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STUDIES ON THE MOSQUITO PATHOGENIC HYPHOMYCETE *CULICINOMYCES CLAVISPORUS*

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ABSTRACT

In 1982, the hyphomycete *Culicinomyces clavisporus* was isolated from larvae of *Culiseta inornata* collected from a pond in Alberta, Canada. Previously, the fungus was known only from areas around Chapel Hill, North Carolina, and Sydney, Australia. *Culiseta inornata* is a new host record for this fungus. The Canadian isolate was compared with isolates from the U.S. and Australia with regard to growth rate on solid media, colonial morphology and pigmentation, and growth in a liquid medium. The Canadian and Australian isolates were more similar to each other than to the American isolate. Observations by scanning electron microscopy and light microscopy revealed that all three isolates produce conidia of two sizes, and that the conidia are produced from phialides having more than one neck (polyphialides). In 1983, *Culicinomyces clavisporus* was isolated again from larvae of *Culiseta inornata* collected in a marsh adjacent to the first site.

Key Words: *Culicinomyces clavisporus*, mosquito pathogen, entomopathogen, Hyphomycetes.

In 1972, observations of a fungal pathogen causing high mortality in laboratory-reared mosquito larvae were made independently in Sydney, Australia, and Chapel Hill, North Carolina (Sweeney *et al.*, **1973**; Couch *et al.*, **1974**). The American isolate was named *Culicinomyces clavisporus* by Couch, Romney and Rao (**1974**) (as "*clavosporus*," see discussion under nomenclature). Sweeney and his co-workers in Australia considered their isolate to be congeneric (Sweeney, **1975**; Sweeney and Panter, **1977**), but only recently confirmed its identity as *C. clavisporus* (Sweeney *et al.*, **1982**). In this study, they compared the original isolate from Sydney with one from Chapel Hill and concluded that they were conspecific. Although they noted some differences between the strains, they were unable to evaluate these differences until more isolates could be studied.

Sweeney and his associates studied *Culicinomyces clavisporus* as a potential biocontrol agent for mosquitoes. Its host range, mode of pathogenesis, efficacy in field trials, and potential pathogenicity to other insects and animals have been well documented (Sweeney, **1981a**, **b**; **1983**). Its geographic range and occurrence in nature are less well known.

In 1982, we isolated *Culicinomyces clavisporus* from field-collected larvae of *Culiseta inornata* (Williston) in Alberta, Canada. Prior to this, the fungus was known only from two widely separated geographic regions. Infections in field-collected mosquitoes have been known previously only in *Aedes rupestris* larvae (Russell *et al.*, **1978**; Frances, pers. comm.). *Culiseta inornata* is a new host record for this fungus.

The discovery of *Culicinomyces clavisporus* from Canada coincided with an announcement (Anon., **1982b**) that an American and an Australian isolate were available from the American Type Culture Collection (ATCC). This provided an

opportunity for a comparative study of isolates from the three geographic regions. From this comparison, we concluded that the three strains are conspecific and that the form-genus *Culicinomyces* should be maintained for now.

MATERIALS AND METHODS

Isolation of the Canadian strain.—Between June 15 and October 6, 1982, approximately 12 ground pools and ponds in the Devon, Alberta, area were monitored for mosquito pathogens. Records of mosquito density, water pH, temperature, and conductivity were kept for each body of water. Larvae collected weekly by dipping were placed in pans in the laboratory at 20 C and observed daily. Dead larvae were removed and examined microscopically for invasion by fungal hyphae. Infected larvae were placed in 200 ml Bates' medium S (McLintock, **1952**) containing 20 laboratory-reared larvae of *Culiseta inornata.* Laboratory-reared larvae which became infected were bathed for 5 min in 0.12% sodium hypochlorite (2% household bleach), and then 5 min in 50 μg/ml chloromycetin before being placed on agar plates. Hyphal growth on the agar was subcultured until pure cultures were obtained, but it was difficult to obtain a culture free from bacterial contaminants.

A culture and dried specimens are maintained at the University of Alberta Mold Herbarium as UAMH 4618. Subcultures were deposited at the ATCC (52635), the Commonwealth Mycological Institute (280342), the Centraalbureau voor Schimmelcultures (755.83) and the USDA-ARS Insect Pathology Research Unit, Boyce Thompson Institute, Ithaca, N.Y. (RS 964).

Other isolates.—American and Australian isolates were obtained from the ATCC as 38490 (=UAMH 4658) and 46258 (=UAMH 4659), respectively. Four additional isolates from North Carolina were received from the USDA-ARS Insect Pathology Research Unit. Their numbers are UAMH 4848 (RS 372) mosquito, 4849 (RS 582) *Anopheles quadrimaculatus,* 4850 (RS 584) *Anopheles quadrimaculatus,* all three from Chapel Hill, and 4851 (RS 706) mosquito, from North Carolina. One additional isolation from Alberta was made from *Culiseta inornata* collected in July, 1983, and numbered UAMH 4854. All of the last five isolates were obtained too late to be included in the comparative growth studies, but they were examined microscopically.

Growth studies.—Media consisted of Pablum mixed cereal agar without chloromycetin (PCA), potato dextrose agar (PDA), Sabouraud dextrose agar (SDA) containing 50 μg/ml chloromycetin, oatmeal-salts agar (OAT) (all recipes in Padhye *et al.,* **1973**), cornmeal agar with yeast and dextrose (CM^+, Sweeney *et al.,* **1982**), and nutrient agar (NA) and broth (NB, Difco). Small fragments of growth (approximately 1 mm^3) from one-week-old cultures on OAT were transferred to Petri plates containing each of the media listed above. Each strain was inoculated to three replicate plates and the colony diam were measured after 21 da. The cultures were incubated at 25 C and exposed to fluorescent ceiling lights on an irregular basis (usually 8–10 h/da, 5 da/wk).

One liter flasks containing 500 ml of NB were inoculated with conidia from each of the three strains. The cultures were incubated at 20 C and agitated at approximately 170 oscillations/min on a Burrell wrist action shaker. Observations on sporulation and development of pellicle were made daily for 28 da.

Scanning electron microscopy.—Techniques used for preparation of specimens were adapted from Brown and Brotzman (**1979**). Small blocks of agar with mycelial growth were removed from 47 da cultures on OAT and placed onto glass micro-

scope slides. Each specimen was vapor-fixed for 2 da at room temperature in a sealed dish containing 4 ml of 2% osmium tetroxide in distilled water, then quick-frozen in liquid nitrogen and lyophilized overnight. Infected larvae were prepared by two methods: (1) vapor fixation as described above but air-dried for 6 da rather than lyophilized, and (2) fixation in 1% osmium tetroxide, 2% Kodak Photoflo in distilled water 2 da at 4 C, dehydration through a graded ethanol series to 95% ethanol followed by air drying for 4 da.

All dehydrated specimens were affixed to SEM stubs using conductive silver paint and sputter-coated with gold in a Nanoteck-Semprep 2. Observations were made on a Cambridge S 250 SEM operated between 10–20 kV.

RESULTS AND DISCUSSION

Nomenclature.—Originally, the epithet given to this species was "*clavosporus.*" Recently, the orthographic variant "*clavisporus*" has been adopted by some authors (Onions, **1979**; von Arx, **1981**; Hall, **1982**; Sweeney *et al.*, **1983**). In 1982, Hall claimed that the correct spelling is "*clavisporus.*" However, roots of both Latin and Greek origin are combined, and there is some disagreement about the proper vowel to use in such cases. Classical mycologists used "*clavisporus*" for this combination (for example, see Saccardo, **1886**, p. 631); for this reason, we have adopted and recommend the usage of "*clavisporus*" rather than "*clavosporus.*"

History of the ATCC isolates.—Couch *et al.* (**1974**) deposited the type specimen of *C. clavisporus* at the University of North Carolina Herbarium, but no mention was made of deposition of a living culture. Strain data for ATCC 38490 (Anon., **1982a**) are J. N. Couch, mosquito, *Anopheles quadrimaculatus.* This strain was not deposited at ATCC until 1979 (Jong, pers. comm.). During the period from 1973 to 1979, several isolations of *C. clavisporus* have been made from infected larvae at Chapel Hill (Humber, pers. comm.; Panter, pers. comm.). ATCC 38490 is presumably one of Couch's early isolates, but precise information on the source of some of the Chapel Hill isolates is lacking. It appears that the isolate on which the original description was based is no longer available (Humber, pers. comm.).

Strain data for ATCC 46258 (Anon., **1983**) are R. C. Russell S1 #235, mosquito, *Anopheles amictis hilli.* This strain was deposited at ATCC January 18, 1982 (Jong, pers. comm.). According to Panter (pers. comm.), this is the original Sydney isolate, and it is the strain used by Sweeney *et al.* (**1982**) in their comparative study of the Australian and American strains. In their publication, no strain numbers were given for either strain, but the American strain was one of Couch's isolates (Panter, pers. comm.).

Habitat and distribution.—*Culicinomyces clavisporus* was first observed causing infection in laboratory-reared mosquito larvae in two widely separated localities, Sydney, Australia, and Chapel Hill, N.C. Presumably, the fungus was introduced with the water used to rear the larvae. In the U.S. (Couch *et al.*, **1974**) the water came from University Lake, a man-made reservoir of several acres near Chapel Hill (Sweeney, **1983**). In Australia, the water originated from McCarr's creek, a small perennial stream near Sydney (Russell *et al.*, **1978**; Sweeney, **1983**).

Russell *et al.* (**1978**) provided the first report of an infection in field-collected mosquitoes: larvae of *Aedes rupestris* Dobrotworsky, breeding in small rock pools in the partially dried bed of McCarr's creek. Further isolations have been made from *Aedes rupestris* occurring in rock pools of varying sizes at Nattai River near Mittagong (100 km south of Sydney) (Frances, pers. comm.).

Debenham and Russell (**1977**) demonstrated that an infection of *Culicinomyces*

clavisporus originating in larvae can be carried into the adult stage. Infection of the adult mosquito may be important in dispersal of the fungus.

In Alberta, *Culicinomyces clavisporus* was found on larvae of *Culiseta inornata* occurring in a permanent pond north of Devon (Winterburn area). The pond measures approximately 25 × 50 m; it has a muddy bottom with a grassy periphery and was covered with duck weed (*Lemna* sp.) by mid-summer. Infection in *Culiseta inornata* was detected only in larvae collected on August 12 and 19 1982. Properties of the water on the two collecting dates were: August 12—15 C, 8.1 pH, 410 μmhos/cm conductivity at 22 C; August 19—17 C, 7.8 pH, 412 μmhos/cm conductivity at 22 C.

The two samples consisted of 30 dips taken from the pond periphery in the grassy area. Each dip yielded approximately 6–7 larvae/350 ml. On August 12, 215 larvae (40 first, 44 second, 70 third, and 61 fourth instar) and 5 pupae were collected. Of 55 larvae identified, 64% were *Culiseta minnesotae* Barr, 32% were *Culex territans* Walker and 4% were *Culiseta inornata.* Of 170 larvae (12 first, 102 second, 24 third, and 32 fourth instar) and 11 pupae collected on August 19, 71 were identified; 63% were *Culiseta inornata* and the remainder consisted of approximately equal numbers of *Culex territans* and *Culiseta minnesotae.*

From the collection of August 19, we observed a larva of *Culiseta inornata* in which hyphae had penetrated the external cuticle to form conidiophores bearing terminal and lateral flask-shaped phialides (FIGS. 1, 2). Obovate conidia were produced from the tip of the phialide. The fungus was tentatively identified as *Culicinomyces clavisporus* and later confirmed by comparison with the two isolates obtained from ATCC.

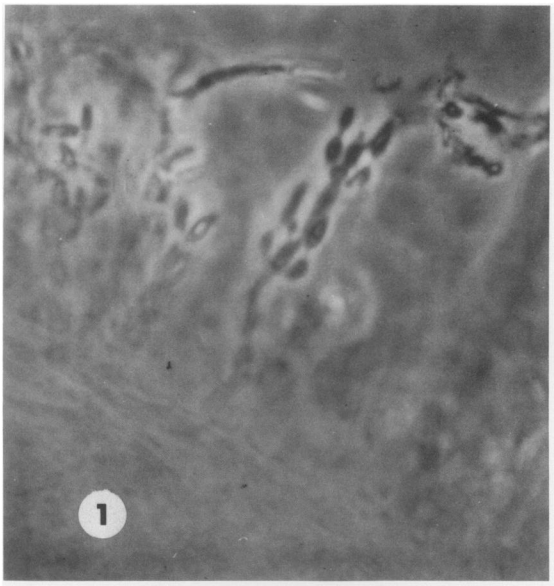
Only larvae of *Culiseta inornata* were infected with *Culicinomyces clavisporus,* 4 from the August 12 sample and 10 from the August 19 sample. However, more larvae may have been infected than diagnosed since many dead larvae disintegrated or were devoured by other larvae. Conversely, infection in some of the field-collected larvae may have occurred following exposure to other infected larvae in the laboratory, since the infected larvae did not die until 8–9 da after being collected. Consequently, the ratio of infected to collected larvae may not reflect the infection rate in the field.

Infection by *Culicinomyces clavisporus* occurred when the population of *Culiseta minnesotae* and *Culex territans* in the pond was being replaced by *Culiseta inornata. Culiseta inornata* was present in the pond until October 6, 1982. A second instance of infection in *Culiseta inornata* in Alberta was found in August, 1983, in a marsh near the pond.

The recovery of *Culicinomyces clavisporus* from a permanent pond and a marsh broadens the range of its known aquatic habitats (rock pools, streams, ponds and lakes), and its geographic distribution (USA, Australia, Canada). *Culiseta inornata* is a new record for a mosquito host infected in nature; only *Aedes rupestris* has been previously reported.

Growth studies.—The appearance of colonies of *Culicinomyces clavisporus* growing on solid agar media has been described previously (on NA and CM^+ by Sweeney *et al.,* **1982**; on malt agar and PDA by Onions, **1979**). We compared growth rates of the three isolates on six media (TABLE I, FIG. 3). The U.S. strain grew slightly faster except on SDA (FIG. 3b). It also grew well on CM^+ (FIG. 3c), the medium on which it has been routinely maintained since its isolation (Sweeney *et al.,* **1982**). In contrast, the Australian strain grew slowest on NA (FIG. 3d), the medium used to maintain the stock culture in Sydney (Sweeney *et al.,* **1982**).

The Canadian and Australian strains had similar growth rates but colonies of the latter were frequently glabrous and tough, sometimes splitting the agar after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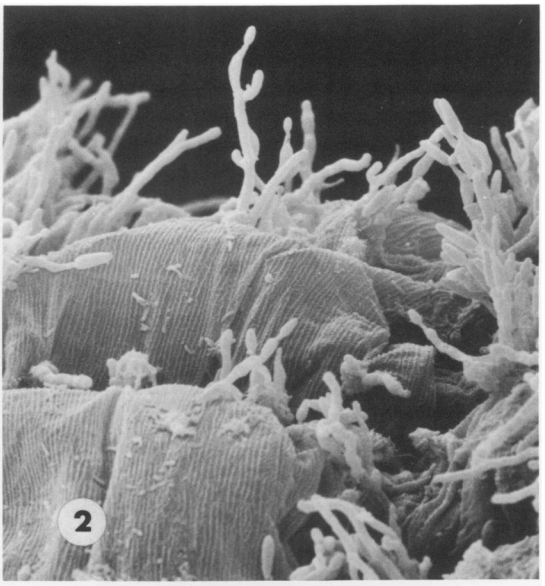
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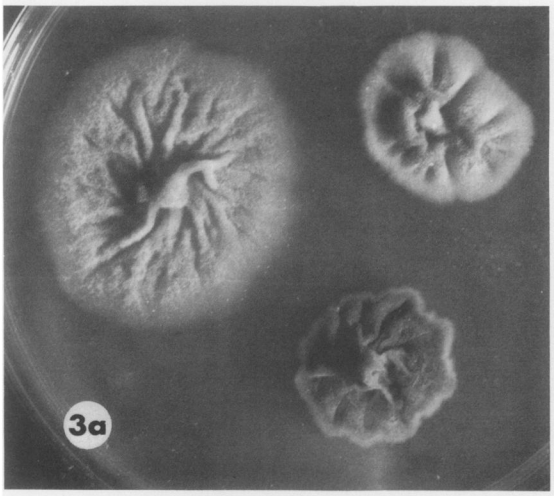
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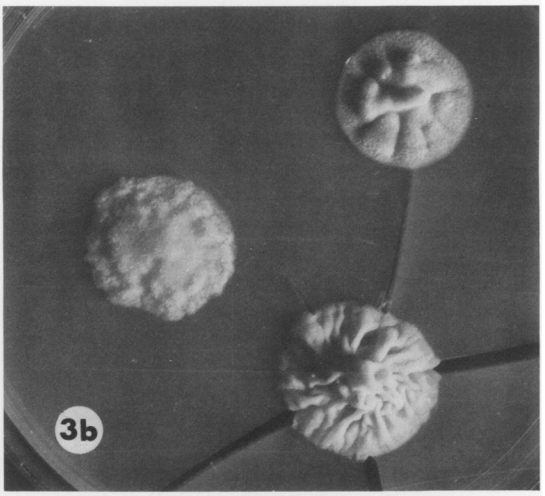
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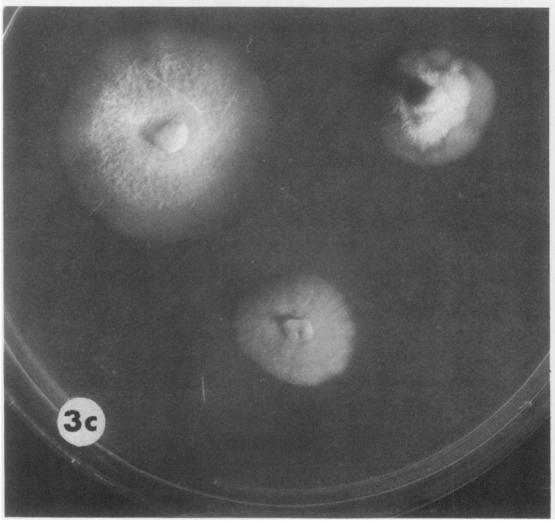
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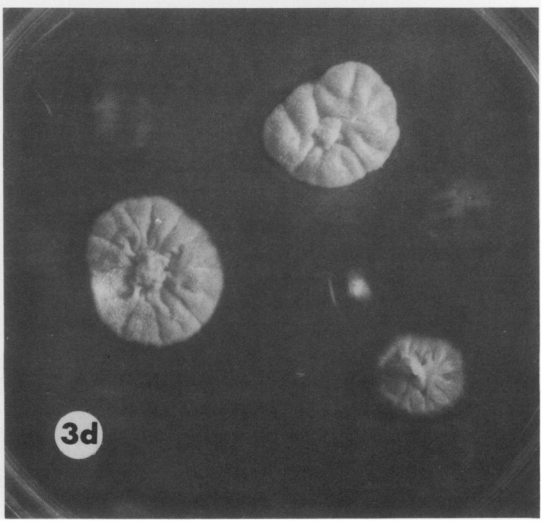
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TABLE I

MEAN COLONY DIAMETERS (IN CM) OF THREE *Culicinomyces clavisporus* ISOLATES GROWN FOR 21 DA AT 25 C ON SIX SELECTED MEDIA

Strain	Media					
	PDA	PCA	CM+	OAT	NA	SDA
American (ATCC 38490)	3.8	3.0	2.8	3.1	1.8	1.8
Australian (ATCC 46258)	1.9	1.6	1.6	1.9	1.0	2.1
Canadian (UAMH 4618)	2.3	1.7	1.6	1.9	1.5	1.8

several weeks' growth (FIG. 3b). Colonies of the first Canadian isolate developed abundant aerial mycelium, but colonies of the new isolate, UAMH 4854, are more glabrous and aerial mycelium develops slowly. On some media (PDA, PCA), colonies of both the Australian and Canadian strains developed a darkly pigmented surface mycelium (FIG. 3a).

Culicinomyces clavisporus is unique among the entomopathogenic Hyphomycetes in its ability to produce conidia on larvae which are submerged (Roberts and Humber, **1981**; Roberts and Sweeney, **1982**). Other fungi produce conidia only when infected larvae float to the surface and the mycelium is exposed to air.

In liquid shaker culture (NB), the American strain produced more abundant hyphae. The mycelial growth of the American and Australian isolates appeared darkly pigmented by macroscopic observation whereas the mycelium of the Canadian strain was not pigmented. The Australian strain sporulated readily, the Canadian strain moderately, and the American strain sparsely under these conditions. A similar observation was reported by Roberts and Sweeney (**1982**) for the American and Australian isolates.

Conidium development.—Conidiogenesis in *Culicinomyces clavisporus* is characterized by the formation of terminal and lateral phialides which are borne either singly (FIG. 4), or in complex whorls at irregular intervals along the conidiophore (FIGS. 5, 6, 8), or in penicillate structures which may be simple (FIG. 7) to complexly branched. Phialides are flask-shaped, 8–15 × 2–3 μm, tapering at the neck to a diameter of 0.5–1 μm (FIGS. 5, 6) sometimes with a minute collarette. Occasionally, lateral phialides are more swollen at the base, tapering abruptly at the neck; these shorter phialides measure 8 μm or less (FIG. 10). The conidia are obovate, 5–7.5 × 1.5–3 μm, and they accumulate in slimy masses (FIG. 9).

We also noted two aspects of conidiogenesis which have not been previously illustrated. The first is the formation of conidia of a second type which are oval to cylindrical, unicellular, 2–3 × 1–2 μm. These smaller conidia occur on phialides adjacent to ones producing the larger obovate conidia (FIGS. 13, 14). They also accumulate in slimy masses.

In addition to simple phialides we observed polyphialides (FIGS. 4, 11, 12)

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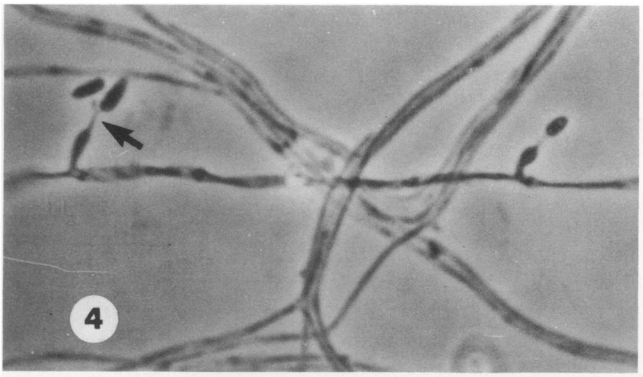
FIGS. 1–3. *Culicinomyces clavisporus.* (1, 2—UAMH 4618). 1, 2. Hyphae emerging through cuticle of *Culiseta inornata* larva to form conidiophores bearing terminal and lateral, flask-shaped phialides bearing obovate conidia; 1, ×770, 2, ×800. 3. Colonies of three strains grown on different media at 25 C for 21 da; a. on PDA, b. on SDA, c. on CM+, d. on NA. American strain (UAMH 4658) at left; Canadian strain (UAMH 4618) at upper right; Australian strain (UAMH 4659) at lower right. All ×1.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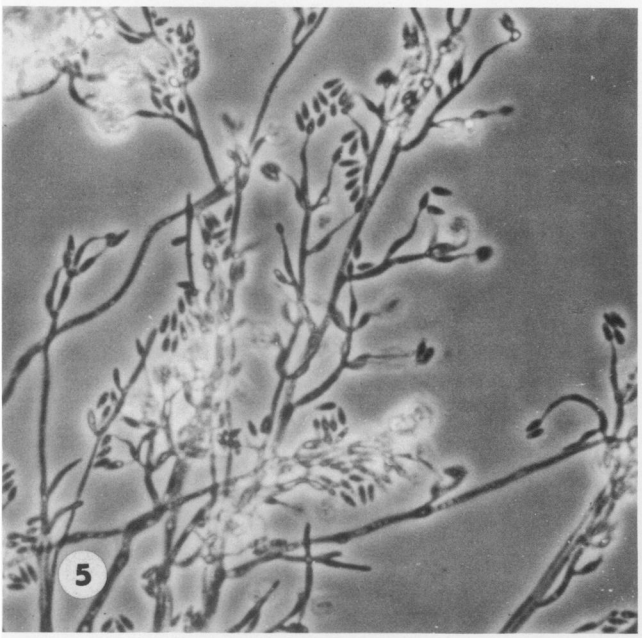
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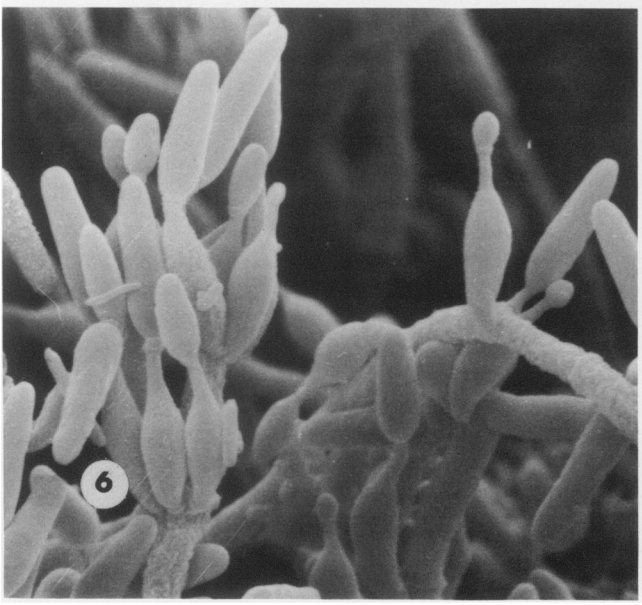
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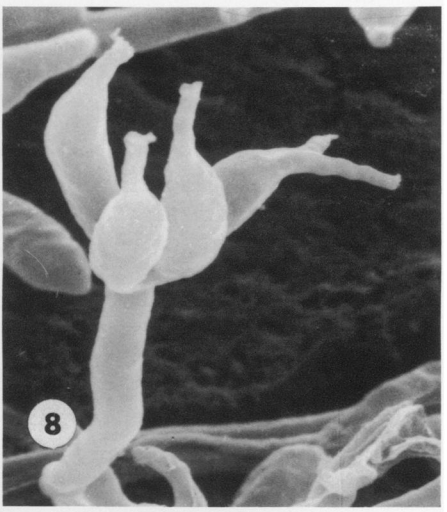
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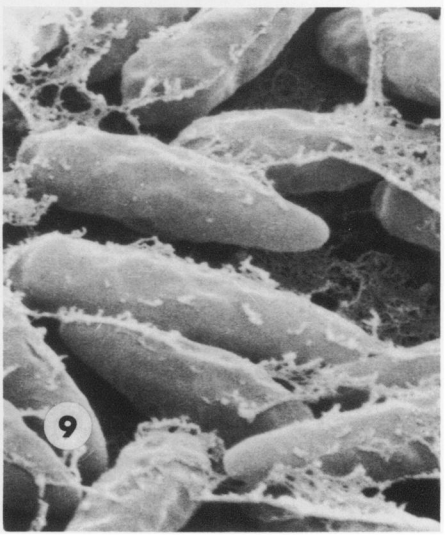
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with two, or rarely more than two, openings. Inman and Bland (**1983**) also observed polyphialidic development.

Strain variation.—In **1982**, Sweeney *et al.* compared the growth and sporulation of an American and an Australian isolate in agar culture and on larvae, summarized the differences, and concluded that they were conspecific.

Our study has confirmed the variability among three isolates of *C. clavisporus.* The strains vary in their colonial morphology and growth rates (TABLE I, FIG. 3) and in their conidium development. Conidial dimorphism occurred in all three strains but the small conidia were more abundant in the American and Australian strains. Roberts and Sweeney (**1982**) reported globose rather than obovate conidia in some batches of conidia of the Australian strain grown in 750 liter fermenters. They stated that the round conidia were less virulent to mosquito larvae. It may be that the small "conidia" are spermatia. We saw the small conidia in agar slide culture using PCA as the medium, but not in submerged broth culture. Polyphialides occurred more commonly in the American strain. The four additional isolates from North Carolina also developed conidia of both types, and polyphialides.

Neither we nor Sweeney *et al.* (**1982**) have found the penicillate, complexly branched structures which were produced by the original American isolate growing on *Aedes atropalpus epactius* Dyar (Couch *et al.*, **1974**: fig. 18). Sweeney *et al.* (**1982**) observed only sparse development of phialides which occurred singly or in whorls of 2 or 3.

Taxonomy of Culicinomyces.—*Culicinomyces* is a monospecific genus having taxonomic affinities to several other hyphomycete genera. The endoparasitic nematode-destroying fungus *Meria coniospora* Drechsler (**1941**) produces conidia similar in size (4–7 × 1.8–2.5 μm) and shape, but its conidia sometimes terminate in small bud-like protrusions (FIG. 16) which are adhesive structures. The conidia of *M. coniospora* are produced successively from small pegs (FIGS. 15, 16, arrows) which develop laterally near the apex of each conidiogenous cell. These pegs appear to elongate during formation of successive conidia.

The size, shape, and arrangement of conidiogenous structures in *Beauveria* Vuill., *Tolypocladium* Gams, *Verticillium* Nees and *Paecilomyces* Bain. are similar to *Culicinomyces.* Each of these genera also contains entomopathogenic species. *Beauveria* differs in forming a sympodially proliferating conidiogenous axis. From each node on the geniculate rachis, only a single conidium is produced. In *Tolypocladium,* the conidiogenous structures are phialides which are borne singly or in false whorls on narrow, loosely branched conidiophores (Bissett, **1983**). Frequently, the phialides are grouped in dense clusters. The phialides are short and swollen basally, narrowing abruptly to a filiform neck which is frequently bent. In his comprehensive review, Bissett (**1983**) has enlarged *Tolypocladium* by the addition of several species. Two species formerly treated in *Verticillium* including *V. balanoides* (Drechl.) Dowsett *et al.* (**1982**) and *V. microsporum* Jaap are described as having subulate phialides arranged in verticils. By this treatment, the distinction between *Tolypocladium* and *Verticillium* becomes less clear, and these

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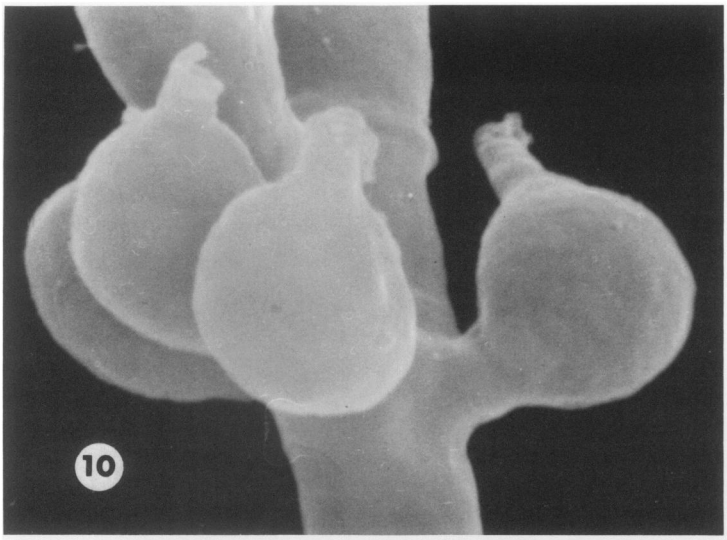
FIGS. 4–9. *Culicinomyces clavisporus.* (4—UAMH 4658; 5–8—4618; 9—4659). 4. Simple phialide and polyphialide (arrow) borne singly, ×770. 5, 6. Phialides borne in whorls at irregular intervals along the conidiophore; 5, ×460, 6, ×2900. 7. Phialides arranged in penicillate structures bearing obovate conidia in slimy masses, ×770. 8. Phialides borne in single whorl, ×3000. 9. Obovate conidia covered in slime, ×6700.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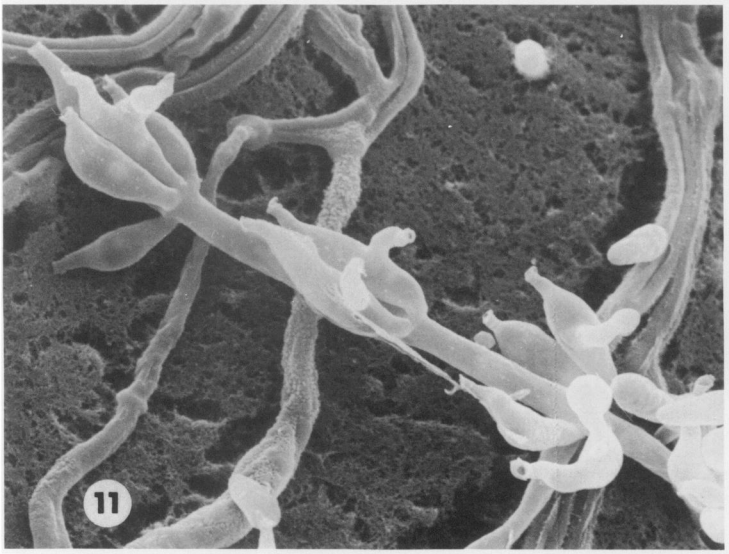
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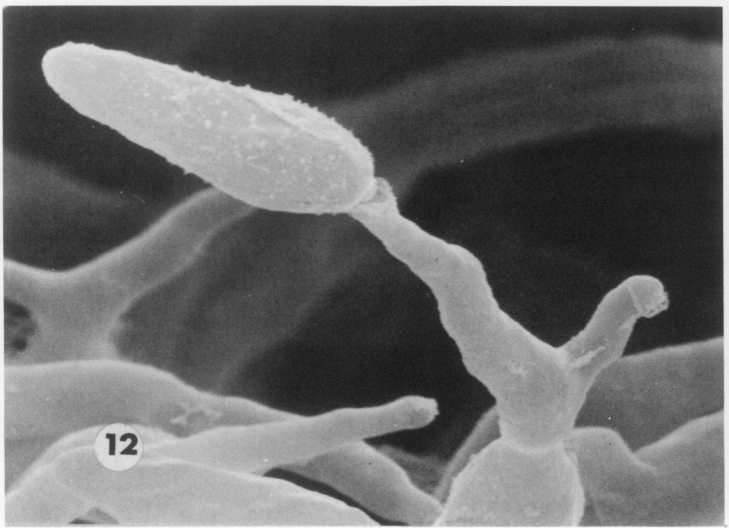
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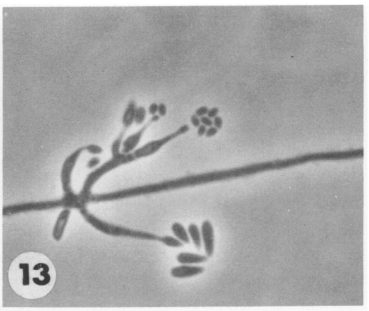
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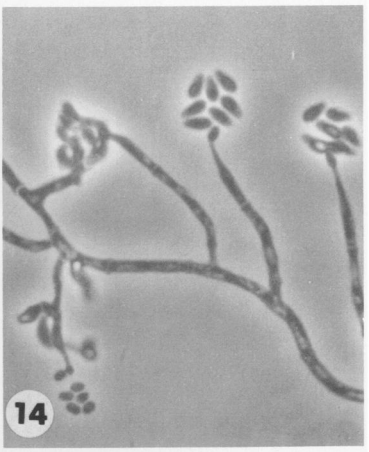
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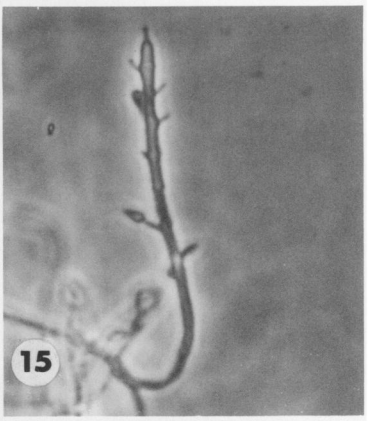
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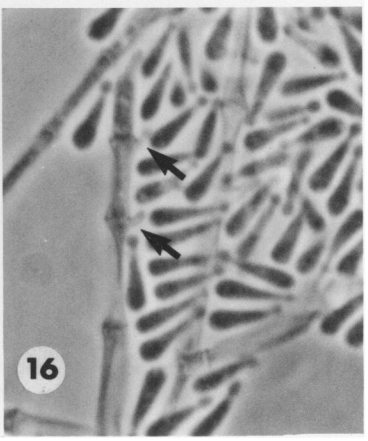
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species are probably better left in *Verticillium*. Bissett separates the genera on the basis of the broader conidiophore main axis in *Verticillium*.

The concept of *Verticillium* was broadened by Gams (**1971**) in his Section *Prostrata* to include many species which display erect conidiophores, but a more recent treatment (Domsch *et al.*, **1980**) indicates that the genus is not well defined. We consider *Verticillium* to be characterized by aculeate phialides without collarettes. The phialides are divergently arranged in verticils and borne at intervals along the length of undifferentiated or well-developed, erect, sometimes demataiaceous conidiophores. Conidia are borne in slime. Von Arx (**1981**) treated *Culicinomyces* under *Verticillium*, but in *Culicinomyces* the phialides are adpressed in whorls rather than divergently arranged.

Paecilomyces is similar to *Culicinomyces* in the arrangement and shape of phialides, but in *Paecilomyces* the conidia are formed in true chains (Gams, **1978**; Subramanian, **1979**; Minter *et al.*, **1983**). *Culicinomyces* is further differentiated from both *Paecilomyces* and *Verticillium* by its polyphialides.

Hirsutella Pat. species occur on arthropods and many have teleomorphs in the Clavicipitaceae. Polyphialides, which occur occasionally in *Culicinomyces*, are more common in *Hirsutella*. Both mono- and polyphialidic conidiogenous cells are borne laterally or terminally in synnemata, but mononematous forms are also included (Minter and Brady, **1980**; Samson *et al.*, **1980**; Evans and Samson, **1982a, b**). The mononematous forms of *Hirsutella* can be differentiated from *Culicinomyces* by the phialides which are usually borne singly, arising from undifferentiated hyphae more or less at right angles. None of the species develop the adpressed whorls of phialides characteristic of *Culicinomyces*. The phialides are swollen basally and taper gradually or abruptly at the neck which may be twisted in a helical rotation. Furthermore, conidia are borne singly or in small groups, frequently in a characteristic mucous sheath which may be pigmented. In the majority of mononematous species, the conidia are ellipsoidal or in the shape of an orange segment (*fide* Minter and Brady, **1980**). Further investigation may show that *Culicinomyces clavisporus* could be accommodated in *Hirsutella*; we are reluctant to propose a transfer at this time.

Culicinomyces clavisporus differs from all other entomopathogenic species of Hyphomycetes in its aquatic rather than terrestrial habitat. It has been found in nature only as a pathogen of mosquito larvae and certain other aquatic Diptera. It is unique in its ability to produce and disperse conidia under water.

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FIGS. 10–14. *Culicinomyces clavisporus*. (10—UAMH 4658; 11, 12, 14—4618; 13—4659). 10. Basally swollen, short phialides borne in whorl, ×7000. 11. Simple phialides and polyphialides borne in whorls at intervals on conidiophore, ×2100. 12. A polyphialide with two openings, ×6200. 13, 14. Small, oval to cylindrical and larger obovate conidia borne in slimy masses from adjacent phialides, ×770. Figs. 15, 16. *Meria coniospora*. (UAMH 4730). 15, 16. Obovate conidia produced from small pegs (FIG. 16, arrows) borne laterally near apex of each conidiogenous cell. Conidia are equipped with bud-like protrusions which are adhesive structures; 15, ×770, 16, ×1500.

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