyceps* species.

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