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The determination of the partial 18 S ribosomal DNA sequences of *Cordyceps* species

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Y. ITO AND T. HIRANO. 1997. *Cordyceps* species, which are used in Chinese traditional medicines, are fungal parasites of insects. In this study the partial nucleotide sequences of 18 S ribosomal DNA from four *Cordyceps* species were determined and compared with the sequences of published ascomycetes. The sequence data support the concept that *Cordyceps* species belong to the pyrenomycetes. Based on sequence data the phylogenetic tree was constructed using the neighbor-joining (NJ) method. Diversity in the phylogenetic tree was found for *Cordyceps* species. A new classification of *Cordyceps* species can be constructed based on the phylogenetic information obtained from such rDNA sequences.

INTRODUCTION

Cordyceps species are parasitic fungi belonging to the Hypocreales of the ascomycetes. They infect the larva or imago of insects, kill them, and then form a fruit body on the insect. They generally show a high level of host specificity, i.e. a species that infects a cicada does not infect other types of insects. Some *Cordyceps* species are used in Chinese traditional medicine in Japan and China. Recently it has been reported that *Cordyceps* species could produce many kinds of bioactive compounds (Kneifel *et al*. 1977 ; Furuya *et al*. 1983 ; Fujita *et al*. 1994). About 400 species of *Cordyceps* are known, and they are classified by colour and shape of fruit body or spore, shape of ascus, and kind of host insect (Shimizu 1994).

Among bacteria and eukaryotes, the comparison of rRNA (rDNA) sequences is the most useful method for deducing phylogenetic relationships (Woese 1987 ; Gutell 1993 ; Wilmotte *et al*. 1993). For *Cordyceps* this method has still not been applied and their phylogenetic relationship with other fungi is not known. For the purpose of analysing the DNA sequence, it is necessary to amplify it. Therefore the polymerase chain reaction (PCR) is the most useful method because it can amplify the particular DNA sequence region by use of a pair of primers (Medlin *et al*. 1988 ; Boettger 1989 ; Edwards *et al*. 1989). Since Ito and Hirano (1996) recently designed new primers for amplification of the DNA from *Cordyceps* species and could amplify nearly the entire 18 S rDNA, in this study the partial 18 S rDNA sequences of four species were determined to infer their phylogenetic relationship with other ascomycetous fungi.

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MATERIALS AND METHODS

Fungal strains

Isaria japonica Yasuda (teleomorph, *Cordyceps takaomontana* Yakushiji et Kumazawa), *Hymenostilbe odonatae* Y. Kobayasi (teleomorph, *Cordyceps odonatae* Kobayasi), and *Cordyceps tuberculata* (Leb.) Maire f.moelleri (Henn.) Y. Kobayasi were obtained from insects captured in Ibaraki Prefecture in Japan. *Cordyceps sinensis* (Berk.) Sacc. was purchased as dried material at a Chinese drug store in Tokyo.

DNA preparation

DNA was isolated from 100 mg of the fruit body of the fungi by use of an ISOPLANT DNA extraction kit (NIPPON GENE Co., Tokyo, Japan) based on the method of Jhingan (1992). The isolated DNA was suspended in 20 μl of TE buffer (10 mmol l^{-1} Tris hydrochloride, 1 mmol l^{-1} EDTA, pH 8·0).

PCR amplification

All 18 S rDNAs were amplified with newly designed primers (Ito and Hirano 1996). The condition of PCR amplification consisted of an initial denaturation step of 94°C for 5 min

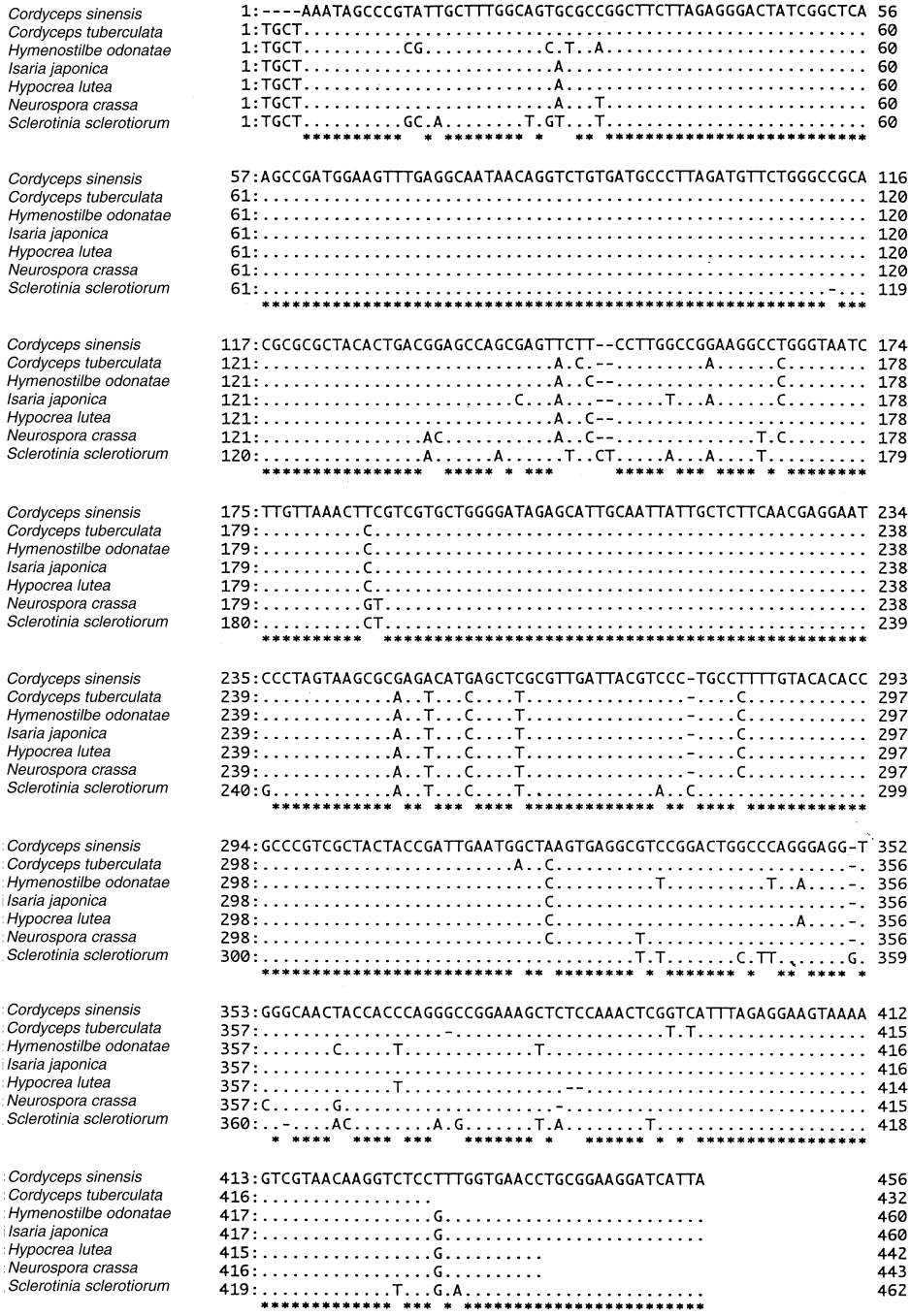

Fig. 1 Alignment of 18 S rDNA sequences starting from position 1337 for *Cordyceps*, *Isaria*, *Hymenostilbe*, *Neurospora*, *Hypocrea* and *Sclerotinia* species. The sequence of *C. sinensis* was used as a reference. The dots indicate nucleotides identical to the nucleotides in the reference species, and the dashes indicate deletions. The asterisk indicates identity among all species

Table 1 18 S rDNA sequence similarity among seven ascomycetes

	% rDNA sequences similarity						
Organism	1	2	3	4	5	6	7
1. *Cordyceps sinensis*	100						
2. *C. tuberculata*	95·7	100					
3. *Hymenostilbe odonatae*	94·3	96·2	100				
4. *Isaria japonica*	96·1	98·7	96·5	100			
5. *Neurospora crassa*	94·4	95·9	95·5	96·9	100		
6. *Hypocrea lutea*	95·8	97·0	97·1	98·0	96·7	100	
7. *Sclerotinia sclerotiorum*	90·1	90·2	91·2	91·4	91·3	90·0	100

followed by 30 cycles for 94°C for 0·5 min, 55°C for 0·5 min, and 72°C for 1·5 min, and a final extension step of 72°C for 10 min. The amplifications were performed in a Perkin–Elmer temperature controller. The amplification products were purified by agarose gel electrophoresis before sequencing.

Sequencing

The sequencing reaction was carried out with the cycle sequencing method described by Pharmacia LKB Biotechnology (Uppsala, Sweden). Two primers annealing to evolutionarily conserved areas were used to sequence both strands of approximately 460 bp regions that were located upstream from the 3′ end of 18 S rDNA. Sequence primer 1 was 5′GTTGGTGGAGTGATTTGTCTGC3′ (corresponding to the sequence at positions 1284–1306 in *Saccharomyces cerevisiae*); and sequence primer 2, 5′TAATGATC-CTTCCGCAGGTT3′ (corresponding to the sequence at positions 1766–1785 in *S. cerevisiae*). Sequences were analysed by use of an automated laser fluorescence DNA sequencer (ALF ; Pharmacia LKB Biotechnology AB).

Multiple alignments were carried out with the GENETYX ver.8 program (Software Development Co. Ltd, Tokyo, Japan) and Clustal W version 1.5 software (Thompson *et al.* 1994). The phylogenetic distances were calculated by the NJ method (Saitou and Nei 1987).

Other sequences used for phylogenetic tree construction were taken from the EMBL or the GenBank nucleotide sequence library. The EMBL accession number is J01353 for *S. cerevisiae* and X69850 for *Sclerotinia sclerotiorum*, and the GenBank accession number is U00IFOC for *Hypocrea lutea* and NCRRNAS for *Neurospora crassa*.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession numbers: D86053 for *C. sinensis*, D86054 for *C. tuberculata*, D86055 for *Hymenostilbe odonatae* and D86056 for *I. japonica*.

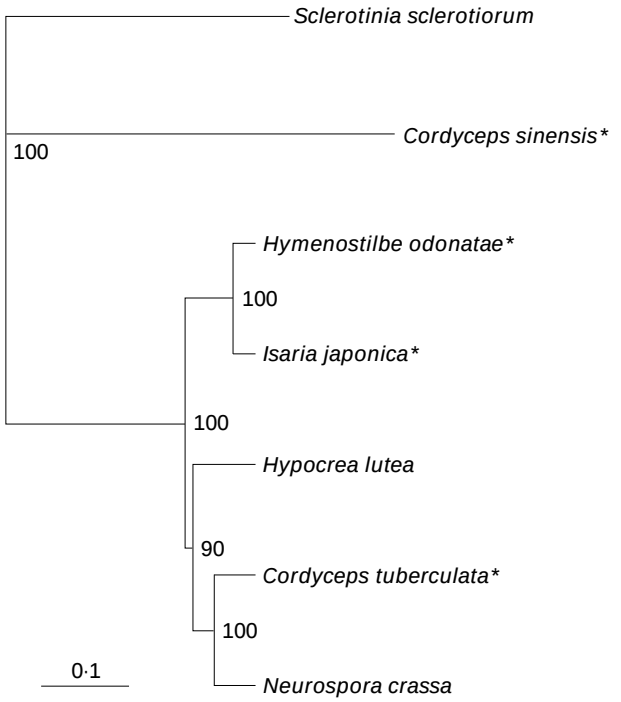

Fig. 2 Phylogenetic relatedness among the seven ascomycetous fungi assessed by the NJ method. The strains determined in the present study are indicated by an asterisk. Numbers that indicate bootstrap values based on 100 replications of these data are shown at the internal nodes. The distance that corresponds to 0·1 sequence divergence is indicated by the bar

RESULTS

The partial nucleotide sequences of the 18 S rDNA from four *Cordyceps* species were determined for the first time in this study. The region of the determined sequences contained the variable regions V7–9 corresponding to the nucleotide sequence of *S. cerevisiae*. It has been reported that the nucle-

otide sequences in the variable regions differ among fungi (Neefs *et al*. 1993). Aligned sequences including those of three other ascomycetous fungi, *N. crassa*, *H. lutea*, and *S. sclerotiorum*, are shown in Fig. 1. The sequence of *C. sinensis* was used as a reference. The seven sequences were very similar to each other. Alignment of the sequences from the seven fungi showed a similarity level of 90·0–98·7% (Table 1). The sequence of *Cordyceps* species showed a similarity of 94·4–97·0% with those of *N. crassa* and *H. lutea*.

The phylogenetic relationships among the seven ascomycetes are shown in Fig. 2. This phylogenetic tree was calculated by the NJ method. It was constructed based on three sequences obtained from the sequence databank and the four species in this study. The tree showed two clusters: *C. tuberculata*, *N. crassa* and *H. lutea*; and *Hymenostilbe odonatae* and *I. japonica*. This indicates that *C. tuberculata* is closely related to *N. crassa*, which belongs to Sordariales, and *H. lutea*, which belongs to Hypocreales. Further, the phylogenetic relationship between *Hymenostilbe odonatae* and *I. japonica* was close. These fungi are anamorphs of this *Cordyceps* genus (Shimizu 1994; Hawksworth *et al*. 1995). Thus it is confirmed that this *Cordyceps* species belongs to Hypocreales from the genetic analysis. Interestingly, *C. sinensis* was phylogenetically distant from the other *Cordyceps* species.

DISCUSSION

Cordyceps species display a diversity of morphological properties (Shimizu 1994), and the phylogenetic relationship among these species is unknown. The 18 S rDNA sequences of four of these species were determined and the phylogenetic tree constructed including three other ascomycetes fungi. The tree suggests that *Cordyceps* species are not a monophyletic group. Recently, it was reported that the extent of rRNA sequence diversity among the parasitic fungal species of *Metschnikowia* is large (Mendonça-Hagler *et al*. 1993), and it was suggested that the parasitic associations could have resulted in greater selective pressure to adapt to the host's immune response in highly specific niches. The present results support this speculation. *Cordyceps* species are known to be parasitic on many kinds of insects, and host selectivity is very severe. It is possible that a new classification of *Cordyceps* species could be constructed according to accumulated phylogenetic information obtained from rDNA sequences.

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