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Cordyceps bassiana and Production of Stromata *in vitro* Showing *Beauveria* Anamorph in Korea

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A *Cordyceps* species was found with a *Beauveria* anamorph state on larval insect cadavers on Obong mountains in Gangwon Provinces, Republic of Korea. Cultures from discharged ascospores formed an anamorph identifiable as *Beauveria bassiana*. This teleomorph-anamorph connection was also confirmed by the *in vitro* production of fertile ascomata from conidial cultures with morphology like that of field-collected specimen. This is the first report of *in vitro* production of a teleomorph for any *Beauveria* species. The *Cordyceps* species has been conspecified as *Cordyceps bassiana*, a species described from China with *B. bassiana* anamorph.

KEYWORDS: ***Beauveria bassiana*, *Cordyceps bassiana*, Neotype, Systematics, Teleomorph-anamorph connection**

Beauveria bassiana (Bals.) Vuill. (Hypocreales: anamorphic Clavicipitaceae) is a nearly ubiquitous fungal pathogen of insects (Humber, 2000) and the fungal biocontrol agent most widely used against insect pests (Ferron, 1981; McCoy, 1990; Feng *et al.*, 1994). Feng *et al.* (1994) summarized recent progress and achievements in the mass production, formulation, and application of *B. bassiana* as a microbial pesticide.

Beauveria bassiana was widely utilized in East Asia for its medicinal value long before being used for insect biocontrol. Traditional medicinal uses of *Beauveria*-infected silkworm pupae include treating infantile convulsions, epilepsy, stroke, sore throat, and in external poultices to treat erisypelas and many sorts of wounds (Ying *et al.*, 1987). The *Dongeuibogam* ('The Precious Book of Eastern Medicine'), a much revered work on traditional Korean herbal medicine, reported the use of this fungus to cure paralysis and cancer.

Entomopathogenic hyphomycetes from more than twenty genera including *Beauveria* (Sung *et al.*, 2001) have been placed, or are suspected to belong to Clavicipitaceae and are potentially linked with the genus *Cordyceps* (Humber, 2000). Despite its cosmopolitan distribution and long history in mycology (Bassi, 1835; Balsamo-Crivelli, 1835a, b), the teleomorphic state of *B. bassiana* has been remained unsure. The first indisputable record of teleomorph-anamorph connection in *Beauveria* species was of *B. brongniartii* (Sacc.) Petch, an anamorph of *Cordyceps brongniartii* Shimazu *et al.* (1988). Schaerffenberg (1955) reported the clavicipitaceous teleomorph of *B. bassiana*, but with little credible evidence. Recently, the new species *Cordyceps bassiana* Li *et al.* was described from China on a carpenterworm larva (Lepidoptera: Cossidae), and linked unequivocally to *B. bassiana* (Li *et al.*, 2001).

Here, we report the evidence of the teleomorph-anamorph connection in a *Cordyceps* specimen collected in Republic of Korea, it is similar to *Cordyceps bassiana*, based on its original description (Li *et al.*, 2001). This finding not only provides an additional insight on the systematic of *Cordyceps*, but also confirms that its anamorph is the morphological species *B. bassiana* by producing its entire life cycle in artificial culture.

Materials and Methods

Field collection and isolation of cultures. Frequent collecting trips by the first author and students from Kangwon National University constitute an ongoing, intensive survey of *Cordyceps* and other insect pathogenic fungi on many sites in northeastern Republic of Korea (Sung, 1996). The most productive habitats for *Cordyceps* species are usually in well drained but moist litter adjacent to stream beds in primary forests on mountain slopes. The methods used to recover and to handle *Cordyceps* specimens follow Sung (1996).

Cultures were isolated from freshly collected *Cordyceps* stromata EFCC C-783 by either of two methods. The usual approach used ascospores discharged from a fresh

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stroma taped to the inner surface of a petri dish lid and then placed over a sterile Petri dish bottom. Suspensions of discharged ascospores in sterile water were streaked onto Sabouraud dextrose agar + 1% (w/v) yeast extract (SDAY). Also, perithecia were removed from stromata, crushed in a drop of sterile water, and the macerate streaked on SDAY. All plates were incubated at 25°C, and the resultant colonies were transferred to fresh medium to avoid contaminants. In order to identify and characterize conidiophores slide cultures were made by placing small blocks of SDAY on slides, inoculating the corner, and topping the blocks with a sterile coverslip; these were incubated in a damp chamber. These slide cultures were deposited in culture collections EFCC (Entomopathogenic Fungal Culture Collection, Kangwon National University, Chuncheon) and ARSEF (ARS Collection of Entomopathogenic Fungal Cultures, Ithaca).

Morphological characters of the stromatic fungus and conidial isolate. Morphological characters of stromata were determined from intact stromata; microscopic characters were observed primarily by hand sectioning of a stroma to determine the arrangement and sizes of perithecia, and dimensions of asci and ascospores. As is typical of *Cordyceps*, ascospores are multicellular; they disassociate at the septa to produce part-spores, or secondary ascospores. These were measured immediately after being shot onto the surface of water agar plates. Measurements of macro-structures (stromata, etc.) were obtained by converting the vernier calibrations of the microscope stage position to actual distances; this method of measuring can be more accurate for macro-structures than the use of an ocular micrometer.

Production of stromata on brown rice and silkworm pupae by mycelia grown on liquid cultures. *Cordyceps* isolate EFCC C-783 was grown in cotton-plugged, 1100 *ml* plastic bottles on rice/silkworm medium, an autoclaved mix of 70 g unmilled brown rice, 30 g silkworm pupal tissue, and 100 *ml* water. The inoculated rice/silkworm cultures were incubated at 24°C under 1000 lux of continuous fluorescent light. After 7~10 d, primordia of stromata became apparent and continued to grow. After these primordia were 5~10 mm tall, the bottles were transferred to 20°C, 80~90% RH, and 1000 lux of illumination under standard fluorescent lamps. In order to maintain a high local relative humidity for the cultures, the outsides of the culture bottles were repeatedly sprayed with sterilized water for several days after transfer to these cooler growing conditions. Developing stromata were regularly observed for contamination during the continued growing period. By 50~60 d after the initial inoculation, ascomata reached a mature height of 10~12 cm and were freeze-dried to retain their shape and size.

Results

Identifications. After numerous trips to collect *Cordyceps* species and other entomopathogenic fungi throughout the northeastern Republic of Korea, we collected a *Cordyceps* specimen (EFCC C-783) which possesses the characteristics of teleomorphic and anamorphic states. The nonstromatic and mycelial mats on the host surface spread into the surrounding substratum and produce an anamorph that can be readily diagnosed as *B. bassiana*. The examination of the cultures from either ascospores or macerated tissue from the stromata of the specimen (EFCC C-783) confirmed that *B. bassiana* was anamorph of the *Cordyceps* and this connection was not due to dual infections by different fungi. The *Cordyceps* specimen grew from unidentified subterranean insect larvae (order Lepidoptera) at and somewhat below the level of the soil and litter in which the infected larvae died. The mature stroma were simultaneously formed with other immature stromata and a loose hyphal weft bearing the *B. bassiana* anamorph (Sung 1996). The perithecial stromata of this *Cordyceps* species is sulfur yellow and up to 45 mm long. The stroma consisted of a slightly swollen, irregularly shaped fertile part, 17 × 4 mm, with a smooth to folded surface contour, and stipe, up to 28 mm long, with an indistinct separation between the stipe and fertile part (Figs. 1~2). Perithecia were immersed in the stroma, and heavily distributed over the fertile part (Figs. 3~4) with only the slightly dark colored ostioles emergent.

The only *Cordyceps* species whose overall morphologies resembled those of the Korean collections was *C. bassiana* (Li *et al.*, 2001). Moreover, our study of the type specimen of *C. bassiana* (Research Center on Entomogenous Fungi, Anhui Agric. Univ., China) reveals it is conspecific with the Korean collections, including the nonstromatic production of a *B. bassiana* anamorph. The perithecia of *C. bassiana* appeared to have more papillate ostioles and were slightly larger (at 610~720 × 230~320 μm) than those of the Korean collections. In view of the overall similarities, the *Cordyceps* specimen of our collections is considered as *C. bassiana*.

***In vitro* fruiting of *Cordyceps* isolate EFCC C-783.** Cultures from the Korean collection isolated from ascospores and tissue macerates were similar to each other. The cultures are fast growing; they cover the surface of the brown rice/silkworm pupa medium within 7 d. The mycelium is initially light gray, but gradually changes to a deep, dark yellow. About two weeks after inoculation the silkworm pupae are completely filled with mycelium and light yellow mycelial masses begin to form on the surface of the pupae, and initials of synnemata become visible. Synnemata are prominent on the rice/silkworm medium; they were 5~6 cm long (Fig. 9), after 30 d. Conidia cov-

Figs. 1~8. *Cordyceps bassiana* (EFCC C-783). 1~2. Habit of *Cordyceps bassiana* on infected insects. 3. Surface of field-collected stroma showing slightly darker, slightly erumpent ostioles of the perithecia. 4. Perithecial ostioles from field-collected stroma. 5~6. Germination of ascospores on water agar. 7. Conidiogenous cells and conidia of the *Beauveria* state produced in culture. 8. Perithecia of *C. bassiana* produced in artificial culture after inoculation by the *Beauveria* state; note that these are more elongated than those produced on naturally occurring stromata on the insect host.

ered the synnemata and formed abundantly on the mycelial mat which spread on the substratum. After another 30~40 d a culture of the stromatic initials produces erect columnar stromata with swollen fertile apices and mature perithecia. The overall process takes 50~60 d from the time of inoculation to the production of mature perithecia. Fertile stromata formed *in vitro* and *in vivo* are morphologically comparable. In one instance a culture of EFFC C-783 produced stromata and perithecia (Figs. 9~11) on brown rice/silkworm medium. The perithecia in the stromata produced *in vitro* were flask-shaped, somewhat larger ($362 \pm 81 \times 196 \pm 27\ \mu m$), and more prominently emergent from the stromatic context than those in the stromata formed in the field on a natural host (Figs. 1~5). The size and shape of naturally occurring perithecia ($530\sim550 \times 290\sim300\ \mu m$, sesame seed) differed from those produced *in vitro* ($195\sim470 \times 155\sim235\ \mu m$, more elongated and clavate).

Description of the species. On insect larvae (unidentified species of Lepidoptera) immersed in soil/detritus or decaying wood, simultaneously forming both stromata and conidia. Stromata sulfur yellow, 40~45 mm long with apical fertile part somewhat swollen, 17×4 mm, indistinctly separated from stipe, 10~30 mm long (Figs. 1, 2). Perithecia immersed, ovoid (frequently shaped like sesame seeds; Fig. 5), $530\sim550 \times 290\sim300\ \mu m$, and densely distributed throughout fertile part (Figs. 3, 4). Ascospores filiform,

400~450 × 1~1.5 μm, dissociating to form part-spores (Fig. 6) that, in turn, each produce a globose secondary conidium on an unbranched sterigma (Fig. 6); this sterigma (Fig. 7) not observed to branch sympodially or to become a denticulate rachis. Stromata produced in culture (Figs. 8, 10) with fertile part less swollen, less distinct from the stipe (Fig. 9), bearing perithecia less completely immersed (Fig. 11).

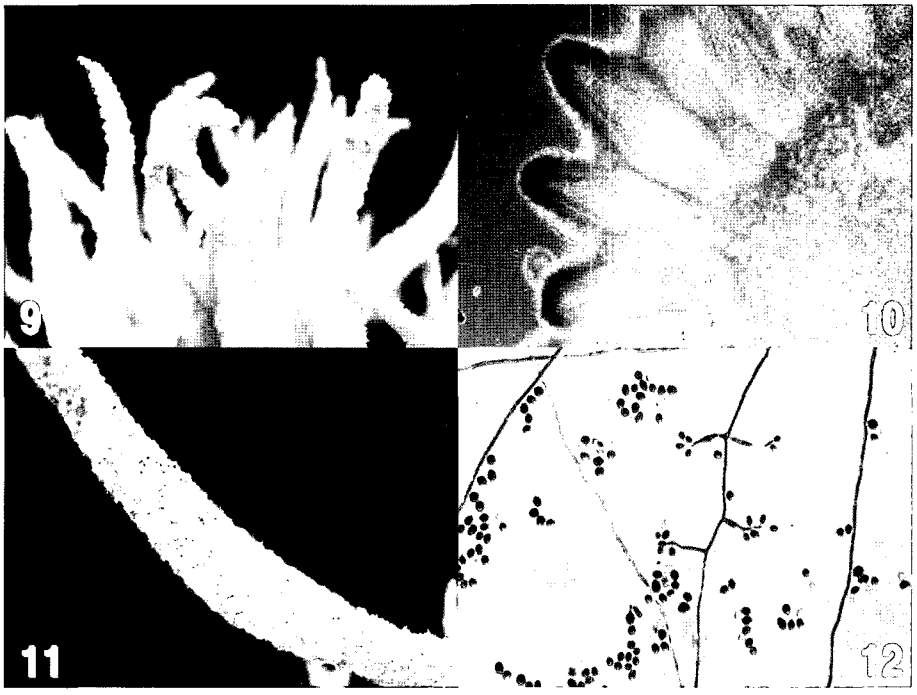
Anamorph appearing as a dense covering of white balls of conidia on the surface of the hosts. Conidiogenous cells usually forming in dense clusters on short lateral branches or borne singly on vegetative hyphae. Conidiogenous cells producing a single conidium at each denticulate locus before proliferating sympodially several times to form a narrow rachis; ultimately the conidiogenous cell comprising a subglobose to flask-shaped base from which a denticulate rachis extends; clustered conidiogenous cells and conidia forming conspicuous, white balls. Cultures on SDAY producing creamy white colony. Conidial synnemata not forming on SDAY, usually conidial synnemata and occasionally perithecial stromata forming on brown rice/silkworm medium (Fig. 9); Conidia and conidiogenous cells as formed in nature.

All isolates from the ascospores and perithecia had the same appearance and produced a light brown pigment in SDAY. They did not produce stromata on SDAY but did sometimes produce conidial synnemata on brown rice/silkworm medium (Fig. 9); all isolates produced conidia sympodially on elongated rachis on conidiogenous cells (Fig. 12) typical of *Beauveria* species. Conidiogenous cells subglobose to flask-shaped and often in clusters. Conidia short ellipsoid to globose, 2.6~3.4 × 1.2~1.9 μm (Figs. 7, 12). These morphological characters correspond completely with *B. bassiana* as characterized by Hoog (1972) and are indistinguished from *B. bassiana* as commonly detectable in soil or from insects throughout the world.

Specimen examined: Republic of Korea, Kangwon Province, near Chuncheon, adjacent to stream at base of Mount Samak; insect larva (order Lepidoptera); 16 Sept 1995, EFCC C-783, preserved in EFCC, Kangwon National University, Chuncheon; stromata produced *in vitro* (from axenic culture, EFCC C-783, established from ascospores discharged from specimen EFCC C-783).

Discussion

To clarify the life history of a *Cordyceps* species by reliably connecting teleomorphic and anamorphic states, the teleomorph - anamorph connection was made for *C. bassiana*. The linkage of a *Beauveria* species with a *Cordyceps* teleomorph, while no longer complete surprising, is still a major advance for the extraordinarily rich spectrum of hyphomycete genera that are anamorphs of



Figs. 9~12. *Cordyceps bassiana* in culture. 9. Habit of ascomatous culture; conidial synnemata are out of focus in the background, while large, fertile stromata are visible in the foreground. 10. Perithecia produced on stromata *in vitro*; note that these perithecia are more elongated than those in the stroma produced in the field. 11. High magnification of surface of cultured stromata; note that perithecia are less completely immersed than in those on field-collected stromata. 12. Conidiogenous cells with extended, denticulate raches and globose conidia produced *in vitro*.

Cordyceps species. The range of entomopathogenic hyphomycetes associated with *Cordyceps* states comprises nearly twenty genera, including species in such genera as *Beauveria*, *Metarhizium*, *Paecilomyces*, *Lecanicillium* (until recently included in *Verticillium*), *Hirsutella*, *Hymenostilbe*, and *Nomuraea* (Kobayasi and Shimizu, 1983; Samson *et al.*, 1988; Humber, 2000; Zare and Gams, 2001).

The production of *Cordyceps* ascomata in culture has been reported most often for *C. militaris* (Kobayasi, 1941; Basith and Madelin, 1968), but *in vitro* production of stromata seems to be easier for *C. militaris* than other *Cordyceps* species using the brown rice/silkworm medium and growing conditions discussed here. Sung (1996) obtained stromata of *C. militaris*, *C. pruinosa* Petch, and *C. scarabaeicola* Kobayasi, and excellent production of synnemata of *Paecilomyces tenuipes* (Peck) Samson. If the conditions allowing cultures of *B. bassiana* or *B. brongniartii* to produce ascomata in culture could be determined, it might be possible to discover why the teleomorphs of these two globally dispersed, the teleomorphic species of *Beauveria* seem to be restricted to eastern Asia and are very rare, even in their indigenous ranges. Many ascomycetes might lose the capacity for sexual reproduction due to the loss or incapacitation of their mating type genes (Sharon *et al.*, 1996; Turgeon, 1998; Yun *et al.*, 1999), but the presence of such genes has not yet been demonstrated for any entomopathogenic clavicipitaceous anamorphs. Due to limited evidence of the sexual reproduction, it is not yet known whether the *Beauveria* species is clonal or reproductive throughout their global distribution. The heterokaryosis and parasexual recombination also appears to be reasonably possible for *B. bassiana* since both vegetative compatibility groups (Couteaudier and Viaud, 1997) and parasexuality (Paccola-Meirelles and Azevedo, 1991; Bello and Paccola-Meirelles, 1998) have been demonstrated for *B. bassiana.*

Conidial synnemata could form on rice/silkworm medium after two weeks of growth; perithecial stromata formed after the synnemata in the single instance when stromata of *Cordyceps* isolate EFCC C-783 formed *in vitro*. Species of *Beauveria*, *Metarhizium* and some other common entomopathogenic hyphomycetes do not usually produce synnemata but can occasionally do so either in culture or on infected hosts in the field. Synnemata are common or usual, but not necessarily always formed, for many entomopathogenic species in *Hirsutella*, *Paecilomyces*, and other genera. While *Hirsutella* contains numerous synnematous species, some species produce no synnemata on infected hosts or in culture (Minter and Brady, 1980; Minter *et al.*, 1983; Hodge, 1998), but cultures of one variety of a normally mononematous species, *H. thompsonii* Fisher var. *synnematosa* Samson (1980) can produce large synnemata with a nearly stromatic texture. Many *Paecilomyces* species usually form synnemata on their hosts (Samson, 1974) but in culture progressively lose the ability to form them. The synnemata of entomopathogenic *Cordyceps* anamorphs seem to reflect an inherent genomic capacity to produce erect stromata, little is understood about the conditions influencing the formation of such erect fascicles of hyphae or even the fundamental development differences between more loosely organized conidial synnemata and erect stromata bearing perithecia and asci. The phenomenon that *C. bassiana* may produce its *Beauveria* anamorph on synnemata is an interesting but one without taxonomic significance.

The ability to obtain the *in vitro* production of ascomata by such entomopathogens as *Beauveria* species enables significant new studies of the ecology, mating system, developmental biology, biochemistry, and pathogenensis of these fungi that would be difficult or impossible to approach by any other means. Production of *Cordyceps* stromata in culture could also open possibilities for entirely new frontiers in the study and, indeed, practical exploitation of these fungi for biocontrol by breeding new isolates, for medicinal purposes, and for development of cottage industries to produce *Cordyceps* stromata especially for use in traditional Asian medicine.

Until recently, the only *Beauveria* species for which a teleomorphic connection was indisputably confirmed was *B. brongniartii* (Shimazu *et al.*, 1988), although this hyphomycete was shown in culture to be the anamorph of both *C. brongniartii* and *C. scarabaeicola*. In analyses of small and large subunit rDNA from the Clavicipitaceae by Sung *et al.* (2001), all samples of *Beauveria* species or their teleomorphs clustered together. The pairings of *B. caledonica* and *C. scarabaeicola* in the dendrogram in Sung *et al.* (2001) is no indication of anamorphic-teleomorphic connection since ascospore cultures from Japanese and Korean *C. scarabaeicola* collections formed only *B. brongniartii* (Shimazu *et al.*, 1988; Sung, 1996) but might indicate the possible synonymy of *B. caledonica* (isolated from Scottish soil, Bissett and Widden, 1988) with the morphologically very similar entomopathogen, *B. brongniartii*. The distribution of *B. brongniartii* and one of its two teleomorphs onto two branches within a clade does confirm that *B. brongniartii* probably needs to be split into at least two species (M. Shimazu, personal communication).

The data presented here for *C. bassiana* and by Sung (1996) comparing ascomata of the same fungus produced on field-collected hosts and in culture cast doubt on the value of some traditional structural criteria to distinguish among similar *Cordyceps* species. These findings underscore the ultimate importance of complementing and extending traditional data about morphology, development, pathobiology, anamorph-teleomorph connections, biogeography, and other characters with molecular data (Liu *et al.*, 2002) to allow a long needed comprehensive taxonomic revision of this large, cosmopolitan, and complex genus.

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