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CULICINOMYCES BISPORALIS, A NEW ENTOMOPATHOGENIC HYPHOMYCETE FROM LARVAE OF THE MOSQUITO *AEDES KOCHI*

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ABSTRACT

In 1984 and 1985, a fungus was observed parasitizing larvae of *Aedes kochi* collected from leaf axils of *Colocasia* sp. in a rainforest in Queensland, Australia. In axenic culture, the fungus produces single-celled, cylindrical and cuneiform conidia which are borne in slime from subulate phialides. Based on its development of conidia of two types, its parasitism of mosquito larvae, and its underwater conidiation, the fungus is described as the new species, *Culicinomyces bisporalis*. The fungus is compared with similar fungi belonging to several genera. *Tolypocladium parasiticum* Barron, the only species of *Tolypocladium* known to produce conidia underwater, is also shown to produce conidia of two types, and is redisposed as *Culicinomyces parasiticus* Sigler, *comb. nov.* *Tolypocladium* is maintained as a genus distinct from *Beauveria*.

Key Words: *Culicinomyces, Tolypocladium, Beauveria,* mosquito pathogen, entomopathogen, Hyphomycetes.

In 1984, Goettel *et al.* reported the isolation in Canada of *Culicinomyces clavisporus* Couch, Romney & Rao from field-collected larvae of *Culiseta inornata* (Williston). Based on a detailed examination of the three available isolates of *C. clavisporus*, the only species of the genus, and a comparison with similar entomopathogenic Hyphomycetes, they concluded that the form-genus was sufficiently distinct to warrant its maintenance. *Culicinomyces* is characterized by the development of slimy single-celled conidia from predominantly subulate phialides arranged singly, in adpressed whorls or in more complex penicillate structures. In *C. clavisporus*, both mono- and polyphialides occur and produce conidia of two types. The obclavate conidia are most prominent and are produced by the fungus on the surface of parasitized mosquito larvae. The smaller oval conidia have been found only when the fungus is grown in artificial culture.

Mosquito larvae appear to be the predominant natural hosts for *Culicinomyces clavisporus*. Infections in field-collected larvae were known previously only from *Aedes rupestris* Dobrotworsky (Russell *et al.*, **1978**) and *Culiseta inornata* (Goettel *et al.*, **1984**), but recent studies have broadened the natural host range to include *Ae. rubrithorax* (Macquart), *Culiseta inconspicua* Lee, and *Aedes* sp., in addition to larvae of Ceratopogonidae (*Dasyhelea* sp.) and Chironomidae (Frances *et al.*, **1985a**; Frances, **1986**). Infections

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in other species of mosquito have either been laboratory-acquired, or introduced into a population by application of conidia during field trials (Sweeney *et al.*, **1973**; Couch *et al.*, **1974**; Russell *et al.*, **1983**; Frances *et al.*, **1985b**; Sweeney, **1985**). *Culicinomyces clavisporus* infections occur predominantly in larvae of aquatic Diptera which feed on or near bottom sediments (Frances *et al.*, **1985a**). The fungus is unusual among the entomopathogenic Hyphomycetes in being able to produce and disperse its conidia underwater.

In 1984, the second author observed a fungus on moribund *Aedes kochi* (Donitz) larvae collected from the leaf axils of a taro in northern Queensland, Australia. When isolated in agar culture, the fungus bore many similarities to *C. clavisporus* but its conidia were different in size and shape. In June, 1985, a second isolate of the fungus was obtained from field-collected larvae of *Ae. kochi* obtained from the same site.

The similarity in the morphology of the fungus to *C. clavisporus,* in addition to its pathogenicity for mosquito larvae, suggest that the new fungus could be accommodated in *Culicinomyces.* In this report we describe and illustrate the new species, *Culicinomyces bisporalis.* The biology and pathology of the new species will be discussed in a second report.

MATERIALS AND METHODS

Collection and isolation.—On June 4, 1984, 60 *Aedes kochi* larvae were collected from the leaf axils of a taro (*Colocasia macrorhiza* Schott.) in a rainforest near Millaa Millaa Falls (145°30′E, 17°30′S), northern Queensland, Australia, and returned to the laboratory for examination. Four to 7 da post-collection, 6 moribund larvae with internal hyphal growth were observed. Infected larvae were placed onto 9 cm Petri plates containing NUTRANS agar (BBL Nutrient Agar diluted 2:1 with Oxoid Lab Lemco Broth, and with 100 ppm streptomycin sulphate, 20 ppm neomycin sulphate and 500 ppm chloramphenicol) (Frances *et al.*, **1985a**). Hyphal growth on the agar was subcultured until a pure culture was obtained (Isolate MM-1).

On June 8, 1985, a total of 1314 *Ae. kochi* larvae were collected from about 100 *Colocasia* plants from the same collection site and placed into a 1 L plastic cup. Moribund larvae were examined microscopically for signs of fungal infection. One infected moribund larva was observed but attempts to obtain an axenic culture failed. Subsequently, another isolate (Isolate MM-2) of the fungus was obtained from a second infected larva which died 20 da after collection.

In 1985, the two isolates were sent for deposit to the University of Alberta Microfungus Collection and Herbarium (UAMH) and to the Plant Protection Research Unit, Ithaca, New York (ARSEF). The accession numbers of the two isolates are MM-1 = UAMH 5174 = ARSEF 1948 and MM-2 = UAMH 5175 = ARSEF 1949. Herbarium specimens consisting of dried colonies are preserved at UAMH.

At UAMH, the isolates were grown on a variety of media including potato dextrose agar (PDA, Difco), phytone yeast extract agar (PYE, BBL), and Pablum cereal agar (CER) (without antibiotics) (Padhye *et al.*, **1973**).

Scanning electron microscopy.—Infection was induced in *Ae. aegypti* (L.) larvae by transferring approximately 50 second instar laboratory-reared larvae into a tray containing 200 ml distilled water and adding growth from a 34 da old colony of the fungus on cornmeal agar (CM Oxoid). Dead larvae were observed 7 da post challenge; external sporulation occurred at 9 da. Nine da after challenge, 10 dead larvae were prepared for SEM by fixation in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.3) for 24 h. The specimens were then washed and stored in cacodylate buffer. The larvae were critical-point dried, and coated with gold in a Magnatron sputter coater. Observations were made on a JEOL JSM-35C SEM, using a back-scattered electron image. Three of 10 larvae examined by SEM showed no evidence of mycosis.

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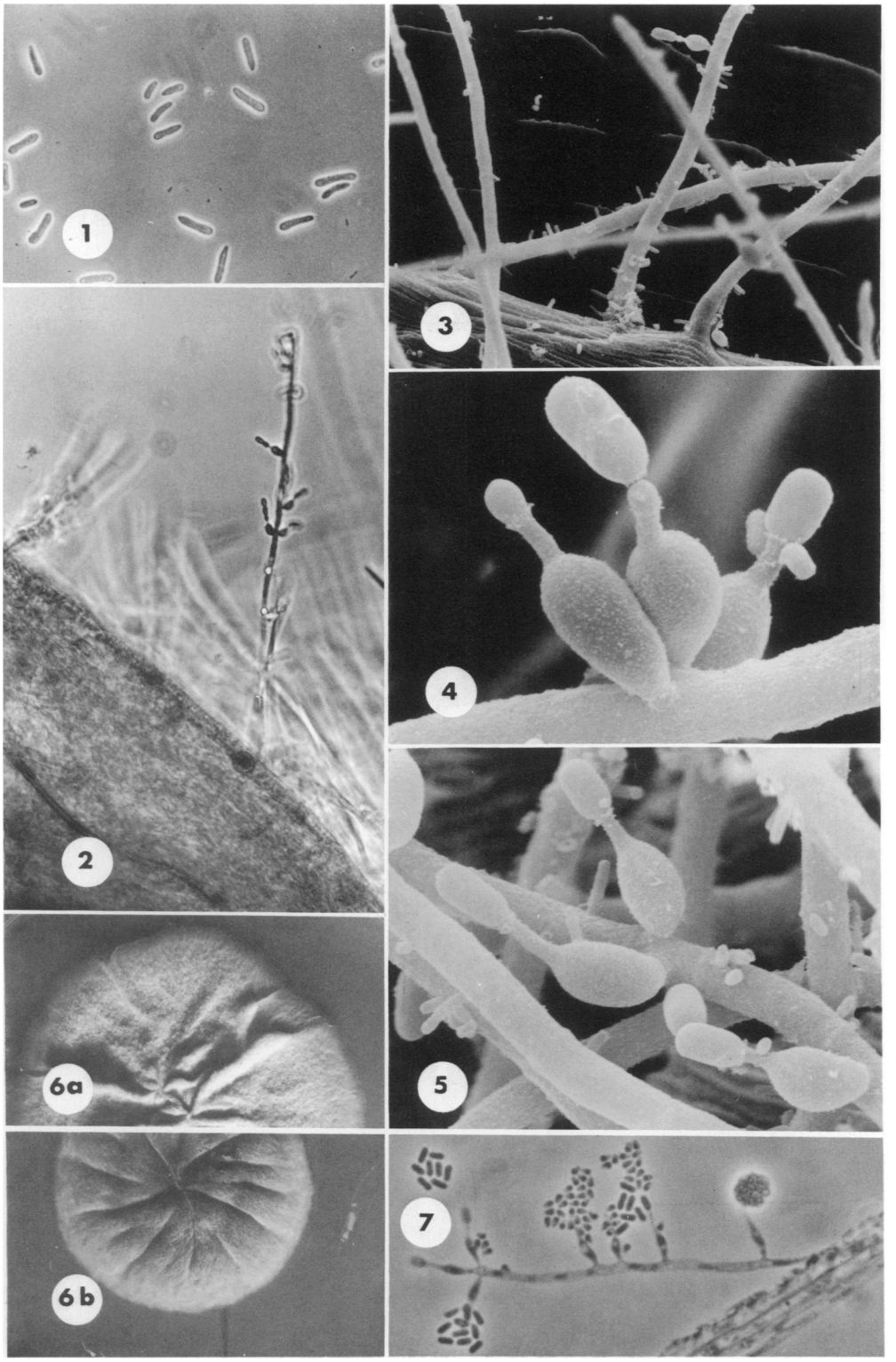
FIGS. 1–7. *Culicinomyces bisporalis.* (6, UAMH 5174; 7, 5175.) 1. Blastic conidia ("blastospores") in hemocoel of infected cadaver of *Ae. kochi* larva, ×240. 2–5. Hyphae emerging from *Ae. aegypti* larva to form conidiophores bearing flask-shaped phialides bearing cylindrical conidia. 2, ×450; 3, ×320; 4, ×7050; 5, ×4230. 6. Colonies at 25 C; a. on cellophane on PDA at 5 wk, b. on PYE without cellophane at 4 wk. Both ×1.6. 7. Flask-shaped phialides bearing cylindrical and cuneiform conidia, ×720.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TAXONOMY

Culicinomyces bisporalis Sigler, Frances & Panter, *sp. nov.* FIGS. 1–11

Corpora hyphalia in hospite 10–25 × 2.5–5 μm. Coloniae in agaro ad 25 C tarde crescunt, flavidae, vel cremeae, sparsae, planae vel undatae. Incrementum tardum ad 30 C, nullum ad 37 C.

Hyphae hyalinae, 2 μm latae. Conidiophora simplicia vel complexa; phialides solitariae, aut verticillatae vel penicillatae, magnitudine et forma variae; prope basim inflatae et subglobosae, 5–8 × 2.5–3 μm, ad collum abrupte angustatae; plerumque subulatae, 9–16(35) × 1.5–2 μm, prope collum 0.5 μm; collare minutum vel indistinctum. Conidia hyalina, 0-septata, cylindrica, 3–4 × 1.2–1.5 μm, et cuneiforma, 2–2.5 × 1.5–2 μm, in capitulis mucosis. Polyphialides et chlamydosporae absunt. Teleomorphosis ignota est.

HOLOTYPUS: Colonia exsiccata ex conidio singulari isolata in herbario UAMH (UAMH 5174A). Cultus ex larvae *Aedis kochi* (ex Queensland, Australia) a S. Frances, 1984.

Description on the host.—On field-collected *Ae. kochi* larvae, hyphae had ramified through the buccal cavity and foregut region and penetrated the external cuticle within 14 da. No external sporulation was observed but cylindrical blastic conidia ("blastospores" or hyphal bodies) measuring 10–25 × 2.5–5 μm were observed in the hemocoel of some dissected cadavers (FIG. 1).

Aedes aegypti larvae became infected 4–7 da following challenge; dead larvae sank to the bottom of the tray. Nine days after challenge, hyphae emerged through the cuticle producing a sparse layer bearing phialides singly or in whorls. Phialides were short, flask-shaped with swollen bases, measuring 4–6 × 2.3–2.5 μm, tapering abruptly at the neck to 0.5 μm (FIGS. 2–5). Conidia produced on submerged cadavers, single-celled, cylindrical, tapering slightly at the base, 2.5–3 × 1.2–1.5 μm (FIGS. 4, 5).

Description in vitro.—Colonies (FIG. 6) on PDA and PYE at 25 C similar in growth rate, slow-growing, 50–55 mm diam at 5 wk, flat with radial folds from center to margin, initially glabrous with pale yellow surface mycelium, gradually developing cream colored aerial growth, margin paler, flat, lobate or entire. On PDA aerial growth more abundant than on PYE; on PYE in older cultures darkening at center to rusty brown. Colonies on CER slower-growing, 30–35 mm diam at 5 wk, similar in color and texture, but developing few radial folds. No growth at 37 C, restricted growth at 30 C.

Hyphae septate, narrow, 2 μm diam. Conidial apparatus more complex *in vitro*. Phialides borne singly (FIG. 7), in whorls on short conidiophores (FIG. 8) or in branched penicillate structures (FIG. 11). Phialides variable in size and shape, ranging from short, flask-shaped phialides, as on the host, tapering abruptly at the neck, measuring 5–8 × 2.5–3 μm (FIG. 7); more commonly subulate, tapering gradually from base to neck, measuring 9–16(35) × 1.5–2 μm, at the neck 0.5 μm (FIGS. 9, 10). Collarette minute or indistinct. Conidia of 2 shapes, borne on separate but adjacent phialides: (1) cylindrical, measuring 3–4 × 1.2–1.5 μm; and (2) cuneiform, measuring 2–2.5 × 1.2–1.5 μm (FIGS. 9, 10). Both types of conidia hyaline, single-celled, produced in slimy masses. Polyphialides and chlamydospores not observed. Teleomorph unknown.

HABITAT: On mosquito larvae.

DISCUSSION

In 1984, Goettel *et al.* compared the genus *Culicinomyces* with *Beauveria* Vuill., *Hirsutella* Pat., *Paecilomyces* Bain., *Tolypocladium* Gams, and *Verticillium* Nees, since the size and shape, or arrangement of the conidiogenous structures were similar, and each genus included some entomopathogenic species. They concluded that the criteria for delimitation of some of the genera were not well defined and recommended maintaining the anamorph genus *Culicinomyces* pending further investigation. The addition of a second species having both a morphological similarity and an apparent biological relationship to the type species adds support to the argument

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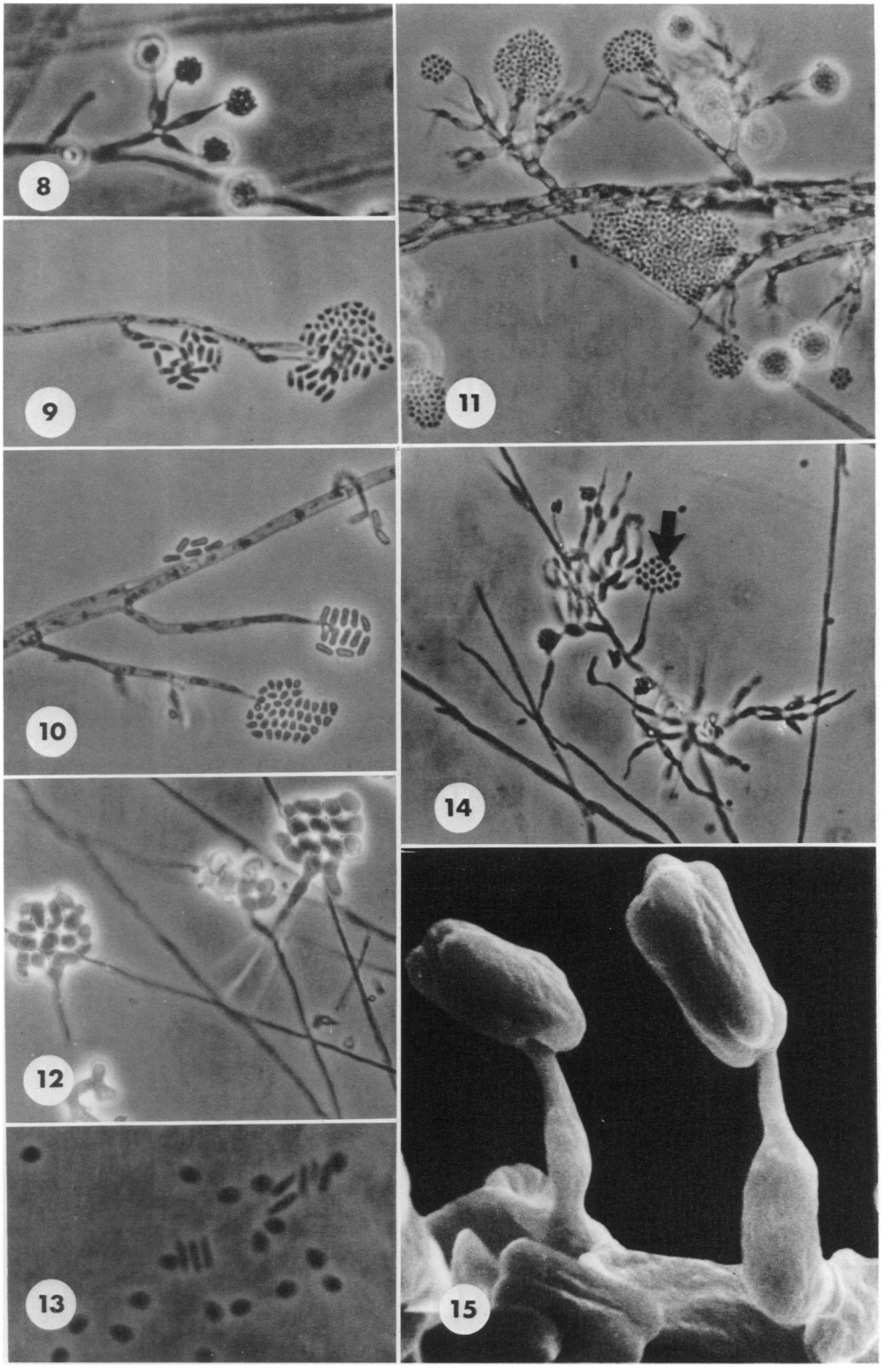
FIGS. 8–11. *Culicinomyces bisporalis*. (8, 9, 11, UAMH 5174; 10, 5175.) 8. Phialides in whorls bearing gloeoid conidia, ×720. 9, 10. Cylindrical and cuneiform conidia borne from adjacent subulate phialides, ×720. 11. Subulate phialides borne in more complex penicillate structures, ×570. FIGS. 12–14. *Culicinomyces parasiticus* (DAOM 184880). 12. Multicellular bulbil-like bodies, ×570. 13. Oval and ellipsoidal conidia, ×1790. 14. Subulate phialides bearing oval and ellipsoidal conidia (arrow), ×570. FIG. 15. *Tolypocladium cylindrosporum* (UAMH 4561). Flask-shaped phialides bearing gloeoid cylindrical conidia, ×7050. Figure 15 copyright 1987 by Mr. M. Goettel.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for maintaining the genus, although species within an anamorph genus need not be phylogenetically related.

Culicinomyces clavisporus and *C. bisporalis* have several features in common: (1) they have been found in nature parasitizing mosquito larvae, (2) they are able to produce and disperse their conidia underwater, and (3) in agar culture they produce two types of gloeoid conidia from subulate phialides, and the colonies are often glabrous with little aerial mycelium. *Culicinomyces bisporalis* can be readily distinguished from *C. clavisporus* by its conidia which are cylindrical and cuneiform rather than obovate and oval. The phialides of the two species are similar in size and shape, but polyphialides have not been seen in *C. bisporalis.*

Three other fungi similar to *Culicinomyces* species are *Paecilomyces ampullaris* Matsushima, *Tolypocladium parasiticum* Barron and *Verticillium balanoides* (Drechsler) Dowsett *et al.* In Matsushima's illustration of *P. ampullaris* (**1971**, Fig. 114.2), subulate phialides are arranged singly or in whorls on short conidiophores and appear remarkably similar to the shape and arrangement of phialides in *Culicinomyces.* A polyphialide is also illustrated, but the conidia are described as dry and occurring in fragile chains. A culture from the type obtained from Dr. Matsushima (MFC 2716 = UAMH 5300) was somewhat degenerate. We observed subulate mono- and polyphialides arranged predominantly singly, and slimy rather than dry conidia. Some conidia were 1-septate. *Paecilomyces ampullaris* has been isolated only once from soil. Preliminary laboratory challenge tests determined that *P. ampullaris* and *Tolypocladium parasiticum* (described below) were not infective to *Ae. aegypti* larvae (Goettel, pers. comm.). Although *P. ampullaris* does not appear to be well placed in *Paecilomyces,* we await additional isolations and further investigation into its biological activity before proposing its transfer to *Culicinomyces.*

Barron (**1980**) described *T. parasiticum* as an endoparasite of bdelloid rotifers in a semi-aquatic environment. The fungus was capable of causing epidemics in the rotifer population and it is the only species of *Tolypocladium* known to produce conidia under-water. *Tolypocladium cylindrosporum* Gams and other entomopathogenic Hyphomycetes of aquatic larvae produce conidia only when infected larvae float to the surface and are exposed to air (Roberts, **1975**; Samson and Soares, **1984**). Similarly, *T. trigonosporum* Barron, another parasite of bdelloid rotifers, produces conidia sparingly or not at all on the submerged host, but prolifically on dead rotifers which float to the surface of the water (Barron, **1981**).

Barron (**1980**) noted an unusual feature of *T. parasiticum* parasitizing rotifers. This was the presence of variably shaped, multicellular resting spores which enlarged by budding of individual cells. In his review of *Tolypocladium,* Bissett (**1983**) examined a culture from the type of *T. parasiticum* (DAOM 184880) and reported the presence of chlamydospores, but he could not confirm the development of multicellular spores. He also noted that the size of the phialides of the fungus grown in agar culture was considerably smaller than the size of the phialides from the host, as reported by Barron.

The senior author examined DAOM 184880 (=UAMH 5325) and observed the multicellular bulbil-like bodies (FIG. 12) in slide cultures of the fungus using CER as the agar medium. Measurements of the phialides are close to those reported by Bissett (*op. cit.*), but the phialides are somewhat variable in shape and arrangement. When solitary on the conidiophore, the phialides appeared shorter and were flask-shaped, with swollen bases, but when in aggregates, in more complexly branched conidiophores, the phialides were subulate, 7.5–18 μm long, tapering gradually to a narrow neck, 0.5 μm wide, with a minute collarette. The conidia are slimy and single-celled; on the host, globose to subglobose, 3–4.5 × 2.5–3 μm (Barron, *op. cit.*); in culture, oval with apiculate base, 2.2–2.5 × 1.5–1.8 μm (FIGS. 13, 14). From the subulate phialides occurring on the more complexly branched conidiophores, conidia of a second type were observed, which are ellipsoidal, measuring 3.5 × 0.5–1 μm (FIGS. 13, 14 arrow).

Tolypocladium parasiticum is distinct from other species of *Tolypocladium* in producing conidia of two types, in sporulating underwater and in its slow growth rate. The species is therefore redisposed in *Culicinomyces.*

Culicinomyces parasiticus (Barron) Sigler, *comb. nov.* [BASIONYM: *Tolypocladium parasiticum* Barron, *Canad. J. Bot.* **58**: 439. 1980 ≡ *Beauveria parasitica* (Barron) von Arx, *Mycotaxon* **25**: 156, 1986].

KEY TO THE SPECIES OF *CULICINOMYCES*

1. In culture, conidia of two shapes, obovate 5–7.5 × 1.5–3 μm and oval to cylindrical, 2–3 × 1–2 μm ..... *C. clavisporus*
1. Conidia of other shapes 2
   2. Conidia cuneiform 2–2.5 × 1.2–1.5 μm and cylindrical, 3–4 × 1.2–1.5 μm .. *C. bisporalis*
   2. Conidia oval with apiculate base, 2.2–2.5 × 1.5–1.8 μm and ellipsoidal, 3.5 × 0.5–1 μm ..... *C. parasiticus*

Culicinomyces bisporalis is similar, but easily distinguished from *Verticillium balanoides* (Drechsler) Dowsett *et al.* The nuciform or acorn-shaped conidia of *V. balanoides* are similar in shape, but slightly larger than the cuneiform conidia of *C. bisporalis.* Further, only one type of conidium has been observed in *V. balanoides,* and the phialides occur singly or in divergent verticils of two or three along the conidiophore (Dowsett *et al.,* **1982**), rather than in penicillate structures. Although Bissett (**1983**) transferred *V. balanoides* to *Tolypocladium,* this species differs from other species of *Tolypocladium* by the shape and divergent arrangement of the phialides.

Tolypocladium differs from *Culicinomyces* in having phialides which are short, mostly 9 μm or less in length, swollen basally and tapering abruptly to a narrow neck which is frequently bent. Characteristically, the phialides are grouped in dense clusters, similar in arrangement to the conidiogenous cells of *Beauveria* Vuill. When Gams (**1971**) described *Tolypocladium,* he selected *T. inflatum* as type species, with CBS 824.70 designated as type. Since its original publication, this industrially important fungus, known for its production of potent immunosuppressant cyclosporins, has been renamed twice. Bissett (**1983**) took up the name *Pachybasium niveum* Rostrup because the description appeared similar, even though the phialides as described by Rostrup were larger than those of *T. inflatum,* and no type material could be located. Bissett made his decision, in part, on the basis of his examination of a specimen, DAOM 63095, which had been identified by Brewer (**1958**) as *Pachybasium niveum* and which Bissett considered to be conspecific with *T. inflatum.* In the absence of extant type material of *P. niveum,* Bissett selected as neotype DAOM 167322, from alpine soil.

Recently, von Arx (**1986**) transferred *T. niveum* and most other species of *Tolypocladium* to *Beauveria* based on the general appearance of the conidiogenous structures and on his observation of sympodial or percurrent elongation of the conidiogenous axis in species of *Tolypocladium.*

In von Arx's drawing of *T. niveum* [as *Beauveria nivea* (Rostrup) von Arx], based on a "fresh isolate," the conidia are depicted as developing sympodially, leaving minute scars on the rachis. The apex of the conidiogenous structure in *T. niveum,* and in other species of *Tolypocladium sensu stricto,* is narrow, about 0.5 μm diam, and the presence of minute scars is extremely difficult to detect by light microscopy. By courtesy of M. Goettel, we present a SE micrograph of *Tolypocladium cylindrosporum* (FIG. 15) in which several conidia remain *in situ* at the apex of the conidiogenous cell. No denticles or scars can be seen. There can be no disagreement that the conidia of *Beauveria* are borne on short denticles which occur on a sympodially proliferating rachis. The argument for inclusion of the species of *Tolypocladium* in *Beauveria* does not at this time appear to be well supported by conclusive evidence.

An additional entomopathogenic species of *Tolypocladium, T. extinguens* Samson & Soares (**1984**) was not among the species transferred to *Beauveria* by von Arx. This fungus also produces conidia of different shapes from both mono- and polyphialides. Since we have not examined the fungus, and its ability to sporulate underwater is not known, we do not propose to transfer this species to *Culicinomyces* at this time.

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