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Tolypocladium inflatum is the anamorph of *Cordyceps subsessilis*

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Abstract: A collection of *Cordyceps subsessilis* is documented. Axenic cultures of single part ascospores produced an anamorph attributable to the common soil hyphomycete *Tolypocladium inflatum* (= *T. niveum*). Efrapeptins were identified in culture filtrates of the anamorph. The efrapeptin profile of the *C. subsessilis* anamorph was found to be similar to that of other isolates of *T. inflatum*. This is the first report of a teleomorph for this important anamorph genus.

Key Words: Clavicipitales, *Cordyceps facis*, cyclosporin, efrapeptin, teleomorph, *Tolypocladium niveum*

Two interesting specimens of a *Cordyceps* species were collected by students of a field mycology course led by R. P. Korf in the autumn of 1994 in Michigan Hollow State Forest in Danby, New York. Each specimen comprised a small, elongated stroma arising from the remains of a beetle larva (FIG. 1).

The fruiting bodies (CUP 63485) are about 1.5 cm tall and are constructed of loosely woven hyaline hyphae. The perithecia have prominent yellow necks and are partially immersed in a small head at the apex of the stipe. On one of the specimens several small heads are produced from the same stipe which branches near the apex (FIG. 1). The asci (FIG. 9, 10) are long and slender with an apical cap approximately 2 μm long and contain smooth, hyaline ascospores which break into many part spores measuring on average 4.0 × 1.0 μm (FIG. 2–5, 9). Although no information on substrate was recorded, the specimens appear to have been buried to just below the fertile head in loose organic soil or dung, particles of which adhere to the stipe. The hosts were identified by Dr. J. K. Liebherr (pers. comm.) as larvae in the coleopteran family Scarabaeidae, subfamily Aphodiinae, the larvae of which typically inhabit dung.

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The specimens were identified as *Cordyceps subsessilis* Petch (Ascomycota: Clavicipitales), which Petch (1937) described based on a pair of specimens collected by R. Thaxter. The holotype (FH 6145) occurs on a beetle larva identified as Scarabaeidae: Rutelinae by J. K. Leibherr (pers. comm.). Mains (1958) contributed further details based on a reexamination of Thaxter's specimens and two additional specimens collected by A. H. Smith. *C. subsessilis* has been reported only from Tennessee, North Carolina, Michigan, New York, Washington, and Japan.

C. subsessilis bears a close ressemblance to *C. facis* Kobayasi et Shimizu, to which these specimens were originally attributed (Hodge and Krasnoff, 1995), and which also occurs on beetle larvae. Unfortunately the type specimen of *C. facis* was not available for comparison, but based on the terse diagnosis in Kobayasi (1982), a watercolor illustration (Kobayasi and Shimizu, 1983), and a color photograph (Imazeki et al., 1988), it appears likely that *C. facis* is a later synonym of *C. subsessilis*.

Single spore isolation.—Cultures were made by hydrating and dissecting a perithecium and removing the centrum elements in a drop of sterile water on a siliconized slide, then washing them in a second drop. The drop, which contained liberated part ascospores as well as entire asci, was pipetted onto water agar (WA) and spread aseptically with an inoculating loop. As individual part ascospores germinated, they were removed under a microscope using a fine needle and transferred to malt extract agar (MEA, Difco). Twenty-two of the twenty-five isolated spores yielded cultures of the same hyphomycetous fungus; three failed to grow. The isolates were deposited as accessions 4877 through 4898 in ARSEF (USDA-ARS Collection of Entomopathogenic Fungal Cultures, Plant Protection Research Unit, U.S. Plant, Soil, and Nutrition Laboratory, Tower Rd., Ithaca, NY 14853). ARSEF 4884 is also accessioned as DAOM 209986 and CBS 305.95.

Characteristics in culture.—Germination of part ascospores was observed on WA-coated slides held in a moist chamber at room temperature (FIGS. 2–5). The

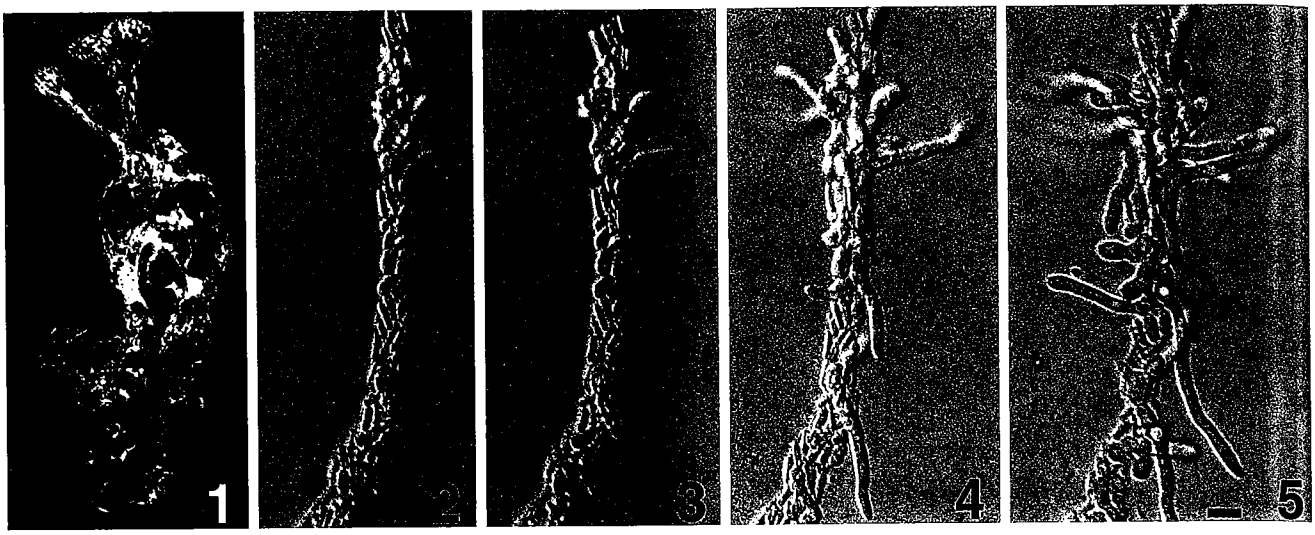

FIG. 1–5. *C. subsessilis* stroma and ascospore germination. 1. Stroma of *C. subsessilis* (CUP 63485) on scarabaeid beetle larva. Bar = 1 mm. 2–5. Germination of *C. subsessilis* part ascospores in an intact ascus. 2. 12 h after hydration. 3. 14 h. 4. 18 h. 5. 24 h. Bar = 5 μm.

cylindrical spores swelled to become ellipsoid before producing bipolar germ tubes within 24 h. In slide culture and on the aerial mycelium of young colonies slender *Acremonium*-like phialides were observed three d after germination (FIG. 6). In older cultures these simple phialides were not observed, but copious conidia (FIG. 7) were produced in slime from basally swollen phialides on simple or sparingly branched conidiophores (FIG. 8).

On cornmeal agar (CMA, Difco) and MEA the colonies were pale cream with sparse aerial hyphae and concentric zones of conidial production with profuse slime. At approximately 22 C the average colony diameter after 10 d was 30 mm or 35 mm on CMA and MEA, respectively.

The anamorph was identified as *Tolypocladium inflatum* W. Gams (= *T. niveum* (O. Rostrup) Bissett) (Hyphomycetes). The name *T. inflatum* is now the correct name for this fungus because the Committee for Fungi of the International Association for Plant Taxonomy has accepted (Gams, 1996) the proposal of Dreyfuss and Gams (1994) to reject its earlier synonym *Pachybasium niveum* O. Rostrup.

The morphology of the anamorph was compared to published descriptions (Bissett, 1983; Gams, 1971b) and to ARSEF 3280 (= DAOM 167322), a culture derived from the neotype of *T. niveum* (Bissett, 1983). The chief differences were the appearance of *Acremonium*-like phialides as noted above and the appressed character of the cultures.

No evidence of an anamorph was detected on the specimens from which ascospores were taken (CUP 63485) nor on the type specimen (FH 6145). Patches of *T. inflatum* were found, however, on the host body and in cavities in the wood surrounding the host in a specimen of *C. subsessilis* collected by A. H. Smith in Oakland County, Michigan (MICH 7724).

Efrapeptin analysis.—Many species of *Tolypocladium* produce efrapeptins, compounds with demonstrated insecticidal and antifungal activity (Krasnoff et al., 1991). A culture (ARSEF 4884) derived from a *C. subsessilis* part ascospore was tested for efrapeptin production to further characterize its affinity to *T. inflatum* isolates derived from soil.

Plugs from agar cultures were used as inoculum for 100 mL volumes of Czapek Dox broth (Difco) supplemented with 0.5% Bacto-peptone. Cultures were grown under ambient laboratory light conditions at 22 C on a rotary shaker at 160 rpm for 11 d, then harvested by filtration. The filtrate was extracted in methylene chloride following the procedure described by Krasnoff and Gupta (1991). Extracts were dissolved in methanol (5 mg/mL), filtered through a 0.40 μm filter, and 10 μL aliquots were analyzed by reversed phase HPLC (Supelcosil® LC-8, 0.46 × 15 cm, 5 μm particle size) run in isocratic mode at 1.0 mL/min with a mobile phase consisting of acetonitrile : 12.5 mM ammonium sulfate, 70 : 30. Detection was by UV absorption at 225 nm (0.2 area units full scale). Efrapeptins in samples were identified by comparison of retention times and co-chromatography with a reference mixture of efrapeptins C, D, E, F and G purified from *T. inflatum* ARSEF 616 (Gupta et al., 1991).

HPLC analyses of culture filtrates of ARSEF 4884

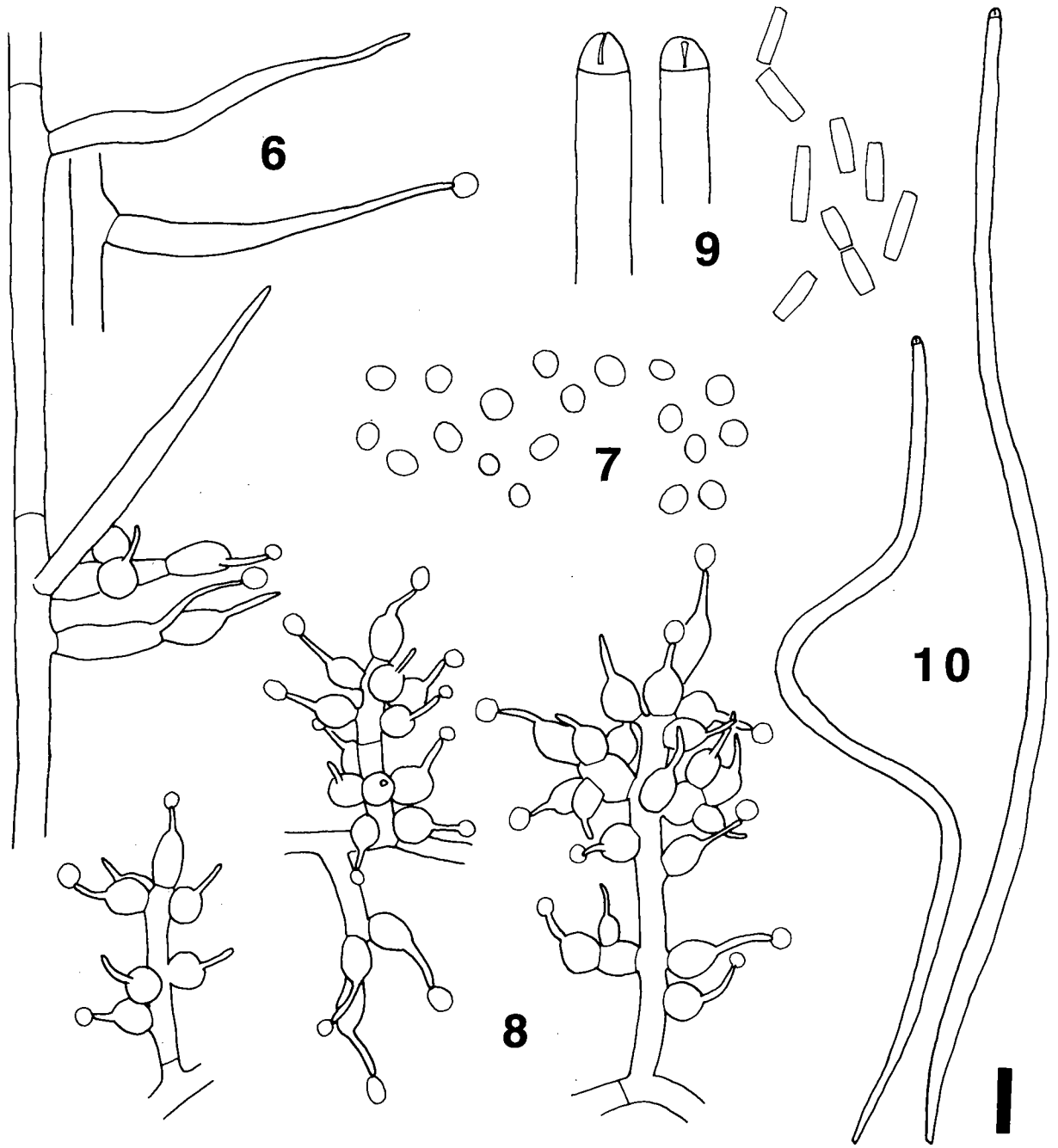

FIG. 6–10. Morphology of *C. subsessilis* (CUP 63485) and its *T. inflatum* anamorph (ARSEF 4884). 6. *Acremonium*-like phialides produced in three d-old colonies on CMA. 7. Conidia on CMA. 8. Conidiophores on CMA. 9. Ascal apices and part ascospores of *C. subsessilis*. 10. Asci of *C. subsessilis*. Bar = 10 μm.

indicated that it produced efrapeptins C, D, and E in a ratio of 15 : 65 : 20 (based on an average of 6 replicates). The profile of efrapeptins produced by ARSEF 4884 resembles most *T. inflatum* isolates previously analyzed (Krasnoff and Gupta, 1991; 1992) in the distinct predominance of efrapeptin D, with efrapeptin E representing the most abundant secondary component. There was no evidence that ARSEF 4884 produces efrapeptins F and G, which are typically produced by other *T. inflatum* isolates (Krasnoff and Gupta, 1992).

Discussion.—Tolypocladium W. Gams was first described for three soil-inhabiting species (Gams, 1971b). It presently includes a variety of species known from soil (Bissett, 1983), from rotifers (Bar-

ron, 1980, 1981), and from insect hosts (Samson and Soares, 1984; Weiser et al., 1991). No teleomorph has previously been reported for any species of *Tolypocladium*. That *Tolypocladium* species might have clavicipitalean teleomorphs has been suggested by Dreyfuss and Gams (1994) and von Arx (1986) and implied by Bissett (1983), who noted their similarity to other clavicipitalean anamorphs. Here we demonstrate that *Cordyceps subsessilis* has an anamorph attributable to *T. inflatum*, the type species of *Tolypocladium*.

Although first and best known as a soil fungus, some isolates of *T. inflatum* have been shown to display entomopathogenic properties (Weiser, 1987). The discovery of an insect-pathogenic teleomorph for *T. inflatum* suggests that this species might have ancestrally been an insect pathogen that has secondarily become successful as a facultative soil saprobe. Samson and Soares (1984) proposed that *Tolypocladium* species commonly found in soil may have a nematode alternate host; this hypothesis has yet to be tested, and *T. inflatum* has not been reported to affect nematodes.

T. inflatum isolates produce two important groups of metabolites, cyclosporins and efrapeptins. The immunosuppressive compound cyclosporin A is used extensively to prevent rejection of transplanted organs and has potential application in the treatment of various autoimmune diseases (Borel, 1986; Borel and Kis, 1991). Cyclosporins also display antifungal (Dreyfuss et al., 1976) and insecticidal (Weiser and Matha, 1988) properties. Efrapeptins, potent inhibitors of mitochondrial and other prokaryotic ATPases, also have antifungal and insecticidal activity (Krasnoff et al., 1991). The role of these powerful compounds in the ecology of *Tolypocladium* species is little understood.

Cordyceps is a large genus that includes some 280 described species (Kobayasi, 1982) which are pathogens of insects and other arthropods as well as parasites of fungi. Known anamorphs of *Cordyceps* species belong to hyphomycetous genera including *Acremonium, Akanthomyces, Beauveria, Hirsutella, Hymenostilbe, Metarhizium, Nomuraea, Paraisaria, Paecilomyces, Pseudogibellula, Sporothrix, Tilachlidium,* and *Verticillium* (Roberts and Humber, 1981; Shimazu et al., 1988; Liang et al., 1991; Gams, 1971a). Reports of anamorphs referred to *Stilbella* by Kobayasi (1941) are probably erroneous, being based on mycoparasites growing on *Cordyceps* stromata (Seifert, 1985); the same may be true of some *Sporothrix* species. The anamorphs of most *Cordyceps* species remain unknown; cultural studies in this difficult genus are badly needed.

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