Mycoscience (2009) 50:261–272
DOI 10.1007/s10267-008-0480-y

FULL PAPER

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Teleomorph–anamorph relationships and reclassification of *Cordyceps cuboidea* and its allied species

Received: July 16, 2008 / Accepted: December 26, 2008

Abstract Based on morphological characteristics and molecular phylogeny, we reclassified *Cordyceps cuboidea* and allied species *C. alboperitheciata*, *C. prolifica*, and *Ophiocordyceps ryogamiensis*. We investigated their teleomorph-anamorph relationships and revealed that these four species have *Hirsutella*-like anamorphs with morphological differences between them. By analyzing their molecular phylogeny, inferred from DNA sequences of internal transcribed spacer (ITS) and large subunit (LSU) D1/D2 region of rDNA, they were separated into four close-knit clades. Although *C. prolifica* and *O. ryogamiensis* formed their own clades, isolates of *C. cuboidea* separated into two clades, i.e., a true *C. cuboidea* clade and one resembling a new species, the *O. paracuboidea* clade. The latter two species are distinguished by the fruiting region of the stroma. In addition, *C. alboperitheciata* is regarded as a synonym of *C. cuboidea.* From the morphology, teleomorph-anamorph relationships, and molecular phylogeny, we concluded these species should be assigned to the genus *Ophiocordyceps*.

Key words Insect fungi · *Ophiocordyceps paracuboidea* · Ophiocordycipitaceae · Taxonomy

Introduction

Cordyceps (Fr.) Link sensu lato (s. l.) (Clavicipitaceae s. l., Ascomycota), including 400–500 species (Index Fungorum; http://www.speciesfungorum.org/Names/Names.asp), has been known as pathogens to ten orders of arthropods and an ascomycete, *Elaphomyces* (Kobayasi 1941, 1982). Kobayasi (1941) classified *Cordyceps* s. l. to three subgenera and seven subsections based on the morphological characteristics. Although these characters and host preferences are still important for identification to the species, molecular phylogenetic studies in the last decade have proposed a new taxonomy system (Artjariyasripong et al. 2001; Sung et al. 2001; Stensrud et al. 2005). Sung et al. (2007) divided, on the basis of phylogenetic analysis using several genes, *Cordyceps* s. l. into four genera in three families: *Cordyceps* sensu stricto (s. s.), *Metacordyceps* G. H. Sung et al., *Elaphocordyceps* G. H. Sung & Spatafora, and *Ophiocordyceps* (Petch) G. H. Sung et al. These four genera are characterized by morphology of their ascostroma and the anamorphs. However, 175 species are still suspended as *incertae sedis* because of insufficient information about both morphology and molecular phylogeny. These lacks are mainly caused by the fact that many holotype specimens were lost or preserved in formalin, by which the DNA of those specimens has been damaged and is unusable for molecular phylogeny study.

Cordyceps cuboidea Kobayasi & Shimizu, *C. alboperitheciata* Kobayasi, and *C. prolifica* Kobayasi remained as *incertae sedis*. To establish the taxonomy of these *Cordyceps* s. l. and related taxa, analyses of the morphology of specimens and living strains, teleomorph-anamorph relationships, ecology, and molecular phylogeny are required. In this article, we describe anamorphs of the three species mentioned above and *Ophiocordyceps ryogamiensis* (Kobayasi & Shimizu) G. H. Sung et al. by cultivating newly obtained materials. To clarify the taxonomy of these closely related species, we examined their phylogenetic relationships using sequences of two regions of rDNA [internal transcribed spacer (ITS)1–5.8S-ITS2, large subunit (LSU) D1/D2] and compared the morphology of both the teleomorphs and anamorphs.

Material and methods

Fungal materials and isolation

The specimen numbers, collecting dates, and other details are shown in Table 1. Table 2 shows the morphological characters of *C. cuboidea* and its allied species, on which we based the identification of the specimens. The holotype specimen of *C. cuboidea* (TNS-F-195457), preserved in the

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Table 1. Species, strains, and specimens used in this study

Strain no.	Specimen no.	Host	Locality of source	Collecting date	GenBank/DDBJ accession no.	
					ITS	LSU D1/D2
Cordyceps cuboidea						
NBRC 100941	NBRC H-12377	Hyperparasited stroma of *C. stylophora* Berk. & Broome (1857) on larva of beetle	Settsu-kyo, Takatsuki, Osaka	31 Oct. 2004	AB378666	AB378646
NBRC 100942	NBRC H-12383	Larva of beetle	Ryu-sen-kyo, Ibaraki, Osaka	08 Dec. 2004		AB378647
NBRC 101742	NBRC H-12471	Larva of beetle	Shimoseya, Miyazu, Kyoto	16 Oct. 2005	AB378667	AB378648
NBRC 101739	NBRC H-12411	Larva of beetle	Ryu-sen-kyo, Ibaraki, Osaka	14 Jul. 2005	AB378668	AB378649
NBRC 103834	NBRC H-12569	Larva of beetle	Ryu-sen-kyo, Ibaraki, Osaka	09 Sep. 2006	AB378669	AB378650
NBRC 103835	NBRC H-12600	Larva of beetle	Higashi-kakegawa liver, Kameoka, Osaka	12 Oct. 2006	AB378670	AB378651
C. alboperitheciata						
NBRC 101740	NBRC H-12407	Larva of beetle	Settsu-kyo, Takatsuki, Osaka	23 Jun. 2005	AB378671	AB378652
NBRC 103836	NBRC H-12545	Larva of beetle	Niryo, Takatsuki, Osaka	24 Jun. 2006	AB378672	AB378653
Ophiocordyceps ryogamiensis						
NBRC 101751	NBRC H-12452	Larva of beetle	Ryu-sen-kyo, Ibaraki, Osaka	26 Aug. 2005	AB378673	AB378654
NBRC 103837	NBRC H-12599	Larva of beetle	Ryu-sen-kyo, Ibaraki, Osaka	09 Oct. 2006	AB378674	AB378655
C. prolifica						
NBRC 100744	NBRC H-12364	Larva of *Tanna japonensis*	Zen-nouji, Oomiya, Kyoutango, Kyoto	13 Sep. 2004		AB378656
NBRC 101750	NBRC H-12436	Larva of *T. japonensis*	Settsu-kyo, Takatsuki, Osaka	01 Aug. 2005	AB378675	AB378657
NBRC 103838	NBRC H-12581	Larva of *T. japonensis*	Miyazu, Kyoto	10 Sep. 2006	AB378676	AB378658
NBRC 103839	NBRC H-12583	Larva of *T. japonensis*	Miyazu, Kyoto	10 Sep. 2006	AB378677	AB378659
White synnema / conidial mycelia						
NBRC 103840	NBRC H-12546	Stroma of immature *C.* cf. *cuboidea* on larva of beetle	Settsu-kyo, Takatsuki, Osaka	13 Jul. 2006		AB378660
NBRC 103841	NBRC H-12564	Stroma of *C. prolifica* on larva of *T. japonensis*	Settsu-kyo, Takatsuki, Osaka	19 Aug. 2006		AB378661
NBRC 103842	NBRC H-12568	Stroma of immature *C. ryogamiensis* on larva of beetle	Ryu-sen-kyo, Ibaraki, Osaka	09 Sep. 2006	AB378678	AB378662
NBRC 103843	NBRC H-12570	Stroma of immature *C.* cf. *cuboidea* on larva of beetle	Ryu-sen-kyo, Ibaraki, Osaka	09 Sep. 2006		AB378663
NBRC 103844	NBRC H-12576	Stroma of *C. prolifica* on larva of cicada	Miyazu, Kyoto	10 Sep. 2006		AB378664
NBRC 103845	NBRC H-12581	Stroma of *C. prolifica* on larva of cicada	Miyazu, Kyoto	10 Sep. 2006		AB378665

ITS, internal transcribed spacer; LSU, large subunit

Table 2. Characteristics of teleomorphs of *Cordyceps cuboidea* and its allied species according to original descriptions

Species	*C. cuboidea*	*C. alboperitheciata*	*Ophiocordyceps ryogamiensis*	*C. prolifica*
Reference	Kobayasi and Shimizu (1980)	Kobayasi and Shimizu (1982)	Kobayasi and Shimizu (1983)	Kobayasi and Shimizu (1963)
Host	Larva of Coleoptera	Larva of Coleoptera	Larva of Coleoptera	Larva of Cicada
Stromata	Cylindrical attenuate, ochre yellow	White, pale dark brown at the base	Fleshy, white, palely darkened, glabrate at the base	Thin cylindrical, glabrous, brown, partly covered with grayish mycelia
Perithecia	Superficial, lemon-shaped, glabrate	Superficial, ovoid, white	Superficial, ovoid, central clustered on stroma	Superficial, ovoid or ellipsoid, grayish brown
Size (μm)	450–500 × 200–270	500–550 × 270–300	320–430 × 200–230	550–580 × 300–310
Part spores (μm)	1.5 × 1	1.5–2 × 1	1.5–2.5 × 1	1.5 × 1

National Museum of Nature and Science, Japan, was also examined for comparison with collected specimens. Multiple part spores from a mature perithecium were isolated using the Skerman's micromanipulator. Either TrePY agar (trehalose 20 g, peptone 3 g, Bacto yeast extract 1 g, distilled water 1 l, agar 15 g; pH 6.7) or potato sucrose agar (PSA: decocted extract of 200 g potato, sucrose 20 g, distilled water 1 l, agar 20 g; pH 5.6) was used for isolation and cultivation. From the anamorphic state, multiple conidia were isolated in the same way as above. Cultures were incubated at room temperature (approximately 25°C). All specimens were dried and deposited into the NITE Biological Resource Center (NBRC) Herbarium, and all cultures were deposited into the NBRC culture collection.

Morphological observations

Morphological observations were conducted by light microscopy (LM) and scanning electron microscopy (SEM). To measure teleomorphic structures such as perithecia, asci, and part spores, fresh materials were mounted in one drop of water on glass slides and observed with a LM Eclips E600 (Nikon, Tokyo). Anamorphs formed on TrePY agar or PSA were observed by SEM. To prepare the SEM specimens, agar blocks with mycelia were fixed with 1% OsO_4 solution at 4°C overnight, then dehydrated in an ethanol series, and finally substituted with isoamyl acetate. After critical-point drying and coating with platinum-palladium, the specimens were observed under a JSM-6060 (JEOL, Tokyo, Japan) operated at 15 kV.

Phylogenetic analysis

For DNA isolation, cultures were incubated on TrePY agar or PSA for 1–4 weeks at room temperature. Total DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) or Nucleon PhytoPure plant DNA extraction kit (Amersham Bioscience, Piscataway, NJ, USA) according to the manufacturers' instructions.

The internal transcribed spacer (ITS) and LSU D1/D2 regions of rDNA were amplified by polymerase chain reaction (PCR) using *TaKaRa Ex Taq* HS (TaKaRa Bio, Otsu, Shiga, Japan) as a single fragment with the standard primer pairs ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) for ITS region (White et al. 1990), and NL1 (5′-GCATATCAATA AGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTG TTTCAAGACGG-3′) for LSU D1/D2 regions (O'Donnell 1993). Amplification of the desired fragment was performed with a T-gradient thermocycler (Whatman Biometra, Göttingen, Germany) with the following program: 3 min for 95°C, then 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C, extension for 1 min at 72°C, incubation 5 min at 72°C, and soaking at 4°C. Amplified DNA was purified using Agencourt AMPure (Agencourt Bioscience, Beverly, MA, USA), then used as a template DNA for the sequencing amplification. The BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was applied for the reaction under the following thermal cycler program: 3 min for 96°C, then 30 cycles of 10 s at 96°C, 5 s at 50°C, 2 min at 60°C, followed by a 4°C soak. Nucleotide sequences were determined in both directions using the same primers as for PCR. Sequences were analyzed with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Sequences were assembled using ATGC ver. 4.0.10 (Genetyx, Tokyo, Japan), and edited in multi-alignment using the BioEdit Sequence Alignment Editor ver. 7.0.5.3 (Hall 1999) and Clustal X ver. 1.83 software (Thompson et al. 1997) package. The Clustal X was used to generate the evolutionary distances (the K_{nuc} value; Kimura 1980) and the similarity values and to perform the neighbor-joining (NJ) analysis (Saitou and Nei 1987) from K_{nuc} values. The bootstrap resampling method (Felsenstein 1985) with 1000 replicates was performed for evaluating the topology of the phylogenetic tree. The NJPlot (Perrière and Gouy 1996) was used for plotting the phylogenetic tree. The alignment was deposited in TreeBASE (http://www.treebase.org/treebase/index.html) under the study number S2272. The sequence data obtained in this study were registered to the DDBJ/EMBL/GenBank nucleotide sequence database; their accession numbers are shown in Table 2. Eighteen sequences of *Ophiocordyceps* species, 7 sequences of *Elaphocordyceps*, 1 sequence of *Metacordyceps*, 5 sequences of *Cordyceps* sensu strict (s. s.), 3 sequences of *Cordyceps* s. l., 3 sequences of *Hypocrea*, and *Hypomyces* (as an outgroup) were retrieved from the database (see Figs. 1, 2) and employed for phylogenetic analysis.

Results and discussion

The phylogenetic tree based on the partial sequences of LSU D1/D2 region (504 bp) of 38 species belonging to *Cordyceps* s. l. shows that 15 strains of the 4 species (*C. cuboidea*, *C. alboperitheciata*, *C. prolifica*, and *O. ryogamiensis*) aggregate into one large cluster as a sister group of *Ophiocordyceps* species (Fig. 1). These species were found to be phylogenetically closely related to each other. For detailed analysis of the strains in this cluster, an NJ tree was constructed using the ITS region (13 sequences, 606 bp data sets; Fig. 2). The 15 strains are subdivided into four clades, which are supported with relatively high bootstrap values. The branching topologies of the 15 strains are almost the same in the above two trees. *Cordyceps cuboidea* (NBRC 100941, NBRC 103834, NBRC 103835) and *C. alboperitheciata* (NBRC 101740, NBRC 103836) form clade 1, *C. ryogamiensis* and the strain from conidia on immature *Cordyceps* sp. NBRC 103842 form clade 2, three strains identified as "*C. cuboidea*" (NBRC 100942, NBRC 101739, NBRC 101742) form clade 3, and four strains of *C. prolifica* form clade 4.

Based on culturing, we verified teleomorph-anamorph relationships of *C. cuboidea*, *C. alboperitheciata*, *C. prolifica*, and *O. ryogamiensis*. Morphological characteristics of both teleomorphs and anamorphs of the four clades were

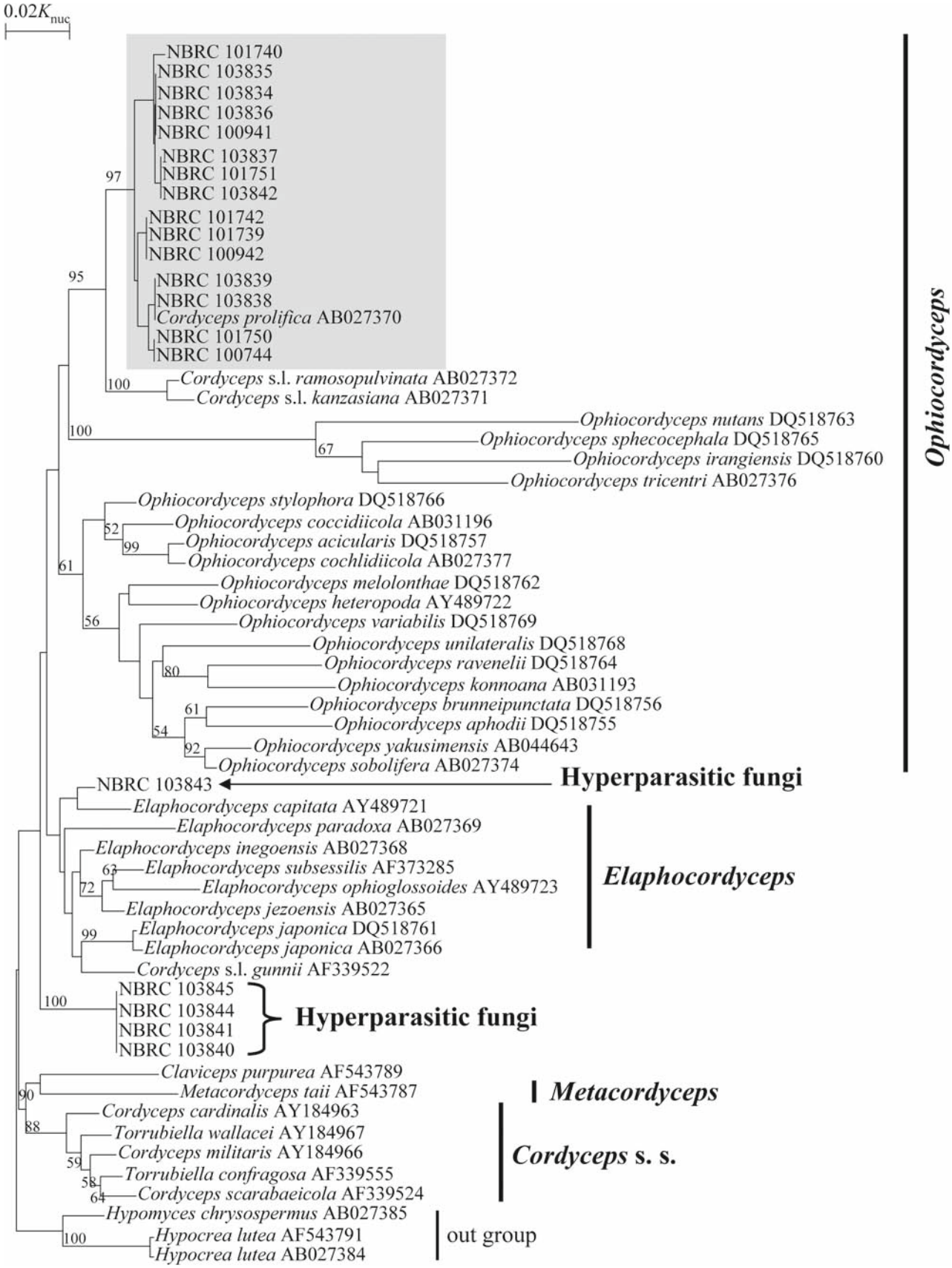

Fig. 1. Neighbor-joining tree derived from large subunit rDNA D1/D2 region sequences of our isolates of *Cordyceps cuboidea* and its allied species and of other species that were retrieved from GenBank. *Hypomyces chrysospermus* (AB027385) and *Hypocrea lutea* (AB027384, AF543791) were specified as outgroup. Bootstrap values ≥50% are shown *above branches*

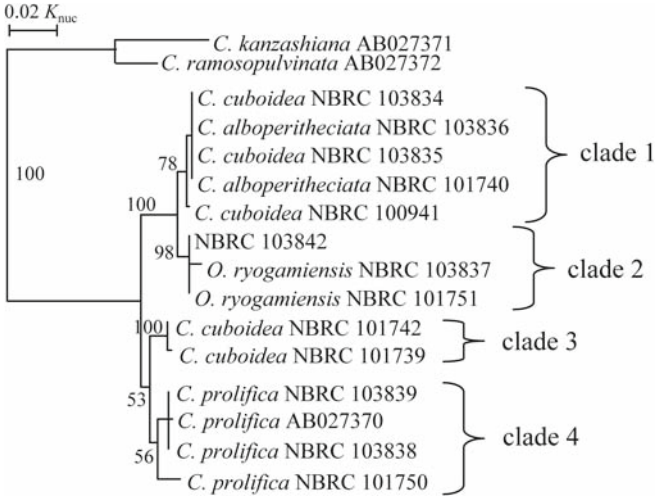

Fig. 2. Neighbor-joining tree derived from internal transcribed spacer (ITS) region sequences of isolates of *Cordyceps cuboidea* and its allied species and of other species that were retrieved from GenBank

observed and compared (Table 3). Two types of phialides (i.e., *Acremonium*-like, tapering slender phialide, and *Hirsutella*-like phialide with broad base and slender neck) are formed by all taxa. Branching of phialides is irregular or quasi-verticillate. Distinctive characteristics of each species are indicated with boldface letters in Table 3. The relatively clear difference in the teleomorphs is the position of the fruiting perithecia. In anamorphs, *O. ryogamiensis* is distinctive in forming longer conidia and mostly slender-shaped conidiogenous cells.

Molecular phylogeny analysis revealed that our isolates of *C. cuboidea* contained two different lineages (clades 1 and 3). We found these two clades were distinguishable by morphological characters, although they are similar in overall appearance of stroma and having the same host. The clade 1 fungus produces perithecia at the upper half of the aerial part of stroma (Fig. 3a, b). The clade 3 fungus, however, forms perithecia over almost the whole stroma (see Fig. 5a, b). This characteristic causes a difference in the length from the position of surface of the wood to the lowest perithecium: in clade 1, this is 2–17 ($\bar{x}$ = 7.0) mm, whereas in clade 3 it is 0–1.5 ($\bar{x}$ = 0.9) mm. Their anamorphs also show a slight difference: the clade 3 fungus abundantly produces *Hirsutella*-like phialides, while the clade 1 fungus produces *Hirsutella*-like phialides only in the sporodochia formed in the aged part of the colony. Branching of conidiophores also differs, irregular versus quasi-verticillate, between the two clades. These morphological differences indicate that these *C. cuboidea*-like fungi are divided into two distinct taxa. To elucidate which is the true *C. cuboidea*, we examined the holotype specimen of *C. cuboidea* (TNS-F-195457). The holotype specimen has perithecia positioned at the upper half of the aerial part of the stroma (Fig. 3a). This ascomata character is also concordant with the clade 1 fungus. Thus, we concluded the clade 1 fungus is the true *C. cuboidea* and that clade 3 is an undescribed species.

Two specimens of *C. alboperitheciata* clustered in clade 1. We observed the color of the perithecia changed from white to orangish yellow and to brown as they matured and became identical with *C. cuboidea*. Furthermore, no difference was found between the two species in the following morphological characters: distribution of perithecia on the stroma, size of part spores, shape and length of conidiogenous cells, branching of phialides and size of conidia. These facts suggest that the white color of perithecia of *C. alboperitheciata* is not a species-delimiting character from *C. cuboidea*, in contrast to the suggestion by Kobayasi and Shimizu (1982). We conclude *C. alboperitheciata* is conspecific with *C. cuboidea* and should be treated as a synonym of the latter species.

Ophiocordyceps ryogamiensis, which formed clade 2, is distinct from other species by its long part spores and conidia (Table 3). In addition, its anamorph grows on the surface of stroma concurrently with perithecia in nature (Fig. 4b). The sequence of the ITS region of NBRC 103842, which was isolated from conidia on the immature stipe of *O. ryogamiensis*, shows 100% similarity with *O. ryogamiensis* NBRC 101751. This case indicates the anamorphic state of the *Ophiocordyceps* was growing on the stroma. We observed this phenomenon only in *O. ryogamiensis*, but not in the other three species.

We found *C. prolifica*, which formed clade 4 in the molecular analysis, has a *Hirsutella* anamorph forming phialides with a cylindrical basal part and a slender neck. Kobayasi and Shimizu (1976), however, illustrated synnematous structures with acro-pleurogenous phialides on the stroma as a conidial state of *C. prolifica*. It is likely that the synnematous fungus was not a true anamorph of *C. prolifica* because the type of phialides is different from those of *Hirsutella*. We also found and isolated synnematous anamorphic fungi (NBRC 103841, NBRC 103844, NBRC 103845) from the stroma of *C. prolifica*. Molecular analysis clearly shows the three strains are far apart from *C. prolifica* (see Fig. 1). These findings suggest the original description of the anamorph of *C. prolifica* by Kobayasi and Shimizu (1976) was based on a misunderstanding.

Synnematous anamorphic fungi (NBRC 103840, NBRC 103843) were also isolated from the stromata of *C. cuboidea* as well as from that of *C. prolifica*. These fungi are clearly different from *C. cuboidea* or *C. prolifica*, based on culture studies and molecular data. Thus, these fungi were not the anamorph of the *Cordyceps* species but possibly hyperparasitic fungi. Our molecular phylogenetic study suggested that the possible hyperparasitic fungi were included in Hypocreales. These findings may show that such hyperparasitic fungi usually grow over the stroma of *Cordyceps* species.

Sung et al. (2007) described that one of the distinctive morphological characters of the genus *Ophiocordyceps* was fibrous, wiry to pliant, darkly pigmented stroma. *Hirsutella* and *Hymenostilbe* were recognized as the dominant anamorphs. Sung et al. assigned *C. ryogamiensis* to *Ophiocordyceps* without molecular phylogenetic analysis, probably because it has wiry and dark pigmented stromata. We found this species has a *Hirsutella*-like anamorph, which supports

Table 3. Morphology of specimens and cultures of the four clades of *Cordyceps cuboidea* and its allied species

	C. alboperitheciata (clade 1)	*C. cuboidea* (clade 1)	*Ophiocordyceps ryogamiensis* (clade 2)	*O. paracuboidea* (clade 3)	*C. prolifica* (clade 4)
Typical host	Larva of beetle	Larva of beetle, other *Cordyceps*	Larva of beetle	Larva of beetle	**Larva of cicada**
Habitat	In decayed wood	In decayed wood	In decayed wood	In decayed wood	**On the ground**
Stromata (mm)	8.8–29.9 (19.9) × 0.9–1.8 (1.4)[a]	3.2–18.1 (13.7) × 0.3–7.4 (3.1)	16.4–29.1 (11.8) × 0.3–1.7 (1.1)	3.2–38.4 (11.8) × 0.3–1.7 (1.1)	70.9–140.0 (101.1) × 0.8–2.2 (1.5)
Position of fruiting perithecia	**Upper side on aerial part**	**Upper side on aerial part**	**Middle of stroma, sparsely produced**	**Almost whole stroma**	**Upper side to middle of aerial part**
Perithecia (μm)	350–550 (446) × 230–400 (297)	400–500 (443) × 250–330 (291)	430–600 (513) × 260–390 (332)	400–600 (507) × 290–400 (336)	320–530 (431) × 200–340 (274)
Asci length (μm)	400–520 (475)	250–570 (401)	450–610 (549)	225–400 (288)	430–650 (548)
Ascus apex (μm)	3.5–5.0 (4.2)	3.0–5.0 (4.0)	3.75–5.0 (4.4)	3.0–6.25 (4.1)	3.0–5.0 (4.2)
Part spores (μm)	1.0–3.0 (2.2) × 1.0–2.0 (1.4)	1.5–3.0 (2.1) × 1.0–1.5 (1.1)	2.5–5.0 (3.7) × 1.5–2.0 (1.9)	1.3–2.5 (2.2) × 1.0–2.0 (1.4)	2.0–3.0 (2.4) × 1.0–2.0 (1.8)
Anamorph					
On stroma in nature	Absent	Absent	**Present, *Hirsutella*-like**	Absent	Absent
On the medium					
Conidia	Globose, fusiform	Globose, fusiform	**Ellipsoid**	Aubglobose, fusiform	Globose, fusiform
Size (μm)	1.3–2.4 (1.8) × 1.1–1.5 (1.3)	1.9–3.7 (2.7) × 1.3–2.3 (1.7)	2.5–3.9 (3.1) × 1.0–1.4 (1.2)	1.3–1.9 (1.8) × 1.0–1.9 (1.4)	1.5–3.5 (2.5) × 1.1–1.8 (1.4)
Conidiogenous cells	Slender, cylindrical to *Hirsutella*-like, broad base with long tapering neck	Slender, cylindrical to *Hirsutella*-like, broad base with long tapering neck	Mainly slender, long cylindrical, but shorter with broad base on the sporodochial part	Mainly *Hirsutella*-like, broad base with one tapering neck, partially slender, cylindrical	Slender, cylindrical to *Hirsutella*-like, broad base with long tapering neck
Acremonium-like	On the edge of colony	On the edge of colony	**Mainly this type**	Partially slender shape	On the edge of colony
Size (μm)	18.9–22.2 (20.3) × 0.8–1.1 (1.0)	18.0–46.8 (27.0) × 0.4–1.9 (1.2)	17.5–55.2 (27.5) × 0.8–2.7 (1.3)	10.9–20.9 (15.3) × 0.5–1.1 (0.8)	19.6–30.6 (24.6) × 0.7–1.7 (1.2)
Hirsutella-like	On the sporodochial part	Sometimes at the aged part	On the aged and sporodochial part	**Mainly this type**	On the aged and arising synnema
Size (μm)	Length: 17.7–27.8 (21.8) Base: 1.1–2.4 (1.9) Neck: 0.5–0.9 (0.6)	Length: 12.9–25.0 (19.0) Base: 1.0–1.6 (1.3) Neck: 0.4–0.7 (0.5)	Length: 17.4–25.7 (21.5) Base: 1.3–1.6 (1.4) Neck: 0.3–0.7 (0.5)	Length: 10.9–21.5 (16.1) Base: 1.2–1.6 (1.4) Neck: 0.4–0.8 (0.5)	Length: 10.8–16.3 (14.2) Base: 1.5–1.7 (1.6) Neck: 0.5–0.7 (0.6)
Branching	Irregular	Irregular	Quasi-verticillate	Quasi-verticillate	Quasi-verticillate

Boldface type shows distinctive characters
[a] Values in parentheses show the average

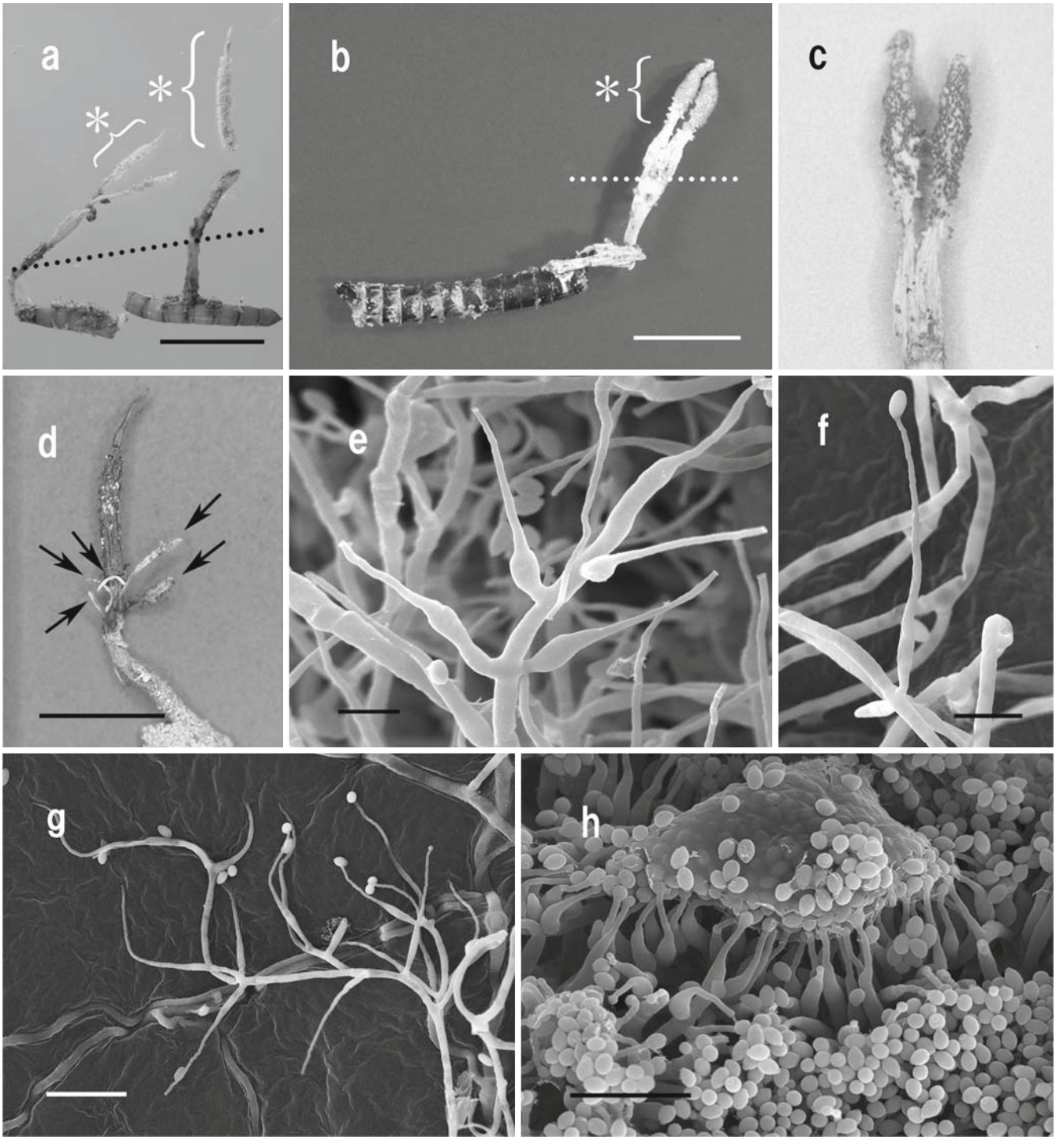

Fig. 3. *Ophiocordyceps cuboidea* (= *Cordyceps cuboidea*, clade 1): holotype specimen TNS-F-195457 (**a**); specimen NBRC H-12545 (**b**, **c**); specimen NBRC H-12377 (**d**); culture NBRC 103834 (**e–g**); culture NBRC 101740 (**h**). **a, b** The *dotted lines* show the position of the surface of the wood. The upper half of the aerial part is fertile (*). **c** Enlargement of stroma of **b**. **d** Several stromata (*arrows*) of *C. cuboidea* hyperparasitizing a stroma of *C. stylophora*. **e** *Hirsutella*-like phialides in culture. **f** *Acremonium*-like phialide. **g** Conidiophore developing phialides irregularly. **h** Conidia and conidiogenous cells aggregating as a sporodochium at the center of the colony. *Bars* **a, b** 1 cm; **d** 5 mm; **e, f** 5 μm; **g, h** 10 μm

the taxonomic treatment by Sung et al. (2007). The other three taxa, *C. cuboidea* (clade 1), the new species (clade 3), and *C. prolifica* (clade 4) have similar characters to the other *Ophiocordyceps*, i.e., wiry stroma and *Hirsutella*- or *Acremonium*-like anamorphs. From these findings in the phylogenetic analysis and morpho-logical characters, we conclude that the four species of the *C. cuboidea* group should be assigned to the genus *Ophiocordyceps*.

Taxonomy

Ophiocordyceps cuboidea (Kobayasi & Shimizu) S. Ban, Sakane & Nakagiri, emend. & comb. nov. Fig. 3

Basionym: *Cordyceps cuboidea* Kobayasi & Shimizu Bull. Natn. Sci. Mus., Ser. B. 6(4):131 (1980).

Synonym: *Cordyceps alboperitheciata* Kobayasi & Shimizu Bull. Natn. Sci. Mus., Ser. B. 8(3):84 (1982).

Species description by Kobayasi and Shimizu (1980) is emended in fertile part of stroma, the color of perithecia, and anamorphic state, as follows.

The hosts are larvae of Coleoptera living in decayed wood. Sometimes it hyperparasitizes other *Cordyceps* fungi. Stroma cylindrical or sometimes flattened, wiry, hard, cream to pale yellow, 3.2–29.9 × 0.3–7.4 mm ($\bar{x}$ = 16.8–2.3), on coleopteran larva living in decayed wood. Perithecia superficial, on the upper part of stroma, pale orangish yellow, sometimes white when young, and changing to pale orangish yellow or brown at maturity, lemon-shaped, 350–550 × 230–400 µm ($\bar{x}$ = 444 × 294). Asci 250–570 × 3.0–5.0 µm ($\bar{x}$ = 438 × 4.1). Ascospores needle-shaped, hyaline, finely segmented into part spores. Part spores 1.0–3.0 × 1.0–2.0 µm ($\bar{x}$ = 2.2 × 1.3). Colonies effuse, growing 2.5–3.5 cm/25°C 2 weeks on PSA, pale yellowish brown to cream. Mycelium immersed and superficial, hyaline to cream, pale brown. Synnemata not formed. Conidiogenous cells phialidic, hyaline, mostly slender, *Acremonium*-like, 18.9–22.2 × 0.8–1.1 µm ($\bar{x}$ = 20.3 × 1.0), sometimes *Hirsutella*-like with broader base, particularly formed in the sporodochium, 17.7–27.8 µm ($\bar{x}$ = 21.8) long, 1.1–2.4 µm ($\bar{x}$ = 1.9) wide at the base, 0.5–0.9 µm ($\bar{x}$ = 0.6) wide at the neck. Conidia hyaline, globose to elliptical, 1.3–3.7 × 1.1–2.3 µm ($\bar{x}$ = 2.2 × 1.3), forming a spore drop with fewer than 10 spores at the apex of phialide.

Anamorph: *Acremonium*-like/*Hirsutella*-like.

Holotype: TNS-F-195457.

Specimens and strains examined: NBRC H-12377 (NBRC 100941), NBRC H-12383 (NBRC 100942), NBRC H-12569 (NBRC 103834), NBRC H-12600 (NBRC 103835), NBRC H-12407 (NBRC 101740), and NBRC H-12545 (NBRC 103836).

Notes: In the specimen of *C. cuboidea* (NBRC H-12377, NBRC 100941) a clump of stromata of *C. cuboidea* occur from the stipe of *O. stylophora* (Berk. & Broome) G. H. Sung et al. on coleopteran larva (Fig. 3d). This observation suggests *C. cuboidea* can be hyperparasitic on another fungus.

Ophiocordyceps ryogamiensis (Kobayasi & Shimizu) G. H. Sung, J. M. Sung, Hywel-Jones & Spatafora in Sung, Hywel-Jones, Sung, Luangsa-ard, Shrestha & Spatafora, Stud. Mycol. 57: 46 (2007). Fig. 4

The hosts are coleopteran larvae living in decayed wood. Stroma appear through segments of larva. One to four stromata occur on one host (Fig. 4a), and sometimes each stroma branches at the aerial part above. Stromata are cylindrical to flat, usually curved, 16.4–29.1 × 0.3–1.7 mm ($\bar{x}$ = 11.8 × 1.1), dark brown at the basal part, light to dark brown at the fruiting middle part, white at the upper part. Perithecia appear sparsely on the middle of stroma, superficial, brown except the base covered with hyphae, 430–600 × 260–390 µm ($\bar{x}$ = 513 × 332). Asci are 450–610 µm ($\bar{x}$ = 549) long, 3.8–5.0 µm ($\bar{x}$ = 4.4) wide at the apical cap. Ascospores divide into part spores, which are cylindrical, 2.5–5.0 × 1.5–2.0 µm ($\bar{x}$ = 3.7 × 1.9). Part spores derived from the both ends of ascospore are longer, 6.2–7.5 µm, conical shape. The anamorph often occurs on the stroma even when mature perithecia are present. Conidiogenous cells are phialidic and have a broad base and slender neck similar to *Hirsutella* (Fig. 4b). Conidia are ellipsoid, 2.5–3.9 × 1.0–1.4 µm ($\bar{x}$ = 3.1 × 1.2). Phialides developed on the agar surface are slender, cylindrical, 17.5–55.2 × 0.8–2.7 µm ($\bar{x}$ = 27.5 × 1.3) (Fig. 4c). These phialides branch in quasi-verticillate fashion. Sporodochial aggregates are sometimes produced with *Hirsutella*-like phialides, 17.4–25.7 µm ($\bar{x}$ = 21.5) long, 1.3–1.6 µm ($\bar{x}$ = 1.4) wide at the base, 0.3–0.7 µm ($\bar{x}$ = 0.5) wide at the neck (Fig. 4d,e). Conidia are ellipsoid, 2.5–3.9 × 1.0–1.4 µm ($\bar{x}$ = 3.1 × 1.2). Several conidia accumulate at the apex of the phialide.

Anamorph: *Acremonium*-like/*Hirsutella*-like.

Holotype: TNS-F-197971.

Specimens examined: NBRC H-12452 (NBRC 101751), NBRC H-12599 (NBRC 103837).

Notes: The anamorph of *O. ryogamiensis* was newly found and described above. Two types of conidiogenous cells, *Hirsutella*-like and *Acremonium*-like, are produced on the media. Only the *Hirsutella*-like conidial state has been found on the stroma in nature.

Ophiocordyceps paracuboidea S. Ban, Sakane & Nakagiri, sp. nov. Fig. 5

Stromata solitaria vel nonnulla, cylindracea, non-ramulosa, ex larva Coleoptera. Pars sterilis in ligno albo, interdum radicina. Perithecia conferta, tegentia holostroma, superficialia, perpendiculariter collocata, citriformia, pallide aurantio-brunnea, 400–600 × 290–400 µm ($\bar{x}$ = 507 × 336). Asci hyalini, cylindracei, 8-spori, 225–400 × 3.0–6.25 µm ($\bar{x}$ = 288 × 4.1), cum apice conspicue inspissato. Ascosporae aciformes, hyalinae. Articuli ascosporarum breves, 1.3–2.5 × 1.0–2.0 µm ($\bar{x}$ = 2.2 × 1.4). Coloniae in PSA effusae, lente crescentes, pallidae ochraceae vel cremeae. Cellulae conidiogenae monophialidicae, hyalinae, verticillatae cum 2–3 phialidibus, ut plurimum *Hirsutella*-formes, 10.9–21.5 µm ($\bar{x}$ = 16.1) longae, 1.2–1.6 µm ($\bar{x}$ = 1.4) latae ad basim, 0.4–0.8 µm ($\bar{x}$ = 0.5) latae ad collum; raro*Acremonium*-formes, partim graciles, cylindricae, 10.9–20.9 × 0.5–1.1 µm ($\bar{x}$ = 15.3 × 0.8). Conidia hyalina, aseptata, subglobosa vel fusoidea, 1.3–1.9 × 1.0–1.9 µm ($\bar{x}$ = 1.8–1.4).

Habitatio: Coleoptera larva.

Nom. Jap.: Kuchiki-awanomitake.

Holotypus: NBRC H-12471.

Etymology: para + cuboidea means false *O. cuboidea*.

Stromata solitary or several, cylindrical, not branched, on larva of Coleoptera living in decayed wood. Sterile part inside wood white, sometimes root-like. Perithecia crowded

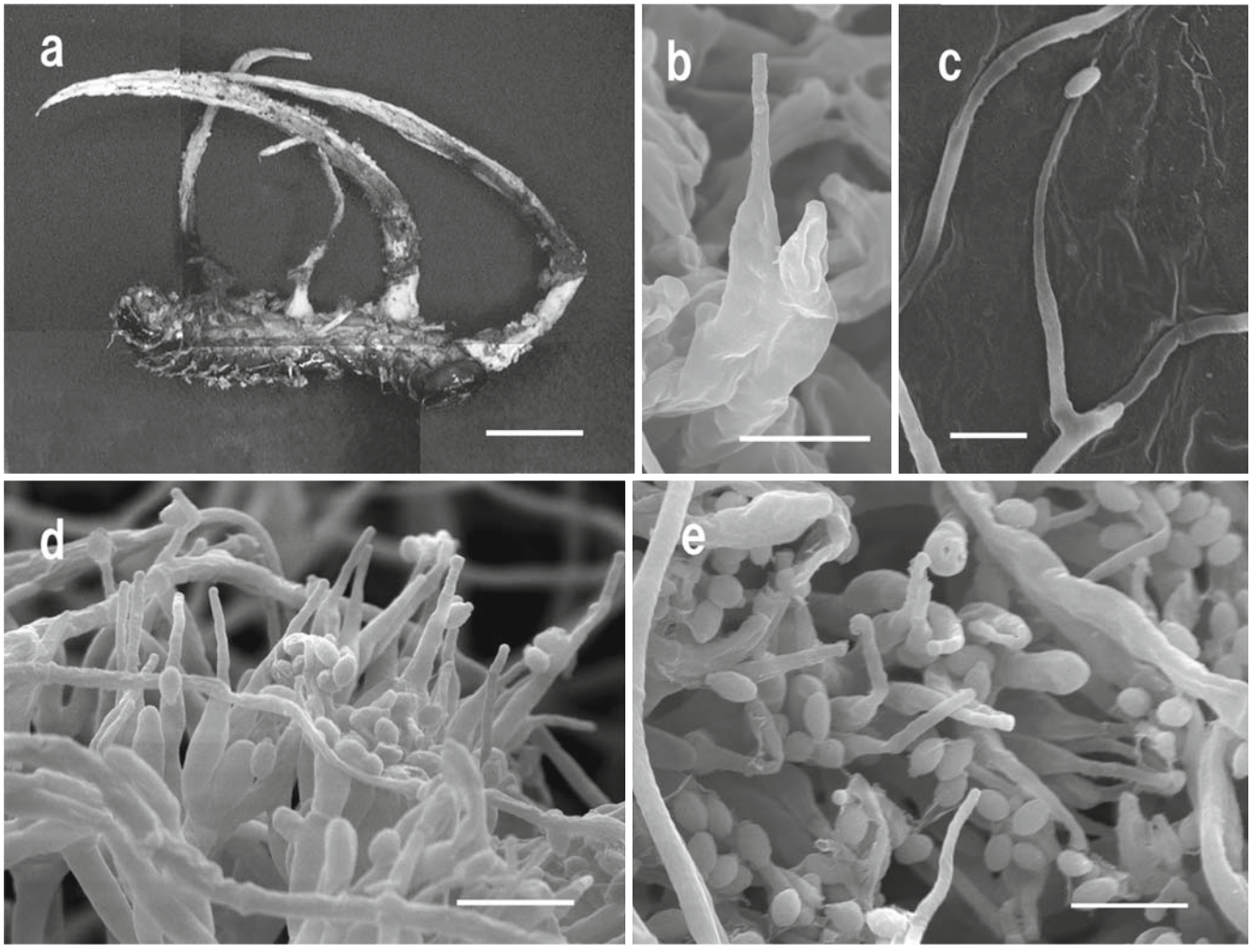

Fig. 4. *Ophiocordyceps ryogamiensis* (clade 2). **a** Specimen NBRC H-12452. **b** Phialide on the stroma (dried specimen NBRC H-12599). **c–e** Phialides produced in culture, NBRC 103842: *Acremonium*-like phialide (**c**); *Hirsutella*-like phialide (**d**); conidia and conidiogenous cells aggregating as a sporodochium at the center of colony (**e**). *Bars* **a** 0.5 cm; **b–e** 5 μm

and covering whole stroma, superficial, vertically placed, lemon-shaped, pale orangish brown, 400–600 × 290–400 μm ($\bar{x}$ = 507 × 336). Asci hyaline, cylindrical, 8-spored, 225–400 μm ($\bar{x}$ = 288), possessing a conspicuously thickened apical cap, 3.0–6.3 μm ($\bar{x}$ = 4.1) in diameter. Ascospores needle-shaped, hyaline, finely segmented into part spores. Part spores cylindrical, 1.3–2.5 × 1. 0–2.0 μm ($\bar{x}$ = 2.2–1.4). Colonies effuse, slowly growing 2.5–3 cm/25°C 2 weeks on PSA, pale yellowish brown to cream. Mycelium immersed and superficial, hyaline to cream, pale brown. Synnemata none. Conidiophores erect, produced in aerial hyphae. Conidiogenous cells phialidic, hyaline, verticillate with 2–3 phialides, mainly *Hirsutella*-like, 10.9–21.5 μm ($\bar{x}$ = 16.1) long, 1.2–1.6 μm ($\bar{x}$ = 1.4) wide at the base, 0.4–0.8 μm ($\bar{x}$ = 0.5) wide at the neck; rarely *Acremonium*-like, slender, cylindrical, 10.9–20.9 × 0.5–1.1 μm ($\bar{x}$ = 15.3 × 0.8). Conidia hyaline, subglobose to fusiform, 1.3–1.9 × 1.0–1.9 μm ($\bar{x}$ = 1.8–1.4).

Anamorph: mainly *Hirsutella*-like, rarely *Acremonium*-like.

Holotype: NBRC H-12471 (ex-holotype strain is NBRC 101742) on coleopteran larva in decayed wood, broadleaf forest, Shimoseya, Miyazu, Kyoto Pref., Japan. 16 October, 2005, collected by T. Sakane, dried specimen; deposited in Herb. NBRC.

Other specimens examined: NBRC H-12411 (NBRC 101739), NBRC H-12383 (NBRC 100942).

Notes: *Ophiocordyceps paracuboidea* produces *Hirsutella*-like phialides and quasi-verticillate branching of conidiophores (Fig. 5h–j), which differ from *C. cuboidea*. The shorter part spore [1.3–2.5 × 1.0–2.0 μm ($\bar{x}$ = 2.2 × 1.4)] distinguishes this fungus from other similar species parasitizing a coleopteran host, such as *O. clavate* Kobayasi & Shimizu (5.0–9.0 × 1.5 μm), *O. geniculata* (Kobayasi & Shimizu) G. H. Sung et al. (5.0–6.0 × 1.0 μm), and *Elaphocordyceps subsessilis* (Petch) G. H. Sung et al. (3.0–5.0 × 1.0 μm). From all these facts and molecular data, we propose to describe this species as a new species.

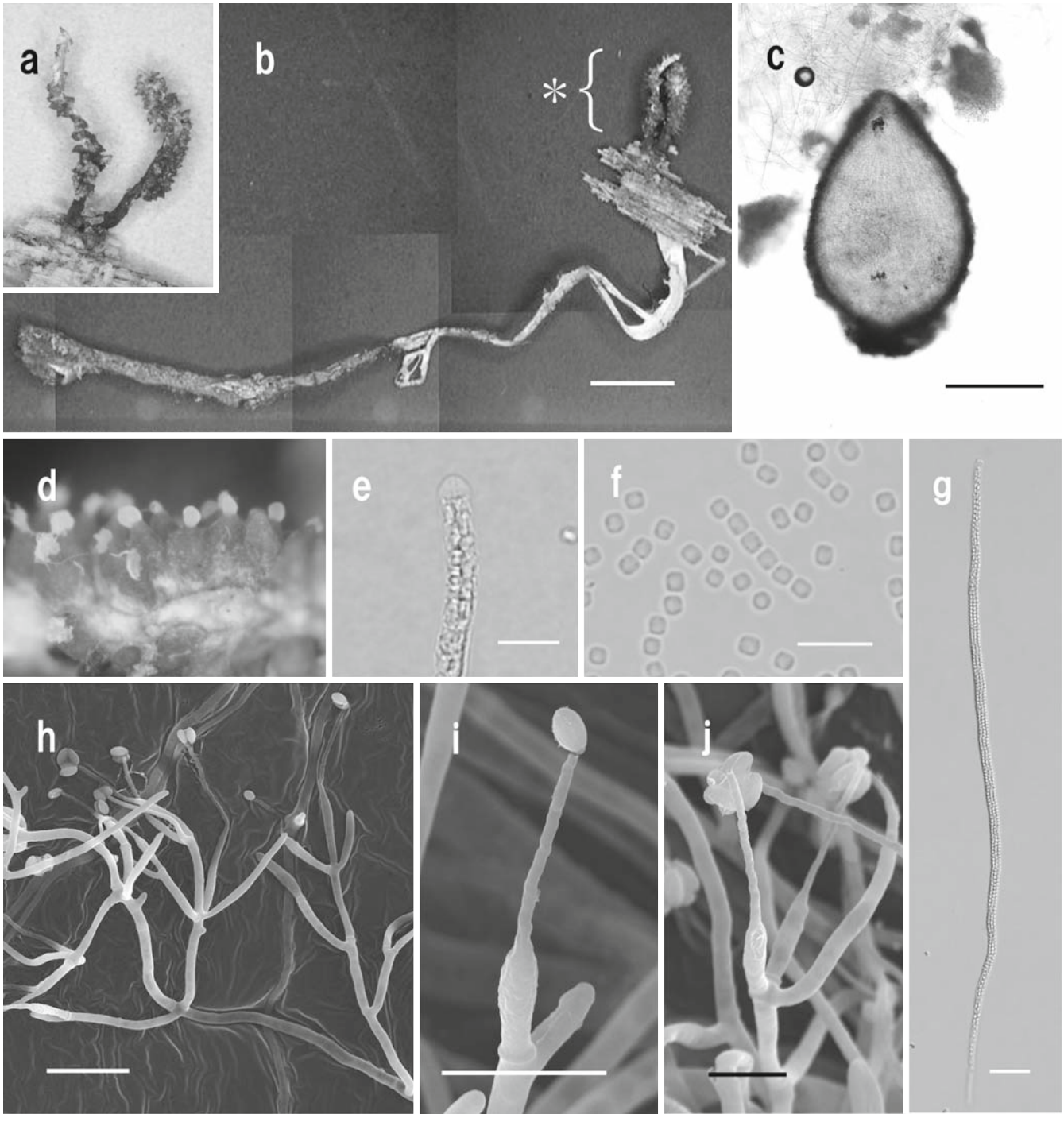

Fig. 5. *Ophiocordyceps paracuboidea* (clade 3): specimen NBRC H-12471 (**a–g**); culture NBRC 101739 (**h–j**). **a** Enlarged picture of stroma of **b**. **b** The remaining cortex shows the borderline between inside wood and aerial part. Note almost the whole aerial part is covered with perithecia (*). **c** Perithecium. **d** Perithecia on fertile part of stroma. Note drops of ejected ascospores are deposited on the apex of the perithecia. **e** Apical cap of ascus. **f** Part spores. **g** Ascus. **h** Conidiophores developing quasi-verticillate phialides. **i, j** *Hirsutella*-like phialides and conidia. *Bars* **b** 0.5 cm; **c** 200 µm; **e, f, h** 10 µm; **g** 20 µm; **i, j** 5 µm

Ophiocordyceps prolifica (Kobayasi) S. Ban, Sakane & Nakagiri, comb. nov. Fig. 6

Basionym: *Cordyceps prolifica* Kobayasi & Shimizu Bull. Nat. Sci. Mus., 6(3):289 (1963).

The hosts are larvae of cicada living under the ground. This character is apparently different from the members of the above three clades. Stromata with 1–2 branches occur from the host body and form 2–3 branches above the aerial part. The total length is 70.9–140.0 × 0.8–2.2 mm ($\bar{x}$ = 101.1 × 1.5), with ~10–30 mm aerial part (Fig. 6a). Perithecia occur on the upper to middle part of stroma, superficial, oblong to lemon-shaped, 320–530 × 200–340 µm ($\bar{x}$ = 431 × 274), pale reddish yellow, sometimes covered

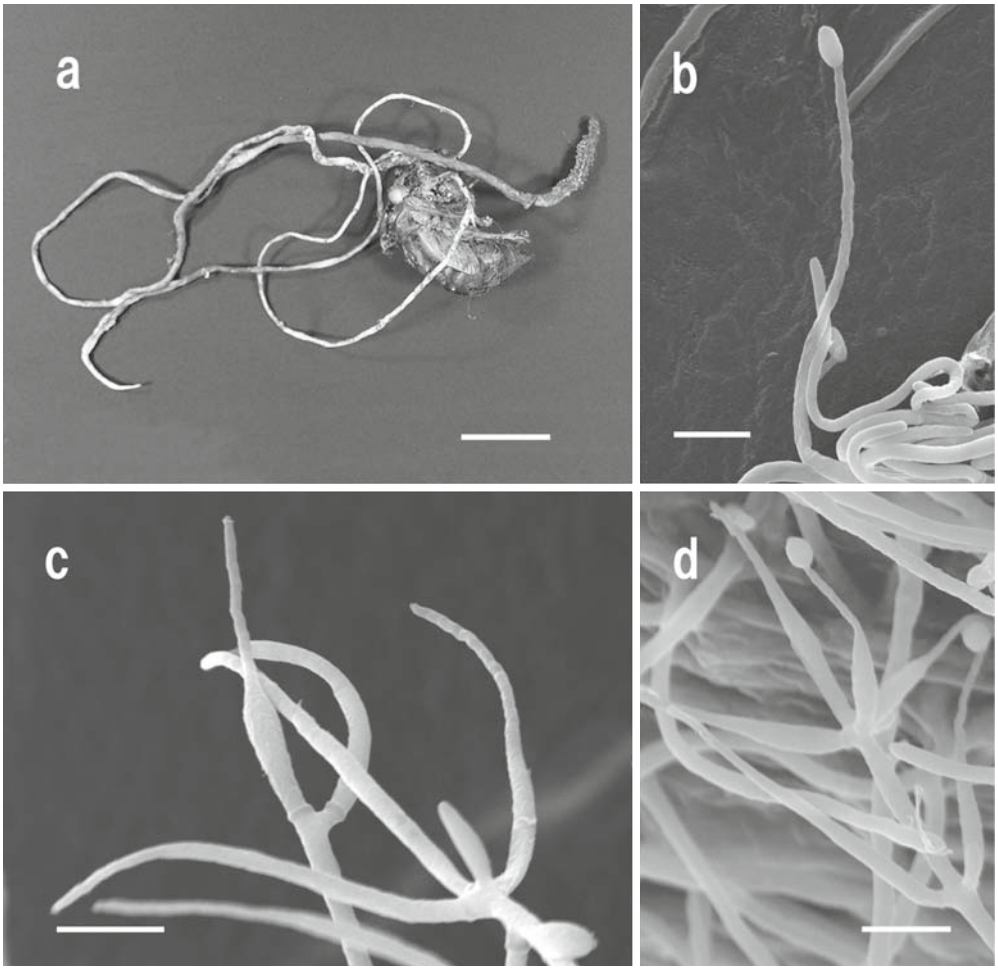

Fig. 6. *Ophiocordyceps prolifica* (= *Cordyceps prolifica*, clade 4): specimen NBRC H-12436 (**a**); culture NBRC 103839 (**b–d**). **a** Stromata growing on a larva of *Tanna japonensis*. **b** *Acremonium*-like phialide. **c** *Hirsutella*-like phialides. **d** Conidiophore developing quasi-verticillate phialides. *Bars* **a** 1 cm; **b–d** 5 μm

with white hyphae. Asci are 430–650 μm ($\bar{x}$ = 548) long, 3.0–5.0 μm ($\bar{x}$ = 4.2) wide at the apical cap. Ascospores divide into part spores, 2.0–3.0 × 1.0–2.0 μm ($\bar{x}$ = 2.4 × 1.8). The conidial ontogeny is phialidic. Two types of phialides are found, an *Acremonium*-like slender type developed at the edge of colony (Fig. 6b) and a *Hirsutella* type having a broad base and slender neck with unclear border developed on the aged colony (Fig. 6c). Synnema-like structures with *Hirsutella*-like phialides are sometimes produced in culture. *Acremonium*-like phialides are 19.6–30.6 × 0.7–1.7 μm ($\bar{x}$ = 24.6 × 1.2). *Hirsutella*-like phialides are 10.8–16.3 μm ($\bar{x}$ = 14.2) long, 1.5–1.7 μm ($\bar{x}$ = 1.6) wide at the base, 0.5–0.7 μm ($\bar{x}$ = 0.6) wide at the neck. Two to five phialides are formed in quasi-verticillate fashion (Fig. 6d). Conidia are globose to fusiform, 1.5–3.5 × 1.1–1.8 μm ($\bar{x}$ = 2.5 × 1.4). Fewer than 5 spores make a drop at the apex of a phialide.

Anamorph: *Hirsutella*-like (see described above).

Holotype: TNS-F-230300.

Specimens examined: NBRC H-12364 (NBRC 100744), NBRC H-12436 (NBRC 101750), NBRC H-12581 (NBRC 103838), and NBRC H-12583 (NBRC 123839).

Notes: *Ophiocordyceps prolifica* is unique in parasitizing cicada larvae among the four species closely related and looks very different in having long stroma, probably a consequence of the deep underground occurrence of the host.

Acknowledgements We sincerely thank Dr. Hiroki Sato for allowing use of the photograph of the type specimen and Dr. Gi-Ho Sung for his kind advice. We are also grateful to Mr. S. Kato, Mr. T. Kumada, and Mr. T. Ito, members in the Tango-no-shizen-wo-tanoshimu-kai, for supporting us by collecting specimens.

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