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NOTE

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Note on *Cordyceps brongniartii* Shimazu collected from the wild in Japan

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Abstract We collected *Cordyceps brongniartii* from the wild associated with coleopteran larvae for the first time in Japan. Morphological comparisons of *C. brongniartii* with the type specimen showed slight morphological difference, whereas it showed considerable differences from those collected in the wild in China. PCR-RFLP and ITS sequence analyses corroborated the teleomorph–anamorph relationship between *C. brongniartii* and *Beauveria brongniartii*.

Key words Entomopathogenic fungi · rDNA-ITS

Cordyceps brongniartii Shimazu is the only *Cordyceps* species originally described from artificially induced fruit bodies (Shimazu et al. 1988). *Cordyceps brongniartii* from the wild has only been reported in China (Liu and Liang 1993), although its anamorph *Beauveria brongniartii* (Sacc.) Petch has been found in many regions of the world (Piatti et al. 1998; Choo et al. 2002; Dolci et al. 2006; Hadapad et al. 2006).

The Chinese material differs morphologically from the specimen described by Shimazu et al. (1988) in perithecial arrangement, sizes of perithecia and asci, and host species. Yahagi et al. (2004) reported that the morphological characteristics of cultivated *C. militaris* fruit bodies differ from those of the wild type in perithecial arrangement. Thus, it is possible that wild specimens and cultured *C. brongniartii* may also differ morphologically.

In August 2004, we collected *C. brongniartii* fruit bodies associated with coleopteran larvae in a forest. This is the first report of wild *C. brongniartii* in Japan. In the present article, we describe the environmental remarks for fruit body formation and compare our fungus with the holotype specimen morphologically. Based on molecular data, we corroborate an anamorph–teleomorph relationship.

Twenty-one fruit bodies were collected in Toyoura-cho, Abuta-gun, Hokkaido, Japan. Of these 21 fruit bodies, 8 were not associated with insect carcasses and 8 were immature or damaged. Therefore, the remaining 5 intact fruit bodies were used for isolation and observation. Isolates were obtained from fruit body tissues using the surface-sterilization method of Sasaki et al. (2004). To observe the anamorph, isolates were incubated on Sabouraud glucose agar medium [SGA: 20 g agar, 10 g peptone, 20 g glucose, and 1 l distilled water (pH 6.5)] at 25°C in the dark for approximately 1 month.

Macroscopic features of the fruit bodies were examined by naked eye and under a dissecting microscope. Colors of fruit bodies were assessed according to Munsell (1990). Collected specimens were then oven-dried for 72 h at 60°C. For microscopic observation, the dried samples were rehydrated in 0.05% Triton X-100 solution (Hywel-Jones 1995). Twenty perithecia and asci, 30 partspores, and conidia were observed under a differential interference microscope. The holotype of *C. brongniartii* (I-22-N-3) was borrowed from Dr. Shimazu of the Forestry and Forest Products Research Institute, Ibaraki, Japan.

The polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and internal transcribed spacer (ITS) sequence data were used to supplement the morphological identifications. Samples for analyses were obtained from each dried stipe sample (approximately 3 mm long) and each piece of cultured mycelium (approximately 3 × 3 × 3 mm). DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For the PCR-RFLP analysis, the ITS region was amplified with the primer pair PN3 and PN16 (Neuvéglise et al. 1994) and digested with *Acc*II enzyme according to Wada et al. (2003). For sequencing, the ITS region was amplified with the primer pair ITS1f (Gardes and Bruns 1993) and ITS4 (White et al. 1990). After amplification, samples were purified with a LaboPass PCR purification Kit (Cosmo Genetech, Seoul, Korea). These ITS1f/4 primers were also used for sequencing reactions with an

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Table 1. Morphological comparisons of *Cordyceps brongniartii*

Part	Specimen of Shimazu et al. (1988)	Liu and Liang (1993)	Specimens of present study
Host	Coleoptera	Lepidoptera	Coleoptera
Perithecium			
Arrangement	Semi-immersed	Superficial	Semi-immersed
Shape	Obovoid to clavate	Ovoid to obpyriform	Obovoid to clavate
Dimension (μm)	334–534 × 168–318	562–837 × 310–500	394–580 × 163–310
Ascus			
Dimension (μm)	170–375 long[a]	250–350 long	180–412 × 2.3–4.0
Cap breadth (μm)	3.4–4.3	4.5–4.8	2.4–4.3
Partspore			
Dimension (μm)	2.6–6.0 × 0.8–1.4	2.5–8.4 × 0.7–1.2	4.0–15.9 × 0.9–1.4

[a]Ascus dimension is quoted from Shimazu et al. (1988)

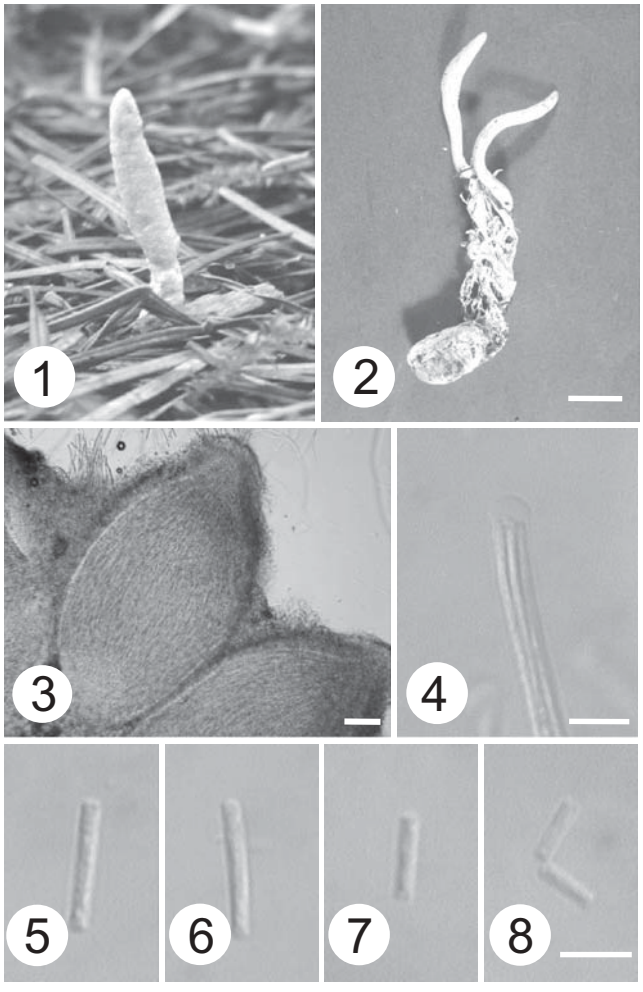
ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Sequences were detected using an ABI autosequencer 3730 (Applied Biosystems) and aligned using CLUSTAL W (Thompson et al. 1994). The resulting sequence was compared to database sequences in DDBJ using the BLAST program. All specimens and cultures were deposited in the Laboratory of Forest Resource Biology (FORB), Hokkaido University, Hokkaido, Japan.

The morphology of the examined fungus agreed with the original description (Shimazu et al. 1988). The morphology of all isolates agreed to the description by de Hoog (1972). Nevertheless, our samples showed slight morphological differences compared to the holotype. The lengths of the perithecia, asci, and partspores of the fruit bodies showed slightly wider ranges than the holotype specimen (Table 1). In particular, the maximum sizes of the partspores in our samples were more than twice longer than those of Shimazu et al. (1988). Yahagi et al. (2004) reported that the perithecia of *C. militaris* shifted from semi-immersed to superficial with cultivation. This phenomenon was not recognized in *C. brongniartii*. To clarify the morphology of the specimen obtained from the wild, description is given for the *C. brongniartii* specimens obtained in the present study.

Cordyceps brongniartii Shimazu, Trans. Mycol. Soc. Japan 29: 328, 1988.

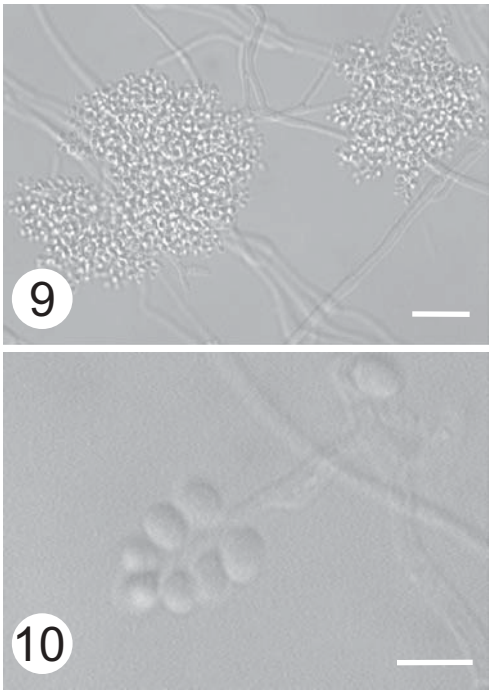
Anamorph: *Beauveria brongniartii* (Sacc.) Petch, Trans. Br. mycol. Soc. 10: 249, 1924.

Stromata clavate, furrowing on basal part, fleshy, 16–42 mm long, 1.4–3.7 mm wide (mean, 29 × 3.4 mm), protruding from the ground and connecting the host insect to the soil with whitish mycelial strands (Figs. 1, 2). Whitish soft mycelia covering the host (Fig. 2). Perithecial head slightly fertile or not, becoming attenuate from fertile part, yellow (10YR7/8–10YR8/8), 7–21 mm long, 2.2–4.5 mm wide (mean, 15 × 3.7 mm). Color of the stalk yellow to olive-yellow (2.5Y8/8–10YR7/8). Perithecia semi-immersed, translucent-walled, obovoid to clavate, and 394–580 × 163–310 μm (mean, 455 × 232 μm) (Fig. 3). Ostioles prominent, visible on the outer surface. Asci cylindrical, hyaline, 180–412 × 2.3–4.0 μm (mean, 324 × 3.1 μm), with hemispheric cap 2.4–4.3 μm in diameter (mean, 3.6 μm) (Fig. 4). Ascospores filiform, multiseptate, and easily disarticulated to partspores. Partspores cylindrical, truncate at both ends, 4.0–15.9 × 0.9–1.4 μm (mean, 6.9 × 1.2 μm) (Figs. 5–8).



Figs. 1–8. *Cordyceps brongniartii*. **1** A stroma protruding from litter layer. **2** Stromata growing from coleopteran larva. **3** Perithecia. **4** Ascus with a semispheric cap. **5–8** Partspores. *Bars* **2** 10 mm; **3** 50 μm; **4** 5 μm; **5–8** 5 μm

Anamorph: Mycelium hyaline. Conidium arrangement sympodial (Figs. 9, 10). Conidia produced along zigzag elongations of flask-shaped or subcylindrical conidiogenous cells, often in clusters, hyaline, ellipsoidal (FORBc04002 and FORBc04005) or oval (all other isolates), 2.3–3.2 ×



Figs. 9, 10. *Beauveria brongniartii* isolated from tissue of *Cordyceps brongniartii*. **9** Conidiogenous structures. **10** Conidia with conidiogenous cell. *Bars* **9** 40 µm; **10** 5 µm

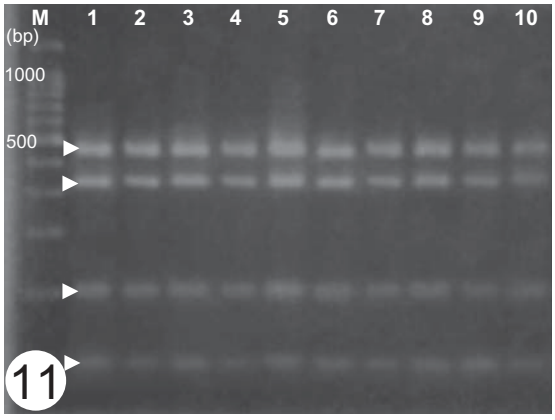


Fig. 11. Restriction patterns of internal transcribed spacer (ITS) regions of *Cordyceps brongniartii* fruit bodies and their isolates digested by *Acc*II. *Lanes 1, 3, 5, 7,* and *9* are *C. brongniartii* fruit bodies (*1*, FORBs04001; *3*, FORBs04006; *5*, FORBs04007; *7*, FORBs04008; *9*, FORBs04010). Lanes *2, 4, 6, 8,* and *10* are isolates (*2*, FORBc04001; *4*, FORBc04002; *6*, FORBc04003; *8*, FORBc04004; *10*, FORBc04005). *Lane M* is a 100-bp ladder DNA marker

0.8–2.2 µm (mean, 2.7 × 1.8 µm) and 2.7–3.8 × 1.1–1.7 µm (mean, 3.2 × 1.4 µm), respectively.

All isolates grew well and formed conidia on SGA medium. Colonies appeared lanose to velvety or powdery with red pigment. Production of red pigment in culture was congruent with previous reports (Kawakami 1978; Wada et al. 2003).

Habitat: Solitary or gregarious in small groups on thick litter layer in *Abies sachalinensis* (Fr. Schm.) Masters plantation forest in association with carcasses of coleopteran larva. Located on a gentle slope at approximately 250 m elevation. The annual average temperature is approximately 7°C, and the annual average precipitation is approximately 1200 mm (Japan Meteorological Agency; http://www.jma.go.jp/jma/index.html).

Fungal materials examined: Toyoura-cho, Abuta-gun, Hokkaido, Japan, August 22, 2004, collected by F. Sasaki and Y. Nishihara, FORBs04001 (isolate FORBc04001), FORBs04006 (isolate FORBc04002), FORBs04007 (isolate FORBc04003), FORBs04008 (isolate FORBc04004), FORBs04010 (isolate FORBc04005).

In contrast to the slight differences observed between the type specimen and our samples, considerable differences were noted in comparison with Liu and Liang (1993; see Table 1). The samples in Liu and Liang (1993) exhibited superficial perithecia that were considerably larger than those of our samples. The ascus cup widths in Liu and Liang (1993) were also markedly larger than our samples. In addition, the host was lepidopteran pupae in bamboo groves.

From the PCR-RFLP analysis, identical patterns representing four bands of approximately <100, 120, 330, and 470 bp were obtained in all isolates and all fruit body samples (Fig. 11). Wada et al. (2003) found that PCR-RFLP analysis with *Acc*II of three *Beauveria* species showed four patterns for the following fungal groups or species: *B. brongniartii* on scarab beetles, *B. brongniartii* on longicorn beetles, *B. bassiana*, and *B. amorpha*. Our PCR-RFLP pattern was identical to that of *B. brongniartii* on scarab beetles (Wada et al. 2003).

One selected isolate (FORBc4005) was used for sequencing. Our resulting sequence (accession no. AB235200) exhibited 100% homology with the sequences of *B. brongniartii* in GenBank (accession nos. AB027381, AB106649, AB237659, AB258367, AB258368, AY245628, and AY336941). The rDNA analyses corroborated the teleomorph–anamorph relationship of *C. brongniartii* and *B. brongniartii*.

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