Interkingdom Host Jumping Underground: Phylogenetic Analysis of Entomoparasitic Fungi of the Genus Cordyceps

Naruoh Nikoh*† and Takema Fukatsu*

*National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukuba, Japan; and †Bio-Oriented Technology Research Advancement Institution, Omiya, Japan

Most members of the ascomycetous genus Cordyceps are endoparasitic fungi of insects and other arthropods, but about 20 of the 300 described species are parasitic to hart's truffles, Elaphomyces spp. In order to understand the evolution of host specificity and the process of interkingdom host jumping in Cordyceps, we investigated the phylogenetic relationships of 22 representatives, including 4 truffle parasites and 18 insect parasites, based on nuclear and mitochondrial rDNA sequences. Five monophyletic groups were identified in both nuclear and mitochondrial phylogenies. In three of the five clades, the members utilized hosts from the same insect group, suggesting that the endoparasite-host connections have been conserved to some extent. On the other hand, it was also shown that major host shifts between distantly related insects must have occurred repeatedly. Notably, phylogenetic analyses strongly suggested that parasites of hart's truffles originated from parasites of cicada nymphs during the evolution of the Cordyceps. The common habitats of cicada nymphs and hart's truffles, deep underground and associated with tree roots, suggest that the interkingdom host jumping from Animalia to Fungi might have been promoted by the overlapping ecological niche of the unrelated hosts. This finding provides an impressive case of a drastic host shift in favor of the host habitat hypothesis.

Introduction

There are a number of taxa whose members are exclusively endoparasitic to other organisms: parasitoid wasps (Godfray 1994; Hawkins 1994), parasitic helminths (Desponnier and Karapelou 1987; Kearn 1998), entomopathogenic fungi (Samson, Evans, and Largé 1988; Tanada and Kaya 1993; Shimizu 1994), parasitic protozoans (Kreier 1993; Coombs et al. 1998), rickettsial endoparasitic bacteria (O’Neill, Hoffman, and Werren 1997), and many others. In general, these endoparasites show more or less strict host specificity, in which an endoparasite can infect and utilize a particular range of host organisms, or sometimes only a particular species of host. The host specificity is likely to be the natural outcome of the endoparasitic life, in which endoparasites have to develop highly specific and sophisticated mechanisms to recognize the host, to enter the host body, to avoid the host’s immune system, to survive and proliferate under special endoparasitic environments, to synchronize their life cycle parameters to those of the host, etc. (Bianco and Maizels 1989). Under these biological constraints, it is expected that the connection between endoparasites and their hosts should be conservative to some extent in the course of endoparasite evolution. On the other hand, they have to explore and infect new host individuals by horizontal transmission in order to survive and reproduce. During horizontal transmission, they must frequently encounter various nonhost organisms, which may sometimes lead to the establishment of new endoparasite-host relationships. Therefore, the pattern of host specificity currently observed in a particular endoparasitic taxon is an evolutionary product of two components: maintenance of already established endoparasite-host connections (association by descent) and occasional host shifts (association by colonization) (Futuyma and Slatkin 1983; Brooks and McLennan 1991).

To understand the evolution of endoparasite-host relationships, it is important to consider what factors have been involved in the acquisition of new hosts. In this context, two principal hypotheses have been formulated to address the factors that dominate the pattern of host shifts (Norton and Carpenter 1998; Shaw 1988; Brooks and McLennan 1991). “The host relatedness hypothesis” suggests that host shifts tend to follow the host’s phylogenetic lines on the grounds that related hosts will provide the most similar internal environments for endoparasitism. “The host habitat hypothesis” suggests that host shifts tend to follow the host’s microhabitat or feeding habitat lines on the grounds that the probability of encounter can be a dominant factor in the endoparasite-host association. These hypotheses, although not necessarily exclusive to each other, are applicable to patterns of host shifts at different levels. When various endoparasitic taxa are examined for host specificity, the pattern is universally found that a group of related endoparasites utilize a group of related hosts, particularly at lower taxonomic levels (Hafner and Nadler 1988; Shaw 1988). These cases favor the host relatedness hypothesis. On the other hand, at higher taxonomic levels of the endoparasites, it is frequently found that a group of related endoparasites utilize distantly related organisms (Shaw 1988; Maslov et al. 1996). The host relatedness hypothesis cannot be applied to these cases, but the host habitat hypothesis should be considered. As far as we know, however, although a number of reports have suggested the involvement of ecological overlaps based on obscure circumstantial evidence (Klassen and Beverley-Burton 1988; Shaw 1988; Durette-Desset, Beveridge, and Spratt 1994), few studies...
have presented convincing phylogenetic analyses of host shifts that favor the host habitat hypothesis. Notably, drastic host shifts between absolutely unrelated organisms have sometimes occurred in the evolutionary history of endoparasitic taxa. For example, in the trypanosomatid protozoans, most species are parasitic to vertebrates, but only one genus, *Phytomonas*, utilizes plants (Vickerman 1994). In the endoparasitic bacteria of the genus *Wolbachia*, there are distinct but closely related lineages, of which some are parasitic to insects and other arthropods and the others are associated with filarial nematodes (O’Neill, Hoffman, and Werren 1997; Bandi et al. 1998). In the life cycle of endoparasitic protozoans and helminths, it is commonly found that phylogenetically unrelated hosts are utilized at different life stages (Gibson and Bray 1994; Vickerman 1994). Such remarkable “host jumping” must have expanded the host range, pioneered novel ecological niches, and had a great impact on the radiation and diversification in various endoparasitic groups. However, it is generally not easy to trace and reconstruct the evolutionary process of such host jumping events (Maslov et al. 1996; Bandi et al. 1998). Because remarkable host-jumping events usually occurred anciently in the history of the endoparasitic taxa, the status before and after the host jumping cannot be surely assigned based on the characters of extant organisms. Conversely, if detailed molecular phylogenetic analysis is conducted on an endoparasitic taxon in which a drastic host-jumping event occurred recently, we may be able to understand an important aspect of endoparasitic evolution.

In these contexts, ascomycetous fungi of the genus *Cordyceps* are an interesting research subject. *Cordyceps* is placed in the family Clavicipitaceae of the order Clavicipitales in the class Pyrenomycetes, whose members are known to be exclusively endoparasitic to insects (Mains 1957; Shimizu 1994). In general, members of *Cordyceps* show strict host specificity, although the degree of specificity differs from species to species. Some parasitize only a single host species (e.g., *Cordyceps sobolifera*, strictly on the nymph of *Platyleura kaempferi* in Japan), while others utilize a range of hosts from a particular taxonomic group (e.g., *Cordyceps militaris*, on the pupa of various moths) (Shimizu 1994). The majority of *Cordyceps* species utilize insect hosts from various orders such as Homoptera, Lepidoptera, Coleoptera, Hymenoptera, and Diptera, suggesting that major host shifts between insect orders have occurred in the genus. A relatively small number of species are known to parasitize spiders, and notably, about 20 of the 300 described *Cordyceps* species are parasitic on harp’s truffles, hypogeous fungi of the genus *Elaphomyces* (Mains 1957; Shimizu 1994). Therefore, it is suggested that during the evolution of a single genus, *Cordyceps*, an interkingdom host-jumping event between Animalia and Fungi must have occurred. So far, several molecular phylogenetic analyses of *Cordyceps* and related fungi have been reported (Spatanora and Blackwell 1993; Ito and Hirano 1997; Suh et al. 1998). In these studies, however, close intrageneric relationships in *Cordyceps* could not be resolved based on the relatively small amount of sequence data. In addition, these studies included only small numbers of species, insufficient to conduct comparative analyses on the evolution of host specificity.

In the present study, in order to reconstruct the evolution of host specificity and the process of interkingdom host jumping, we investigated the phylogenetic relationships between 22 representatives, including 4 truffle parasites and 18 insect parasites, of the genus *Cordyceps* based on nuclear and mitochondrial rDNA sequences.

**Materials and Methods**

**Materials**

Information on fungal materials examined in this study is presented in table 1. Most of the *Cordyceps* species were collected in the field in Japan, except *C. japonica*, (IFO9647), from the culture collection at the Institute for Fermentation, Osaka, Japan. The entomoparasitic deuteromycetes *Beauveria bassiana* (IFO4848), *Beauveria brongniartii* (IFO5299), *Metarhizium anisopliae* (IFO5940), and *Paecilomyces tenuipes* 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 1989; Fukatsu, Sato, and Kuriyama 1997). *Hypomyces chrysospermus* (IFO6817) and *Hypocreia lutea* (IFO9061) were used as outgroup taxa.

**DNA Extraction**

Fruit bodies of the field-collected materials were surface-sterilized in 80% ethanol and cut by a clean razor to obtain uncontaminated fungal tissue from the core region. The tissue was ground well into powder in a mortar in the presence of liquid nitrogen, from which DNA was extracted with the QIAamp tissue kit (QIAGEN). Fungal isolates from the culture collection were cultivated in YMMD medium (3g/liter yeast extract, 3g/liter malt extract, 5g/liter polypeptone, 10g/liter dextrose) and subjected to DNA extraction in the same way.

**PCR, Cloning, and Sequencing**

From the whole fungal DNA, a DNA segment containing almost the entire length of the nuclear small-subunit ribosomal DNA (SSU rDNA) was amplified by PCR using primers NS1 (5’-TACGCTATGCTT-GGTCCTCTCAG-3’) and FS2 (5’-TAG-GATTCCTCGTGAAGA-3’). A DNA segment containing the 3′ end of the nuclear SSU rDNA, ITS1, 5.8S rDNA, and ITS2 and the 5′ region of nuclear large-subunit rDNA (LSU rDNA) was amplified using primers CFS2 (5’-TCTTCACGGGAATGCCTATCAG-3’) and NLB1 (5’-TACCCTACGTTACATGAG-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 nearly the entire length of the mitochondrial SSU rDNA was amplified using primers...
Table 1
List of Materials and DNA Sequence Accession Numbers

<table>
<thead>
<tr>
<th>HOST</th>
<th>SPECIES</th>
<th>ORIGIN</th>
<th>COLLECTION DATE</th>
<th>COLLECTOR</th>
<th>DNA Sequence Accession Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hart's truffles ................</td>
<td>Cordyceps capitata</td>
<td>Takamatsu, Kagawa</td>
<td>Feb. 3, 1995</td>
<td>S. Mitani</td>
<td>AB027318, AB027364, AB027340</td>
</tr>
<tr>
<td></td>
<td>Cordyceps jezoensis</td>
<td>Miyazu, Kyoto</td>
<td>Oct. 21, 1997</td>
<td>Y. Nakano</td>
<td>AB027319, AB027365, AB027341</td>
</tr>
<tr>
<td></td>
<td>Cordyceps japonica</td>
<td>IFO9647</td>
<td></td>
<td></td>
<td>AB027320, AB027366, AB027342</td>
</tr>
<tr>
<td></td>
<td>Cordyceps ophioglossoides</td>
<td>Takamatsu, Kagawa</td>
<td>Feb. 3, 1995</td>
<td>S. Mitani</td>
<td>AB027321, AB027367, AB027343</td>
</tr>
<tr>
<td>Cicada nymphs ...............</td>
<td>Cordyceps inegoensis</td>
<td>Muroran, Hokkaido</td>
<td>Aug. 18, 1997</td>
<td>Y. Nishihara</td>
<td>AB027322, AB027368, AB027344</td>
</tr>
<tr>
<td></td>
<td>Cordyceps paradoxa</td>
<td>Amami Island, Kagoshima</td>
<td>Sep. 1, 1997</td>
<td>K. Fujimoto</td>
<td>AB027323, AB027369, AB027345</td>
</tr>
<tr>
<td></td>
<td>Cordyceps proliferata</td>
<td>Saita, Tokushima</td>
<td>Jun. 23, 1997</td>
<td>H. Manabe</td>
<td>AB027324, AB027370, AB027346</td>
</tr>
<tr>
<td></td>
<td>Cordyceps kansashiana</td>
<td>Irimote Island, Okinawa</td>
<td>Apr. 21, 1994</td>
<td>D. Shimizu</td>
<td>AB027325, AB027371, AB027347</td>
</tr>
<tr>
<td></td>
<td>Cordyceps ramosopulvinata</td>
<td>Tsuruoka, Yamagata</td>
<td>Jul. 13, 1994</td>
<td>K. Takeda</td>
<td>AB027326, AB027372, AB027348</td>
</tr>
<tr>
<td></td>
<td>Cordyceps heteropoda</td>
<td>Miyazu, Kyoto</td>
<td>May 15, 1994</td>
<td>S. Konishi</td>
<td>AB027327, AB027373, AB027349</td>
</tr>
<tr>
<td></td>
<td>Cordyceps sobolifera</td>
<td>Matsusaka, Mie</td>
<td>Jul. 16, 1997</td>
<td>T. Okuda</td>
<td>AB027328, AB027374, AB027350</td>
</tr>
<tr>
<td></td>
<td>Cordyceps sp. 1a</td>
<td>Amami Island, Kagoshima</td>
<td>May 31, 1997</td>
<td>K. Fujimoto</td>
<td>AB027329, AB027375, AB027352</td>
</tr>
<tr>
<td>Moth pupae ........................</td>
<td>Cordyceps cochliidicola</td>
<td>Miyazu, Kyoto</td>
<td>Jul. 20, 1997</td>
<td>T. Fukatsu</td>
<td>AB027331, AB027377, AB027355</td>
</tr>
<tr>
<td></td>
<td>Cordyceps sp. 2b</td>
<td>Kanonji, Kagawa</td>
<td>Jun. 23, 1997</td>
<td>H. Manabe</td>
<td>AB027332, AB027378, AB027356</td>
</tr>
<tr>
<td></td>
<td>Cordyceps militaris</td>
<td>Tsuruoka, Yamagata</td>
<td>May 10, 1997</td>
<td>K. Takeda</td>
<td>AB027333, AB027379, AB027357</td>
</tr>
<tr>
<td></td>
<td>Paecilomyces tenuipes</td>
<td>Miyazu, Kyoto</td>
<td>Jul. 20, 1997</td>
<td>T. Fukatsu</td>
<td>AB027334, AB027380, AB027358</td>
</tr>
<tr>
<td>Anamorphic generalist .......</td>
<td>Beauveria bronginartii</td>
<td>IFO5299</td>
<td></td>
<td></td>
<td>AB027335, AB027381, AB027359</td>
</tr>
<tr>
<td></td>
<td>Beauveria bassiana</td>
<td>IFO4848</td>
<td></td>
<td></td>
<td>AB027336, AB027382, AB027360</td>
</tr>
<tr>
<td></td>
<td>Metarhizium anisopliae</td>
<td>IFO5940</td>
<td></td>
<td></td>
<td>AB027337, AB027383, AB027361</td>
</tr>
<tr>
<td>Spittlebugs ........................</td>
<td>Cordyceps tricentri</td>
<td>Saita, Tokushima</td>
<td>Jun. 23, 1997</td>
<td>H. Manabe</td>
<td>AB027330, AB027376, AB027353</td>
</tr>
<tr>
<td>Scale insects ...............</td>
<td>Cordyceps cocccidiicola</td>
<td>Miyazu, Kyoto</td>
<td>Jul. 20, 1997</td>
<td>T. Fukatsu</td>
<td>AB031195, AB031196, AB031197</td>
</tr>
<tr>
<td>Beetle larvae ..................</td>
<td>Cordyceps kumoana</td>
<td>Muroran, Hokkaido</td>
<td></td>
<td></td>
<td>AB031192, AB031193, AB031194</td>
</tr>
<tr>
<td>Outgroup ........................</td>
<td>Hypocreus lutea</td>
<td>IFO9061</td>
<td></td>
<td></td>
<td>AB027338, AB027384, AB027362</td>
</tr>
<tr>
<td></td>
<td>Hypomyces chrysospermus</td>
<td>IFO6817</td>
<td></td>
<td></td>
<td>AB027339, AB027385, AB027363</td>
</tr>
</tbody>
</table>

a This species has not been described but is illustrated in Shimizu (1994) under the Japanese name 'Amamisemitake' with species number 15.
b This species has not been described but is morphologically similar to Cordyceps pruinosa.
Molecular Phylogenetic Analyses

Multiple alignments were conducted according to the methods of Feng and Doolittle (1987) and Gotoh (1993) using a computer. The final alignment was inspected and corrected manually. Ambiguously aligned nucleotide sites were excluded from the phylogenetic analysis. Nucleotide sites that included alignment gaps were also omitted from the aligned data set. The aligned sequences were analyzed by the following methods to infer phylogenetic relationships. A neighbor-joining (NJ) tree (Saitou and Nei 1987) was constructed with Kimura's (1980) two-parameter distance using the program package CLUSTAL W (Thompson, Higgins, and Gibson 1994). A maximum-likelihood quartet puzzling (ML-PUZZLE) tree was constructed using the program PUZZLE, version 4.0.2 (Strimmer and von Haeseler 1996). A maximum-parsimony (MP) tree was constructed using the program PAUP*, version 4.0b2 (Swofford 1996). A maximum-likelihood quartet puzzling (NJ) tree (Saitou and Nei 1987) was constructed with 1,000 resamplings for NJ analysis and 100 resamplings for the ML-PUZZLE and MP analyses.

Statistical Analysis of the Monophyly of Truffle Parasites

To test the monophyly of four truffle parasites, maximum-likelihood (ML) analysis was conducted (Kishino and Hasegawa 1989). The total rDNA sequence data of four truffle parasites, Cordyceps capitata, Cordyceps japonica, Cordyceps jezoensis, and Cordyceps ophioglossoides, and two cicada parasites, Cordyceps inegoensis and Cordyceps paradoxa, were analyzed with those of four outgroup taxa, Cordyceps kanzashiana, Cordyceps prolifica, H. chrysospermus, and H. lutea. Because the phylogenetic relationship of the outgroup taxa was confident (see fig. 3), the topology was fixed in the analysis. Log-likelihood scores for all possible topologies of ingroup taxa were calculated, and an ML tree, the difference of log-likelihood between each tree and the ML tree, and the variance of the difference were estimated using the program package MOLPHY, version 2.3 (Adachi and Hasegawa 1996).

Results

Nuclear and Mitochondrial rDNA Sequences from the Cordyceps Fungi

In order to analyze the evolution of host specificity in the genus Cordyceps, nuclear and mitochondrial rDNA fragments from 24 representatives of Cordyceps and related fungi (table 1) were amplified by PCR, cloned, and sequenced.

Nuclear SSU rDNA Segment

The size of the amplified fragment using primers NS1 and FS2 is expected to be approximately 1.5 kb. From 16 of the 24 species, however, longer PCR products (1.9–3.3 kb) were obtained. Sequence analyses revealed that the increases in length were due to the insertions of group I introns in the nuclear SSU rDNAs. These introns were excluded from the data set for phylogenetic analysis.

Nuclear LSU rDNA Segment

The size of the amplified product using primers CFS2 and NLB1 is expected to be around 1.7 kb. From all 24 species, PCR products of nearly the expected size were obtained.

Mitochondrial SSU rDNA Segment

The size of the product using primers MSA0 and MSX3 is expected to be approximately 1.4 kb. From 10 of the 24 species, however, longer PCR products (2.7–7.3 kb) were obtained. These variations in length were attributed to the insertions of group I and II introns in the mitochondrial SSU rDNAs. These introns were excluded from the data set for phylogenetic analysis.

Phylogenetic Analysis Based on Nuclear rDNAs

The nuclear SSU rDNA segment and the nuclear LSU rDNA segment from the Cordyceps fungi were concatenated and subjected to phylogenetic analysis. Figure 1 shows the NJ tree based on an unambiguously aligned data set of 3,014 nucleotide sites. Notably, four truffle parasites (C. capitata, C. japonica, C. jezoensis, and C. ophioglossoides) formed monophyletic groups supported by a 92.7% bootstrap value, with two cicada parasites (C. inegoensis and C. paradoxa). Hereafter, we call this monophyletic group “the truffle-cicada clade.” Next to the truffle-cicada clade, six cicada parasites, a beetle parasite, a scale parasite, and a moth parasite constituted a poorly supported group. In this group, three cicada parasites (C. kanzashiana, Cordyceps ramosopulvinata, and C. prolific), two cicada parasites (Cordyceps sobolifera and Cordyceps sp. 1), and a scale parasite and a moth parasite (Cordyceps coccidicola and Cordyceps cochlidicola) formed monophyletic groups with nearly 100% bootstrap values. Hereafter, we call
Evolution of Host Specificity in *Cordyceps* 633

**Fig. 1.**—Phylogenetic relationship of the *Cordyceps* fungi based on nuclear rDNA sequences. A total of 3,014 unambiguously aligned nucleotide sites were subjected to neighbor-joining analysis. Bootstrap values obtained with 1,000 resamplings are shown at the nodes. Host organisms are presented in parentheses. Names of the clades are shown on the right side.

These clades “cicada clade A,” “cicada clade B,” and “the scale-moth clade,” respectively. In addition, there was a well-defined monophyletic group, supported by a 100% bootstrap value, which was constituted by three moth parasites (*C. militaris, Cordyceps* sp. 2, and *P. tenuipes*) and two anamorphic generalists (*B. bassiana* and *B. bronginartii*). Hereafter, we call this monophyletic group “the moth clade.”

Phylogenetic Analysis Based on Mitochondrial SSU rDNA

Next, the mitochondrial SSU rDNA sequences from the *Cordyceps* fungi were subjected to phylogenetic analysis. Figure 2 shows the NJ tree based on an unambiguously aligned data set of 1,258 nucleotide sites. As in the nuclear rDNA phylogeny, the truffle-cicada clade, supported by a 89.0% bootstrap value, was identified in the mitochondrial rDNA phylogeny. Also, cicada clade A, cicada clade B, and the moth clade were supported by nearly 100% bootstrap values in the mitochondrial phylogeny. The scale-moth clade was also identified, although the bootstrap support was low (68.8%). In conclusion, the nuclear rDNA phylogeny (fig. 1) and the mitochondrial rDNA phylogeny (fig. 2) were reasonably concordant, although some discrepancies were found, particularly where statistical supports for the groupings were not significant.

Phylogenetic Relationships of the *Cordyceps* Fungi

To infer the intrageneric relationships of the closely related *Cordyceps* fungi as confidently as possible, the nuclear and mitochondrial rDNA data were combined to produce a large data set, which was subjected to detailed molecular phylogenetic analyses. Figure 3 shows the strict-consensus tree of three methods, NJ, ML-PUZZLE, and MP, based on an unambiguously aligned data set of 4,272 nucleotide sites. As in figures 1 and 2, the truffle-cicada clade, cicada clade A, cicada clade B, the scale-moth clade, and the moth clade were identified with high bootstrap support. In the truffle-cicada clade,
C. japonica and C. jezoensis formed a monophyletic group supported by fairly high bootstrap values. In the moth clade, the relationships (C. militaris, P. tenuipes, (B. bassiana, B. bronginartii)) were supported with confidence. The other clades were nonsignificantly supported, although they appear in figure 3.

Phylogenetic Analysis of the Truffle-Cicada Clade

In the truffle-cicada clade, it was tested by ML analysis whether the truffle parasites are monophyletic. The number of possible phylogenetic relationships of six fungi, four truffle parasites, and two cicada parasites is 945. Log-likelihood scores for all of the topologies were calculated by the ML method based on the unambiguously aligned 4,272 nucleotide sites of nuclear and mitochondrial rDNAs. Table 2 shows the five trees with the best log-likelihood scores. In the tree with the best score (tree 1), the four truffle parasites were not monophyletic. Monophyly of the truffle parasites was found in the tree with the second best score (tree 2). However, the difference in log-likelihood between them was only 0.3 ± 8.3, indicating that these topologies are almost equally likely. From these results, monophyly of the truffle parasites was neither supported nor rejected by the present data.

Discussion

In the present study, we conducted molecular phylogenetic analyses of 22 species of endoparasitic fungi from the genus Cordyceps. As far as we know, this is the first comprehensive phylogenetic study on this fungal group. Five monophyletic groups were identified among the fungi examined: the truffle-cicada clade, cicada clade A, cicada clade B, the scale-moth clade, and the moth clade. Two independent molecular phylogenies based on nuclear rDNAs (fig. 1) and mitochondrial SSU rDNA (fig. 2) consistently supported these clades, indicating the reliability of the result. Based on the total sequence data of more than 4,200 unambiguously aligned nucleotide sites, we presented a phylogenetic tree of the Cordyceps fungi (fig. 3), which provided us...
Evolution of Host Specificity in *Cordyceps* 635

Fig. 3.—Phylogenetic relationship of the *Cordyceps* fungi based on the total sequence data. A tandemly concatenated nuclear and mitochondrial rDNA data set (4,272 nucleotide sites) was subjected to neighbor-joining (NJ), maximum-likelihood quartet puzzling (ML-PUZZLE), and maximum-parsimony (MP) analyses. The strict-consensus tree of the three analyses is presented. Numbers at the nodes are bootstrap values (%) obtained by the NJ (left), ML-PUZZLE (center), and MP (right) methods, respectively. Shown on the right side are host organisms, names of the clades, and morphological types of the stromata.

with a credible phylogenetic framework to understand the evolutionary aspects in this group. At the maturity of the *Cordyceps* fungi, fruiting bodies (or stromata) of a spectacular shape grow out of the host insect. In their shape, size, and coloration, in combination with the host species, the morphology of fruiting bodies shows a wide variety, although morphological characters are generally stable in a particular species (Kobayashi 1982; Samson, Evans, and Largé 1988; Shimizu 1994). When the shape of the fruiting body was arranged on the phylogenetic tree, it was evident that members of the same clade form fruiting bodies of similar shapes (fig. 3). Therefore, the five clades proposed by the molecular data were also supported by morphological characters, reinforcing the reliability of the phylogeny.

In three of the five clades, cicada clade A, cicada clade B, and the moth clade, the members utilize hosts from the same insect group, which suggests that the endoparasite-host connections have been conserved to a considerable extent during the evolution of the *Cordyceps* fungi. These results favor the host relatedness hypothesis. The conservativeness of the host specificity can be realized through two processes that are not necessarily incompatible (Futuyma and Slatkin 1983; Brooks and McLennan 1991). One process is cospeciation based on tight endoparasite-host connections. The other process is minor host shifts between phylogenetically related hosts based on relatively loose endoparasite-host connection. In *Cordyceps*, cicada parasites generally parasitize only one or a few cicada species (Shi...
Fig. 4.—Hypothesis: evolutionary process and promoting factors of interkingdom host jumping from cicada nymph to hart’s truffle in the genus *Cordyceps*.
In the cicada clades, therefore, the former process should be taken into account in addition to the latter process. On the other hand, it is known that moth parasites utilize a wide variety of lepidopteran larvae and pupae in general (Samson, Evans, and Largé 1988; Shimizu 1994). In the moth clade, it seems quite likely that the latter process is overwhelming. To discuss these ideas with certainty, however, reliable information on the host range of the endoparasites and the phylogenetic relationships of the hosts is required.

At the same time, figure 3 shows that major host shifts between distantly related host insects must have occurred repeatedly in the evolutionary course of *Cordyceps*. In the scale-moth clade, for example, it is suggested that a major host shift between Lepidotera and Homoptera took place. In this study, we examined 22 fungi whose host organisms included Homoptera, Coleoptera, Lepidotera, and *Elaphomyces* fungi. To date, 10 insect orders, Homoptera, Heteroptera, Coleoptera, Coleoptera, Hymenoptera, Diptera, Odonata, Isoptera, Orthoptera, and Dictyoptera, have been recorded to be hosts of *Cordyceps* species (Shimizu 1994). In addition, some species are known to parasitize spiders (Shimizu 1994). Therefore, there appears to be no doubt that occasional major host shifts have expanded the host range, pioneered new ecological niches, and driven the diversification and speciation of the *Cordyceps* fungi. However, our results failed to reconstruct the evolutionary process of major host changes between insect orders with satisfactory resolution. More sequence data and more extensive sampling of *Cordyceps* species are necessary to analyze these processes.

The most important finding in this study is the phylogenetic placement of truffle parasites in *Cordyceps*. All four truffle parasites examined were placed in a well-supported monophyletic group, the truffle-cicada clade, together with two cicada parasites. Located outside the clade were a number of cicada parasites but no truffle parasites. These phylogenetic relationships strongly suggest that (1) the ancestral host of the truffle-cicada clade is the cicada, (2) hart’s truffle was acquired secondarily after the divergence of the truffle-cicada clade, and therefore (3) interkingdom host jumping from cicada nymph to hart’s truffle must have occurred inside the clade. As far as we know, this is the first report that has definitively demonstrated the evolutionary process of interkingdom host jumping in a single endoparasitic genus.

Why from cicada nymph to hart’s truffle? What factors have promoted the jump between absolutely unrelated hosts? When we consider the life cycle and ecology of these organisms, a very interesting point in common emerges. Cicada nymphs feed on the xylem fluid of tree roots almost throughout a lifetime of several years underground (Yoshimura 1997). Hart’s truffles are the mycorrhizal associates of tree roots and are completely subterranean throughout their lives (Trappe 1979). Their habitats are often as deep as 10 cm under the ground, where few organisms as large as them are found. Although speculative, it is conceivable that the overlapping niches of cicada nymphs and hart’s truffles, both deep under the ground and associated with tree roots, may have promoted this drastic host jumping (fig. 4). We regard this finding as impressive evidence in favor of the host habitat hypothesis.

How many times have truffle parasites evolved from cicada parasites in *Cordyceps*? Parsimoniously, a single origin is assumed. However, given the ecological factors that may facilitate the host-jumping event, the possibility of multiple origins due to convergent evolution should be considered. To assess which of the alternative hypotheses is more likely, statistical analysis was conducted on the phylogenetic relationship in the truffle-cicada clade (table 2). Unfortunately, the monophyly of the truffle parasites was neither supported nor rejected, because the relationship in the clade is too close to be resolved by the rDNA sequence data. Phylogenetic analysis using faster-evolving molecules will give an answer to this question.

When did the host jumping happen? Berbee and Taylor (1993) analyzed the evolutionary history of radiation of the true fungi, in which the nucleotide substitution rate of fungal 18S rDNA was estimated to be around 1% per 100 MYA. By superimposing this rate on our data, the age of the host switching, presumably at the base of the truffle-cicada clade, was calculated to be 43 ± 13 MYA.

Although drastic host changes must have occurred in various host-endoparasite systems, it is generally difficult to trace and reconstruct the evolutionary process in detail. The case of the *Cordyceps* fungi provides us with an opportunity to gain insights into how novel host-endoparasite relationships have been established.

**Acknowledgments**

We thank A. Sugimura, S. Kumagai, and K. Sato for their technical and secretarial assistance; M. Izawa for photographs of *Cordyceps* fungi; K. Fujimoto, S. Konishi, H. Manabe, S. Mitani, Y. Nakano, Y. Nishihara, T. Okuda, D. Shimizu, and K. Takeda for materials; and T. Okuda, D. Shimizu, and K. Takeda for photographs of *Cordyceps* fungi; K. Fujimoto, S. Konishi, H. Manabe, S. Mitani, Y. Nakano, Y. Nishihara, T. Okuda, D. Shimizu, and K. Takeda for materials; and J. W. Spatafora, T. Wilkinson, and D. L. Stern for reading the manuscript. This research was supported by the Program for Promotion of Basic Research Activities for Innovation Biosciences (ProBRAIN) of the Bio-Oriented Technology Research Advancement Institution, Japan.

**LITERATURE CITED**


Naruya Saitou, reviewing editor

Accepted December 21, 1999