Evolutionary Dynamics of Multiple Group I Introns in Nuclear Ribosomal RNA Genes of Endoparasitic Fungi of the Genus *Cordyceps*

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A large number of group I introns were discovered in coding regions of small and large subunits of nuclear ribosomal RNA genes (SSU rDNA and LSU rDNA) in ascomycetous fungi of the genus *Cordyceps*. From 28 representatives of the genus, we identified in total 69 group I introns which were inserted at any of four specific sites in SSU rDNA and four specific sites in LSU rDNA. These group I introns reached sizes of up to 510 bp, occurred in up to eight sites in the same organism, and belonged to either subgroup IB3 or subgroup IC1 based on their sequence and structure. Introns inserted at the same site were closely related to each other among *Cordyceps* fungi, whereas introns inserted at different sites were phylogenetically distinct even in the same species. Mapped on the host phylogeny, the group I introns were generally not restricted to a particular lineage, but, rather, widely and sporadically distributed among distinct lineages. When the phylogenetic relationships of introns inserted at the same site were compared with the phylogeny of their hosts, the topologies were generally significantly congruent to each other. From these results, the evolutionary dynamics of multiple group I introns in *Cordyceps* fungi was inferred as follows: (1) most of the group I introns were already present at the eight sites in SSU and LSU rDNAs of the ancestor of the genus *Cordyceps*; (2) the introns have principally been immobile and vertically transmitted throughout speciation and diversification of *Cordyceps* fungi, which resulted in the phylogenetic congruence between the introns at the same site and their hosts; (3) in the course of vertical transmission, the introns have repeatedly been lost in a number of lineages independently, which has led to the present sporadic phylogenetic distribution of the introns; and (4) a few acquisitions of new introns, presumably through horizontal transmission, were identified in the evolutionary history of the genus *Cordyceps*, while no transpositions were detected. Losses of group I introns in SSU rDNA have occurred at least 27 times in the evolutionary course of the 28 *Cordyceps* members.

Introduction

Group I introns are characterized by conserved RNA secondary structures essential for splicing and are capable of self-splicing or splicing assisted by protein factors for excision (Cech 1988; Lambowitz and Belfort 1993; Saldanha et al. 1993). To date, group I introns have been identified in organellar and nuclear genomes of diverse organisms, including green algae, higher plants, and fungi, and in the genomes of some eubacteria and phages (Dujon 1989; Gargas, DePriest, and Taylor 1995). Phylogenetic distribution of group I introns is widespread and often sporadic, which strongly suggests that these sequences are mobile genetic elements capable of horizontal transmission between evolutionarily distinct lineages (Dujon 1989). In fact, several molecular mechanisms have been identified or postulated to be involved in the mobility of group I introns (for review, see Dujon 1989; Lambowitz and Belfort 1993; Belfort and Perlman 1995). Some group I introns contain open reading frames that encode endonucleases to mediate their sequence-specific transposition at the DNA level into homologous, intronless coding regions, a process called ''homing'' (Jacquier and Dujon 1985; Macreadie et al. 1985; Colleaux et al. 1986). Group I intron mobility may also occur at the RNA level from the reversal of the splicing reaction in which an excised intron recognizes a short 5′ flanking sequence in the coding region and inserts itself into the RNA. Reverse transcription of the intron-containing coding region followed by general recombination with the intronless genomic copy would result in the horizontal transfer of the group I intron (Woodson and Cech 1989; Roman and Woodson 1998). On the other hand, these acquisition processes are thought to be countered by the loss of group I introns in which reverse transcription of an intronless RNA is followed by general recombination with the intron-containing genomic copy of the coding region (Gargouri, Lazowska, and Slonimski 1983; Merlos-Lange et al. 1987).

To date, there has been no report on advantageous effects of group I introns on host organisms (see also Belfort 1990; Edgell, Fast, and Doolittle 1996). The superfluous insertion sequences in rDNAs, which are one of the most abundant genes in the genome, are likely to be neutral or slightly deleterious to the host's fitness. Therefore, group I introns are regarded as a selfish genetic element maintained on the basis of its capability to replicate and move within and across lineages of the host. If so, the widespread and sporadic distribution of group I introns must have been achieved through a dynamic equilibrium between gains due to their mobility and losses due to drift and/or selection. The following processes are thought to underlie the gain, maintenance, and loss of group I introns: (1) horizontal transmission, in which introns move between different species; (2) transposition, in which introns move to a different location in the host genome; (3) vertical transmission, in

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Table 1
List of Materials and DNA Sequence Accession Numbers Determined in this Study

Species	Origin	Collection Date	Collector	DDBJ/EMBL/GenBank Accession No.		
				Nuclear SSU rDNA	Nuclear 3′ SSU 5′ LSU rDNA	Nuclear 3′ LSU rDNA
Cordyceps pruinosa	Miyazu, Kyoto	July 20, 1997	T. Fukatsu	AB044629	AB044635	
Cordyceps sp. 3[a]	Miyazu, Kyoto	June 1, 1997	Y. Nakano	AB044630	AB044636	
Cordyceps takaomontana	Miyazu, Kyoto	August 27, 1997	Y. Nakano	AB044631	AB044637	
Cordyceps yakushimensis	Amami Island, Kagoshima	September 1, 1997	K. Fujimoto	AB044632	AB044643	
Paecilomyces sp.[b]	Miyazu, Kyoto	July 20, 1997	T. Fukatsu	AB044633	AB044644	
Tolypocladium inflatum	IFO31669			AB044634	AB044645	
Beauveria bassiana	IFO4848					AB044638
Cordyceps kanzashiana	Iriomote Island, Okinawa	April 21, 1994	D. Shimizu			AB044639
Coryceps prolifica	Saita, Tokushima	June 23, 1997	H. Manabe			AB044640
Cordyceps sp. 2[c]	Kanonji, Kagawa	June 23, 1997	H. Manabe			AB044641
Paecilomyces tenuipes	Miyazu, Kyoto	July 20, 1997	T. Fukatsu			AB044642

Note.—For all the materials and DNA sequences analyzed in this study, see also table 1 in Nikoh and Fukatsu (2000).

[a] This species is not yet described but is morphologically similar to *Cordyceps hepialidicola.*

[b] This species is not yet described but is illustrated in Shimizu (1994) under the Japanese name ''Mayudamatake'' with species no. 123.

[c] This species is not yet described but is morphologically similar to *Cordyceps pruinosa.*

which introns do not move and are stably maintained through generations of the host; (4) degeneration, in which maintained introns accumulate mutations, eventually become incapable of splicing, and finally degenerate into a junk sequence; and (5) loss, in which introns are excised out and lost. To explain the sporadic distribution patterns of mobile selfish genetic elements, a hypothesis has been proposed: the cyclical model of invasion, degeneration, and loss, followed by reinvasion (Hurst and McVean 1996; Goddard and Burt 1999). However, there has been only one study in which the evolutionary processes were phylogenetically reconstructed in detail and the frequency of the processes was quantitatively estimated (Goddard and Burt 1999).

In investigating the evolutionary dynamics of mobile genetic elements, the strategy of taxa sampling is very important. When comparison is made between distantly related taxa, it is expected that most evolutionary events will be untraceable and drastic horizontal transmission events will be preferentially detected. In contrast, when we analyze closely related taxa, few evolutionary events such as horizontal transmissions and losses may be detected. In this context, it would be ideal if we could examine a small and well-defined organismal group in which many group I introns exhibited a remarkable variety of insertion patterns.

An ascomycetous fungal genus, *Cordyceps* (family Clavicipitaceae), embraces some 300 described members which are exclusively endoparasitic to insects and other organisms (Kobayashi 1982; Samson, Evans, and Largé 1988; Spatafora and Blackwell 1993; Shimizu 1994; Nikoh and Fukatsu 2000). In the present study, we demonstrate that nuclear rDNAs of *Cordyceps* fungi contain a large number of group I introns. Surprisingly, as many as 69 group I introns were identified from SSU and LSU rDNAs of 28 representatives of the genus. The evolutionary dynamics of the multiple group I introns in the genus was investigated in detail.

Materials and Methods

Materials

Information on *Cordyceps* and related fungi examined in this study is presented in table 1. Most materials were collected in the field in Japan, while some were from the culture collection at the Institute for Fermentation, Osaka, Japan. In addition to the rDNA sequences listed in table 1, the following rDNA sequences of *Cordyceps* fungi reported in Nikoh and Fukatsu (2000) were subjected to the analyses in this study: *Cordyceps capitata* (AB027318, AB027364), *Cordyceps jezoensis* (AB027319, AB027365), *Cordyceps japonica* (AB027320, AB027366), *Cordyceps ophioglossoides* (AB027321, AB027367), *Cordyceps inegoensis* (AB027322, AB027368), *Cordyceps paradoxa* (AB027323, AB027369), *Cordyceps prolifica* (AB027324, AB027370), *Cordyceps kanzashiana* (AB027325, AB027371), *Cordyceps ramosopulvinata* (AB027326, AB027372), *Cordyceps heteropoda* (AB027327, AB027373), *Cordyceps sobolifera* (AB027328, AB027374), *Cordyceps* sp. 1 (AB027329, AB027375), *Cordyceps tricentri* (AB027330, AB027376), *Cordyceps cochlidiicola* (AB027331, AB027377), *Cordyceps* sp. 2 (AB027332, AB027378), *Cordyceps militaris* (AB027333, AB027379), *Paecilomyces tenuipes* (AB027334, AB027380), *Beauveria brongniartii* (AB027335, AB027381), *Beauveria bassiana* (AB027336, AB027382), *Metarhizium anisopliae* (AB027337, AB027383), *Cordyceps konnoana* (AB031192, AB031193), and *Cordyceps coccidiicola* (AB031195, AB031196). The entomoparasitic deuteromycetes *Beauveria bassiana, Beauveria brongniartii, Metarhizium anisopliae, Paecilomyces tenuipes, Paecilomyces* sp., and *Tolypocladium inflatum* are regarded as anamorphs of the *Cordyceps* spp. based on molecular phylogenetic and ecological lines of evidence (Shimazu, Mitsuhashi, and Hashimoto 1988; Liang, Liu, and Liu 1991; Hoge, Krasnoff, and Humber 1996; Fukatsu, Sato,

and Kuriyama 1997; Nikoh and Fukatsu 2000). *Hypomyces chrysospermus* (AB027339, AB027385) and *Hypocrea lutea* (AB027338, AB027384) were used as outgroup taxa.

Molecular Biological Procedures

The whole DNA of the fungi was extracted from the materials as previously described (Nikoh and Fukatsu 2000). A DNA segment containing almost the entire length of nuclear SSU rDNA was amplified by PCR using primers NS1 (5′-GTAGTCATATGCTTGTCTC-3′) (White et al. 1990) and FS2 (5′-TAGGNATTCCTC-GTTGAAGA-3′). A DNA segment containing the 3′ end of the nuclear SSU rDNA, ITS1, the 5.8S rDNA, ITS2, and the 5′ region of nuclear LSU rDNA was amplified using primers CFS2 (5′-TCTTCAACGAGG-AATNCCTA-3′) and NLB1 (5′-TTCGCTTTACCT-CATAAAACTGAG-3′). Since primers FS2 and CFS2 are complementary to each other, these two rDNA segments can be connected to give a long nuclear rDNA segment. In addition, a DNA segment containing the 3′ region of nuclear LSU rDNA was amplified using primers ILA1 (5′-GCCAGAAAGTGRTGTTGACGCAAT-3′) and ILB1 (5′-GRTRACATTCATCAGYAGGGT-AAA-3′). PCR was conducted using TaKaRa LA *Taq* DNA polymerase (TaKaRa) under a temperature profile of 94°C for 2 min, followed by 30 cycles of 98°C for 10 s, 55°C for 1 min, and 68°C for 4 min, and final extension at 60°C for 5 min. The amplified product was purified by the Geneclean II kit (BIO 101 Inc.) and cloned with pT7 Blue vector (Novagen) and *Escherichia coli* strain DH5α competent cells. Plasmids containing the PCR product were isolated using the QIAprep-Spin Miniprep kit (QIAGEN), subjected to dye-terminator labeled cycle sequencing reaction with amplifying and internal primers using the BigDye DNA sequencing kit (Perkin Elmer), and analyzed with an ABI PRISM 377 DNA sequencer (Perkin Elmer). Sequences of internal primers were as follows: ILA2, 5′-CGCGCATGAATG-GATTAACGA-3′; ILA3, 5′-CAGGTGGGGAGTTTGG-CTGGG-3′; ILA4, 5′-GTGGCRGCCAAGCGTTSATAG-CGA-3′; ILB2, 5′-AACGCTTACCGAATTCTGCTT-3′; ILB3, 5′-GGAGATTTCTGTTYTCCATGAGCC-3′; ILB4, 5′-MRKGYCTTCTTTCCCCGCTGATT-3′; ITS2, 5′-GCTGCGTTCTTCATCGATGC-3′; ITS4, 5′-TCCTCCGCTTATTGATATGC-3′; NS2, 5′-GGC-TGCTGGCACCAGACTTG-3′; NS2X, 5′-AGCTGG-AATTACCGCGGCTGCTGG-3′; NS2Y, 5′-CCA-GACTTGCCYTCYAATTRTTCC-3′; NSA1, 5′-AGCAGGCGCGCAAATTACCCAATC-3′; NSA2, 5′-AGAGTGCTCCAGGCAGGCYTATGC-3′; NSA3, 5′-AGTATGGTCGCAAGGCTGAAACTT-3′; NSA4, 5′-AAGCCGATGGAAGTTTGAGGCAAT-3′; NSAX1, 5′-CCTGCGGCTTAATTTGACTCAAC-3′; NSB3, 5′-CCAGAACATCTAAGGGCATCACAG-3′; NSB4, 5′-CCTGGTGGTGCCCTTCCGTCAATT-3′; NSB5, 5′-GCGGTCCTAGAAACCAACAAAATA-3′; NSBX1, 5′-GACCTGGTGAGTTTCCCCGTGTT-3′; NSBX2, 5′-ACCTGTTATTRCCTCAAACTTCC-3′; NSFI1, 5′-CTGTCAATCCTCATTGTGTCTGGC-3′; NLA1, 5′-CGGAGGAAAAGAAACCAACAGGAT-3′; NLA2, 5′-GAAACACGGACCAAGGAGTCGTC-3′; NLB2, 5′-ACCRKACGGGRYTYTYACCCTC-3′. Positions of the amplifying and internal primers are shown in figure 1. Determined nucleotide sequences were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases. Accession numbers are listed in table 1.

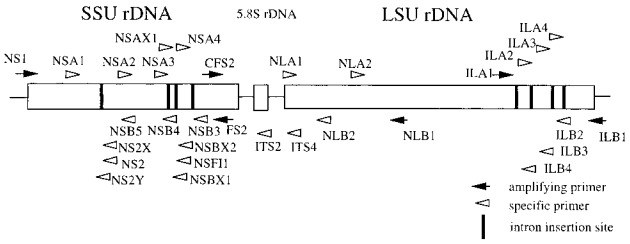

FIG. 1.—Diagrammatic representation of primers used in this study and insertion sites of group I introns on ribosomal RNA genes of *Cordyceps* fungi.

Molecular Phylogenetic Analyses

Multiple alignments were conducted according to the methods of Feng and Doolittle (1987) and Gotoh (1993). The final alignment was inspected and corrected manually. To align group I introns with certainty, we constructed a putative secondary structure of the introns (Michel and Westhof 1990) and considered the secondary structure in addition to the primary one. Ambiguously aligned nucleotide sites were excluded from the phylogenetic analysis. Nucleotide sites that included alignment gaps were also omitted from the aligned data set. Aligned sequence data are available on request from the corresponding author. Phylogenetic relationships were inferred by the neighbor-joining (NJ) method (Saitou and Nei 1987) with Kimura's (1980) two-parameter distance using the program package CLUSTAL W (Thompson, Higgins, and Gibson 1994), by the maximum-likelihood quartet puzzling (ML-PUZZLE) method using the program package PUZZLE (Strimmer and von Haeseler 1996), and by the maximum-likelihood (ML) method using the program package MOLPHY (Adachi and Hasegawa 1996). A bootstrap test (Felsenstein 1985) was conducted with 1,000 resamplings for NJ trees and 100 resamplings for ML-PUZZLE trees, respectively.

Statistical Evaluation of Congruence Between Phylogenetic Trees

To estimate the congruence between phylogenetic trees, Brooks parsimony analysis (Brooks 1981; Brooks and McLennan 1991) was performed using the program package MacClade, version 3.07 (Maddison and Maddison 1992). In this method, all terminal taxa and internal nodes of a phylogenetic tree were converted into a matrix of binary characters which represented the tree topology. Then, a test tree (in this study, an intron tree) was fitted to a binary-coded given tree (in this study, an rDNA tree) under the criteria of Wagner's parsimony.

The congruency index between the trees was calculated by (number of characters in binary-coded matrix for a given tree)/(total number of characters mapped on a test tree). To statistically evaluate the level of congruence, the observed congruency index was compared with the null distribution of the index. We generated 100,000 random bifurcate trees, fitted them to the matrix of a given (rDNA) tree, calculated congruency indices for the 100,000 trees, and obtained a null distribution of the index.

Results

Variable Lengths of Nuclear rDNA Sequences in the *Cordyceps* Fungi

In order to analyze the phylogenetic relationship between endoparasitic fungi of the genus *Cordyceps*, nuclear rDNA fragments from 28 representatives of *Cordyceps* and related fungi (table 1 and *Materials and Methods*) were amplified by PCR. Unexpectedly, the amplified products were not always of typical size but varied drastically in length between species.

SSU rDNA Segment

The size of the amplified fragment using primers NS1 and FS2 was expected to be approximately 1.5 kb. From 22 out of 28 species, however, longer PCR products (1.9–3.3 kb) were obtained.

5′ Region of LSU rDNA Segment

The size of the amplified product using primers CFS2 and NLB1 was expected to be around 1.7 kb. From all 28 species, PCR products of nearly the expected size were obtained.

3′ Region of LSU rDNA Segment

The size of the amplified fragment using primers ILA1 and ILB1 was expected to be approximately 0.8 kb. From 21 out of 28 species, longer PCR products (1.2–2.5 kb) were obtained.

Multiple Insertions Responsible for the Length Variety in the Nuclear rDNA Segments

We determined and analyzed the sequences of the SSU rDNA segment and the 5′ region of the LSU rDNA segment from six species of fungi (table 1) in addition to the sequences from 22 species described in Nikoh and Fukatsu (2000). The sequences of the 3′ region of the LSU rDNA segment from five representative species (table 1) in which evidently longer PCR products were identified were also determined. The lengths of the segments ranged from 1,520 to 3,300 bp in the SSU rDNA, from 1,630 to 1,781 bp in the 5′ region of LSU rDNA, and from 1,215 to 2,549 bp in the 3′ region of LSU rDNA.

Alignment of the sequences revealed that the remarkable length variety in the SSU rDNA and the 3′ region of the LSU rDNA segments was due to multiple insertion sequences in these regions.

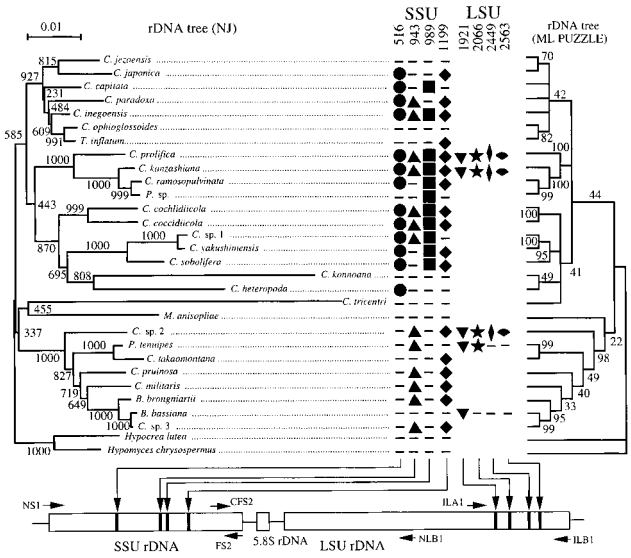

FIG. 2.—Phylogenetic relationship of *Cordyceps* fungi based on nuclear rDNA sequences, and insertion patterns of group I introns. A total of 3,013 unambiguously aligned nucleotide sites were subjected to molecular phylogenetic analysis. Shown at the left is a neighbor-joining tree; in the middle, the presence/absence of group I introns at eight sites in rDNAs; at the right, a maximum-likelihood quartet puzzling tree; at the bottom, insertion sites of group I introns. Eight different figures (circle, triangle, square, diamond, reverse triangle, star, thin diamond, fan) indicate the presence of group I introns at the positions, whereas dashes indicate intronless positions. The blanked positions were not sequenced. Bootstrap values obtained are shown at the nodes.

SSU rDNA Segment

In the SSU rDNA segment, four insertion sites were identified and named SSU516, SSU943, SSU989, and SSU1199, respectively, after the corresponding nucleotide sites in *E. coli* rRNA (Gutell 1993). Of 28 sequences determined, 13 were inserted at SSU516, 13 were inserted at SSU943, 11 were inserted at SSU989, and 17 were inserted at SSU1199 (fig. 2). When all these insertions were removed, the size of the resultant SSU rDNA segments became, as expected, around 1.5 kb. In *C. kanzashiana*, *C. prolifica*, *C. inegoensis*, *C. cochlidiicola*, and *C. coccidiicola*, all four sites were occupied by insertions. As a result, for example, the sizes of the SSU rDNA segment from *C. kanzashiana* and *C. prolifica* were 3,217 and 3,300 bp instead of the uninserted sizes 1,520 and 1,521 bp, respectively.

3′ Region of LSU rDNA Segment

In the 3′ region of the LSU rDNA segment, four insertion sites were identified and named LSU1921, LSU2066, LSU2449, and LSU2563, respectively. Out of five sequences determined, five were inserted at LSU1921, four were inserted at LSU2066, three were inserted at LSU2449, and three were inserted at LSU2563 (fig. 2). When all of these insertions were removed, the size of the resultant LSU rDNA segments was, as expected, around 0.8 kb. In *C. kanzashiana*, *C. prolifica*, and *Cordyceps* sp. 2, all four sites were occupied by insertions. As a result, for example, the sizes

of the LSU rDNA segments from *C. kanzashiana* and *C. prolifica* were 2,385 and 2,549 bp, respectively, instead of the uninserted sizes of 789 and 790 bp.

Phylogenetic Distribution of the Multiple Insertions

In order to demonstrate the evolutionary patterns of the insertions, we conducted molecular phylogenetic analysis of the *Cordyceps* fungi based on their rDNA sequences. The sequences of SSU rDNA, ITS1, 5.8S rDNA, ITS2, and the 5′ region of LSU rDNA, from which all of the insertion sequences were removed, were concatenated and subjected to the analysis (fig. 2). The NJ tree and the ML-PUZZLE tree showed a good overall agreement in their topology, although minor discrepancies were found where statistical supports of the groupings were weak. As previously reported (Nikoh and Fukatsu 2000), in the *Cordyceps*, several well-supported monophyletic groups were identified, such as cicada clade A (*C. ramosopulvinata, C. kanzashiana, C. prolifica, Paecilomyces* sp.), cicada clade B (*C. sobolifera, C. yakushimensis, Cordyceps* sp. 1), the truffle-cicada clade (*C. japonica, C. jezoensis, C. capitata, C. ophioglossoides, C. inegoensis, C. paradoxa, T. inflatum*), the moth clade (*C. militaris, C. pruinosa, C. takaomontana, Cordyceps* sp. 2, *Cordyceps* sp. 3, *P. tenuipes, B. bassiana, B. brongniartii*), the scale-moth clade (*C. coccidiicola, C. cochlidiicola*) and others. As shown in figure 2, the insertions were generally not phylogenetically restricted to a particular lineage, but, rather, widely and sporadically distributed among distinct lineages. Notably, presence/absence patterns of the insertions were quite complicated, even in most of the well-supported monophyletic groups in the *Cordyceps*.

Characterization of the Insertion Sequences

DNA database searches revealed that all of the insertions showed significant sequence similarity to group I introns. Moreover, all of the insertions contained sequence elements P, Q, R, and S, which are thought to be needed for formation of the secondary structure of group I introns (fig. 3; Cech 1988).

Group I introns described to date have been classified into 11 subgroups (IA1–IA3, IB1–IB4, IC1–IC3, and ID) on the basis of comparative sequence analysis (Michel and Westhof 1990). We examined the group I introns of the *Cordyceps* fungi for their placement in the subgroups. Based on sequence similarity, all of the group I introns inserted at the six sites SSU516, SSU989, SSU1199, LSU2066, LSU2449, and LSU2563 were estimated to belong to subgroup IB3. The group I introns inserted at the other two sites, SSU943 and LSU1921, were inferred to be members of subgroup IC1. Notably, introns inserted at the same site always belonged to the same subgroup. No open reading frames of considerable size were identified in the group I introns in nuclear rDNAs of the *Cordyceps* fungi.

Phylogenetic Analysis of the Multiple Group I Introns Inserted in the rDNAs of the *Cordyceps* Fungi

Molecular phylogenetic analyses were performed to examine the relationships between the multiple group

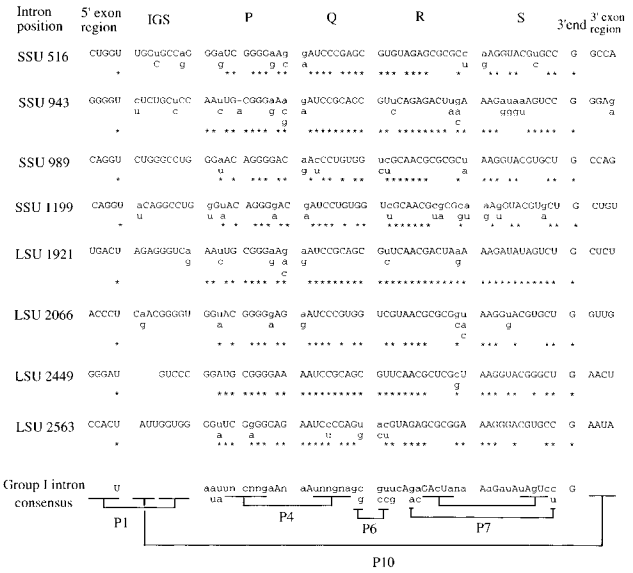

FIG. 3.—Consensus sequence motifs of group I introns of *Cordyceps* fungi. The following motifs, identified in the group I introns at eight sites (SSU516, SSU943, SSU989, SSU1199, LSU1921, LSU2066, LSU2449, and LSU2563), are shown: the 5′ exon, the internal guide sequence (IGS), conserved sequence elements P, Q, R, and S, the 3′ end of the intron, and the 3′ exon. Capital letters indicate 100% identity at the nucleotide site, whereas lowercase letters imply that two or three nucleotides occupy the site. Asterisks indicate nucleotide sites in accordance with the consensus sequence of group I introns (Cech 1988) shown at the bottom. Five of 10 base-pairing interactions characteristic of group I introns (Cech 1988) are also shown.

I introns of the *Cordyceps* fungi. Since the introns of the *Cordyceps* fungi were shown to belong to either subgroup IB3 or subgroup IC1, group I introns of these subgroups were collected from DNA databases and subjected to analysis together with the introns of the *Cordyceps*.

Subgroup IB3

Figure 4 is the molecular phylogenetic tree of group I introns of the subgroup IB3. Notably, the group I introns of the *Cordyceps* fungi inserted at the same sites constituted distinct phylogenetic groups. The introns at SSU516 and those at LSU2066 formed good monophyletic groups with high bootstrap values of 99.9% and 97.4%, respectively. The introns at SSU989 and those at LSU2449 constituted monophyletic groups with moderate bootstrap values of 83.5% and 82.9%, respectively. The introns at LSU2563 also formed a monophyletic group, although statistical support of the clade was low. The introns at SSU1199 were also grouped into a cluster, although the cluster contained an alien sequence, a group I intron of *Nectria galligena*. It should be noted, however, that (1) *Nectria* is phylogenetically closely related to the *Cordyceps* and placed in the same group, Hypocreales/Clavicipitales (Spatafora and Blackwell 1993); (2) the *Nectria* intron is inserted at the same site, SSU1199; and (3) judging from the tree topology and statistical supports, it appears possible that the *Nectria* intron might be the most basal branch of the cluster in spite of the tree shape inferred.

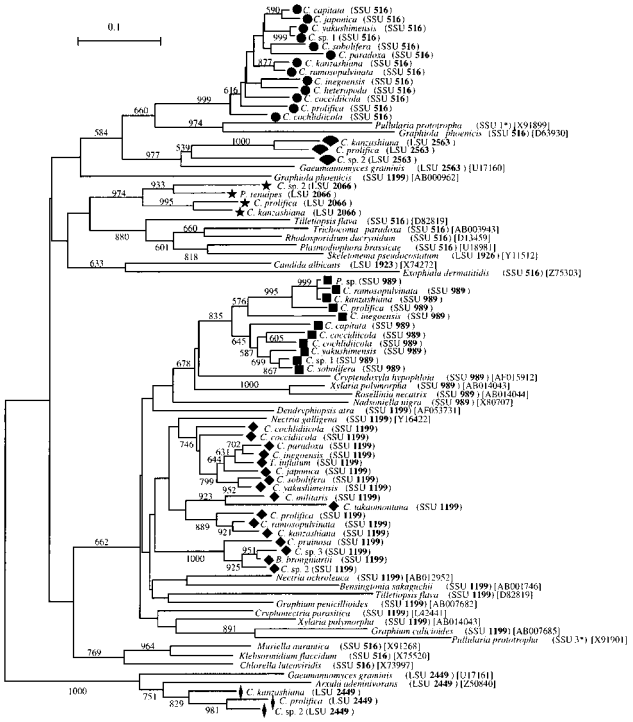

FIG. 4.—Phylogenetic placement of IB3 group I introns inserted at the SSU516, SSU989, SSU1199, LSU2066, LSU2449, and LSU2563 sites in rDNAs of *Cordyceps* fungi. The sequences were analyzed with group I introns of other organisms in DNA databases belonging to subgroup IB3. A total of 196 unambiguously aligned nucleotide sites were subjected to neighbor-joining analysis. Bootstrap values obtained with 1,000 resamplings are shown at the nodes, although values of less than 500 are omitted. Insertion sites of the group I introns are shown in parentheses. Asterisks indicate that inserted positions of introns were not identified. Accession numbers are in brackets. Group I introns of *Cordyceps* fungi inserted at the same sites are indicated with the same symbols as in figure 2. This tree is arbitrarily rooted.

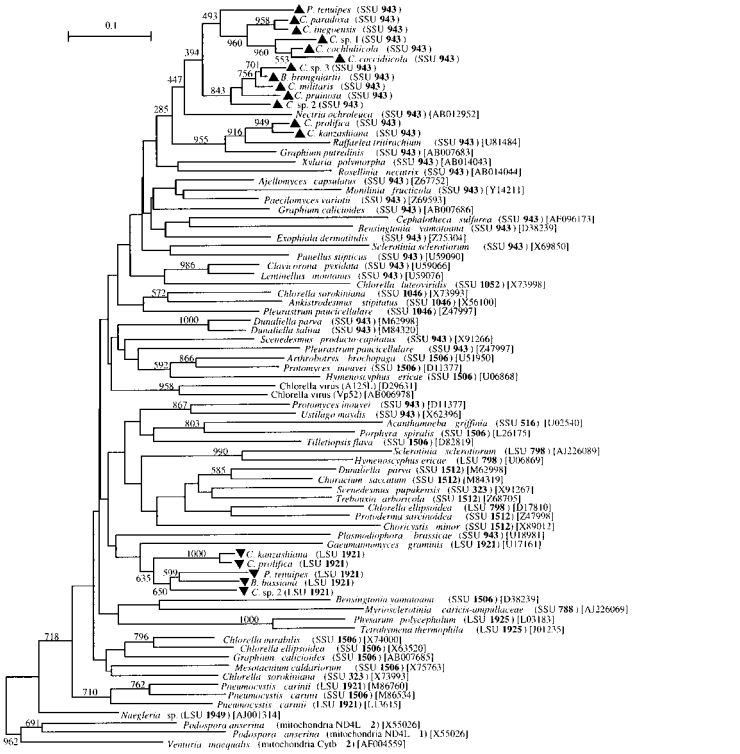

FIG. 5.—Phylogenetic placement of IC1 group I introns inserted at SSU943 and LSU1921 sites in rDNAs of *Cordyceps* fungi. The sequences were analyzed with group I introns of other organisms in DNA databases belonging to subgroup IC1. A total of 197 unambiguously aligned nucleotide sites were subjected to neighbor-joining analysis. Bootstrap values obtained with 1,000 resamplings are shown at the nodes, although values of less than 500 are omitted. Insertion sites of the group I introns are shown in parentheses. Accession numbers are in brackets. Group I introns of *Cordyceps* fungi inserted at the same sites are indicated with the same symbols as in figure 2. This tree is arbitrarily rooted.

Subgroup IC1

Figure 5 shows the molecular phylogenetic tree of group I introns of subgroup IC1. The group I introns of the *Cordyceps* at LSU1921 formed a monophyletic group, although statistical support of the clade was not significant. The introns at SSU943 were grouped into a monophyletic cluster except for the introns of *C. prolifica* and *C. kanzashiana*. These two exceptional introns of the *Cordyceps* were placed in a well-supported clade with group I introns of *Raffaelea tritirachium* and *Graphium putredinis.* This phylogenetic relationship was also statistically supported by an ML analysis (data not shown).

Analysis of Phylogenetic Congruence Between the rDNAs and the Inserted Group I Introns

The phylogeny of the rDNAs is expected to reflect the evolutionary history of host organisms. On the other hand, the phylogenetic trees of the group I introns are, considering that they are a mobile genetic element, likely to be affected by gains and losses in vertical and horizontal transmission processes in the evolutionary course of the hosts. Thus, in order to estimate evolutionary dynamics of the group I introns in the *Cordyceps*, we compared the rDNA tree with the trees of the group I introns.

SSU516 Introns

Figure 6*A* shows the comparison between the tree of SSU516 introns and the tree of rDNAs. The topologies of the two trees exhibited a good overall congruence. However, several discrepancies were observed in the truffle-cicada clade (*C. japonica*, *C. capitata*, *C. inegoensis*, *C. paradoxa*) and in the placement of *C. heteropoda.*

SSU943 Introns

Figure 6*B* shows the comparison between the tree of SSU943 introns and the tree of rDNAs. The SSU943 introns of *C. prolifica* and *C. kanzashiana* were omitted from this analysis because they were shown to belong to a distinct lineage (see fig. 5). The topologies of the two trees showed a good overall congruence, although a relatively slight discrepancy was found in the placement of *P. tenuipes.*

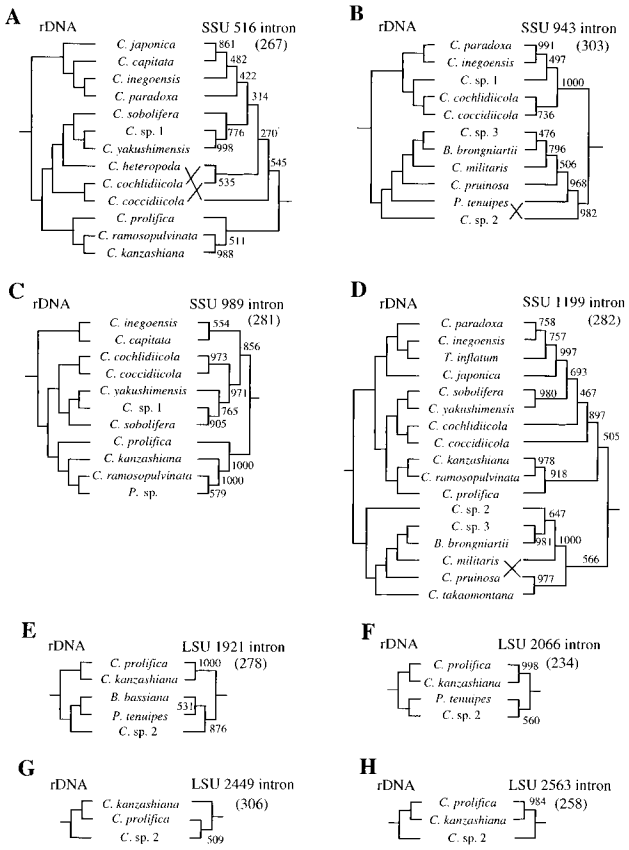

FIG. 6.—Phylogenetic congruence between inserted group I introns and flanking rDNAs in the genus *Cordyceps*. *A*, SSU516 introns. *B*, SSU943 introns. *C*, SSU989 introns. *D*, SSU1199 introns. *E*, LSU1921 introns. *F*, LSU2066 introns. *G*, LSU2449 introns. *H*, LSU2563 introns. Numbers on the intron trees are bootstrap values obtained with 1,000 resamplings. Topologies of the rDNA trees reflect the neighbor-joining tree in figure 2. In parentheses are the numbers of unambiguously aligned nucleotide sites used for phylogenetic analysis of the group I introns. Outgroups of the trees are as follows: (*A*) *Graphiola phoenicis* SSU516 intron and *Pullularia prototropha* SSU intron 1. (*B*) *Ajellomyces capsulatus* SSU943 intron and *Xylaria polymorpha* SSU943 intron; (*C*) *Cryptendoxyla hypophloia* SSU989 intron and *X. polymorpha* SSU989 intron; (*D*) *Graphium penicillioides* SSU1199 intron and *Dendryphiopsis atra* SSU1199 intron; (*E*) *Gaeumannomyces graminis* LSU1921 intron; (*F*) *Plasmodiophora brassicae* SSU516 intron and *Tilletiopsos flava* SSU516 intron; (*G*) *Arxula adeninivorans* LSU2449 intron and *G. graminis* LSU2449 intron; (*H*) *G. graminis* LSU2563 intron.

SSU989 Introns

Figure 6*C* represents the comparison between the tree of SSU989 introns and the tree of rDNAs. The topologies of the two trees showed very good overall congruence, with a discrepancy only in the position of *Cordyceps* sp. 1.

SSU1199 Introns

Figure 6*D* shows the comparison between the tree of SSU1199 introns and the tree of rDNAs. The topologies of the two trees showed a good overall congruence, although a number of local discrepancies were observed.

LSU1921 Introns

Figure 6*E* represents the comparison between the tree of LSU1921 introns and the tree of rDNAs. The topologies of the two trees agreed perfectly.

LSU2066 Introns

Figure 6*F* shows the comparison between the tree of LSU2066 introns and the tree of rDNAs. The topologies of the two trees were perfectly congruent, although the possibility of congruence by chance is not negligible when only four taxa are analyzed.

LSU2449 Introns

Figure 6*G* represents the comparison between the tree of LSU1921 introns and the tree of rDNAs. The topologies of the two trees were discrepant. Considering that *C. prolifica* and *C. kanzashiana* are closely related (fig. 2; Nikoh and Fukatsu 2000), the evolutionary history of LSU2449 introns might be not parallel to that of flanking rDNAs. However, because the statistical support of the grouping of *C. prolifica* and *Cordyceps* sp. 2 was very weak, it might be possible that the apparent discrepancy was due to limited resolution of the phylogenetic analysis.

LSU2563 Introns

Figure 6*H* shows the comparison between the tree of LSU2563 introns and the tree of rDNAs. The topologies of the two trees were perfectly congruent, although congruence by chance is possible when only three taxa are analyzed.

Statistical Evaluation of the Phylogenetic Congruence

As shown in figure 6, the group I intron trees and the corresponding rDNA trees appeared to be congruent in their topologies. In order to objectively evaluate the degree of congruence, SSU516, SSU943, SSU989, and SSU1199 introns were subjected to Brooks parsimony analysis (fig. 7). In the distributions of congruency indices of 100,000 randomly generated trees, the indices of the observed intron trees exhibited very high values at a statistically significant level ($P < 10^{-5}$), indicating that the congruencies between the group I intron trees and the corresponding rDNA trees could not occur by chance.

Discussion

Thus far, there have been a number of evolutionary studies on group I introns from a wide array of organismal groups. In most of these reports, group I introns were observed more or less sporadically in distant lineages of the hosts without reflecting host phylogeny, which suggests drastic horizontal transmissions between distinct lineages (e.g., Sogin et al. 1986; Nishida, Blanz, and Sugiyama 1993; Gargas, DePriest, and Taylor 1995; Turmel et al. 1995; Hibbett 1996; Cho et al. 1998; Nishida, Tajiri, and Sugiyama 1998; Watanabe et al. 1998;

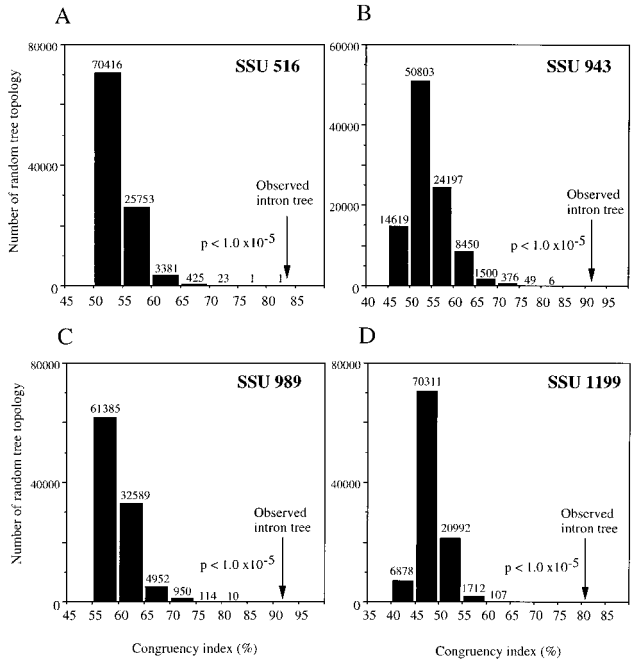

FIG. 7.—Statistical evaluation of the phylogenetic congruence between inserted group I introns and flanking rDNAs in the genus *Cordyceps*. *A,* SSU516 introns. *B,* SSU943 introns. *C,* SSU989 introns. *D,* SSU1199 introns.

Cho and Palmer 1999; Holst-Jensen et al. 1999; Perotto et al. 2000). Only a few studies have provided convincing evidence that supports stable maintenance of particular group I introns over long periods of evolutionary time (Xu et al. 1990; Kuhsel, Strickland, and Palmer 1990; Bhattacharya et al. 1994). These reports highlight the characteristics of group I introns as mobile genetic elements. In contrast, when studies are focused on lower taxonomic levels (i.e., intrafamilial or intrageneric), other aspects of the evolutionary dynamics of group I introns often emerge, such as vertical transmission and occasional loss (Turmel et al. 1993; Bhattacharya, Friedl, and Damberger 1996; Goddard and Burt 1999). The different patterns are, at least in part, probably artifactual. When comparisons are made between distantly related taxa, it is expected that most evolutionary events will be untraceable and occurrences of drastic horizontal transmission will be emphasized. Therefore, it would be ideal if we could examine a small and well-defined organismal group in which many group I introns exhibited a remarkable variety of insertion patterns. In the present study, we discovered a surprisingly large number of group I introns exhibiting highly polymorphic insertion patterns in a single fungal genus, *Cordyceps*, which provided us with an unprecedentedly suitable subject with which to investigate the evolutionary dynamics of the element in detail.

In total, 69 group I introns were identified in nuclear SSU and LSU rDNAs of 28 endoparasitic fungi representing the genus *Cordyceps*: 54 introns at 4 specific sites in 28 sequences of SSU rDNA, and 15 introns at 4 specific sites in 5 sequences of LSU rDNA (fig. 2). If LSU rDNAs of the remaining 23 taxa were sequenced, the number of introns would further increase. To date, several organismal groups have been reported to contain large numbers of group I introns, such as lichen-forming fungi (Gargas, DePriest, and Taylor 1995), ericoid mycorrhizal fungi (Perotto et al. 2000), chloroplasts of *Chlamydomonas* algae (Turmel et al. 1993), and others. As far as we know, the *Cordyceps* fungi are the most prominent reservoir of group I introns so far described.

In filamentous fungi, nuclear rDNA genes are present in tandem repeats ranging from 60 copies in *Coprinus* (Cassidy et al. 1984) to 220 in *Neurospora crassa* (Russell et al. 1984). It should be noted, therefore, that group I introns may not necessarily be found in all rDNA repeats within the genome (Hibbett 1996; Perotto et al. 2000). In fact, we observed a weak intronless rDNA band coamplified with the thick intron-containing rDNA band from several samples examined in this study (data not shown). If group I introns occur at a low frequency in the rDNA repeats, we may fail to detect inserted rDNA copies. In addition, multiply inserted long rDNAs are much less efficiently amplified by PCR than intronless short rDNAs. Taking these factors into account, the number of group I introns identified in this study might still be an underestimate.

The group I introns of the *Cordyceps* fungi exhibited very interesting physical and phylogenetic distribution patterns. First, the introns were inserted at highly specific sites, four sites in SSU rDNA and four sites in LSU rDNA (figs. 1 and 2). Second, introns inserted at the same site were almost always closely related between different species, whereas introns inserted at different sites were distantly related even in the same species (figs. 4 and 5). Third, when the phylogenetic relationship of introns at the same site was compared with the phylogeny of their hosts, the topologies were generally highly congruent to each other (figs. 6 and 7). Fourth, mapped on the host phylogeny, the introns were not restricted to a particular lineage, but, rather, widely and sporadically distributed among distinct lineages (fig. 2). These patterns strongly suggest that the group I introns were present in the ancestor of the *Cordyceps* fungi, have seldom been mobile but were vertically transmitted through host generations, and have experienced cocladogenesis with speciation of the hosts. Therefore, in the genus *Cordyceps*, the group I introns appear to behave as a vertically transmitted genetic element rather than as an actively mobile genetic element. The polymorphic insertion patterns of the introns observed are best explained by stable vertical transmission of the elements occasionally interrupted by independent losses in a number of lineages.

One of the most impressive results of this study was the phylogenetic congruencies between the group I introns inserted at the same site and the hosts (fig. 6). Although the congruencies were statistically highly significant (fig. 7), it should be noted that the intron trees and the host trees were often not perfectly coincident, but had several local discrepancies. The rDNA trees in figure 6, although presented as bifurcate cladograms, contained a number of clades that received weak statis-

tical support (see fig. 2). Because members of the genus *Cordyceps* are very closely related to each other, intrageneric phylogenetic relationships are sometimes not fully resolved with slow-evolving rDNA sequences (Nikoh and Fukatsu 2000). This is the case for the intron trees in figure 6, because the small sequence size of the group I introns often leads to insufficient resolution and confidence in phylogenetic analysis. Therefore, most of the minor incongruencies between the intron trees and the host trees should be attributed to limited resolution of the phylogenetic analyses. In fact, when poorly supported clades were collapsed, most of the discrepancies were reconciled (data not shown). Of course, the possibility cannot be excluded that part of the discrepancies might come from local horizontal transmissions of group I introns within the genus *Cordyceps*.

Although vertical transmissions and losses are predominant in the evolution of group I introns in the *Cordyceps*, at least one or two horizontal transmission events were detected. SSU943 introns of *C. prolifica* and *C. kanzashiana* did not cluster with SSU943 introns of other *Cordyceps* fungi, but fell in a distinct monophyletic group that contained SSU943 introns from the distantly related ascomycetous fungi *G. putredinis* and *R. tritirachium* (fig. 5). This pattern strongly suggests the possibility that the common ancestor of *C. prolifica* and *C. kanzashiana* horizontally acquired the SSU943 intron from a foreign fungal donor. LSU2449 introns of *C. prolifica*, *C. kanzashiana*, and *Cordyceps* sp. 2 formed a clade, in which introns of *C. prolifica* and *Cordyceps* sp. 2 were related, with LSU2449 introns from the distantly related ascomycetous fungi *Arxula adeninivorans* and *Gaeumannomyces graminis* (fig. 4). However, molecular and morphological lines of evidence consistently supported a phylogenetic affinity between *C. prolifica* and *C. kanzashiana*, which conflicted with the intron phylogeny (Nikoh and Fukatsu 2000; fig. 6*G*). This contradiction might suggest a horizontal transmission event at the LSU2449 site.

In this study, no transposition events were detected. Even in several putative horizontal transmission events described above, it was inferred that foreign group I introns were integrated into the homologous sites in rDNAs of *Cordyceps* members (see figs. 4 and 5). The patterns observed in this study—no transposition, occasional horizontal transmissions into homologous sites, and frequent intron losses—may indicate that homologous recombination is an important mechanism involved in gains and losses of group I introns in nuclear rDNAs of the *Cordyceps* fungi. It should be noted that reverse transcription followed by homologous recombination is believed to be involved in mobility and loss of group I introns (Dujon 1989; Belfort and Perlman 1995). In the group I introns of the *Cordyceps* fungi, we found no open reading frames that encoded proteins for mobility, such as homing endonucleases, which may also be relevant to the scarcity of horizontal transmissions, in contrast to dominating vertical transmissions and losses. Notably, our results unequivocally indicate that the common ancestor of the *Cordyceps* must have experienced drastic acquisitions of many group I introns, although it remains a mystery as to what happened at that time.

What processes underlie the horizontal transmission of group I introns across distinct lineages? At present, no convincing evidence is available. In some cases, spatial and ecological proximities through predation, endoparasitism, endosymbiosis, interspecific hybridization, and other processes have been hypothesized to facilitate the transfer events (Nishida and Sugiyama 1995; Adams et al. 1998). The horizontal transmission route of group I introns in the *Cordyceps* fungi may be linked to endoparasitism. Members of the genus *Cordyceps* are exclusively endoparasitic to various insects and other arthropods (Kobayashi 1982; Samson, Evans, and Largé 1988; Spatafora and Blackwell 1993; Shimizu 1994; Nikoh and Fukatsu 2000). When the same insect is infected with multiple parasitic fungi including *Cordyceps* species, transmission of genetic materials between them might occasionally occur. It is also possible that some introns might have been transferred between the fungi and the host, although no group I introns have so far been reported from insects.

Interestingly, fungal groups that possess many group I introns tend to be highly specialized for symbiotic or parasitic lifestyles, e.g., lichen-forming fungi (Gargas, DePriest, and Taylor 1995), ericoid mycorrhizal fungi (Perotto et al. 2000), yeast-like endosymbionts of anobiid beetles (Noda and Kodama 1996), and entomoparasitic fungi of the genus *Cordyceps*. Are there any causal relationships between upkeep of the introns and endosymbiotic/parasitic lifestyles? Although speculative, we suggest that the slow-growing nature of symbionts/parasites might be responsible. It is expected that the more group I introns rDNAs carry, the less efficient synthesis of rRNAs becomes. Because the ribosome is a copious cellular component essential for protein synthesis, multiple group I introns in rDNAs may result in reduced cell growth and division. If so, heavily inserted rDNAs may have a negative fitness effect on most free-living organisms in which the ability to grow rapidly under favorable conditions is essential for their survival and reproduction. On the other hand, the growth rate of endosymbionts/parasites must be suppressed under a strict control to cope with limited space inside the host body, to ensure survival of the host (at least for a while), to efficiently utilize resources from the host, to synchronize their life cycle parameters with those of the host, etc. (Tanada and Kaya 1993). Therefore, it is expected that the disadvantages due to heavily inserted rDNAs might be relaxed in slow-growing endosymbionts/parasites.

How often have group I introns been acquired and lost in the evolutionary history of the *Cordyceps* fungi? Figure 8 shows a phylogenetic reconstruction of gains and losses of group I introns in SSU rDNA of the *Cordyceps*. Based on the results presented in this study, it was assumed that the SSU516, SSU943, SSU989, and SSU1199 introns were either inherited from or acquired by the ancestor of *Cordyceps* and have subsequently been lost in many lineages independently. Mapped on the host phylogeny based on SSU rDNA sequences, 27

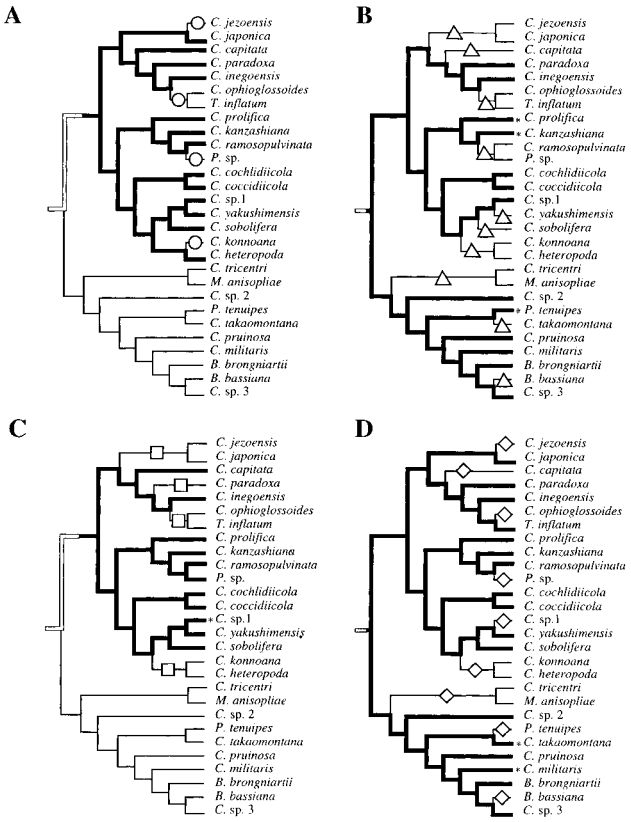

FIG. 8.—Hypothetical reconstruction of gains and losses of group I introns inserted in SSU rDNA on the phylogeny of *Cordyceps* fungi. The tree topology reflects the neighbor-joining tree in figure 2. *A*, SSU516 introns. *B*, SSU943 introns. *C*, SSU989 introns. *D*, SSU1199 introns. Open symbols indicate the estimated branches at which a given intron was lost. Bold and thin lines represent intron-plus and intronless branches, respectively. Open lines imply that the intron may have been descended from the common ancestor of *Cordyceps*. Even when clades supported by bootstrap values of <75% were collapsed into multifurcates, placements of six introns were discrepant between the rDNA and intron trees, which are indicated with asterisks.

intron losses were parsimoniously estimated under this model (fig. 8), although the number may fluctuate due to ambiguity of the tree topology and possible homoplasy. As discussed previously, SSU943 introns of *C. prolifica* and *C. kanzashiana* are likely to be horizontally acquired from a foreign fungal donor (see fig. 5). In addition, four other SSU introns were identified whose phylogenetic placement significantly conflicted with that in the rDNA tree (fig. 8, asterisks), which might suggest local horizontal transfers in the genus *Cordyceps*. On the assumption that these discrepancies are attributable to horizontal transfers, five independent gains of introns through horizontal transfer are parsimoniously estimated on the SSU rDNA phylogeny. If this estimate is accurate, the number of intron losses should increase to 33, because a gain of a new intron must accompany a loss of the original intron at the site in these cases.

In general, group I introns and other mobile selfish genetic elements show widespread and sporadic phylogenetic distribution, which must be a product of dynamic equilibrium between evolutionary gains and losses of the elements. The dynamics of particular elements such as the endosymbiotic bacteria *Wolbachia* and endonuclease-encoding group I introns in yeast mitochondria have been formulated into a hypothetical cyclical model (Hurst and McVean 1996; Goddard and Burt 1999). In the model, a population of uninfected organisms is constantly invaded by selfish genetic elements through horizontal transmission. An element occasionally succeeds in spreading in the population using its special mechanism for selfish propagation. Once perfectly fixed in the population, the mechanism for propagation is of no use and becomes costly. Then, the mechanism begins to accumulate deleterious mutations, which results in the prevalence of degenerate functionless elements. Once a functionless element is fixed in the population, loss of the element proceeds through stochastic and/or selective processes in the absence of intact elements capable of propagation. Finally, the element is completely lost from the population, and the original uninfected status recovers. Although this scenario explains a number of phenomena and appears convincing (Lohe et al. 1995; Hurst and McVean 1996; Goddard and Burt 1999), there have been few works in which the evolutionary processes in the cycle were phylogenetically analyzed in detail. In this context, the group I introns of the *Cordyceps* fungi provide us with interesting and abundant examples to understand what evolutionary events have occurred in the degeneration-loss phase of the cycle.

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