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Moelleriella zhongdongii: stroma development and identification of hirsutella-like and *Aschersonia* synanamorphs

Mariusz TADYCH[a,*], *Priscila CHAVERRI*[b], *Marshall BERGEN*[a], *James F. WHITE*[a]

[a]*Department of Plant Biology and Pathology, Rutgers University, New Brunswick, NJ 08901, USA*
[b]*Department of Plant Sciences and Landscape Architecture, University of Maryland, College Park, MD 20742, USA*

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ABSTRACT

Collections of *Moelleriella zhongdongii* were made at the La Selva Biological Station in Costa Rica. Fresh collections were examined to evaluate developmental stages. Isolations were made from single part-ascospores and *Aschersonia* conidia. *Moelleriella zhongdongii* produces perithecia with evanescent asci and part-ascospores, and both hirsutella-like and *Aschersonia* synanamorphs. Both anamorphs were produced in pure cultures under cultural conditions optimal to induce the respective anamorphs. Low-nutrient conditions favoured production of the hirsutella-like anamorph while high-nutrient conditions favoured development of the *Aschersonia* anamorph. The teleomorph developed on leaves of host plants but were not produced *in vitro*.

Introduction

Species of *Moelleriella* Bres. (*Clavicipitaceae*; *Hypocreales*; *Ascomycota*) are necrotrophic parasites of scale insects (*Lecaniidae* and *Coccidae*; *Homoptera*) and whiteflies (*Aleyrodidae*; *Homoptera*) and biotrophic parasites of plants. The species in this genus and several other allied genera such as *Hypocrella* Sacc. and *Samuelsia* Chaverri, Liu & Hodge typically infect the immobilized immature forms of the insect, consume its body, and continue to develop a stroma using nutrients emerging to the plant surface through the insect's stylet or stylet wound (Bischoff *et al.* 2005; Chaverri *et al.* 2008; Hywel-Jones & Samuels 1998; Koroch *et al.* 2004; Spatafora *et al.* 2007; Sullivan *et al.* 2000; Torres *et al.* 2007).

Moelleriella is associated with production of an aschersonia-like anamorph. Aschersonia-like anamorphs are also associated with genera *Hypocrella* (*Aschersonia* Mont. s. s.) and *Samuelsia*. *Aschersonia* conidia of *Moelleriella* and *Hypocrella* species are fusiform; in contrast, the conidia of *Samuelsia* species are smaller and allantoid (Chaverri *et al.* 2008). Ascospores of *Moelleriella* species disarticulate in the ascus while in the *Hypocrella* and *Samuelsia* species ascospores do not disarticulate in the ascus. The *Aschersonia* anamorph of *Moelleriella* is formed in pits embedded in or in superficial depressions on the surface of stromata. In this synanamorph, moist masses of fusiform enteroblastic conidia are produced from a single tight layer of narrowly-cylindric phialides (Liu *et al.* 2006). The *Aschersonia* anamorph may be an apomorphy of *Moelleriella*, *Hypocrella* and *Samuelsia* and has not been found outside

* Corresponding author.
E-mail address: tadych@aesop.rutgers.edu
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these three genera (Chaverri et al. 2008). References (Chaverri et al. 2008; Evans 1994; Hywel-Jones & Evans 1993; Petch 1921) have described the occurrence of another synanamorph on stromata or emerging from germinating ascospores or conidia that resembled species of the anamorphic genus *Hirsutella* Pat. This synanamorph has slender solitary phialides producing 1–2 conidia at the apex of conidiogenous cells. Liu et al. (2005) described a new species, *Aschersonia insperata* Rombach, M. Liu, Humber & K.T. Hodge, that produced both *Aschersonia* and the hirsutella-like synanamorphs but did not form the teleomorph. It is unclear how prevalent hirsutella-like anamorphs are among species of these three genera, or how these hirsutella-like anamorphs may be induced under cultural conditions.

Recent work at the La Selva Biological Station in Costa Rica resulted in numerous collections of the recently described *Moelleriella zhongdongii* (M. Liu & K.T. Hodge) M. Liu & Chaverri, a pathogen of whiteflies (Chaverri et al. 2008; Liu & Hodge 2005). Preliminary examinations of this fungus demonstrated that it possessed some features that had not been previously known, including the production of both *Aschersonia* and hirsutella-like synanamorphs and evanescent asci. This paper documents the development of the teleomorph and synanamorphs in *M. zhongdongii*.

Materials and methods

Collections of *M. zhongdongii* were made by J. White in Feb. 2007 at the La Selva Biological Station (Heredia; Costa Rica; 10°25′56.46″N, 84°00′33.54″W) along the Camino Experimental Sur trail on leaves of *Piper multiplinervium* [collection 24; Rutgers University Plant Pathology Herbarium (RUTPP; http://herbarium.rutgers.edu/index.html) at the School of Environmental and Biological Sciences, Rutgers University]; the Sendero Chanchera trail (collection 27; RUTPP); and the Camino Experimental Sur trail on leaves of unidentified dicot (collection 25; RUTPP). Fresh collections of stromata were examined microscopically and photographed at the La Selva laboratory to assess structural features of the fungus during its development on leaves beginning with early infection of whiteflies to ascospore maturation and liberation.

Masses of *Aschersonia* conidia and *Moelleriella* part-ascospores were removed from stromata using dissecting needles and placed in vials containing sterile distilled water with a mixture of antibiotics (penicillin 20 mg l^{-1}; streptomycin 40 mg l^{-1}; tetracycline 50 mg l^{-1}). Vials were maintained in a refrigerator at *ca* 5 °C for 7–10 d and then kept at ambient temperature (*ca* 20–25 °C) for 3–5 d. Stromata at various stages of development were also removed from leaves and placed in water with antibiotics. Isolations were made directly from stromata by surface-disinfection with 10 % Clorox for 5 min, followed by washing and plating in Difco potato dextrose agar (PDA) amended with the antibiotic mixture used above. For isolations from part-ascospores and *Aschersonia* conidia, water containing either conidia or part-ascospores was agitated vigorously to separate spores and serially diluted (10×, 100×, and 1000×) in sterile distilled water. The dilutions were spread evenly on agar plates. Plates were sealed with Parafilm and incubated at room temperature for 14–21 d in plastic boxes under diffused light. An isolate of *M. zhongdongii* has been deposited with the ARS Collection of Entomopathogenic Fungi (ARSEF, http://arsef.fpsnl.cornell.edu/; accession number, ARSEF 8800).

To induce the hirsutella-like anamorph in culture, 21d-colonies growing on PDA derived from single part-ascospores or single *Aschersonia* conidia were placed on water agar (WA) for 7–14 d at room temperature. For induction of the *Aschersonia* synanamorph, conidia of *Aschersonia* were placed on PDA and the resulting colonies were grown for 21–28 d at laboratory ambient temperature (20–22 °C) with a 12 h cool white fluorescent light and 12 h darkness. Unsuccessful attempts were made to induce the teleomorph in cultures by making crosses on PDA between single-ascospore-derived colonies using hirsutella-like and *Aschersonia* conidia.

Results

Development on host tissues

In early infections, *M. zhongdongii* formed a white or cream-coloured mycelium on the whitefly body, which eventually completely engulfed the body in the stromal mycelium (Fig 1A). These stromata (0.6–1.8 mm) were circular in outline with a raised peripheral zone (Fig 1B) consisting of short (50–100 × 40–70 μm) synnemata bearing hirsutella-like conidiogenous cells (9.5–19.5 μm long; Fig 1C). Each conidiogenous cell consisted of a broader, more or less cylindrical, basal portion (4.0–9.5 × 2.5–3.0 μm) and a narrow apical extension (5.5–10.0 μm long and < 1.0 μm wide) bearing hirsutella-like conidia (4.0–5.0 × 1.5–2.0 μm; Fig 1D). Hirsutella-like conidiogenous cells and conidia were also seen in more mature perithecial stromata, where they formed sparsely over the surface of stromata. There were numerous instances where phialides were damaged by breaking along the narrow neck region. In the centre of the stromata were pools of moist, yellow masses of *Aschersonia* conidia (Fig 1B). *Aschersonia* conidiomata were composed of a single, closely packed layer of narrow hyaline phialides (36–45 × 1.5–2.0 μm; Fig 1E) bearing a succession of conidia apically. *Aschersonia* conidia were hyaline, fusiform (10.0–15.0 × 1.0–2.5 μm) and contained 3–6 guttules in a linear arrangement (Fig 1F).The *Aschersonia* conidiomatal pit gradually disappeared (Fig 1G-H). In some cases, irregular development of the mycelium of the stroma resulted in partial closure of the central conidiomatal pit, resulting in separation of the central pit into multiple conidiomatal pits. Stromata expanded (0.6–1.8 to 1.0–2.3 mm diam; 0.25–0.8 to 0.6–1.1 mm high) as embedded perithecia (3–40 perithecia per stroma) developed. Perithecia became visible as discrete or confluent hemispherical elevations on the stroma surface (Fig 1I). As perithecia developed, the stroma formed a surface layer of an interwoven network of branching mycelium with thick-walled hyphae measuring 2.5–4.5 μm wide. Ostioles were tan discolourations at the apex of maturing perithecia (Fig 1J). In vertical sections, perithecia were ovate to flask-shaped (380–470 × 140–190 μm), with hyaline plectenchymatous walls (8–14 μm thick). Asci were cylindrical (150–250 × 8–10 μm) with a thickened refractive tip (Fig 1K). The refractive ascus tips collapsed leaving a concave pit at the apex (Fig 1L–M). After

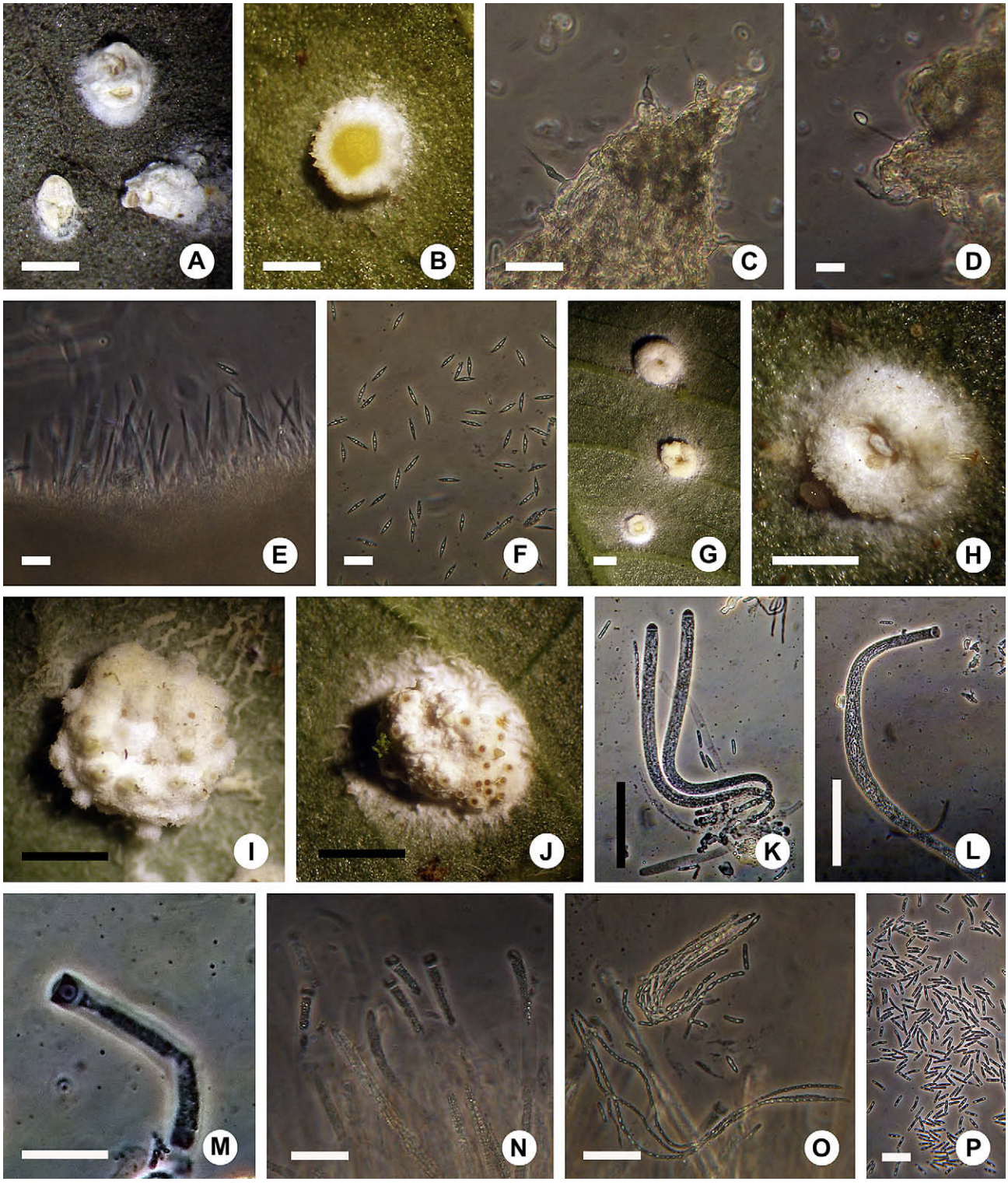

Fig 1 – *Moelleriella zhongdongii*. (A) Young stromata on the leaf of *Piper multiplinervium*. (B) Stroma with peripheral zone of hirsutella-like synnemata and central zone of yellow conidioma of *Aschersonia*. (C) Synnema of hirsutella-like with conidiogenous cells. (D) Conidiogenous cell with conidium of hirsutella-like anamorph. (E) Layer of phialides of *Aschersonia* anamorph. (F) Fusiform conidia of *Aschersonia*. (G) Development of stromata of *M. zhongdongii*. (H) Disappearance of central conidiomatal pit of *Aschersonia*. (I) Early stage of perithecial development on stroma. (J) Ostioles of maturing perithecia on stroma. (K) Asci with refractive tip. (L) Ascus without refractive tip. (M) Concave pit at the ascus apex. (N) Early stage of the ascal wall disintegration. (O) Multiseptate ascospores. (P) Part-ascospores. Bars = (A, B) 1.5 mm; (C, E, F, P) 15 μm; (D) 8 μm; (G–J) 1 mm; (K, L, N) 50 μm; (M, O) 25 μm.

ascospores differentiated, the ascus walls disintegrated beginning with the lateral walls (Fig 1N) and progressing to the tip of the ascus. Filamentous ascospores (eight per ascus) became multi-septate with *ca* 20–22 septa (Fig 1O) and disarticulated at septa to form part-ascospores (Fig 1P). Part-ascospores (8.0–13.0 × 1.0–2.5 μm) were hyaline, cylindrical, slightly curved, and obtuse at both ends; these part-ascospores contained 2–6 refractive guttules, 1–3 located near either extreme. Part-ascospores emerged from ostioles of perithecia in viscous, tan masses. These part-ascospores germinated readily in water suspensions. Examination of part-ascospore masses did not show any remnants of the evanescent ascus walls, refractive tips, or other centrum contents. Sections of mature perithecia demonstrated that cavities were devoid of centrum contents other than masses of part-ascospores.

Cultural studies

After 21 d, part-ascospores germinated from both ends in water suspensions to form septate filaments (Fig 2A). After 21 d, *Aschersonia* conidia swelled (up to 3.5 μm wide), often producing short lateral buds, but no hyphae were present (Fig 2B). When plated onto PDA, both *Aschersonia* conidia and part-ascospores readily developed into colonies. Colonies on PDA after 14 d attained 10–15 mm diam, were white or cream-coloured, raised, hemispherical (Fig 2C), and either smooth superficially with a surface layer of short, radially-oriented hyphae (*Aschersonia* morphology), or tomentose with numerous radiating synnemata (hirsutella-like morphology). Transferring colonies with radiating synnemata to WA plates resulted in production of numerous hirsutella-like conidiogenous cells and conidia (Fig 2D); eventually hirsutella-like colonies also developed at the edge of smooth colonies. Hirsutella-like conidiogenous cells (Fig 2E) were hyaline, elongate (30–120 μm long); each conidiogenous cell had a broader, more or less cylindrical, basal portion (12–25 × 1.5–2.5 μm) at the apex of which was a narrow extension (18–95 long and <1.5 μm wide), and frequently curved at the junction of the broader base and narrow apical extension. Generally, 1–2 conidia (mostly 1) adhered to the apices of conidiogenous cells. Hirsutella-like conidia (3.5–4.0 × 2.0–2.5 μm) were hyaline, lunate (i.e., narrower at each end and curved), with a narrow slime layer visible in phase contrast as a refractive zone surrounding conidia (Fig 2F). Perithecia were not formed in culture despite numerous attempts to make crosses and vary cultural conditions to stimulate teleomorph development.

Discussion

Moelleriella *synanamorphs*

Some *Moelleriella* species have the *Aschersonia* anamorph; other species within this genus have no known anamorph (Chaverri *et al.* 2008). Hirsutella-like anamorphs are rarely described in *Moelleriella*, apparently because of their fragile, ephemeral nature (Chaverri *et al.* 2008; Liu *et al.* 2005). Hirsutella-like anamorphs have been found in *Moelleriella insperata*, *Moelleriella turbinata* (Petch) Chaverri & K.T. Hodge, and *Hypocrella hirsuta* Chaverri & K.T. Hodge (Chaverri *et al.* 2008; Liu *et al.* 2005). Although not well described, Petch (1921) observed a surface layer of scattered conidiophores and conidia on stromata of *Moelleriella reineckiana* Henn. Some previous evidence of their occurrence has emerged from observations of similar states forming from germinating ascospores or *Aschersonia* conidia in several species (Evans 1994; Hywel-Jones & Evans 1993). This process may be due to microcyclic conidiation where smaller secondary conidia may act as a means of additional

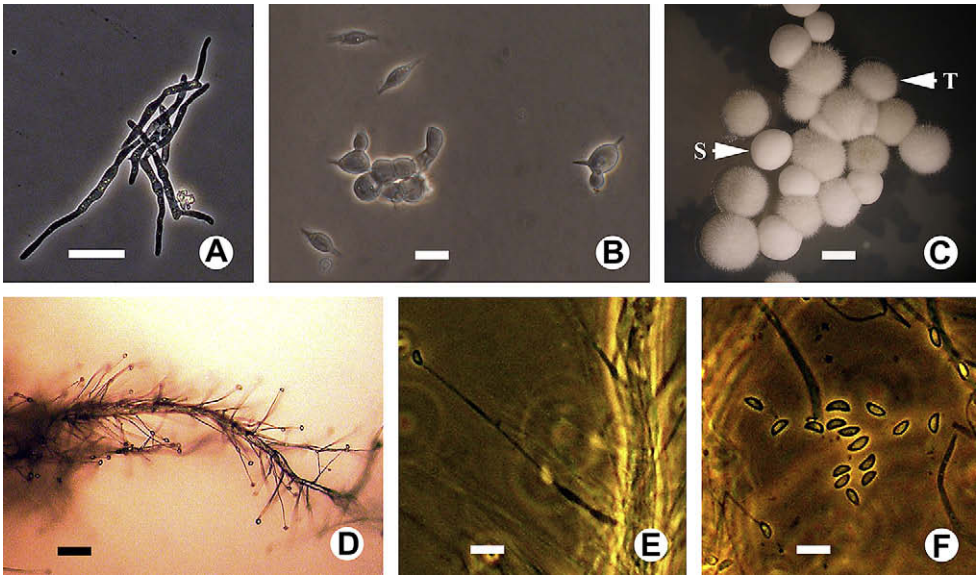

Fig 2 – *Moelleriella zhongdongii*. (A) Part-ascospores germinating after 21 d in water. (B) Swelling conidia of *Aschersonia* anamorph after 21 d in water. (C) 14-day-old smooth (S) and tomentose (T) colonies on PDA. (D) Synnema, conidiogenous cells and hirsutella-like conidia on WA. (E) Hirsutella-like conidiogenous cell with conidium. (F) Hirsutella-like conidia. Bars = (A) 15 μm; (B, F) 5 μm; (C) 10 mm; (D) 30 μm; (E) 8 μm.

dissemination (Bacon & Hinton 1991). Torres *et al.* (2007), using evidence in part derived from phylogenetic data with a small data set, described a new species, *Hypocrella panamensis* M.S. Torres, J.F. White & J.F. Bischoff, that possessed exclusively what was characterized as a lecanicillium-like anamorph. Further phylogenetic analysis needs to be performed to determine the relationship of *H. panamensis* to other *Hypocrella* species as well as to closely related species of *Moelleriella* and *Samuelsia*. Liu *et al.* (2005) described a new species, *Aschersonia insperata*, with both *Aschersonia* and *Hirsutella*. Chaverri *et al.* (2008) placed *A. insperata* in the genus *Moelleriella*. Our study of *M. zhongdongii* is the first to show that *Aschersonia* and hirsutella-like synanamorphs occur on stromata in nature along with the *Moelleriella* teleomorph and that the synanamorphs can both be readily induced in culture from colonies derived from both synanamorphs or the teleomorph.

Implications of pleoanamorphy in Moelleriella

There is growing consensus that the *Aschersonia* anamorph is an apomorphic character of *Hypocrella*, *Moelleriella* and *Samuelsia*, largely because this anamorph morphology is not widely distributed throughout the *Clavicipitaceae*; phylogenetic work supports this hypothesis (Chaverri *et al.* 2005, 2008; Liu *et al.* 2006). The generic limits of *Aschersonia* are uncertain and it is unknown presently whether all species of these holomorphic genera have this anamorph. In all groups of fungi, a unique anamorph with apomorphies can be lost in a subclade or individual species.

If previous observations of hirsutella-like anamorphs in species of *Hypocrella* and *Moelleriella* are correct (e.g., Chaverri *et al.* 2008; Evans 1994; Hywel-Jones & Evans 1993; Petch 1921), hirsutella-like anamorphs may be more common than previously believed. Sung *et al.* (2007) have determined that there are three clavicipitacean clades: i.e., *Cordycipitaceae*, *Clavicipitaceae s. s.*, and *Ophiocordycipitaceae*. The authors retained *Hypocrella* within *Clavicipitaceae*. It is, therefore, probable that the hirsutella-like anamorph also fits within the *Clavicipitaceae*. Speare (1920) described *Hirsutella* as having verruciform synnemata with basally subulate phialides narrowing to slender necks and with a few conidia in a gelatinous matrix. Most species referred to *Hirsutella sensu* Speare are now consider anamorphs of *Ophiocordyceps* Petch (Sung *et al.* 2007); *Ophiocordyceps* is within the newly erected family *Ophiocordycipitaceae*. Sung *et al.* (2007) also found through phylogenetic analysis that other *Hirsutella* species are anamorphs of *Cordyceps* (Fr.) Link or *Torrubiella* Boud. in the family *Cordycipitaceae*. Therefore, *Hirsutella* can be regarded as polyphyletic. The only cordyceps-like fungi remaining in the *Clavicipitaceae* are *Metacordyceps* G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora, teleomorphs of *Metarhizium* Sorokīn. The phylogenetic work of Sung *et al.* (2007) clearly provides evidence that genera within the *Cordycipitaceae* were ancestral to both the *Clavicipitaceae* and the *Ophiocordycipitaceae*. Thus, both plant biotrophs, such as *Claviceps* Tul. and *Epichloë* (Fr.) Tul. & C. Tul. within the *Clavicipitaceae*, and insect-infecting necrotrophs, such as the species of *Ophiocordyceps* within the *Ophiocordycipitaceae*, have likely evolved from insect-infecting necrotrophs of the *Cordycipitaceae*.

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