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Phylogenetic Analysis of Caterpillar Fungi by Comparing ITS 1-5.8S-ITS 2 Ribosomal DNA Sequences

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This study was carried out to identify the phylogenetic relationships among several caterpillar fungi by comparing the sequences of internal transcribed spacer regions (ITS1 and ITS2) and 5.8S ribosomal DNA (rDNA) repeat unit. The sequences of ITS1, ITS2, and the 5.8S rDNA from 10 strains of *Cordyceps* species, 12 strains of *Paecilomyces*, 3 strains of *Beauveria*, 2 strains of *Metarhizium* and 1 strains of *Hirsutella* were amplified, determined and compared with the previously known *Cordyceps* species. The sequences of 5.8S rDNA were more conserved in length and variation than those of ITS regions. Although the variable ITS sequences were often ambiguously aligned, the conserved sites could be found. In the phylogenetic tree, the species generally divided into three clusters, supported by their morphology and/or host ranges. The 5.8S rDNA and ITS1 sequences among 10 species of *Cordyceps militaris* were identical and only one base pair in ITS2 sequence was different. *Cordyceps sinensis* and *Cordyceps ophioglossoides* were also clearly different, although they belonged to the same cluster. The GenBank database search of species revealed sister taxa of an entomogenous fungus. *Metarhizium* was used as an outgroup in all taxa.

KEYWORDS: Caterpillar fungus, *Cordyceps*, Internal transcribed spacers, *Paecilomyces*, Phylogeny

Most of the caterpillar fungi belong to the family Clavicipithaceae and have long been used in Chinese medicine to treat numerous illness, promote longevity, relieve exhaustion and increase athletic prowess (Bok *et al.*, 1999). Especially, *Cordyceps sinensis*, *C. militaris*, and *Paecilomyces japonicus* have been regarded as a celebrated drug in the Chinese Pharmacopeia and used as a tonic. Some polysaccharides isolated from *Cordyceps* species are also known to have potent antitumor activity. The extracts from *C. militaris* and *Isaria felina* showed similar strong negative inotropic effects as those from *C. sinensis* (Yamanaka, 1998). This fungus is known to possess diverse therapeutic effects (Tsunoo *et al.*, 1995). Kinjo *et al.* reported that extracts from cultured mycelia of *C. militaris* had several physiological activities (Kinjo *et al.*, 1996).

In view of insect physiology, microbiology, immunology, and pest control, these fungi are also very important to understand the parasitic and pathogenic mechanism. They infect the host insect, escape its self-defense system, proliferate in its blood or cell, and kill it (Takema *et al.*, 1997). The ascospores of the fruitbodies of *C. militaris* are also used as a biological controlling agent (Sung *et al.*, 1993). Entomogenic deuteromycete fungi of the genera *Cordyceps* and *Paecilomyces*, called 'Dong-chong-xia-cao' (Ascomycotina, Pyrenomycetes, Clavicipitales, Clavicitaceae) in China, form fruiting bodies or sporocarps on their hosts. These fungi were classified by Kobayasi (Kobayasi, 1941). For taxonomic characteristics, they mainly relied on their external features, such as entomopathogenic or mycogenous, or superficial or immersed perithecia, or whether the fertile parts were globose or cylindrical (Sung *et al.*, 1997).

Therefore, it is suggested that during the evolution of a single genus, *Cordyceps*, an interkingdom host-jumping event between Animalia and Fungi had occurred (Nikoh and Fukatsu, 2000).

The entomoparasitic deateromycetes *Beauveria bassiana*, *Metarhizium anisopliae*, and *P. tenuipes* are regarded as anamorphs of the *Cordyceps* spp. based on molecular phylogenetic and ecological lines of evidence (Fukatsu *et al.*, 1997; Liang *et al.*, 1991; Shimazu *et al.*, 1988).

The taxonomy and correct identification of the caterpillar fungi is a hard task. Generally, the taxonomy of these fungi is based on the morphology of the fruiting body and the association with host insect. Besides morphological traits of fruiting bodies, taxonomic characteristics have also been investigated for the systematics of the genus *Cordyceps* (Choi *et al.*, 1999). A various culture studies were conducted to classify the genus *Cordyceps* and *Paecilomyces* (Sung *et al.*, 1993). Isozyme pattern analysis and restriction fragment length polymorphism (RFLP) were carried out by Sung *et al.* (1997). This method developed the new characteristics for taxonomic studies at the species level. However, their methods are variable in the band pattern on the gel so that they cannot be used for lower taxonomical level. Therefore, the detailed classifica-

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tion of caterpillar fungi has brought the complexity to most of taxonomists. Recently, molecular techniques such as DNA/DNA hybridization, electrophoretic karyotyping, RFLP, and DNA sequencing have been used for phylogenetic analysis of various kinds of organisms (Sasamu *et al.*, 1998). PCR direct sequencing method reported by White *et al.* is an excellent method applicable to the fungi because it can be carried out using a small amount of starting material (White *et al.*, 1990). Hirata *et al.* improved the method to determine the rDNA sequences of fungi using tiny amounts of material (Hirata and Takamatsu, 1996). Eukaryotic rDNA is composed of tandemly repeated clusters of 18S, 5.8S, and 28S rRNA genes, which are transcribed as a precursor molecule by RNA polymerase I (Raue and Planta, 1995). The external and internal spacer molecules are then removed in nucleolus before escaping for cytoplasm. The nucleotide sequences of the conservative rRNA coding regions have been widely used for phylogenetic analysis among families or distantly related genera (Berbee and Taylor, 1993; Carmean *et al.*, 1992; Samson, 1974; White *et al.*, 1990). However, the variable ITS regions have an advantage of the phylogenetic analysis and identification of the closely related fungal species (Kim *et al.*, 1999).

In this work, we studied 5.8S rDNA and ITS regions to infer their applicability for the systematics of caterpillar fungi. The objective is to construct the phylogenetic relationship among the regions of ITS1 and ITS2, and 5.8S ribosomal RNA gene of *Cordyceps* and *Paecilomyces* species to compare the interaction between anamorph and telemorph in the genera *Cordyceps* and *Paecilomyces*. To address these aims, we amplified and sequenced the 5.8S rDNA, ITS1 and ITS2. Our results could reveal the detailed phylogenetic relationship among the closely related *Cordyceps* and *Paecilomyces* and related taxa.

Table 1. The list of fungal species and GenBank accession number of the genus *Cordyceps*, *Paecilomyces* and related taxa used in this study

Fungal species	Strains*	Accession No.
Cordyceps nutans	KACC 500169	AF224274
Cordyceps militaris	EFCC-C18	AF153265
Cordyceps militaris	EFCC-C738	AF153264
Cordyceps militaris	MPNU 8001	AF153266
Cordyceps militaris	KACC 500161	AF199590
Cordyceps militaris	KACC 500171	AF199591
Cordyceps ophioglossoides	KCTC 16017	AF199593
Cordyceps ophioglossoides	KCTC 6473	AF208524
Cordyceps scarabaeicola	EFCC-C252	AF199592
Cordyceps sinensis	MPNU 8002	AF291749
Isaria japonica	DGUM 32001	AF200370
Paecilomyces tenuipes	EFCC-C240	AF200368
Paecilomyces tenuipes	EFCC-C660	AF200369
Paecilomyces japonicus	KACC 40503	AF224689
Paecilomyces farinosus	KCTC 16102	AF237664
Paecilomyces sp.	KACC 40219	AF224690
Paecilomyces sp.	KACC 40220	AF224691
Paecilomyces sp.	KACC 40221	AF291750
Paecilomyces sp.	KACC 40222	AF291868
Paecilomyces sp.	KACC 40656	AF224273
Paecilomyces variotii	KACC 40246	AF291869
Paecilomyces variotii	KCCM 60009	AF291870
Beauveria bassiana	KACC 40024	AF291872
Beauveria bassiana	KACC 40218	AF291871
Beauveria bassiana	KACC 40224	AF294646
Metarhizium anisopliae	KACC 40029	AF293842
Metarhizium sp.	KACC 40230	AF293843
Hirsutella thompsonii	KACC 40023	AF293844

*EFCC : Entomopathogenic Fungal Culture Collection, Kangwon National University, Korea. MPNU : Mycological lab. of Pusan National University. KACC : Korean Agricultural Culture Collection, National Institute of Agricultural Science and Technology, Suwon, Korea. KCTC : Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea. DGUM : Microbiology laboratory of Dongguk University, Kyongju, Korea.

Materials and Methods

Fungal strains and cultivation. The 28 strains used in this study were obtained from Korean mountain, and various culture collections (Table 1). The rest strains except the representative in Table 1 were extracted from the GenBank database in NCBI (National Center for Biotechnology Information).

The strains were cultured with shaking in 100 m*l* of PD (Potato Dextrose) broth at 25°C for one week. The mycelia were harvested by filtration or centrifugation, and stored in a freezer until they were used.

DNA extraction. Fungal DNA was extracted from each sample according to the procedure adapted from the benzyl chloride method (Zuker, 1989). Approximately 0.05 g of fungal pellets were suspended in 500 μl of Tris buffer (100 mM Tris-HCl, pH 8.0, 40 mM EDTA), 150 μl of 10% (w/v) sodium dodecyl sulphate (SDS) and 300 μl of benzyl chloride, and then incubated at 55°C for 30 min. The treatment of phenol:chloroform:isoamylalchol (25:24:1) and RNase (1 mg/m*l*) was carried out for the removal of proteins. The DNA was precipitated by adding 2.5 volumes 100% ice-cold ethanol. The pellet was washed with 2 volumes of 70% ethanol and resuspended with distilled water. The purified DNA was kept at -20°C.

PCR amplification and DNA sequencing. The nuclear rDNA region spanning the ITS1, ITS2 and 5.8S rRNA gene was amplified by polymerase chain reaction (PCR) from each strain. Primers ITS5F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4R (5'-TCCTCCGCTTATTGAT ATGC-3') (White *et al.*, 1990) were derived from the conserved region of 18S and 28S rDNA, respectively (Fig. 1). PCR was carried out with Perkin-Elmer model

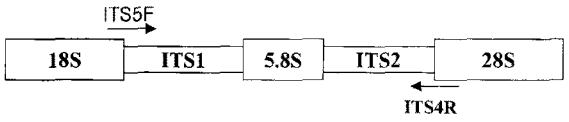

Fig. 1. A map of the ribosomal DNA region containing ITS1 and 2, and the 5.8S rRNA gene. Arrows indicate the position of the primers used for PCR and sequence analysis.

480 thermocycler using the following program: initial denaturation for 3 min at 95°C, 30 cycles of amplification (denaturation for 30 sec at 95°C, annealing for 30 sec at 50°C, and extention for 1 min at 72°C) and final extention of 5 min at 72°C. The PCR products from the amplification were subjected to preparative electrophoresis in a 1.6% agarose gel in TBE buffer. All PCR products yielded only a single visible band. The PCR products were excised from the ethidium bromide-stained gel and purified using a QIAGEN gel elution kit (Qiagen, Wartworth, CA). Direct sequencing of PCR products was done by a Perkin-Elmer Applied Biosystems ABI 377A sequencer and a PRISM Dye Dideoxi Terminator Cycle Sequencing kit (Perkin Elmer) according to the standard protocol (Gyllensten, 1989; Hiraishi, 1992; Smith *et al.*, 1986). Two primers, ITS5F and ITS4R, were used for sequencing in both directions and the DNA sequences were edited and assembled with the program CLONE MANAGER version 4.0 (Scientific Educational Software, Stateline, PA).

Data analysis. The determined rDNA sequences have been deposited in the European Molecular Biology Laboratory (EMBL) data library (Heidelberg, FRG) and accession numbers were indicated in Table 1. They were initially aligned with the sequences of the related genera from the EMBL data library using the multiple alignment program CLUSTAL W (Thomson *et al.*, 1994).

Phylogenetic relationships were inferred by the neighbor-joining method (Saitou and Nei, 1987). The strength of the internal branches from the resulting tree was statistically tested by bootstrap analysis (Felsenstein, 1985) from 1,000 bootstrap replications. The distance matrix was calculated using NucML, and the initial tree based on the neighbor-joining method was reconstructed by NJdist of the PHYLIP 3.5 software package. The G+C content distribution diagrams were obtained by the method of Bibbo *et al.* (Bibbo *et al.*, 1984) using the program GENETYX (Software Development).

Results and Discussion

Sequence alignments. Electrophoresis and direct sequencing of each PCR product confirmed that a single product was amplified and the size of each product corresponded to the expected rDNA. The alignment data of the DNA sequences of ITS1 and ITS2, and 5.8S rDNA using CLUSTAL G were shown in Fig. 2. There is considerable sequence variation in the ITS sequences and little in the regions of the 5.8S rDNA. The sequence variations of the ITS are usually higher in ITS2 than ITS1 in the literature. However, the sequences of ITS1 were more variable in our study than those of ITS2.

The sequence difference of intraspecies in *C. militaris* species appears on one point and base substitution is supposed as the variation of cultural, geographical, environmental conditions, and so on. Unidentified *Paecilomyces* sp. KACC 40219 and KACC 40221 have the similar sequences with *C. nutans* KACC 500169. This property can be described as the close relationships of teleomorph and anamorph between *Paecilomyces* and *Cordyceps*. It is convinced that *I. japonica*, *P. japonica*, and *P. tenuipes* are the same species and are called as another names, because of very similar sequences in multiple alignment of them. Unidentified *Paecilomyces* sp. KACC 40220 and KACC 40656 are thought to be *P. tenuipes* because their sequences are identical.

G+C content and nucleotide length of rDNA ITS regions. The G+C contents and nucleotide lengths of ITS1, ITS2, 5.8S rRNA gene, and whole region (ITS1-5.8S-ITS2) are examined (Table 2). ITS1 of a taxon having GC-rich ITS2 has also a long ITS1 sequence. Torres *et al.* found similar phenomenon in the G+C contents of ITS regions in a wide range of organisms including fungi and called it "GC balance" (Torres *et al.*, 1990). The total G+C contents of ITS1-5.8S-ITS2 in the *Cordyceps* species ranged from 54.6% (*C. scarabaeicola* EFCC-C252) to 56.8% (*C. militaris* species). In *Paecilomyces* species, total G+C content of ITS1-5.8S-ITS2 ranged from 56.0% (*Paecilomyces* sp. KACC 40219) to 61.6% (*Paecilomyces* sp. KACC 40222). *P. tenuipes* EFCC-C240, EFCC-C660, *I. japonica* DGUM 32001, *P. japonica* KACC 40503, and *Paecilomyces* sp. KACC 40220 has the same G+C content as 60.4%. Exceptionally, *Paecilomyces* sp. KACC 40219 has lower G+C content than the other *Paceilomyces* species.

In *Beauveria* species, total G+C content of ITS-5.8S-ITS2 ranged from 54.5% (*B. bassiana* KACC 40224) to 57.4% (*B. bassiana* KACC 40024). In Metarhizium species, it ranged to 50.3% and in Hisutella thompsonii KACC 40023 ranged to 58.8%. This species is similar G+C content with the genus *Cordyceps*. The result suggests that the further studies are necessary for detailed re-identification.

The G+C contents of the 5.8S rRNA gene were stable (44.9~49.0%) among all 28 taxa investigated. On the other hand, the ITS regions revealed relatively high G+C contents: 43.7~63.9% in ITS1 and 59.0~68.1% in ITS2.

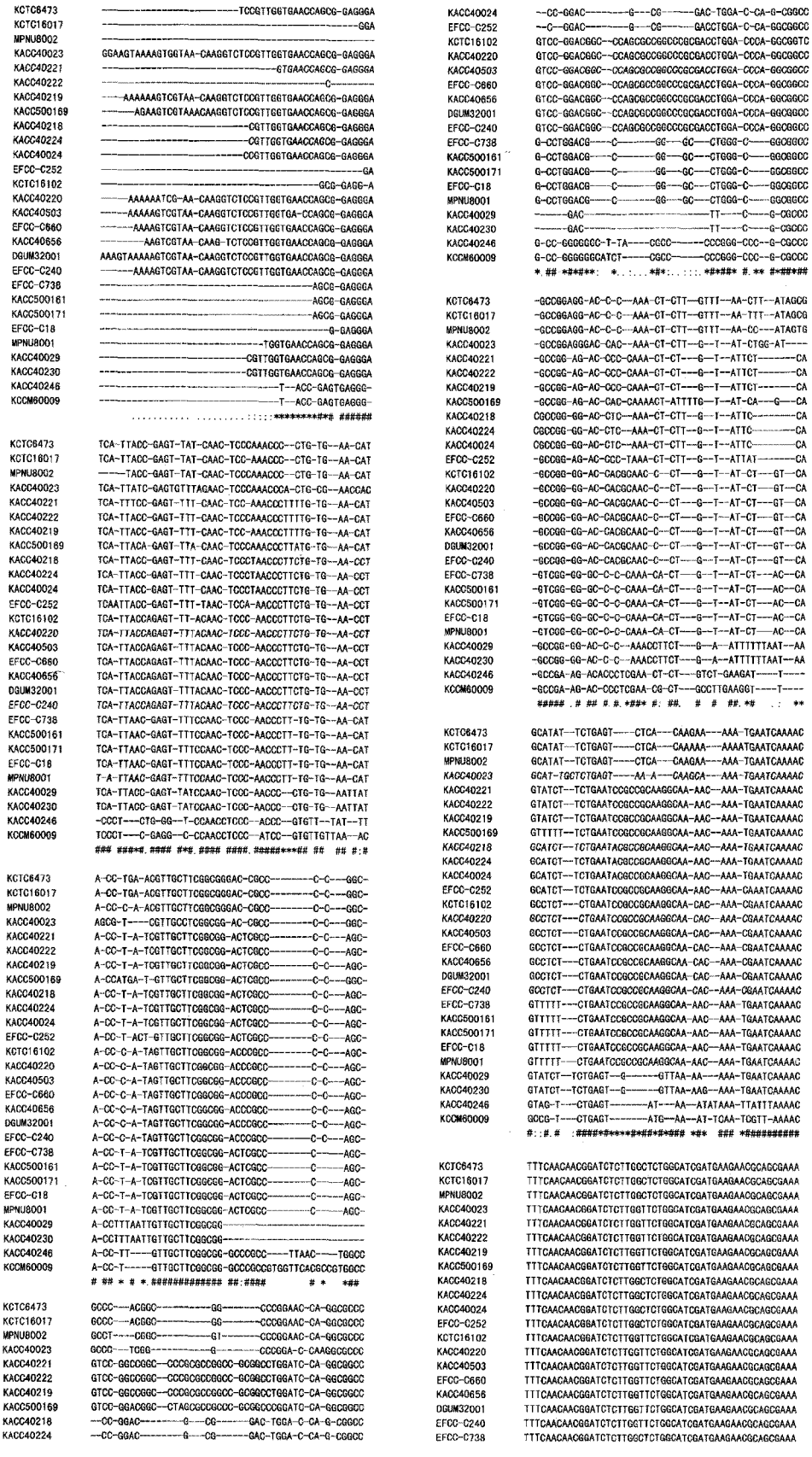

Fig. 2. The alignment data of ITS1, 5.8S rDNA and ITS2 region sequences using CLUSTAL G. A hyphen represents a gap and four characters, '#', '*', ':', and '.', indicate positions that are 80~100%, 60~80%, 40~60% and 20~40% identical, respectively. Each strain number was represented to Table 1.

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KACC500161   TTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAA
KACC500171   TTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAA
EFCC-C18     TTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAA
MPNU8001     TTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAA
KACC40029    TTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA
KACC40230    TTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA
KACC40246    TTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAA
KCCM60009    TTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAA
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KCTC6473     TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
KCTC16017    TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
MPNU8002     TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
KACC40023    TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
KACC40221    TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
KACC40222    TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
KACC40219    TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
KACC500169   TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
KACC40218    TGCGATAAGTAATGTGAATTGCAGAATCCAGTGAATCATCGAATCTTTGA
KACC40224    TGCGATAAGTAATGTGAATTGCAGAATCCAGTGAATCATCGAATCTTTGA
KACC40024    CGCGATAAGTAATGTGAATTGCAGAATCCAGTGAATCATCGAATCTTTGA
EFCC-C252    TGCGATAAGTAATGTGAATTGCAGAATCCAGTGAATCATCGAATCTTTGA
KCTC16102    TGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGA
KACC40220    TGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGA
KACC40503    TGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGA
EFCC-C660    TGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGA
KACC40656    TGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGA
DGUM32001    TGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGA
EFCC-C240    TGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGA
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KACC500161   TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
KACC500171   TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
EFCC-C18     TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
MPNU8001     TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
KACC40029    TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
KACC40230    TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
KACC40246    TGCGATACGTAATGTGAATTGCA-AATTCAGTGAATCATCGAGTCTTTGA
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KCTC16017    ACGCACATTGCGCCCGCCAG-TATTCTGGCGGGCATGCCTGTTCGAGCGT
MPNU8002     ACGCACATTGCGCCCGCCAG-TATTCTGGCGGGCATGCCTGTTCGAGCGT
KACC40023    ACGCACATTGCGCCCGCCAG-TATTCTGGCGGGCATGCCTGTTCGAGCGT
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KACC40023    CATTTC-AACCCTC-GGGCCCCCCCTTCGGGGACGGG--CG-C--G-GTG
KACC40221    CATTTC-AACCCTC--GACTTCCCTTT-G-----GGG-AAATC--G-GCG
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KACC40219    CATTTC-AACCCTC--GACTTCCCTTT-G-----GGG-AAATC--G-GCG
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MPNU8001     CATTTC-AACCCTC--GACGTCCCC-T-G-----GGGGATGTC--G-GCG
KACC40029    CATTAC-GCCCCTC---AAGTCCCC-T-GT----GG--ACTT---G-GTG
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KACC40220    TTGGG--GA-CCGGC-AG----------CA-CCCCG---CCGG----C--
KACC40503    TTGGG--GA-CCGGC-AG----------CA-CCCCG---CCGG----C--
EFCC-C660    TTGGG--GA-CCGGC-AG----------CA-CCCCG---CCGG----C--
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EFCC-C738    TTGGG--GA-CCGGC-AG----------CA-CACCG---CCGC----C--
KACC500161   TTGGG--GA-CCGGC-AG----------CA-CACCG---CCGC----C--
KACC500171   TTGGG--GA-CCGGC-AG----------CA-CACCG---CCGC----C--
EFCC-C18     TTGGG--GA-CCGGC-AG----------CA-CACCG---CCGC----C--
MPNU8001     TTGGG--GA-CCGGC-AG----------CA-CACCG---CCGC----C--
KACC40029    TTGGG--GA-TCGGCGAGGCTGGTTTTCCAGCACAG---CCGT----C--
KACC40230    TTGGG--GA-TCGGCGAGGCTGGTTTACCAGCACAG---CCGT----C--
KACC40246    TTGGGCCC---CGTC----------------CTCCGATTCCGGGGGACGG
KCCM60009    TTGGGTCGA--CGTC----------------C-CCC---CCGGGGGACGG
             #####  ## ##### **             ** #;#*#   ###:    #

KCTC6473     -CCCGAAATGCAGTGGCGAC-CTCG-CCG-CAGCCT-CCCC-TGCG---T
KCTC16017    -CCCTAAATGCAGTGGCGAC-CTCG-CCG-CAGCCT-CCCC-TGCG---T
MPNU8002     -CCCCAAATGCAGTGGCGAC-CTCG-CCG-CAGCCT-CCCC-TGCG---T
KACC40023    -CCCGAAATGCAGTGGCGAC-CCCG-CCG-CGGCCT-CTCC-TGCG---C
KACC40221    -CCCGAAATGAAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---T
KACC40222    -CCCGAAATGAAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---T
KACC40219    -CCCGAAATGAAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---T
KACC500169   -CCCGAAATGGAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---T
KACC40218    -CCTGAAATGGAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---T
KACC40224    --------------------------------------------------
KACC40024    -CCTGAAATGGAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---C
EFCC-C252    -CCTGAAATGGAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---T
KCTC16102    -CCTGAAATGGAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---C
KACC40220    -CCTGAAATGGAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---C
KACC40503    -CCTGAAATGGAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---C
EFCC-C660    -CCTGAAATGGAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---C
KACC40656    -CCTGAAATGGAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---C
DGUM32001    -CCTGAAATGGAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---C
EFCC-C240    -CCTGAAATGGAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---C
EFCC-C738    -CCCGAAATGAAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---T
KACC500161   -CCCGAAATGAAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---T
KACC500171   -CCCGAAATGAAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---T
EFCC-C18     -CCCGAAATGAAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---T
MPNU8001     -CCCGAAATGAAGTGGCGGC-C-CGTCCG-CGGCGA-CCTC-TGCG---T
KACC40029    -CCTTAAATTAATTGGCGGT-CTCG-CCG-TGGCCCTCCTC-TGCGCAGT
KACC40230    -CCTTAAATTGATTGGCGGT-CTCG-CCG-TGGCCCTCCTC-TGCGCAGT
KACC40246    GCCCGAAAGGCAGCGGCGGCAC-CG--CGTC--CGGTCCTCGAGCG---T
KCCM60009    GCCCGAAAGGCAGCGGCGGCGC-CG--CGTC--CGATCCTCGAGCG---T
              ##:######.######### # ##*### #*##** #### ####   *

KCTC6473     A-G-----TA-GCACAAC-CTCGC-ACCGG-AGCG---CGGA-G-AC-G-G
KCTC16017    A-G-----TA-GCACAAC-CTCGC-ACCGG-AGCG---CGGA-G-AC-G-G
MPNU8002     A-G-----TA-GCACAAC--TCGC-ACCGG-AGCG---CGGA-G-AC-G-G
KACC40023    A-G-----TAAGCA-AACGCTCGC-AACG--AGGGACCGGA-GGAC-GAG
KACC40221    A-G-----TAATC-CAAC--TCGC-ACCGG-AAC---CCCGACG--T-G-G
KACC40222    A-G-----TAATC-CAAC--TCGC-ACCGG-AAC---CCCGACG--T-G-G
KACC40219    A-G-----TAATC-CAAC--TCGC-ACCGG-AAC---CCCGACG--T-G-G
KACC500169   A-G-----TAATC-CAACC-TCGC-ACCGG-AAC---CCCGACG--T-G-G
KACC40218    A-G-----TAATA-CAGC--TCGC-ACCGG-AAC---CCCGACG--C-G-G
KACC40224    --------------------------------------------------
KACC40024    A-G-----TAATA-CAGC--TCGC-ACCGG-AAC---CCCGACG--C-G-G
EFCC-C252    A-G-----TAATC-CAAC--TCGC-ACCGG-AAC---CCCGACG--T-G-G
KCTC16102    A-G-----TACAAGCA-C--TCGC-ACCGGGAAC---CC-GACG--C-G-G
KACC40220    A-G-----TACAAGCA-C--TCGC-ACCGGGAAC---CC-GACG--C-G-G
KACC40503    A-G-----TACAAGCA-C--TCGC-ACCGGGAAC---CC-GACG--C-G-G
EFCC-C660    A-G-----TACAAGCA-C--TCGC-ACCGGGAAC---CC-GACG--C-G-G
KACC40656    A-G-----TACAAGCA-C--TCGC-ACCGGGAAC---CC-GACG--C-G-G
DGUM32001    A-G-----TACAAGCA-C--TCGC-ACCGGGAAC---CC-GACG--C-G-G
EFCC-C240    A-G-----TACAAGCA-C--TCGC-ACCGGGAAC---CC-GACG--C-G-G
EFCC-C738    A-G-----TACTC-CAAC--TCGC-ACCGGGAAC---CC-GACG--T-G-G
KACC500161   A-G-----TACTC-CAAC--TCGC-ACCGGGAAC---CC-GACG--T-G-G
KACC500171   A-G-----TACTC-CAAC--TCGC-ACCGGGAAC---CC-GACG--T-G-G
EFCC-C18     A-G-----TACCC-CAAC--TCGC-ACCGGGAAC---CC-GACG--T-G-G
MPNU8001     A-G-----TACCC-CAAC--TCGC-ACCGGGAAC---CC-GACG--T-G-G
KACC40029    A-G-----TAAAG-CA-C--TCGCAACAGG-AGC---CC-GGCG--C-G-G
KACC40230    A-G-----TAAAA-CA-C--TCGCAACAGG-AGC---CC-GGCG--C-G-G
KACC40246    ATGGGGCTT-TGTCA-CC--CGC-TCTG-TAGGC--CCGGCCG-GC---G
KCCM60009    ATGGGGCTT-TGTCA-CG--CGC-TCTGGTAGGG--TCGGCCG-GCTG-G
             # #     ##....##:#  #### #####:#*#    ##.####  . # #

KCTC6473     TCA-C-GCC---GTTAAACG-CCCA-----ACTTC-TCAG-A-GTTGACC
KCTC16017    TCA-C-GCC---GTAAAACG-CCCA-----ACTTC-TCAG-A-GTTGACC
MPNU8002     TCA-C-GCC---GTAAAACG-CCCA-----ACTTC-TCAG-A-GTT----
KACC40023    TC--C-GCC---GTAAAACG-CCCA------GTTCCT-AG-AAGTTGACC
KACC40221    -C-AC-GCC---GTAAAAC--CCCCG----ACTT-CTGA--ACGTTGACC
KACC40222    -CCAC-GCC---GTAAAAC--CCCCG----ACTT-CTGA--ACGTTGACC
KACC40219    -CCAC-GCC---GTAAAAC--CCCCG----ACTT-CTGA--ACGTTGACC
KACC500169   -CCAC-GCC---GTAAAACA-CCCC-----ACTTTCTGA--ACGTTGACC
KACC40218    -CCAC-GCC---GTAAAACA-CCCA-----ACTT-CTGA--ACGTTGACC
KACC40224    --------------------------------------------------
KACC40024    -CCAC-GCC---GTAAAACA-CCCA-----ACTT-CTGA--ACGTTGACC
EFCC-C252    -CCAC-GCC---GTAAAACA-CCCA-----ACTT-CTGA--ACGTTGACC
KCTC16102    -CC-C-GCC---GTGAAACC-CCCA-----ACCT-CTGA--ACGTTGACC
KACC40220    -CC-C-GCC---GTGAAACC-CCCA-----ACCT-CTGA--ACGTTGACC
KACC40503    -CC-C-GCC---GTGAAACC-CCCA-----ACCT-CTGA--ACGTTGACC
EFCC-C660    -CC-C-GCC---GTGAAACC-CCCA-----ACCT-CTGA--ACGTTGACC
KACC40656    -CC-C-GCC---GTGAAACC-CCCA-----ACCT-CTGA--ACGTTGAC-
DGUM32001    -CC-C-GCC---GTGAAACC-CCCA-----ACCT-CTGA--ACGTTGACC
EFCC-C240    -CC-C-GCC---GTGAAACC-CCCA-----ACCT-CTGA--ACGTTGACC
EFCC-C738    -CCAC-GCC---GTAAAACG-CCCA-----AC-T-CTGA--ACGTTGACC
KACC500161   -CCAC-GCC---GTAAAACG-CCCA-----AC-T-CTGA--ACGTTGACC
KACC500171   -CCAC-GCC---GTAAAACG-CCCA-----AC-T-CTGA--ACGTTGACC
```

Fig. 2. Continued.

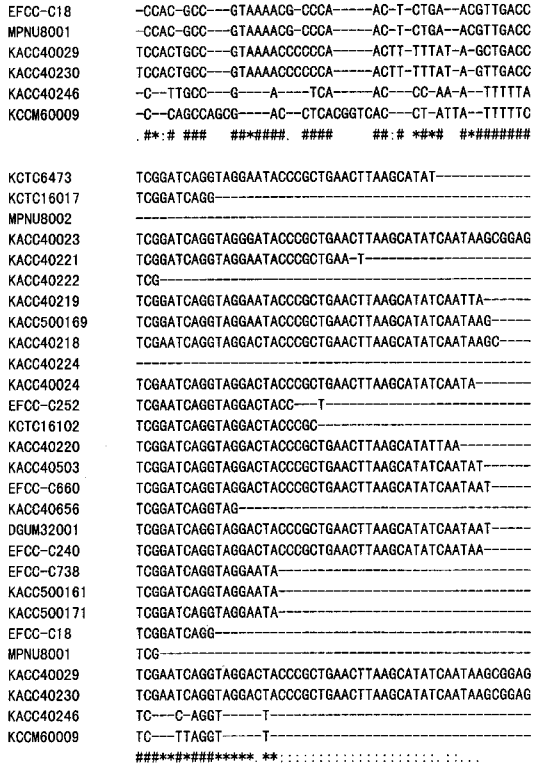

Fig. 2. Continued.

The genus *Paecilomyces* has more than 60% of G+C content except for unidentified strain *Paecilomyces* sp. KACC 40219 (56.0%) and KACC 40221 (56.8%), while the genus *Cordyceps* has below 57% of that through total ITS regions. The survey of G+C content will provide many mycologists to the basic information for the identification of *Paecilomyces* and *Cordyceps*.

The shortest size of ITS1-5.8S-ITS2 was 473 nucleotides in *C. sinensis* species and the longest one was 510 nucleotides in *C. nutans* KACC 500169, among 28 strains sequenced in this study (Table 2). The size of the 5.8S rRNA gene was identical (157 nucleotides) except for 156 nucleotides in *C. scarabaeicola* EFCC-C252, *P. variotii* KACC 40246, *H.* thompsonii) and 158 nucleotides in *Paecilomyces* sp. KACC 40222 among all the tested strains. Most of the tested strains could be divided into three groups, based on the ITS length. One group with short ITS sequence includes *C. militaris* MPNU 8001, EFCC-C18, EFCC-C738, KACC 500161, and KACC500171, *C. scarabaeicola* EFCC-C252, and *C. ophioglossoides* KCTC 16017 and KCTC 6473, and *C. sinensis* MPNU 8002 and *B. bassiana* KACC 40024 and KACC 40218. The other group with long ITS includes *I. japonica* DGUM 32001, *P. tenuipes* EFCC-C240, *P. tenuipes* EFCC-C660, *P.*

Table 2. G+C content and nucleotide of the ITS1, ITS2, and 5.8S rDNA sequences

Fungal strain	ITS1		5.8S rDNA		ITS2		Total	
	GC content (%)	Length (nt)	GC content (%)	Length (nt)	GC content (%)	Length (nt)	GC content (%)	Length (nt)
C. militaris MPNU 8001	55.0	161	45.6	157	64.8	164	56.8	482
C. militaris EFCC-C18	55.0	161	46.6	157	64.8	164	56.8	482
C. militaris EFCC-C738	55.0	161	45.6	157	64.2	164	56.6	482
C. militaris KACC 500161	55.0	161	45.6	157	64.2	164	56.6	482
C. militaris KACC 500171	55.0	161	45.6	157	64.2	164	56.6	482
C. scarabaeicola EFCC-C252	52.0	164	46.4	156	60.9	165	54.6	485
C. ophioglossoides KCTC 16017	52.1	163	45.0	157	65.2	165	55.7	485
C. ophioglossoides KCTC 6473	53.9	163	45.0	157	64.9	164	56.2	484
C. nutans KACC 500169	54.0	186	45.0	157	60.1	167	54.7	510
C. sinensis MPNU 8002	56.3	160	47.1	157	66.7	156	56.7	473
I. japonica DGUM 32001	61.5	183	46.2	157	68.1	162	60.4	502
P. tenuipes EFCC-C240	61.5	183	46.2	157	68.1	162	60.4	502
P. tenuipes EFCC-C660	61.5	183	46.2	157	68.1	162	60.4	502
Paecilomyces sp. KACC 40219	57.1	180	45.0	157	60.9	165	56.0	502
Paecilomyces sp. KACC 40220	61.5	183	46.2	157	68.1	162	60.4	502
Paecilomyces sp. KACC 40221	59.1	181	47.1	157	63.4	164	56.8	502
Paecilomyces sp. KACC 40222	63.9	183	48.7	158	71.3	164	61.6	505
Paecilomyces sp. KACC 40503	61.5	183	46.2	157	68.1	162	60.4	502
Paecilomyces sp. KACC 40656	61.5	183	46.2	157	68.1	162	60.4	502
P. farinosus KCTC 16102	61.3	182	46.2	157	68.1	162	60.3	501
P. variotii KACC 40246	57.4	176	49.4	156	66.7	168	58.0	500
P. variotii KCCM 60009	65.1	186	49.7	157	66.9	181	61.1	524
B. bassiana KACC 40024	56.2	162	49.0	157	66.7	165	57.4	484
B. bassiana KACC 40218	56.2	162	48.4	157	66.1	165	57.0	484
B. bassiana KACC 40224	56.2	162	48.4	157	70.0	45	54.5	364
M. anisopliae KACC 40029	43.7	135	45.9	157	59.0	178	50.3	555
Metarhizium sp. KACC 40230	44.1	136	45.9	157	59.0	178	50.3	555
H. thompsonii KACC 40023	61.7	149	44.9	156	67.1	207	58.8	512

japonica KACC 40503, *Paecilomyces* sp. KACC 40219, KACC 40220, KACC 40221, KACC 40222, KACC 40503 and KACC 40656, and *P. farinosus* KCTC 16102. *Cordyceps nutans* KACC 500169, *H. thompsonii* KACC 40023, *Metarhizium* sp. KACC 40230 and *M. anisopliae* KACC 40029 which have the longest nucleotide on the total ITS regions would be the third group. The lengths of ITS1-5.8S-ITS2 of the shortest group were between 472 to 485 nucleotides, and those of long group were from 501 to 505 nucleotides. The longest group was from 510 to 555 nucleotides. The nucleotide length of the *Paecilomyces* species was longer than those of the *Cordyceps* species.

Phylogenetic analysis of ITS1, ITS2 and the 5.8S rRNA sequences. The morphological traits are subject to environmental influences and can vary substantially from culture to culture (Seady, 1996). Thus, taxonomic considerations, based solely on phenotype, may be subject to ambiguities induced by environmental conditions. Combining molecular and morphological data sets has been discussed by Doyle (Doyle, 1992). Previously, the taxonomy and identification of fungi have been based mainly on morphological characteristics. However, recent molecular techniques have been introduced to provide more objective criteria.

The nucleotide sequence data set obtained from the taxa in Table 1 gave a 510-nucleotide aligned sequences including many ambiguously aligned sites due to the variable nucleotide sequence of the ITS regions (Fig. 2). However, since some conserved sites were found in the ITS regions, the conserved sites of the ITS regions and the 5.8S rRNA gene were used for current analysis. The consensus tree generated by the Neighbor-joining methods of 1,000 bootstrap replications of this data set had an identical topology to that of the most phylogenetic tree and implied that the majority of the branches were favoured at a high level of confidence.

The genus *Cordyceps* and *Paecilomyces* investigated in our study were clearly divided into five groups in the phylogenetic tree based on the ITS1, 2 and 5.8S rDNA sequences (Fig. 3); groups I-V.

The group I included *Paecilomyces* sp. KACC 40220, KACC 40222, KACC 40656, *P. japonica* KACC 40503, *P. tenuipes* EFCC-C660, EFCC-C240, *Isaria japonica* DGUM 32001, and *P. farinosus* KCTC 16102. *Paecilomyces tenuipes*, sometimes referred as *Isaria japonica*, *Paecilomyces japonica*, or other synonyms are known as the telemorph of *C. takaomontana* (Takema *et al.*, 1997). Two *Paecilomyces* sp. KACC 40656 and KACC 40220 strains from KACC(Korean Agricultural Culture Collection) are identified as the same species with *P. tenuipes* because the sequence of the ITS regions are identical. Also, *I. japonica* DGUM 32001, the anamorph of *C. takaomontana* by Shimizu (Shimizu, 1994) and a parasite on pupae of Lepidoptera insects, was used to investigate

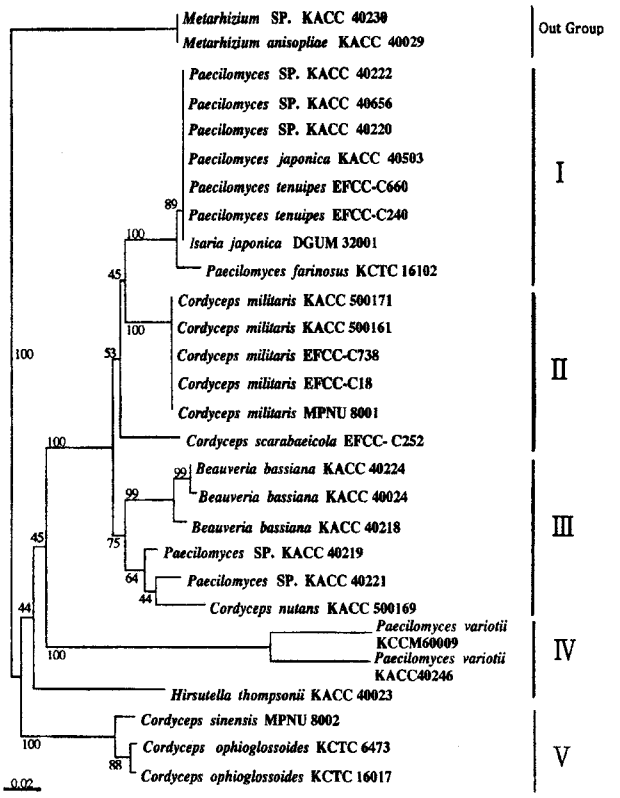

Fig. 3. Phylogenetic tree showing the relationship among the genus *Cordyceps* and *Paecilomyces* related taxa. *Metarhizium* sp. and *Metarhizium anisopliae* were used as outgroup taxa. Bar represented 2 nucleotide substitutions per 1000 nucleotides in ITS1, 2 and 5.8S rDNA sequences. Bootstrap probabilities were indicated at the corresponding branch points.

cultural characteristics under different conditions (Ban *et al.*, 1998). It was reported that the anamorphs of the *Cordyceps* species belong to hyphomycetous genera including *Akanthomyce*, *Beauveria*, *Desmidiospra*, *Hirsutella*, *Hymenostilbe*, *Mariannaea*, *Metarhizium*, *Nomuraea*, *Paraisaria*, *Paecilomyces*, *Tolypocladium*, and *Verticillium* (Evans and Samson, 1984; Gams, 1982; Hodge *et al.*, 1998; Liang, 1991; Main, 1958; Samson, 1974; Samson and Brady, 1983; Shimazu *et al.*, 1988). *Mycoparasites* growing on *Cordyceps* stromata have sometimes been mistaken for anamorphs. This seems to have been the case for putative anamorphs recorded as *Stilbella* species by Kobayasi (Kobayasi, 1941). Many of these have been recognized as species of polycephalomyces by Seifert (Seifert, 1985).

The DNA similarity between ITS1 and ITS2 is shown in Table 3. The standard strains of *P. japonica* and *I. japonica* need to be called as *P. tenuipes* because they have the same similarity value of 100%. *Paecilomyces* sp. KACC 40220 and *Paecilomyces* sp. KACC 40656 are classified as *P. tenuipes* based on Table 3 and Fig. 3, because they had 100% similarity value. Besides, *P. fari-*

Table 3. DNA similarity matrix (excluded gaps) for ITS-5.8S rDNA-ITS2 sequences of the genus *Cordyceps*, *Paecilomyces*, *Beauveria*, *Mearhium* and *Hirsutella*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1																												
2	90.0																											
3	90.3	99.8																										
4	90.0	100	99.8																									
5	90.3	99.8	100	99.8																								
6	90.3	99.8	100	99.8	100																							
7	80.3	76.9	76.7	76.8	76.7	76.7																						
8	82.3	79.0	78.8	79.0	78.8	78.8	99.0																					
9	93.2	89.8	90.0	89.8	90.0	90.0	82.2	81.8																				
10	76.7	79.8	79.5	79.8	79.5	79.5	94.7	95.0	79.2																			
11	88.4	88.5	88.5	88.5	88.5	88.5	76.1	74.8	87.9	75.8																		
12	88.4	88.5	88.5	88.5	88.5	88.5	76.1	74.8	87.9	75.8	100																	
13	88.4	88.5	88.5	88.5	88.5	88.5	76.1	74.8	87.9	75.8	100	100																
14	88.4	88.5	88.5	88.5	88.5	88.5	76.1	74.8	87.9	75.8	100	100	100															
15	89.6	87.9	87.9	87.9	87.9	87.9	76.7	74.1	88.6	75.6	99.8	99.8	99.8	99.8														
16	94.8	88.4	88.6	88.4	88.6	88.6	82.7	80.0	94.4	78.1	87.8	87.8	87.8	87.8	88.4													
17	88.4	88.5	88.5	88.5	88.5	88.5	76.1	74.8	87.9	75.8	100	100	100	100	99.8	87.8												
18	88.4	88.5	88.5	88.5	88.5	88.5	76.1	74.8	87.9	75.8	100	100	100	100	99.8	87.8	87.8											
19	93.3	89.0	89.2	89.0	89.2	89.2	79.3	79.1	91.1	77.7	89.3	89.3	89.3	89.3	89.0	99.8	89.3	89.3										
20	87.2	86.1	86.1	86.1	86.1	86.1	73.9	74.5	85.8	75.4	99.4	99.4	99.4	99.4	99.0	88.8	99.4	99.4	88.7									
21	65.2	66.0	66.3	66.0	66.3	66.3	67.4	67.4	65.1	68.2	65.4	65.4	65.4	65.4	65.4	67.1	65.4	65.4	67.3	65.4								
22	64.4	65.6	65.8	65.6	65.8	65.8	64.9	65.9	64.6	66.2	66.0	66.0	66.0	66.0	65.3	66.7	66.0	66.0	66.1	68.0	85.8							
23	85.8	91.0	91.0	91.0	91.0	91.0	83.2	83.4	94.5	82.7	85.7	85.7	85.7	85.7	85.4	89.6	85.7	85.7	89.5	85.2	69.2	70.9						
24	87.3	90.3	90.6	90.3	90.6	90.6	80.3	80.4	94.7	80.2	86.5	86.5	86.5	86.5	86.3	89.7	86.5	86.5	89.5	86.0	64.6	64.8	99.7					
25	87.6	90.8	91.0	90.8	91.0	91.0	80.7	80.8	95.1	80.7	86.5	86.5	86.5	86.5	86.3	90.1	86.5	86.5	89.9	86.8	65.1	64.5	100	99.6				
26	76.7	74.3	74.5	74.3	74.5	74.5	75.1	75.0	75.8	75.5	75.8	75.8	75.8	75.8	75.5	76.5	75.8	75.8	76.2	75.1	68.1	65.6	82.2	76.3	76.3			
27	77.4	74.3	74.5	74.3	74.5	74.5	75.5	75.1	73.5	75.5	75.8	75.8	75.8	75.8	75.6	76.3	75.8	75.8	76.1	75.4	68.2	64.9	82.2	77.0	77.0	99.2		
28	75.9	76.9	76.9	76.9	76.9	76.9	83.4	83.5	76.6	81.3	75.3	75.3	75.3	75.3	75.0	74.6	75.3	75.3	74.8	74.7	70.3	67.9	83.0	78.7	78.7	76.4	76.4	

The designations of number are 1. *C. nutans* KACC 500169; 2. *C. militaris* EFCC-C18; 3. *C. militaris* EFCC-C738; 4. *C. militaris* MPNU 8001; 5. *C. militaris* KACC 500161; 6. *C. militaris* KACC 500171; 7. *C. ophioglossoides* KCTC 16017; 8. *C. ophioglossoides* KCTC 6473; 9. *C. scarabaeicola* EFCC-C252; 10. *C. sinensis* MPNU 8002; 11. *I. japonica* DGUM 32001; 12. *P. tenuipes* EFCC-C240; 13. *P. tenuipes* EFCC-C660; 14. *P. japonica* KACC 40503; 15. *P. farinosus* KCTC 16102; 16. *Paecilomyces* sp. KACC 40219; 17. *Paecilomyces* sp. KACC 40220; 18. *Paecilomyces* sp. KACC 40656; 19. *Paecilomyces* sp. KACC 40221; 20. *Paecilomyces* sp. KACC 40222; 21. *P. variotii* KACC 40246; 22. *P. variotii* KCCM 60009; 23. *B. bassiana* KACC 40224; 24. *B. bassiana* KACC 40024; 25. *B. bassiana* KACC 40218; 26. *M. anisopliae* KACC 40029; 27. *Mearhizium* sp. KACC 40230; 28. *H. thompsonii* KACC 40023.

nosus KCTC 16102 shows almost similar results in similarity value.

The group II includes *C. militaris* KACC 500171, KACC 500161, EFCC-C738, EFCC-C18, MPNU 8001 and *C. scarabaeicola* EFCC-C252. KACC 40219. As shown in Fig. 3, not only *C. militaris* MPNU 8001 is similar to KACC 500171, KACC 500161 and EFCC-C738 in similarity value but also it has almost same similarity to MPNU 8001 and EFCC-C18. *Cordyceps scarabaeicola* EFCC-C252 had high similarity of 90% compared with the sequences of the *C. militaris* species.

Sung *et al.* reported that *C. scarabaeicola* is the anamorph of *Beauveria* genus based on the morphological characteristics (Sung *et al.*, 1997). Therefore, our result will represent the possibility of the related genus having the similar morphology except anamorph and telemorph.

Beauveria bassiana (anamorph of *Cordyceps* species) also grouped as a same cluster with *Paecilomyces* sp. (KACC 40219, KACC 40221) and *C. nutans* (KACC 500169). However, the species has different sequences with *Paecilomyces* species.

The group IV included *Paecilomyces variotii* (KCCM 6009, KACC 40246) and *Hirutella thompsonii* (KACC 40023). *Paecilomyces variotii* is closely related to other filamentous surface molds such as species of *Penicillium*, *Aspergillus*, and *Trichoderma*. Some of these organisms can inhibit the growth of pathogenic fungi and have been evaluated as biocontrol agents in integrated control systems with chemical preservatives for wood preservation (Ohr *et al.*, 1973). The group V had high bootstrap value compared to the group I and group II. *Cordyceps ophioglossoides* and *C. sinensis* form this group. It is known that *C. sinensis* had the similar immune system with three groups but is differently classified from our analysis.

Conclusion

The nucleotide sequences of ITS regions including the 5.8S rRNA coding gene were obtained from 28 strains of the genus *Cordyceps, Paecilomyces, Beauveria, Metarhizium* and *Hirsutella.* The nucleotide length of these regions varied from 482 bp to 555 bp depending on the taxa. The investigated taxa were divided into two large groups based on the ITS length, i.e., a long-ITS group and a short-ITS group. The phylogenetic tree constructed from the nucleotide sequences supported this grouping. This grouping was somewhat similar to the grouping based on morphological characteristics.

The variable spacer regions of rDNA have been considered to be useful for phylogenetic analysis of closely related genera, interspecies or intraspecies (Carmean *et al.*, 1992). This was also true of the genus *Cordyceps*, and the obtained sequences were sometimes difficult to align among distantly related taxa. However, some conserved sequences were found in the spacer regions, which allowed the phylogenetic analysis of the genus *Cordyceps* using the conserved sequences of the spacers and the coding regions. The separate analyses from ITS1 and ITS2 data set produced nearly similar trees topologically. It is convinced that *I. japonica, P. japonica,* and *P. tenuipes* are the same species and called as another names. The genus *Paecilomyces* has more than 60% of G+C content except unidentified *Paecilomyces* sp. KACC 40219, KACC 40221, while the genus *Cordyceps* has below 57% of that on total ITS regions. The length of ITS1-5.8S-ITS2 of the shortest group was between 473 to 485 nucleotides, and that of long group was from 500 to 524 nucleotides. The longest size of *Cordyceps* genus was 510 nucleotides of *C. nutans* KACC 500169. The nucleotide length of *Paecilomyces* genus was longer than that of the *Cordyceps* genus. The genus *Cordyceps* and *Paecilomyces* investigated in our study were clearly divided into five groups in the phylogenetic tree based on the ITS1, 2 and 5.8S rDNA sequences (Fig. 3); group I-V group.

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References

Ban, K. W., Park, D. K., Shim, J. O., Lee, Y. S., Park, C. H., Lee, J. Y., Lee, T. S., Lee, S. S. and Lee, M. W. 1998. Cultural character is it for inducing fruiting body of *Isaria japonica. Kor. J. Mycol.* **26**: 380-386.

Berbee, M. L. and Taylor, J. W. 1993. Dating the evolutionary radiations of the true fungi. *Can. J. Bot.* **71**: 1114-1127.

Bibbo, M. L., Findieay, P. R. and Jonson, M. W. 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequence. *Gene* **30**: 157-166.

Bok, J. W., Ller, J. C., Klin, H. G. and Neil, G. H. 1999. Antitumor sterols from the mycelia of *Cordyceps sinensis. Phytochemistry* **51**: 891-898.

Bruns, T. D., White, T. J. and Taylor, J. W. 1991. Fungal molecular systematics. *Annual Rev. Ecol. Syst.* **22**: 525-564.

Carmean, D., Kimsey, L. S. and Berbee, M. L. 1992. 18S rDNA sequences and the holometabolous insects. *Mol. Phylogenet. Evol.* **1**: 270-278.

Choi, I. Y., Choi, J. S. and Lee, W. H. 1999. The production of artificial fruiting body of *Paecilomyces japonica. Kor. J. Mycol.* **27**: 87-93.

Doyle, J. J. 1992. Gene trees and species trees: Molecular systematics as one-character taxonomy. *Syst Bot.* **17**: 144-163.

Evans, H. C. and Samson, R. A. 1982. *Cordyceps* species and their anamorphs pathogenic on ants (Formicidae) in tropical forest ecosystems 1. The Cephalotes (Myrmicinae) complex. *Trans. Br. Mycol. Soc.* **79**(3): 431-453.

_____ and _____, 1984. *Cordyceps* species and their anamorphs pathogenic on ants (Formicidae) in tropical forest ecosystems II. the *Camponotus* (Formicinae) complex. *Trans. Brit. Mycol. Soc.* **82**: 127-150.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783-791.

Fukatsu, T., Sato, H. and Kuriyama, H. 1997. Isolation, inoculation to insect host, and molecular phylogeny of an entomogenous fungus *Paecilomyces tenuipes*. *J. Invertebr, Pathol.* **70**: 203-208.

Gams, W. 1982. Generic names for synanamorphs? *Mycotaxon* **15**: 459-464.

Gyllensten, U. B. 1989. Direct sequencing of enzymatically amplified DNA. pp. 45-60. *In* Erlich, H. A., ed. *PCR technology.* Principles and Applications for DNA Amplification. Stockton Press, New York.

Hiraishi, A. 1992. Direct automated sequencing of 16S rDNA by polymerase chain reaction from bacterial cultures without DNA purification. *Lett. Appl. Mcirobiol.* **15**: 210-213.

Hirata. T. and Takamatsu, S. 1996. Nucleotide sequence diversity of rDNA internal transcribed spacers extracted from conidia and cleistothecia of several powdery mildew fungi. *Mycoscience* **37**: 265-270.

Hodge. K. T., Humber, R. A. and Wozniak, C. A. 1998. *Cordyceps* variabilis and the genus synglіocladium. *Mycologia* **90**: 743-753.

_____, Krasnoff, S. B. and Humber, R. A. 1996. *Tolypocladium inflatum* is the anamorph of *Cordyceps subsessilis*. *Mycologia* **88**: 715-719.

Hywell-Jones, N. 1995. *Cordyceps sphecocephala* and a *Hymenostilbe* sp. infecting wasps and bees in Thailand. *Mycol. Res.* **99**: 154-158.

Kim, G. Y., Ha, M. G., Lee, T. H. and Lee, J. D. 1999. Chemosystematics and molecular phylogeny of a new bioflocculant-producing *Aspergillus* stain Isolated from Korean soil. *J. Microbiol. Biotechnol.* **9**: 870-872.

Kinjo, N., Kaizu, Y., Taketomo, N. and Tsunoo A. 1996. Physiological activities of the extracts from cultured mycelia of *Cordyceps militaris* (Vnill.) Fr. *Gen. Educ. Tokyo Med. Dent. Univ.* **26**: 7-14.

Kobayasi, Y. 1941. The genus *Cordyceps* and its allies. *Sci. Rep. Tokyo Bunrika Daigaku, Sect.* **B5**: 53-260

_____. 1982. Keys to the taxa of the genera *Cordyceps* and *Torrubiella*. *Trans. Mycol. Soc. Japan.* **23**: 329-369.

_____ and Shimiza, D. 1983. Iconography of vegetable Wasps and plant worms. Hoikusha Publishing Company Ltd. Osaka, P280.

Liang, Z.-Q. 1991. Verification and identification of the anamorph of *Cordyceps* in northwest area. *Acta Mycol. sin.* **10**: 104-107

_____, Liu, A.-Y. and Liu, J.-L. 1991. A new species of the genus *Cordyceps* and its *Metarhizium anamorph*. *ActaMycol. Mycol. Sin.* **10**: 257-262.

Main, E. B. 1958. North American entomogenous species of *Cordyceps*. *Mycologia* **50**: 169-222.

Nagahama, T., Sato, H., Shimiza, M. and Sugiyama, J. 1995. Phylogenetic divergence of the entomophthoralean fungi: evidence from nuclear 18S ribosomal RNA gene sequences. *Mycologia* **87**: 203-209.

Nikoh, N. and Fukatsu, T. 2000. Interkindom Host Jumping Underground; Phylogenetic Analysis of Entomoparasitic Fungi of the Genus *Cordyceps*. *Molecular biology and Evolution* **17**: 629-638

Ohr, H. D., Munnecke, D. E. and Bricker, J. L. The interaction of *Armillaria mellea* and *Trichoderma* spp. as modified by methyl bromide. *Phytopathology* 1973, **63**: 965-973.

Papierok, B. 1982. Les champignons se develop-pant en cote-d'ivoire sur la fourmi Paltothyreus tarastus F. Relation entre l'hyphomycetes *Tilachlidiopsis catenulata* sp. Nov. et l'ascomycete *Cordyceps myrmecophila* Cesati 1846, *Mycotaxon* **14**: 351-368.

Raue H. A. and Planta, R. J. 1995. The pathway to maturity: processing of ribosomal RNA in *Saccharomyces cerevisiae*. *Gene Expression* **5**: 71-77.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Bio. Evol.* **24**: 189-204.

Samson, R. A. 1974. *Paecilomyces* and some allied hyphomycetes. *Stud. Mycol.* **6**: 1-119.

_____ and Brady, B. L. 1983. *Paraisaria,* a new genus for *Isaria dubia*, the anamorph of *Cordyceps gracilis*. *Trans. Brit. Mycol. Soc.* **81**: 285-290.

Sasamu T., Hirata, T. and Sato, Y. 1998. Phylogenetic analysis and predicted secondary structures of the rDNA internal transcribed spacers of the powdery mildew fungi (Erysiphaceae), *Mycoscience* **39**: 441-453.

Seady, D. A. 1996. Differentiation of *Trichoerma* taxa associated with mushroom production. *Plant Pathology* **45**: 905-912.

Seifert, K. A. 1985. A monograph of stilbella and some allied hyphomycetes. *Stud. Mycol.* **27**: 1-235.

Shimazu, M., Mitsuhashi, W. and Hashimoto, H. 1988. *Cordyceps brongniartii* sp. nov., the teleomorph of *Beauveria brongniartii*. *Trans. Mycol. Soc. Japan* **29**: 323-330.

Shimizu, D. 1994. Color iconography of vegetable wasps and plant warms. P. 226. Seibundo Sinkosha, Tokyo.

Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Conner, C. R., Heiner, C., Kent, S. B. H. and Hood, L. E. 1986. Fluorescence detection in automated DNA sequence analysis. *Nature* **321**: 674-679.

Suh, S. O. and Sugiyama, J. 1994. Phylogenetic placement of the basidomycetous yeasts *Kondoa malvihella* and *Rhodosporidium dacryoidum* and the anamorphic yeast *Sympodiomycopsis paphiopedili* by means of 18S rRNA gene sequence analysis. *Mycoscience* **35**: 367-375.

Sung, J. M., Kim, C. H., Yang, K. J., Lee, H. K. and Kim, Y. S. 1993. Studies on distribution and utilization of *Cordyceps militaris* and *C. nutans*. *Kor. J. Mycol.* **21**: 94-105.

_____, Lee, H. K., Choi, Y. S., Kim, Y. Y., Kim, S. H. and Sung, G. H. 1997. Distribution and taxonomy of entomopathogenic fungal species from Korea. *Kor. J. Mycol.* **25**: 239-252.

_____, Choi, Y. S., Kim, Y. O., Kim, S. H. and Sung, G. H. 1997. *Cordyceps* species collected by Korean entomopathogenic fungal collection. *Kor. J. Mycol.* **21**: 49-60.

Takema, F., Sato, H. and Kuviyama, H. 1997. Isolation, inoculation to insect host and molecular phylogeny of an entomogenous fungus *Paecilomyces tenuipes*. *Inverterate Pathology* **70**: 203-208.

Thomson, J. D., Higgins, D. G. and Gibson, T. J. 1994. Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res.* **22**: 4673-4680.

Torres, R. A., Ganal, M. and Hemleben, V. 1990. GC balance in

the internal transcribed spacers ITS1 and ITS2 of nuclear ribosomal RNA genens. *J. Mol. Evol.* **30**: 170-181.

Tsunoo, A., Taketomo, N., Tsuboi, H., Kamijo, M., Nemoto, A., Sasaki, H., Vchida, M., Yamashita, H., Kinjo, N. and Huang, N. H. 1995. *Cordyceps sinensis*; its diverse effects on mammals in vitro and in vivo. *The 3rd Inter. Sym. Mycol. Soc. Japan. Chiba, Japan*, November 30-December1, pp. 1-10.

White, T., Burns, J., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 482. *In* Innis, M. A. D. H. Gelfand, J. J. Sinsky, and T. J. White. (eds.), *PCR protocols: a guide to methods and applications.* Academic Press, San Diego, California.

Yamanaka, K. 1998, Cultivation Characteristics of *Isaria japonica*. *Mycoscience* **39**: 43-48.

Zuker, M. 1989. On finding all suboptimal folding of an RNA molecule. *Science* **244**: 48-52.