A new species of *Beauveria*, the anamorph of *Cordyceps sobolifera*

Z.Y. Liu*[1], Z.Q. Liang[1], A.J.S. Whalley[2], A.Y. Liu[1] and Y.J. Yao[3]

[1]College of Biological Technology, Guizhou University, Guiyang 550025, Guizhou Province, China; *e-mail: liuzuoyi@public1.gy.gz.cn
[2]School of Bio-molecular sciences, John Moores University, Byrom Street, Liverpool L3 3AF, UK.
[3]Mycology Section, The Royal Botanical Gardens, Kew Richmond, Surrey TW9 3AB, UK.

Liu, Z.Y., Liang, Z.Q., Whalley, A.J.S., Liu, A.Y. and Yao, Y.J. (2001). A new species of *Beauveria*, the anamorph of *Cordyceps sobolifera*. Fungal Diversity 7: 61-70.

Cordyceps sobolifera is one of the earliest species reported in the genus and occurs on Cicada nymphs. There has been no direct evidence to connect its teleomorph to an anamorph, although many researchers have studied putative anamorphs in culture. In this study, the teleomorph-anamorph connection of *C. sobolifera* collected from Sichuan, China, is established based on sequence analysis of the Internal Transcribed Spacer (ITS) regions of ribosomal DNA. The anamorph is described here as *Beauveria sobolifera* sp. nov.

Key words: anamorph, *Beauveria sobolifera*, *Cordyceps sobolifera*, ITS, new species, teleomorph.

Introduction

Cordyceps sobolifera (Hill) Berk. and Broome is one of the earliest species described in *Cordyceps* and occurs on Cicada nymphs. It has been the subject of many studies and it appears in the literature under a range of names. J. Hill described it as *Clavaria sobolifera* Hill based on specimens from Martinique (Watson, 1763), but Berkeley (1843) provided the first formal description of *Cordyceps sobolifera* under the name *Sphaeria sobolifera* (Hill) Berk. Tulasne and Tulasne (1865) refined it to *Torrubia sobolifera* (Hill) Tul. and C. Tul. and a few years later Berkeley and Broome (1875) transferred the species to *Cordyceps* as *C. sobolifera*. Additional synonyms of the species are *C. voeltzkowii* Henn. (1908) based on a specimen from Madagascar and *C. gracilis* Dur. and Mont. on a specimen from Sri Lanka (Petch, 1924). *Cordyceps sobolifera* is widely distributed and is known from China, Cuba, Dominic Republic, Guadeloupe, Japan, Madagascar, Martinique, Mexico and Sri Lanka (Kobayasi, 1982).

There have been some reports of the anamorph of *C. sobolifera*, although there is as yet no direct evidence of the relationship between the anamorph and the teleomorph. Petch (1932) studied the large-spored *Isaria sinclairii* Miquel and considered it to be anamorph of *C. sobolifera*. Petch (1932) however, stated that there was no further evidence for this relationship, except that both grow on Cicada nymphs. Petch (1924, 1931) had previously listed some names related to *Isaria cicadae*, including *Cordyceps sinclairii* Berk., *Cordyceps miquelii* (Tul.) Sacc., *I. arbuscula* Hariot, *I. amorpha* Höhn., *I. harioti* Arnaud, *I. cosmopsaltriae* Yasuda and *I. sinclairii* (Berk.) Petch. Biourge (1923) transferred all species of *Isaria* and *Spicaria* having phialides to *Penicillium* Link. *Isaria* species producing phialides with distinct necks, catenulate conidia and verticillate conidiophores were subsequently placed in the section Isarioidea of *Paecilomyces* (Samson, 1974). Thus *I. cicadae* was combined in *Paecilomyces* as *P. cicadae* (Miquel) Samson. Shing (1975) considered an *Isaria* sp. to be the anamorph of *Cordyceps sobolifera*. Mains (1951) also accepted that *I. cicadae* was the anamorph.

There is presently no direct evidence to confirm that *Isaria* is the anamorph of *C. sobolifera*. It is interesting that a detailed description and illustration of the conidial form of *C. sobolifera* was published by Kobayasi (1939) based on specimens from different regions of Japan, but it is quite different from *P. cicadae*. Kobayasi (1941, 1982) discussed the conidial form of *C. sobolifera*, but gave no name for it.

A survey of entomogenous fungi was made from Dujiangyan, Sichuan, China and fresh mature specimens of *C. sobolifera* were collected. To determine if the culture obtained from ascospores is the true anamorph, the Internal Transcribed Spacer (ITS) sequences of rDNA for both culture and specimen were compared. The results are reported here.

Materials and methods

Collection of specimens and isolation

Specimens of *C. sobolifera* (G97026) were collected from muddy soil under trees in Du Jiang Yan Park, Sichuan, China, in July 1997. To obtain ascospores, the sclerotia of a fresh specimen were wrapped in moist tissue and its fertile head was suspended over a sterile glass slide so that the ascospores were released on to the slide. Unfortunately the ascospores released on the glass slide and then transferred to potato dextrose agar (PDA) failed to germinate because of serious contamination with bacteria, but a few slow-growing colonies were obtained when the abdominal sclerotia were transferred to PDA and incubated at 23 C for 50 d. Morphological characteristics are based

on cultures incubated on PDA and Czapek-Dox agar at 23 C for 14 and 50 d. The culture was labeled as G97007.

Scanning electron microscopy

A piece of a sporulating culture was fixed at room temperature using 2.5% glutaraldehyde in aqueous for 12 h, followed by washing with phosphate buffer twice and treatment with 2% osmium tetroxide for 2 h. Fixed material was washed in distilled water twice, dehydrated in a series of acetone concentrations from 30, 50, 70, 80, 90, 95, and 100%, each time for about 15 min. The sample was placed in a critical point dryer filled with acetone, and dried at 37 C and 1200 bar. A dried sample was loaded on stub and coated with gold for five min.

DNA extraction, amplification and sequencing

Air-dried specimens of *C. sobolifera* stored at 4 C were examined under a dissecting microscope. Debris and surface stains were removed with a fine brush and dissecting knife. Samples (0.002-0.01 g) 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 whirligig head.

Fresh mycelium was obtained by scraping the culture from the surface of PDA plates, which had been incubated at 23 C for 60 d. About 0.05 g 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 from both dry specimens and fresh mycelium were extracted from stromal powder or thick mycelial liquid. 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 chloroform:isoamyl alcohol (24:1) was pipetted into 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 chloroform-isoamyl alcohol extraction was repeated, and 250 µl isopropanal then pipetted into a 1.5 ml Eppendorf tube with the supernatant, mixed by shaking and stored at 20 C overnight for DNA precipitation. The precipitate was centrifuged at 13000 rpm for 15 min, the liquid was discarded and the tube was dried at 65 C for 20 min. Fifty µl of double-distilled water was then added to the DNA preparation.

The PCR reaction mixtures were made up from equal volumes of DNA templates and a total of 100 µl of reaction mixture containing: 10 µl 10 × buffer (10 mM Tris/HCl, pH 8.3), 10 µl 25 mM $MgCl_2$, 0.5 µl 10 mM of each of the four deoxyribonucleotide triphosphates (dNTP), 1 µl 100 ng/µl each of primers (ITS4: 5'-TCCTCCGCTTATT GATATGC-3'; and ITS5: 5'-

GGAAGTAAAAGTCGTAACAAG G-3') (White *et al.*, 1990), 0.5 µl 250 U/ml 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: 97 C premelt for 1 min, followed by 30 cycles at 97 C for 1 min., 48 C for 1 min., 72 C for three min and then at 72 C for 7 min extension. Products were purified using the QIAquick PCR purification Kit (QIAGEN, LTD.). Cycle sequence reactions was performed on the Gene amp PCR system 9600 (Perkin Elmer Corporation) by the cyclic reaction termination method using fluorescently labeled dideoxyribonucleotide triphosphates according to the manufact protocols. The sequencing products were purified according to the instruction for the sequencing kit ($PRISM_{tm}$ Ready Reaction Dyedideoxy Terminator Cycle Sequencing Kit, ABI, Inc.). The electrophoresis and data collection were performed on an ABI_{tm} 377 DNA Sequencer (Perkin Elmer) and the data transferred to a Power Macintosh 7200/90 which was connected to the Sequencer. The raw data were edited with Sequence Navigator and the assembled with AutoAssembler (ABI, Inc.). The GENBANK accession numbers are AJ309325 for G97007, anamorph and AJ309326 for G97026, teleomorph..

Results

Description of* Cordyceps sobolifera *from Sichuan, China (Fig. 1)

The fungus grew on wing-less cicada nymphs. The specimens were collected in moist evergreen forest. Half the stromata and sclerotia (body of nymph) were buried and the other half exposed above the soil surface. The solitary stromata arising from the head of the host measure 5-8 cm high and 4-6 mm wide, yellow to brown and hollow. The heads were clavate and roughened due to the lightly protruding necks of the perithecia. Nodes were either present or absent at the middle of stipes (Fig. 1). No conidia were found on surfaces of the nodes. The perithecia were immersed, narrowly ovoid, 500-600 × 200-250 µm; Asci were cylindrical, 300-450 × 5.7-6.5 µm, with a semiglobose head. The part-spores of ascospores were cylindrical, 7-13 × 1-1.5 µm.

Description of the anamorph

Beauveria sobolifera Z.Y. Liu, Z.Q. Liang, Whalley, A.Y. Liu and Y.J. Yao, **sp. nov.** (Figs. 2-6)

Coloniae in agaro potato dextrosi (PDA) restrictis, primo albus, tandem flavidus. *Hyphae* hyalinae, leves. *Conidiophora* hyalinae, 15-18 µm longa, 2-3.4 µm lata, non aut sparsus ramosus, interdum tumerem, 4-6.5 µm lata. *Conidiiferus-cella* solitariae aut aliqaut in

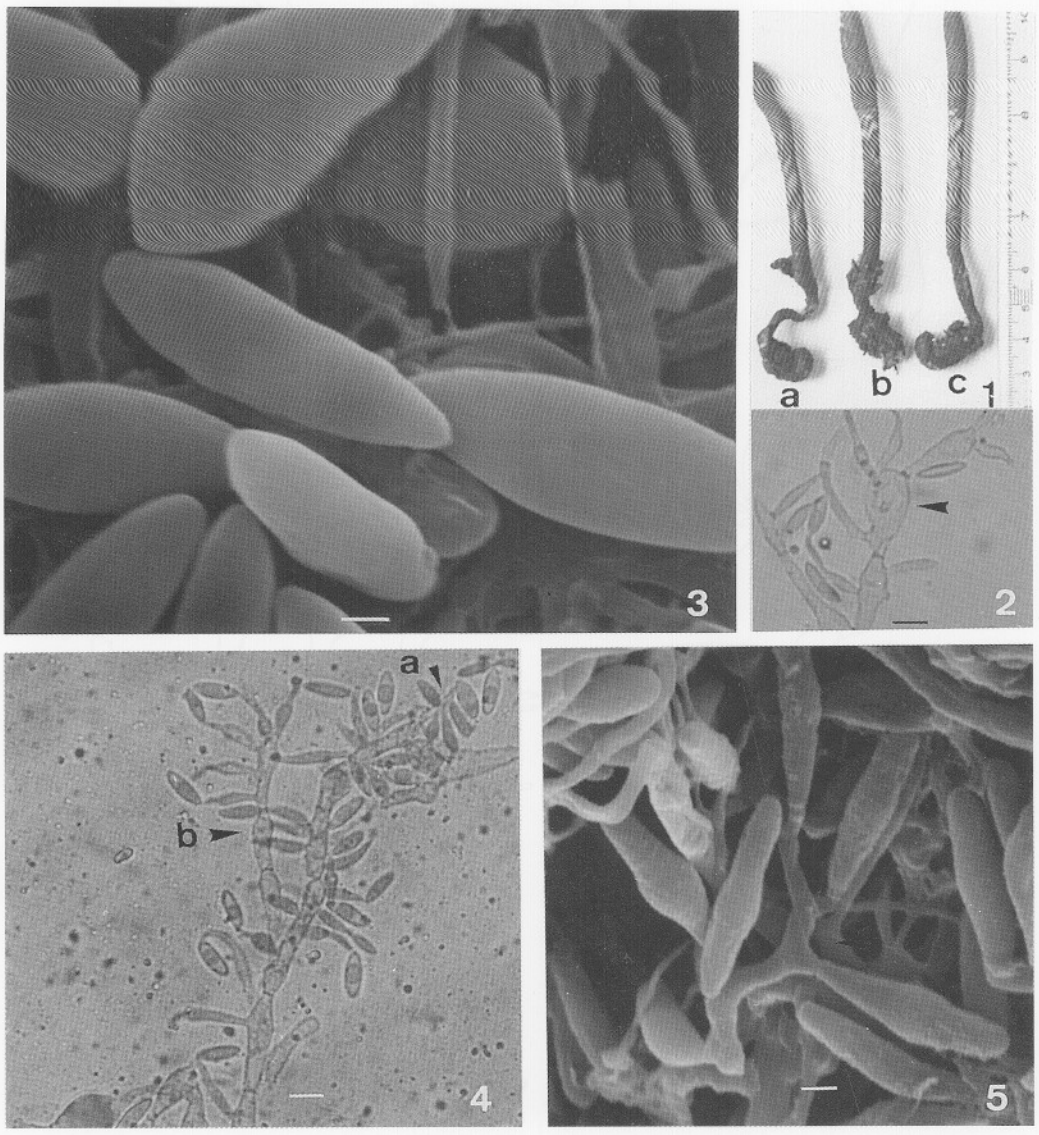

Fig. 1. *Cordyceps sobolifera* (x 0.5). **a.** stroma with nodes on one side. **b.** stroma with nodes. **c.** stroma without nodes. **Figs. 2-5.** *Beauveria sobolifera* **2.** Swollen cell of a conidiophore (arrow) (Photograph). **3.** Conidia (SEM) **4.** Relationship between conidia, conidiogenous cell and conidiophores (Photographs). **a.** arrangement of conidia on conidiogenous cell (arrow). **b.** arrangement of conidiogenous cell on a conidiophore (arrow). **5.** Arrangement of conidia on conidiogenous cell (SEM). Bars: 2, 4 = 5 μm. 3, 5 = 1 μm.

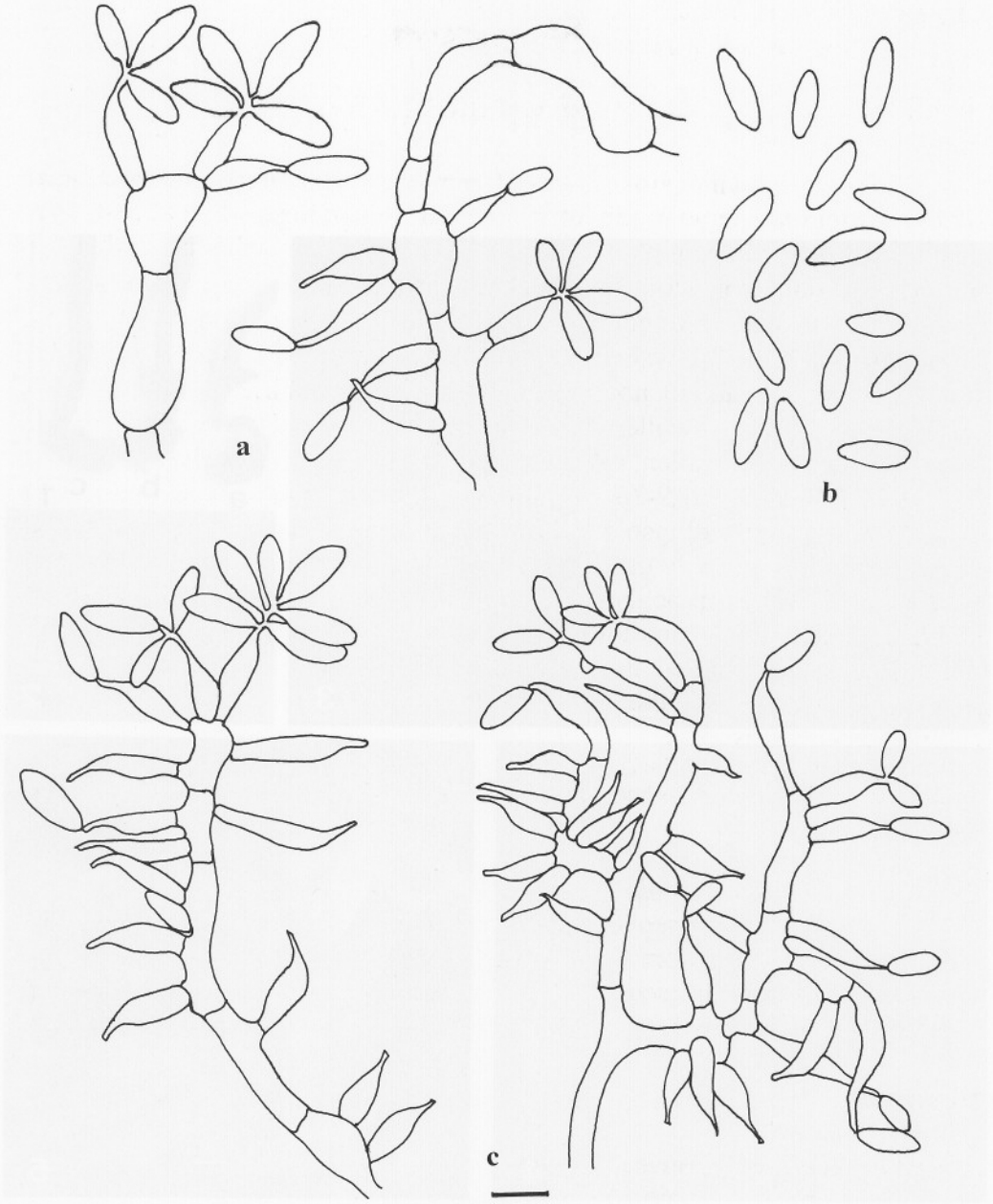

Fig. 6. Conidiogenous structures of *Beauveria sobolifera*. **a.** swollen cell of conidiophores. **b.** conidia. **c.** not branched or sparingly and irregularly branched conidiophores and typical *Beauveria*-type conidiogenous structure (arrow). Bar = 5 μm.

tumidus conidiophoris, (7.6-)8.9-11.5(-15.9) × 1.9-3.2 µm, base ellipsoideae, curvatus collum. *Conidia* hyalinae, leves, ellipsoideae, (5.7-)7.6-9.5(-10.2) × 1.9-3.2 µm, in capitulis mucosis minutis connexa. *Chlamydosporae* absentes.

Culturus siccus typus (G 97007) et culturus vivus, in Guizhou University, Guiyang, Guizhou, China.

Colonies growing slowly, 25-30 mm diam. on potato-dextrose agar (PDA) at room temperature (about 23 C) after 56 d, dense, white at first, later pale yellow, sometimes pink due to conidial colour, hemispherical with a 4-5 mm high region protruding in the centre part of the colony. *Conidia* first produced at the bottom of colonies, then gradually up to the protruding parts. *Hyphae* hyaline, smooth-walled. *Conidiophores* hyaline, 15-80 µm long, 2-3.4 µm wide (Fig. 4, 6a and c), not branched or sparingly and irregularly branched, usually more or less swollen (Figs. 4, 6c), occasionally some cells of the conidiophores very swollen, 4-6.5 µm wide (Figs. 2, 6a). *Conidiogenous cells* (7.6-)8.9-11.5(-15.9) × 1.9-3.2 µm, solitary or with a few, growing densely on swollen cells, mostly ellipsoidal at the lower part, occasionally contracted at the middle, then narrowly tapering into a rachis, terminal conidiogenous cells usually more elongate; rachis straight or bent without clear denticulate scars. *Conidia* produced on conidiogenous cells of sympodial elongation, hyaline, one-celled, smooth-walled, long ellipsoidal, (5.7-)7.6-9.5(-10.2) × 1.9-3.2 µm, aggregating into heads at the tips of the conidiogenous cells (Figs. 4, 5, 6). No chlamydospores observed.

Holotype: CHINA, Sichuan, Du Jiang Yan Park, July 1997, from muddy soil under trees, [College of Biological Technology Guizhou University, Guiyang, Guizhou, G97007; **ex-type** living culture].

The results of molecular biology

The aligned DNA sequence data matrix generated using ITS4 and ITS5 data for *C. sobolifera* from China comprised 560 base pairs. There were no differences between the sequences from the teleomorph (*C. sobolifera*) and anamorph (*B. sobolifera*).

Discussion

Identification of Beauveria sobolifera

Beauveria Vuillemin (1912) was revised several times. Petch (1926) accepted two species, both entomogenous, *B. bassiana* (Bals.) Vuill. and *B. brongniartii* (Sacc.) Petch which were distinct in conidial shape. De Hoog (1972) revised the genus and accepted three species *B. bassiana*, *B. brongniartii* and *B. alba* (Limber) Saccas. *Beauveria alba* is not parasitic on insects and was later transferred to the genus *Engyodontium* as *E. album* (Limber) De Hoog (De Hoog, 1978). De Hoog and Rao (1975) described

another new species *B. vermiconia* De Hoog and Rao, which was later reported to cause an epizootic among larvae of *Hylamorpha elegans* (Coleoptera, Scarabaeidae) (Glare *et al.*, 1993). Three years later *B. felina* (Fr.) Carmichael, Kendrick and Conner was transferred from *I. felina* (Carmichael *et al.*, 1980). The classification of this taxon in *Isaria* or *Beauveria* still is debatable (Von Arx, 1986). Samson and Evans (1982) described a new species, *B. velata* Samson and Evans, found on Lepidoptera larvae, and a new combination, *B. amorpha* (Höhn.) Samson and Evans transferred from *Isaria amorpha* Höhn. Bissett and Widden (1988) reported *B. caledonica* as a new species from moorland soil in Scotland. Morphologically, the genus *Beauveria* is characterised by dry blastic conidia formed on sympodially elongating conidiogenous cells, which are quite different from the slime spores arising from non-percurrent phialides found in *Tolypocladium*. The committee for Fungi of the International Association for Plant Taxonomy accepted the proposal of Dreyfuss and Gams (1994) and considered the type species, *Tolypocladium inflatum* W. Gams as the correct name (Gams, 1996).

Table 1. Comparison of conidia of species in *Beauveria*.

Species	Size	Shape	Habitat	Reference
B. amorpha	3.5-5 × 1.5-2 μm	Cylindrical	Insects	Samson and Evans 1982
B. bassiana	(1.9-)2.4-3.2(-3.6) × 1.9-3 μm	Globose to ellipsoidal	Insects	De Hoog 1972
B. brongniartii	3.2-4.8 × 1.6-2.4 μm	Ellipsoidal to cylindrical	Insects	Shimazu and Hashimota 1988
B. caledonica	(2.4-) 3-5(-6.5) × 1-1.8(-2) μm	Ellipsoidal to cylindrical	Soil	Bissett and Widden 1988
B. velata	3-4 × 3-4 μm	Globose to ellipsoid	Insects	Samson and Evans 1982
B. vermiconia	3.2-4.2 × 3.2-4 μm	Vermiform	Soil or insects	De Hoog and Rao 1975
B. sobolifera	(5.7-)7.6-9.5(-10.2) × 1.9-3.2 μm	Long ellipsoidal	Insects	This paper

Based on light and scanning electron microscopical characters this fungus (isolate G97007) was identified as a new species and named *Beauveria sobolifera* (Figs. 4, 5 and 6). It is characterised by long ellipsoidal conidia (Figs. 3 and 6b) formed on conidiophores lacking obvious conidial scars, and scattered along the cells of the conidiophore (Figs. 4 and 6), and swollen conidiogenous cells (Figs. 3 and 6a). It is very easy to distinguish *B. sobolifera* from other species in *Beauveria* by comparing the size and shape of their conidia (Mugnai *et al.*, 1989). The conidial features of the species accepted by

most mycologists and *B. sobolifera* are compared in Table 1. Only *B. sobolifera* has long ellipsoid conidia. The new species has long conidia [(5.7-)7.6-9.5(-10.2) µm] which are much longer than the other species of the genus, even than those of *B. caledonica* [3-5 (-6.5) µm]. Furthermore *B. caledonica* was isolated from soil, whereas *B. sobolifera* is pathogenic on cicada.

Beauveria sobolifera *is the anamorph of* Cordyceps sobolifera

In this study, *Beauveria sobolifera* has been shown to be the anamorph of *Cordyceps sobolifera*. Many conidial genera were connected to *Cordyceps* but only one species of *Beauveria*, *B. brongniartii* has been reported to be connected to *C. brongniartii* (Shimazu *et al.*, 1988). This is the second report that the genus *Beauveria* is associated with a teleomorph in *Cordyceps*. In addition, the microscopic features of the culture of *B. sobolifera* are very similar to those of the anamorph that Kobayasi (1939) had described. We consider that Kobayasi's anamorph and *B. sobolifera* is the same fungus.

Acknowledgments

This study was supported by the Guizhou Scientific Foundation Committee and partially by the Scientific Fund Committee and Going-abroad Fund Committee of China. We thank K.D. Hyde for his kind suggestions and correcting the paper.

References

Arx, J.A. von (1986). *Tolypocladium*, a synonym of *Beauveria*. Mycotaxon 25: 153-158.

Berkeley, J.M. (1843). On some entomogenous *Sphariae*. Hooker's London Journal of Botany 2: 205-211.

Berkeley, M.J. and Broome, C.E. (1875). On the fungi of Ceylon. *Journal Linnean Society* 14: 110.

Biourge, P.H. (1923). Les Moisissures du groupe *Penicillium* Link. Cellule 33: 7-331.

Bissett, J. and Widden, P. (1988). A new species of *Beauveria* isolated from Scottish moorland soil. Canadian Journal of Botany 66: 361-362.

Carmichael, J.W., Kendrick, W.B., Conners, I.L. and Sigler, L. (1980). *Genera of Hyphomycetes*. University Alberta Press, Edmonton. pp386

Dreyfuss, M.M. and Gams, W. (1994). Proposal to reject *Pachybasium niveum* Rostr. in order to remain the name *Tolypocladium inflatum* W. Gams for the fungus that produces cyclosporin. Taxon 43: 660-661.

Gams, W. (1996). Report of the Committee for Fungi. Taxon 45: 309-311.

Glare, T., Jackson, T.A. and Cisternas, E.A. (1993), *Beauveria vermiconia* is an entomogenous fungus. Mycological Research 97: 336-338.

Hennings, P. (1908). Fungi von Madagaskar den Comoren und Ostafrika. Voeltzkow, Reise in Ostafrica 3: 16-33.

Hoog, G.S.de (1972). The genera *Beauveria*, *Isaria*, *Tritirachium* and *Acrodontium*. Studies in Mycology 1: 1-41.

Hoog, G.S.de and Rao, V. (1975). Some new hyphomycetes. Persoonia 8: 207-212.

Hoog, G.S.de (1978). Notes on fungicolous hyphomycetes and their relatives. Persoonia 10: 33-81.

Ko, K.S., Hong, S.G. and Jung, H.S. (1997). Phylogenetic analysis of *Trichaptum* based on nuclear 18S, 5.8S and ITS ribosomal DNA sequences. Mycologia 89: 727-734.

Kobayasi, Y. (1939). On the genus *Cordyceps* and its allies on cicada from Japan. Bulletin of the Biogeographical Society of Japan 9: 145-176.

Kobayasi, Y. (1941). The genus *Cordyceps* and its allies. Science Reports of the Tokyo Bunrika Daigaku, Section B. 5: 54-207

Kobayasi, Y. (1982). Key to the genera *Cordyceps* and *Torrubiella*. Transactions of the Mycological Society Japan 23: 329-364.

Liang, Z.Q. (1991). Anamorphs of *Cordyceps* and their determination. Journal of Southwest Agriculture. 4: 1-8. (In Chinese)

Mains, E.B. (1951). *Hirsutella*, *Tilachildium* and *Synnematium*. Mycologia 43: 611-18.

Mugnai, L., Bridge, P.D. and Evans, H.C. (1989). A chemotaxonomic evaluation of the genus *Beauveria*. Mycological Research 92: 199-209.

Petch, T. (1924). Studies in entomogenous fungi. Transactions of the British Mycological Society 10: 28-45.

Petch, T. (1926). Studies in entomogenous fungi. VIII. Notes on *Beauveria bassiana*. Transactions of the British Mycological Society 10: 244-271.

Petch, T. (1932). Notes on entomogenous fungi. Transactions of the British Mycological Society 15: 209-245.

Samson, R.A. (1974). *Paecilomyces* and some allied hyphomycetes. Studies in Mycology 6: 1-119.

Samson, R.A. and Evans, H.C. (1982). Two new *Beauveria* from South America. Journal of Invertebrate Pathology 39: 93-97.

Shimazu, M., Mitsuhashashi, W., and Hashimoto, H. (1988). *Cordyceps brongniartii* sp. nov., the teleomorph of *Beauveria brongniartii*. Transactions of Mycological Society of Japan 29: 323-330.

Shing, S.Z. (1975). Classification of *Cordyceps sobolifera* (Hill.) Berk. et Br. and *Cordyceps cicada* Shing sp. nov. Acta Microbiologica Sinica 15: 27-30.

Tulasne, L.R. and Tulasne, C. (1865). Selecta Fungorum Carpologia III, Paris

Vuillemin, P. (1912). *Beauveria*, nouveau genre de Verticilliacees. Bulletin de la Societé Botanique de France 29: 34-40.

Watson, W. (1763). An account of the insect called the Vegetable Fly. Philosophical Transactions of the Royal Society 53: 15-17.

(Received 20 December 2000, accepted 4 April 2001)