Molecular evidence for teleomorph–anamorph connections in *Cordyceps* based on ITS-5.8S rDNA sequences

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The relationship between teleomorphs of *Cordyceps* spp. and their presumed anamorphs have been investigated by analysis of 5.8S and ITS rDNA sequences. The morphological and sequence data confirm that *Paecilomyces hawkesii* is the anamorph of *Cordyceps gunnii*, while *Cordyceps hawkesii* is a synonym of *C. gunnii*, and *P. ginnii* is a synonym of *P. hawkesii*. The following presumed connections are also confirmed: *Beauveria brongniartii* is the anamorph of *C. brongniartii*, *Metarhizium anisopliae* var. *majus* is the anamorph of *C. brittlebankisoides*, *Beauveria sobolifera* is the anamorph of *C. sobolifera*, *Mariannaea pruinosa* is the anamorph of *C. pruinosa*, *Paecilomyces militaris* is the anamorph of *C. militaris*, and *Hirsutella sinensis* is the anamorph of *C. sinensis*. The other isolates sequenced are unlikely to be anamorphs of the teleomorphs from which they were isolated because the sequences from the culture and the teleomorph are quite different. 5.8S and ITS sequences provide useful information for establishing anamorph–teleomorph connections and assisting in the delimitation of species within *Cordyceps*.

**INTRODUCTION**

*Cordyceps* (Clavicipitaceae) is an important genus of entomopathogenic fungi, some of which have been used in traditional Chinese medicine for more than one thousand years. Examples include *Cordyceps sinensis* and *C. sobolifera*, while others such as *C. brongniartii* have potential in the biocontrol of insect pests (Shimazu et al. 1988). *Cordyceps* is a large genus, comprising nearly 500 species or varieties (FUNINDEX; / http: // 194.131.255.3 / cabipages / names/names.asp). Descriptions of at least 400 species are probably validly published as the database includes quite a few names excluded by Kobayasi (1982).

We have collected specimens of *Cordyceps*, which we have isolated in order to investigate their potential as biocontrol agents or medicinal purposes. Because *Cordyceps* species are often isolated from immature stromata and not from single ascospores, it is important to establish if the strain isolated is really a *Cordyceps* and not a contaminant or parasite growing within the stromata. More than 50 species of *Cordyceps* have been connected to anamorphs, representing 26 genera of mitosporic fungi (Kobayasi 1982, Liang 1991a). In some cases, however, a single *Cordyceps* species was reported to be associated with different mitosporic genera. For example, *C. tuberculata* has five anamorph names, while *C. sinensis* has ten (Liang 1991a).

The anamorphs of *Cordyceps* have been studied over a long time. Tulasne & Tulasne (1865) stated that *Isaria farinosus* was the conidial stage of *C. militaris*; *I. farinosus* was later transferred to *Paecilomyces* (Samson 1974). Massee (1895) studied the development of *P. farinosus*, but never found that it could develop into stroma of *C. militaris* on artificial media. Numerous species of *Cordyceps* had no correlated conidial form, and on the other hand many *Paecilomyces* names exist, which are not connected to any known *Cordyceps* (Massee 1895). Tulasne & Tulasne (1865) and Massee (1895) mentioned some conidial stages of other species of *Cordyceps*.

Petch (1921) carried out substantial researches on entomogenous fungi, and since then several mycologists have tried to establish connections between the anamorph and teleomorph of *Cordyceps* species based on morphological and cultural characteristics (Kobayasi

Liang (1991a) revised the methodology for determining the anamorphs of *Cordyceps* and concluded that to induce cultures to produce mature stromata was the best way. This, however, has only been successful in a few species, such as *C. militaris* (Liang 1990) and *C. brongniartii* (Shimazu et al. 1988). It is possible that the nutritional or environmental conditions for producing stromata are not easily met. Microcyclic conidiation is another effective method of determining teleomorph–anamorph connections. In this method conidia and conidiogenous cells produced from ascospores are observed under the microscope. The anamorph of *C. taii* was determined in this way (Liang & Liu 1991). Ascospores of some species, however, do not directly produce conidiogenous cells and conidia. Single ascospore isolation is also not possible from immature specimens. In many cases the stromatal tissues or sclerotia have to be used to isolate *Cordyceps* strains if the stromata are immature or broken.

In nature, it takes *Cordyceps* species some time to infect host insects or spiders, produce mycelium, form stromata, and finally produce mature ascospores. It is therefore possible for the insects and stromata to be contaminated by saprobic, or sometimes parasitic fungi. This is the main reason why a single species of *Cordyceps* has more than one reported ‘anamorph’.

It is now possible to use restriction fragment length polymorphism (RFLP) analysis, random amplified polymorphic DNA analysis (RAPD) or (RAPD-PCR) and amplified fragment length polymorphism (AFLP) as tools for studying genome organisation, molecular evolution, and population genetics of fungi (Mitchell, Roberts & Moss 1995, Vos, Hogers & Bleeker 1996). DNA sequence data, especially of rDNAs, have been widely used for studies of phylogenetic systematics and evolutionary patterns. Ribosomal genes have a mosaic pattern of conserved and variable regions, making them attractive for taxonomic investigations at many levels (Ward & Akrofi 1994, Ko, Hong & Jung 1997). The CODening regions have a pattern of conserved and variable regions, making them attractive for taxonomic investigations at many levels (Ward & Akrofi 1994, Ko, Hong & Jung 1997). The coding regions have evolved slowly and are highly conserved between different species and genera, and can therefore be used for comparing distantly related organisms. Both the internal transcribed spacer (ITS) and intragenic spacer (IGS) have evolved rapidly and can be used for comparison of closely related species and subspecies (Black, McLain & Rai 1989, Chen, Hoy & Schneider 1992). Characterisation with molecular techniques offers a possible aid to identification when morphological, anatomical, and ecological data are unavailable or cannot alone resolve a taxonomic problem. They are particularly helpful in resolving relationships of morphologically simple organisms such as those in *Acremonium*, and in integrating presumably asexual fungi into teleomorph taxonomy at the order, family, genus and species levels (LoBuglio & Taylor 1993, Rehner & Samuels 1995, Glenn et al. 1996, Kuhls et al. 1996, Denman et al. 2000). Nikoh & Fukatsu (2000) and Chen & Hseu (1999) have reported some molecular data on *Cordyceps* species differentiation using RFLP of the 18S rRNA gene and phylogenetic analysis using mitochondrial rDNA sequences. Here ITS and 5.8S rDNA sequence data were used to determine the relationship between the anamorph and teleomorph of several *Cordyceps* species.

**MATERIALS AND METHODS**

**Specimens**

The samples of *C. nepalensis* and *C. multiaxialis* were provided by Mu Zang, co-author of these species. *C. militaris* was collected in Surrey, and *C. robertsi* and *C. gunnii* in Australia. *C. hawkesii* was provided by Ying-lan Guo, and *Xylaria hypoxylon* was obtained from K and used as outgroup. *C. sinensis* was collected from both Baima Snow Mountain and Renzhi Snow Mountain in Yunnan Province. Other *Cordyceps* species were also collected in China. Fuller details of voucher specimens and cultures investigated are in Table 1.

**DNA extraction, amplification and sequencing**

Either dried materials or fresh cultures were used. Air-dried samples were stored at 4 °C, except those from K which were kept in room temperature, and examined under a dissecting microscope. Contaminating debris and surface material were removed with a fine brush and dissecting knife. The samples (2–10 mg) were placed in a 1.5 ml Eppendorf tube with fine sterile sand, mixed by shaking, and ground into powder with a glass stick with a whirligig head.

Dry mycelium of *C. sinensis* was obtained by scraping the culture from the surface of PDA slopes, which had been incubated at 18 °C for 60 d and stored at 4 °C. Fresh mycelia for other cultures were harvested by scraping the surface of PDA plates incubated at 23 °C for 20 d. About 5 mg of dry or fresh samples including some agar, were transferred to 1.5 ml Eppendorf tubes containing sterile sand. The mycelium was then ground with a glass micropestle.

DNA of dry specimens and mycelia was extracted from the stromatal powder and the thick mycelial liquid, respectively, CTAB buffer (600 µl) was added to each Eppendorf tube containing samples and incubated in a 65 °C water bath for 1 h. The same volume of chlorofom: isoamyl alcohol (24:1) was pipetted in the tube, mixed by shaking, and centrifuged at 13000 rpm for 10 min. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube. The chlorofom–isoamyl alcohol extraction was repeated, and 250 µl isopropanol pipetted into a 1.5 ml Eppendorf tube with the supernatant, mixed by shaking and stored at 20 °C overnight to allow the DNA to precipitate. The precipitate was centrifuged at 13000 rpm for 15 min, the liquid drained off and the tube dried at 65 °C for 20 min; 50 µl of double distilled water was then added
Table 1. Specimens studied and the presumed relationship between anamorphs and teleomorphs.

<table>
<thead>
<tr>
<th>Species</th>
<th>Teleomorphs</th>
<th>Reported Anamorphs</th>
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<tr>
<td></td>
<td>Voucher</td>
<td>EMBLno.</td>
</tr>
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<td><strong>Cordyceps multiaxialis</strong></td>
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<td>AJ309359</td>
</tr>
<tr>
<td><strong>C. nepalensis</strong></td>
<td>HKAS28095*</td>
<td>AJ309358</td>
</tr>
<tr>
<td><strong>C. brittlebankioides</strong></td>
<td>G97025*</td>
<td>AJ309332</td>
</tr>
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<td>AJ309348</td>
</tr>
<tr>
<td><strong>C. gunnii</strong></td>
<td>K(M)</td>
<td>AJ309344</td>
</tr>
<tr>
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<td>G97022</td>
<td>AJ309340</td>
</tr>
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<td>AJ309341</td>
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<td>G97034-2</td>
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<td>K(M) 73501</td>
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<td>AJ309336</td>
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<td>K(M) 27083</td>
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<td>L95DS(5137)</td>
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<tr>
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<td><strong>C. tricentri</strong></td>
<td>G97036</td>
<td>AJ309364</td>
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<td><strong>Claviceps purpurea</strong></td>
<td>K(M45581)</td>
<td>AJ309368</td>
</tr>
<tr>
<td><strong>Xylaria hypoxylon</strong></td>
<td>K(M)</td>
<td>AJ309350</td>
</tr>
</tbody>
</table>

*, Type specimen.
to the DNA preparation. The crude extracts containing unquantified DNA amounts were used as templates for PCR amplification.

The PCR reaction mixtures were made up from equal volumes of DNA templates, with a total of 100 µl reaction mixture containing: 10 µl 10 X buffer (10 mM Tris/HC1, pH 8.3), 10 µl 25 mM MgCl2, 0.5 µl 10 mM of each of four dideoxyribonucleotide triphosphates (dNTP), 1 µl 100 ng µl\(^{-1}\) each of primers (ITS4; 5'-TCCTCCGCTTATTGATATGC-3'; and ITS5: 5'-GGAAGTAAAAGTCGTAACAA GG-3'), 0.5 µl 250 U ml\(^{-1}\) Tag polymerase, 1 µl template of DNA and 74.5 µl double distilled water. PCR products were amplified in a Perkin Elmer DNA Thermal Cycler using the following programme 97 °C for 1 min for premelting, followed by 30 cycles at 97 °C for 1 min, 48 °C for 1 min, and 72 °C for 3 min, then at 72 °C for a 7 min extension, and 4 °C for soaking. Amplification products were then purified using QIAquick™ columns according to manufacturer's protocol (Perkin-Elmer Corporation). Cycle sequencing was performed using the following programme for 26 cycles: 10 s denaturation at 96 °C, 5 s annealing at 50 °C, 4 min extension at 60 °C. Fluorescence dye labelled dideoxyribonucleotide triphosphates was used as terminator. Cycle sequencing products of 5 µl were purified by ethanol precipitation. The resuspended sample was run on a PE Applied Biosystems 377 automated DNA sequencer following the manufacturer’s protocols (Perkin-Elmer Applied Biosystems).

Electrophoresis and data collection were performed on the Sequencer and data transferred to a connected Power Macintosh 7200/90. The raw data were edited with Sequence Navigator and the contigs assembled with AutoAssembler (Applied Biosystems). Further alignment of the sequences was manual as alignment was not entirely accurate by computer. Sequence analysis was performed using PAUP4.0b2a (Swofford 1998) with nucleotide substitutions treated as unordered, and alignment gaps treated as missing information. Phylogenetic trees were obtained by an heuristic search with 100 replicates of random taxon addition using equal weights and tree-bisection-reconnection (TBR) branch swapping algorithm, with only four trees held at each step.

The ITS variable positions alone are insufficient to provide evidence for all branching patterns in such a complex phylogenetic tree. 100 bootstrap replicates were performed using the swapping algorithm (TBR) with simple addition of sequences. The tree was rooted with Xylaria hypoxylon, which belongs to a sister order to Cordyceps.

**RESULTS**

**Phylogenetic analysis**

The heuristic analysis of the 36 ITS4 and ITS5 sequences of ribosomal DNA enabled the cladogram in Fig. 1 to be constructed. This showed that 34 sequences of Cordyceps spp. and their isolates were roughly divided into nine groups, based on the relative relationships between teleomorphs and anamorph, with the exception of C. emeiensis, C. tricentri, and C. robertsi. The mitosporic fungi isolated from C. emeiensis, C. tricentri, and C. robertsi were found to be unrelated to their teleomorphs, based on both the heuristic and bootstrap trees, which means each culture is probably not the anamorph of the teleomorph.

The first group comprises five samples, each from either the teleomorph or the anamorph of C. militaris. There are minor sequence differences between the teleomorph and anamorph of C. militaris collected from in Britain (C. militaris\(^{5a}\)) or China (C. militaris\(^{5b}\), C. militaris\(^{5c}\) and Paecilomyces militaris\(^{5d}\)). P. militaris\(^{5c}\) had some sequence differences from other samples in this group. P. militaris\(^{5a}\) and P. militaris\(^{5b}\) were isolated from different stromata collected from Sichuan and Guizhou, China, respectively. Morphological investigation of both the teleomorphs and the cultures revealed no difference between them; P. militaris is therefore the anamorph of C. militaris based on the ITS sequences (Fig. 1).

In groups two, three, and four, the teleomorph shares the same sequence as its anamorph, e.g. C. pruinosa with Marianiana pruinosa, C. brongniartii with Beauveria brongniartii, C. brittlebankisoides with Meta rhizium anisoplae var. majus.

Group five indicates that C. hawkesii and C. gunnii had similar sequences although they were collected from different places, Hunan, and Guizhou, respectively. Their anamorphs, Paecilomyces hawkesii (anamorph of C. hawkesii) and P. gunnii (the anamorph of C. gunnii) also have similar sequences. The sequence data for C. gunnii\(^{6a}\) from Australia was, however, different from the samples from China.

Group six shows that C. sobolifera from China possesses the same ITS sequence as its presumed anamorph, which indicates that this is the anamorph of C. sobolifera. Both samples from China and Kew clustered into a single clade, although there were some differences between them.

In group seven, C. nepalensis and C. multiaxialis, which have similar morphological characteristics to C. sinensis, are shown to have identical or almost identical ITS sequences to that species and its presumed anamorph Hirsutella sinensis (Liu et al. 1989). This confirms that H. sinensis is the anamorph of C. sinensis and that C. nepalensis and C. multiaxialis are synonyms (Liu et al. 2001b).

In groups eight and ten, both Clonostachys rosea isolated from C. robertsi and Clonostachys sp. from C. emeiensis were distant from their presumed teleomorphs, although arranged in a single clade. These mitosporic taxa are thus not the anamorphs of those species.

In the phylogenetic tree in Fig. 2, ten clades with mostly high bootstrap support were obtained. For instance, the isolates of C. gunnii, C. brongniartii,
**Cordyceps** anamorph–teleomorph connections

**Fig. 1.** Phylogram showing relationships between teleomorphs and anamorphs within the genus *Cordyceps*. One of two most parsimonious trees obtained by heuristic researches based on parsimony-informative data of 5.8S rDNA and ITS sequences.

*C. brittlebankisoides*, *C. sobolifera*, *C. pruinosa*, and *C. militaris* have 100% bootstrap support with their teleomorphs (clade 1, 2, 3, 4, 5 and 6). In group five, four samples (*Paecilomyces gunnii*, *P. hawkesii*, *C. gunnii*, and *C. hawkesii*) were placed in a group with 100% bootstrap support apart from *C. gunnii* (clade 5). Based on this bootstrap support, these isolates and presumed anamorphs are related. The relative relationship between *P. gunnii* and *C. gunnii* (*P. hawkesii* and *C. hawkesii*), *B. brongniartii* and *C. brongniartii*, *Metarhizium anisopliae* var. *majus* and *C. brittlebankisoides*, *Beauveria sobolifera* and *C. sobolifera*; *Mariannaea pruinosa* and *C. pruinosa*, *Paecilomyces militaris* and *C. militaris* and *Hirsutella sinensis* and *C. sinensis* are proven. The *Clonostachys rosea* and *Clonostachys* sp. isolated from *C. robertsii* and *C. emeinsis* respectively in group eight, however, were rather different from the teleomorphs in group nine; this means the strains are not anamorphs of *C. robertsii* and *C. emeinsis*.

**DISCUSSION**

*Cordyceps hawkesii* and *C. gunnii*

The original specimens of *Cordyceps hawkesii* in the BM were collected by Hawkes in Tasmania and named after the collector, but was not formally described as there was no definite technical description, and the text...
was privately printed and distributed. Cooke therefore reviewed and redescribed the taxon (Cooke 1858, 1892). Based on the original description of Gray, Cooke (1892) discussed the differences between \textit{C. hawkesii} and \textit{C. gunnii}, including the length, thickness and shape of the stroma, fertile head, and stipe. Two stromata arise together from the same spot in some instances, or from different parts of same caterpillar, and occasionally three or four stromata occur on one individual. The internal structure is undoubtedly the same as in \textit{Cordyceps}. \textit{C. hawkesii} differs from \textit{C. gunnii} in that the stromata are not so thick or dark, and the stipe is irregular, contorted and nodulose, besides being woolly. The species has been recognised only in Tasmania, but \textit{C. gunnii} occasionally appears in Australia (Cooke 1892).

\textit{C. hawkesii} was later treated by Massee (1895), who included literature references, host, and distribution, and by Saccardo (1891) who provided a Latin description of the macroscopic features, but no internal characters. Rodway (1900) pointed out that the stroma was nearly as large as some forms of \textit{C. gunnii}, and had
a bent and often branched stipe, with an abruptly blunt apex. Lloyd (1915), in his synopsis of *Cordyceps* in Australia wrote ‘*Cordyceps hawkesii* appears to me, from the account to be but a short-clubbed (stromata) form of *Cordyceps gunnii*. It was named from Tasmania, grew on the same host, and was distinguished by its short club (stroma) and bearing two fruits near the back or other parts of the larva. Gunn recorded both of these features as occurring exceptionally in his original account of *Cordyceps gunnii*. I found no specimen of *Cordyceps hawkesii* in either of the museums at London’.

Kobayasi (1941) listed this species in his first monograph of *Cordyceps* and its allies with only literature references, but he described the macroscopic and microscopic features in his second monograph (Kobayasi 1982). According to Lloyd, the type specimen of *C. hawkesii* had been missing before 1915, and it is not known where the specimen Kobayasi described came from.

*Cordyceps gunnii*, which grows on caterpillars in Tasmania, was first reported by Berkeley (1843) as ‘*Sphaeria* (*Cordyceps*) *gunnii*’. It has been listed with various authorities but the correct citation is (Berk.) Berk. (1859) as Berkeley (1859) placed this taxon in the genus himself. Berkeley’s (1843) figure shows branched, long and slender, short and thick stipes. The species was found in Tasmania, New South Wales, and Victoria (Massee 1895) and named after its original collector, Gunn.

We examined the type specimen and abundant specimens of *C. gunnii* in K. *C. gunnii* is one of the biggest species in the genus, the stipe (with caterpillar host) is 6–30 cm long and 5–8 mm thick, the stromata are rarely branched, flexuous, rugged below, cylindrical, with the fertile part 4–8 cm long and somewhat thicker than the stipe. The caterpillar is also large, 4–6 cm long with the fertile part 4–8 cm long and somewhat thicker because the stipe (*Clavaria militaris*) from Hunan Province was identified as *C. granulosa* (Kobayasi 1941), *Verticillium militaris* (Gams 1971) or *Paecilomyces militaris* (Liang 1990). Based on the ITS sequences (Figs 1–2), we confirm that *P. militaris* is the anamorph of *C. militaris*.

### Cordyceps militaris and its anamorph

*Cordyceps militaris* is the type species of *Cordyceps*. It has been reported under numerous synonyms, in which different researchers have placed this taxon in different genera based on their recognition of this fungus. Its names include *Clavaria militaris*, *C. granulosa*, *Cordyceps militaris*, *Kentrosporium militaris*, *Sphaeria militaris*, and *Torrubia militaris* (Tulasne & Tulasne 1865). The species furthermore, has different author citations in different publications. (Mains 1939) provided reasons to support the use of *C. militaris* (L.) Link 1833, which in our opinion is correct.

*C. militaris* is an important species with a worldwide distribution and is easily recognisable. The anamorph has been named as *Cephalosporium militaris* (Kobayasi 1941), *Verticillium militaris* (Gams 1971) or *Paecilomyces militaris* (Liang 1990). Based on the ITS sequences (Figs 1–2), we confirm that *P. militaris* is the anamorph of *C. militaris*.

### Cordyceps brongniartii and its anamorph

When Shimazu *et al.* (1988) used *Beauveria brongniartii* from *Anomala costata* (Coleoptera, Scarabaeidae) to carry out bioassays against the larvae of this beetle, they found perithecial stromata developed on some larvae which had been killed by the fungus. When the stromata matured and produced ascospores, this proved to be a new species of *Cordyceps*, described as *C. brongniartii*. The conidiogenous characters of the isolates from the ascospores and perithecia were identical with those of *B. brongniartii*. The anamorph *B. brongniartii* was therefore connected to its teleomorph. *C. brongniartii* was first reported from Yunnan (Liu, Liang & Cao 1993) and the anamorph also connected by microcycle conidiation (Liu *et al.* 1997). In the molecular analysis presented in Figs 1 and 2, *B. brongniartii* is further confirmed as the anamorph of *Cordyceps brongniartii*.

### Notes on other species

*Cordyceps pruinosa*, growing on a cocoon, was described by Petch (1924) based on a specimen collected at...
Nuwara Eliya by Thwaites. This species is distributed widely throughout the world. Liang (1991b) isolated it on rice medium from a fresh specimen and obtained mature stromata, the features of which were basically similar to those of the specimen collected in field. The anamorph was identified as *Marianthae pruinosa*.

*Cordyceps sobolifera*, growing on cicada nymphs, is one of the earliest species reported in the genus. There has been no direct evidence to connect its teleomorph to an anamorph, although presumed related anamorphs have been studied by many researchers. In this study, the teleomorph–anamorph connection of *C. sobolifera* collected from Sichuan, is established based on the ITS rDNA sequence analysis (Figs 1–2). The anamorph is *Beauveria sobolifera* (Liu et al. 2001c).

The relationship between *C. brittlebankioides* and *Metarhizium anisoplae* var. *majus* and between *C. sinensis*, *C. nepalensis*, *C. multiocularis* and *Hirsutella sinensis*, has been discussed by Liu et al. (2001a, 2001b).

In summary, we suggest that ITS sequences can provide useful information in establishing anamorph–teleomorph connections and assisting in the delimitation of species within *Cordyceps*, but there could be differences between anamorphs and teleomorphs if from different locations.

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