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## Harpellales in Newfoundland aquatic insect larvae

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**Abstract:** Among nine Harpellales (Trichomycetes) reported from aquatic insect larvae collected in Newfoundland, Canada, four new species are described: *Harpellomyces abruptus* [living in *Thaumalea verralli* (Diptera: Thaumaleidae)], *Orphella avalonensis* [in *Leuctra ferruginea* (Plecoptera: Leuctridae)], *Stachylina litoralis* [in *Telmatogeton japonicus* (Diptera: Chironomidae)], and an unnamed species of *Smittium* (in Chironomidae) from which an axenic culture was obtained. Other Harpellales were from Simuliidae (*Harpella melusinae*, *Pennella simulii*, *Simuliomyces microsporus*), Chironomidae (*Stachylina robusta*), and an unnamed new genus in caddis fly larvae (Trichoptera). Low vagility of Plecoptera and the occurrence of different species of *Orphella* in the USA and Europe suggest that the genus existed before the North Atlantic formed a barrier as the continents separated. It is hypothesized that vicariant speciation may have occurred in *Orphella* as well as in *Harpellomyces* from Thaumaleidae larvae.

**Key Words:** biogeography, Chironomidae, gut fungi, Leuctridae, Simuliidae, symbiosis, Thaumaleidae, Trichomycetes, Trichoptera

### INTRODUCTION

Newfoundland, Canada, like many areas of the world, is a rich source of aquatic insects infested with symbiotic Harpellales (Trichomycetes), commonly called gut fungi. It is now known that species of some harpellid genera in larval blackflies (Simuliidae) may occasionally grow from the gut into the developing ovaries, resulting in adult blackflies that are sterile but whose ovaries are filled with fungal cysts which the female “oviposits.” This serves as a mechanism for

disseminating the obligate fungal symbionts to new stream sites (Yeboah et al 1984, Moss and Descals 1986, Labeyrie et al 1996). In the process of studying this unusual adaptation, we discovered and report here several new species of Harpellales as well as new hosts and distributions of previously known species.

### MATERIALS AND METHODS

The dipteran insect hosts of Harpellales that we collected included freshwater larvae of Simuliidae, Chironomidae (nonbiting midges), Thaumaleidae (solitary midges), and nymphs of the Plecoptera (stonefly) genus *Leuctra*. Most of our specimens were collected from 18–29 May 1999, and subsequently from 13 April to 30 August 2000, in a number of stream sites in the general vicinity of St. John's on the Avalon Peninsula of Newfoundland (TABLE I). Larvae were taken from streams and other habitats with the aid of nets and strainers, or they were picked directly from substrates such as leaves, sticks, and rocks. Specimens were placed in jars or plastic bags and kept cold until dissected in the laboratory. Living fungi from the dissected gut were studied and photographed using phase-contrast optics. Cultures were attempted by placing exposed fungi in plates of dilute brain-heart infusion agar or a tryptone-glucose-salts agar medium, both containing a shallow distilled water overlayer with added penicillin-streptomycin antibiotic solution (Lichtwardt 1986). Selected fungal specimens were also preserved in CTAB buffer (Gottlieb and Lichtwardt 2001) for subsequent DNA extraction and sequencing.

### DESCRIPTION OF NEW TAXA

**Harpellomyces abruptus** Lichtw., White et Colbo, sp. nov. FIGS. 1–8

Thalli 6–7  $\mu\text{m}$  diametro, plerumque ad basem ramosi, series longas trichosporarum ellipsoidalium, 20–33  $\times$  7–11  $\mu\text{m}$ , sub emissione 2–5 appendiculis ornatas producentes. Zygosporae ignotae. In proctodaeo larvarum Thaumaleidarum (Dipterorum) affixi.

Thalli 6–7  $\mu\text{m}$  diam, often branched at the base, producing long series of ellipsoidal trichospores 20–33  $\times$  7–11  $\mu\text{m}$  that bear 2–5 appendages upon release. Zygosporae unknown. In hindgut of Thaumaleidae (Diptera) larvae.

**Etymology.** *L. abruptus* = steep, for the precipitous cliff where the hosts were collected.

**Specimens examined.** CANADA. NEWFOUNDLAND: Seepage on cliff bordering Southside Rd., St. John's, Newfoundland, 47° 33.23' N, 52° 42.40' W. Microscope slide NF-

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TABLE I. Collection sites on the Avalon Peninsula, Newfoundland, with Harpellales

Site	Stream	Date	Latitude/Longitude
NF-1	Beachy Cove Brook at Hughs Pond	18-V-99	47°37.76' N, 52°50.82' W
NF-2	Beachy Cove Brook at Witch Hazel Rd.	18-V-99	47°35.82' N, 52°50.84' W
NF-3	Goat Cove Brook at Tuckers Hill Rd.	18-V-99	47°36.08' N, 52°52.53' W
NF-4	Little Powers Pond Outlet	21-V-99	47°33.99' N, 52°51.95' W
NF-5	Millers Brook under Millers Rd. near Indian Meal Line	21-V-99	47°37.33' N, 52°49.87' W
NF-6	Clemens Pond Outlet on Bauline Line	21-V-99	47°37.84' N, 52°49.48' W
NF-7	Beachy Cove Brook at Witch Hazel Rd.	21-V-99	47°35.82' N, 52°50.84' W
NF-10	Beachy Cove Brook at Witch Hazel Rd.	22-V-99	47°35.82' N, 52°50.84' W
NF12	Middle Cove cliff at splash zone	23-V-99	47°39.11' N, 52°41.83' W
NF-12a	Middle cove cliff, drip area away from splash zone	23-V-99	47°39.1' N, 52°41.8' W
NF-13	Voiseys Brook at Voiseys Park Recreational Area	24-V-99	47°39.11' N, 52°41.83' W
NF-14	Millers Brook	24-V-99	47°37.33' N, 52°49.87' W
NF-19	Broad Cove River at outlet of Healeys Pond	27-V-99	47°34.41' N, 52°51.01' W
NF-21	Beachy Cove Brook at Hughs Pond	27-V-99	47°37.76' N, 52°50.82' W
NF-22	Seeping cliff at Southside Rd., St. John's	28-V-99	47°33.23' N, 52°42.40' W
NF-23	Seeping cliff at Southside Rd., St. John's	29-V-99	47°33.23' N, 52°42.40' W
NF-26	Goat Cove Brook	29-V-99	47°36.08' N, 52°52.53' W
NF-30	Beachy Cove Brook at Witch Hazel Rd.	13-IV-00	47°35.82' N, 52°50.84' W
NF-31	Beachy Cove Brook at Hughs Pond	17-IV-00	47°37.76' N, 52°50.82' W
NF-32	Forest Field, Salmonier	1-V-00	47°10' N, 53°27' W
NF-33	Beachy Cove Brook at Witch Hazel Rd.	17-VII-00	47°35.82' N, 52°50.84' W
NF-34	Beachy Cove Brook at Witch Hazel Rd.	30-VIII-00	47°35.82' N, 52°50.84' W

23-5 (HOLOTYPE: FH) prepared from a *Thaumalea veralli* Edwards larva (Chironomidae, Thaumaleidae). Other specimens collected from Sites NF-22 and NF-23 (TABLE I) are from same host as the holotype.

The type species, *Harpellomyces eccentricus* Lichtw. & Moss, was described from *Thaumalea* spp. larvae collected in northern Sweden and Wales (Lichtwardt and Moss 1984). It differs from *H. abruptus* primarily by (1) the lack of branching, and (2) the unusual narrowness of parts of the thalli, in some cases measuring only a few micrometers in diameter, with enlargement at the apex where trichospores form. Mature thalli of *H. abruptus* are more uniform in diameter, with narrowing found primarily in the basal branches. Trichospores of *H. eccentricus* measure 20–25 × 6–8 μm and bear three broad appendages upon release from the generative cell. Trichospores of *H. abruptus* have 2–5 appendages and are 20–33 × 7–11 μm; thus they are only slightly larger than those of the European species. Zygospores of *H. eccentricus* (which were not seen in *H. abruptus*) have been found in only one larva from Sweden. Two features of *H. abruptus*—the basal branching of thalli and the more variable number of trichospore appendages—prompt us to emend the description of the genus.

**Harpellomyces** Lichtw. et Moss emend. Lichtw., White et Colbo

Thalli unbranched or branched at the base, producing distally a series of collarless trichospores with 2–5 appendages, or conjugating and producing zy-

gospores attached obliquely and submedially to the zygosporophore. Attached to hindgut cuticle of Thaumaleidae larvae (Insecta, Diptera).

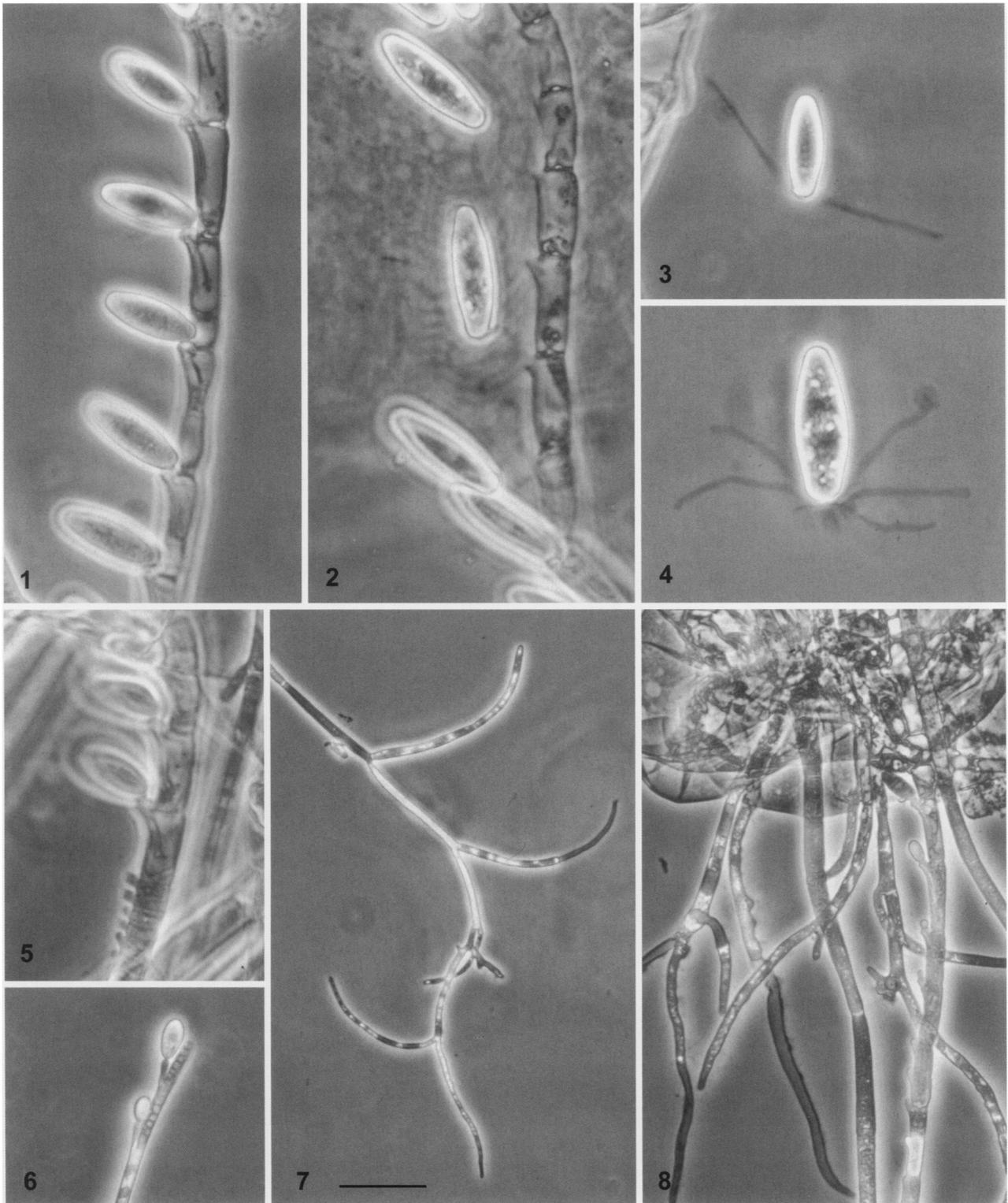
*Harpellomyces* has also been found in Pennsylvania, USA (unpubl), and in Japan (Lichtwardt et al 1987). Comments on those species will be found in the Discussion section.

**Orphella avalonensis** White, Lichtw. et Colbo, sp. nov.

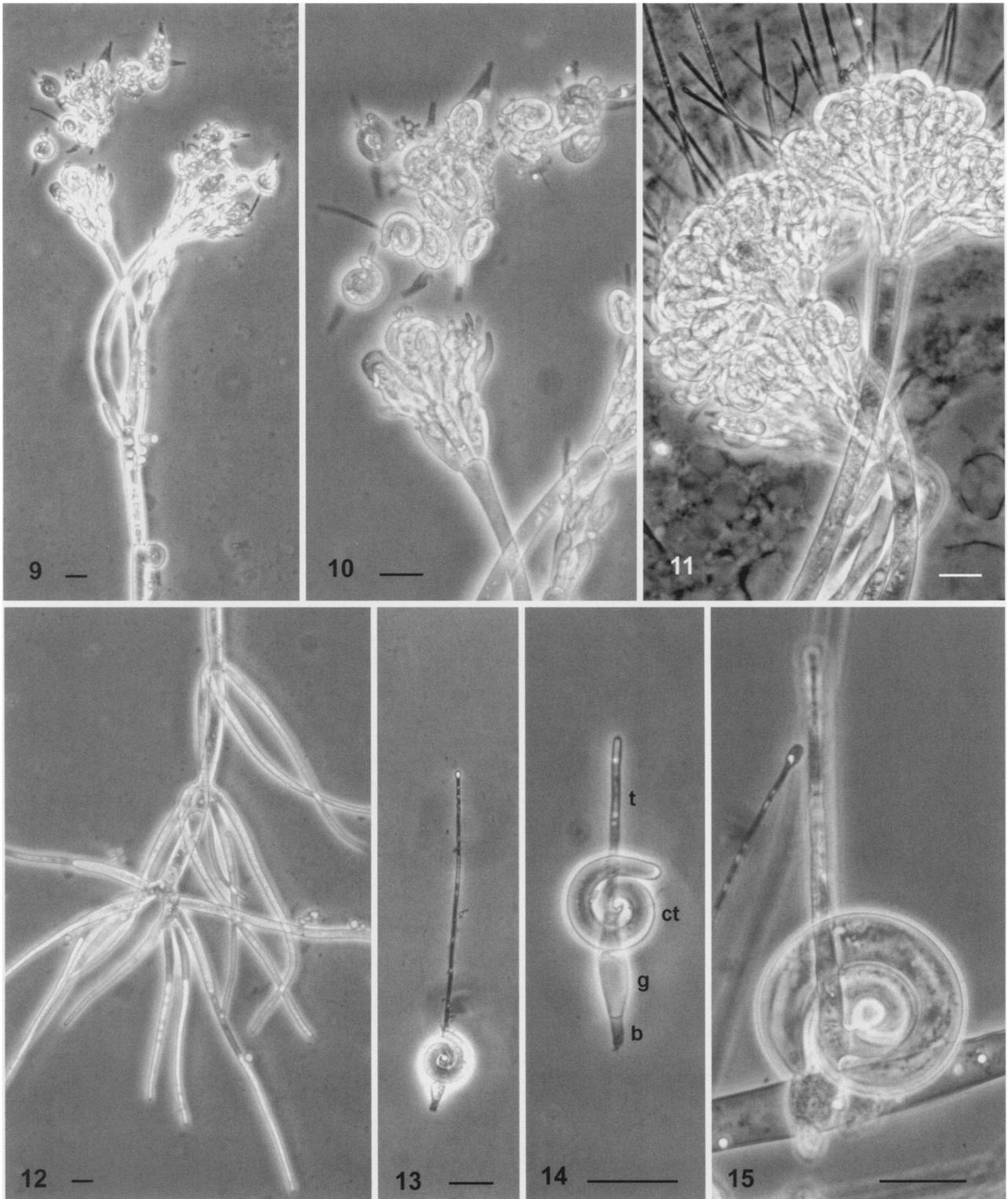
FIGS. 9–15

Thalli usque ad 900 μm longi, in axe principali ad basem ramos multos curtos steriles producentes atque ad apicam aliquot ramos in fasciculos cellularum basaliū producen-tes, quaque cellula basali aliquot cellulis genitalibus atque terminalibus circa 30–95 μm praedita. Trichosporae circa 6 μm diametro, cochleatae, e cellulis genitalibus exorientes 20–22 × ~6 μm. Rarius corpora disseminationis maiora, cellulis genitalibus 28 × 11 μm, cellulis terminalibus 80 μm longi, trichosporis 11 μm diametro, cochleatis. Thalli in proctodaeo nympharum Leuctridarum (Plecopterorum) affixi; pars fertilis ex ano procurrens.

Thalli up to 900 μm long with a main axis bearing multiple short sterile branches at the base and apically producing several branches that terminate in clusters of basal cells, each producing several generative cells with terminal cells about 30–95 μm long. Trichospores about 6 μm wide, coiled, developing from generative cells 20–22 × ~6 μm. More rarely dissemination units much larger, with generative cells 28 × 11 μm, terminal cells 80 μm long, and trichospores 11 μm wide. Attached to hindgut of Leuctri-



FIGS. 1–8. *Harpellomyces abruptus* from a thaumaleid larva. 1, 2. Mature trichospores. 3, 4. Detached trichospores with 2 and 5 basal appendages, respectively. 5, 6. Early stages in development of trichospores from generative cells. 7, 8. Branches produced at bases of thalli. Scale bar = 20  $\mu$ m.



FIGS. 9–15. *Orphella avalonensis* from a stonefly nymph. 9–11. Sporulating heads of thalli and some detached dissemination units with coiled trichospores. 12. Branches at base of main thallic axis. 13, 14. Dissemination units consisting of a generative cell (g) subtended by a small basal cell (b), an extended terminal cell (t), and a coiled trichospore (ct). 15. A rare large dissemination unit. Scale bars = 20 μm.

dae (Plecoptera) nymphs, with fertile part projecting from the anus.

*Etymology.* From the Avalon Peninsula.

*Specimens examined.* CANADA. NEWFOUNDLAND: Beachy Cove Brook at Hughs Pond, 18-V-99, 47° 37.76' N, 52° 50.82' W. Microscope slide NF-3-W7 (HOLOTYPE: FH) prepared from a *Leuctra ferruginea* (Walker) nymph (Plecoptera, Leuctridae). Other specimens collected from Sites 2 and 3 (TABLE I) and from same host as the holotype.

Of the four other species of *Orphella* described, only *O. helicospora* Santam. & Girbal (Santamaria and Girbal 1998) produces tightly coiled trichospores. *Orphella haysii* Lichtw. & M.C. Williams normally produces curved trichospores that are occasionally partly coiled. *Orphella avalonensis* differs from *O. helicospora* in several respects: the base of the thallus is not bifurcate (FIG. 12), the diameter of the trichospore and terminal cell is greater, and the terminal cells are cylindrical rather than clavate as in *O. helicospora*.

The unusually large dissemination units that we found (FIG. 15) may be anomalous. Two detached anomalous dissemination units along with many other units of normal size were found with a thallus kept in a water mount for 3 d. One of us (MMW) has seen similar dissemination units in an *Orphella* from Nova Scotia, Canada.

***Stachylina litoralis* Lichtw., White, and Colbo, sp. nov.**

FIGS. 16–18

Thalli 135–200 × 10–12 μm, in membrana peritrophica per basem subbulbosa affixi, trichosporas 4–8 per thallum producentes, 39–47 × 10–12 μm, colare carentes. In membrana peritrophica larvarum Chironomidarum affixi.

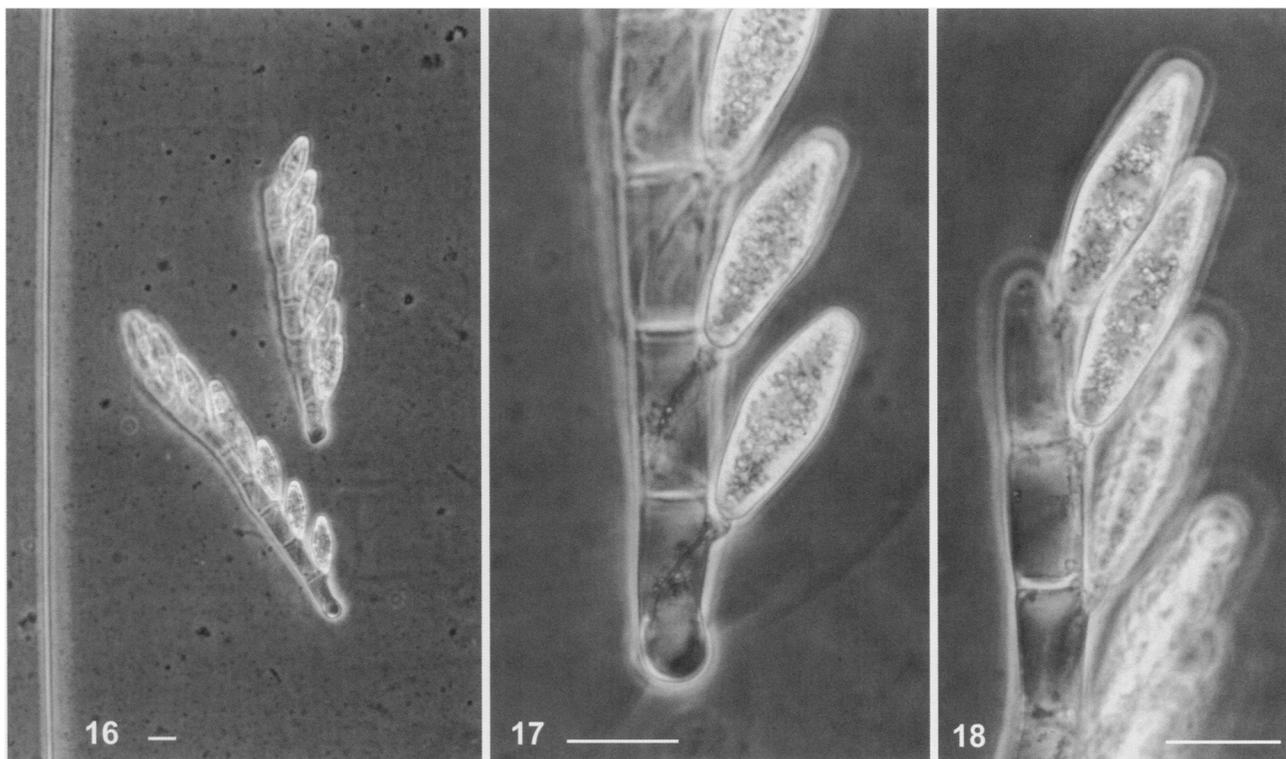
Thalli 135–200 × 10–12 μm, attached to the peritrophic membrane by a slightly bulbous base; 4–8 trichospores produced per thallus, 39–47 × 10–12 μm, without a collar. Attached to peritrophic membrane of Chironomidae larvae.

*Etymology.* *L. litoralis* = of the shore.

*Specimens examined.* CANADA. NEWFOUNDLAND: Middle Cove cliff at high tide splash zone, N of St. John's, Newfoundland, 23-V-99, Site NF-12, 47° 39.11' N, 52° 41.83' W. Microscope slide NF-12-1 (HOLOTYPE: FH) prepared from a *Telmatogeton japonicus* Tokunaga larva (Diptera, Chironomidae).

Of the 30 currently described species of *Stachylina*, *S. litoralis* is most similar to *S. macrospora* Léger & Gauthier. The most obvious distinction is that trichospores of *S. macrospora* are narrower (7–8 μm) than those of *S. litoralis*. Another larva of *T. japonicus* from the same population had a hindgut that was infested with a young, branched harpellid thallus, but with no trichospores to allow for identification.

The host, *T. japonicus*, is one of relatively few Chi-



FIGS. 16–18. *Stachylina litoralis* from a marine chironomid larva. Sporulating thalli within the peritrophic membrane. Scale bars = 20 μm.

ronomidae from marine habitats. Though located in the splash zone of the seashore, the larvae were bathed in fresh water flowing slowly down the cliff, suggesting that while the host larvae tolerate sea water, the gut fungus is not truly marine. Another population of *T. japonicus* larvae, collected on rocks that are submerged at high tide at Maddox Cove south of St. John's, were not infested with any fungus.

#### **Smittium sp.**

FIGS. 19–21

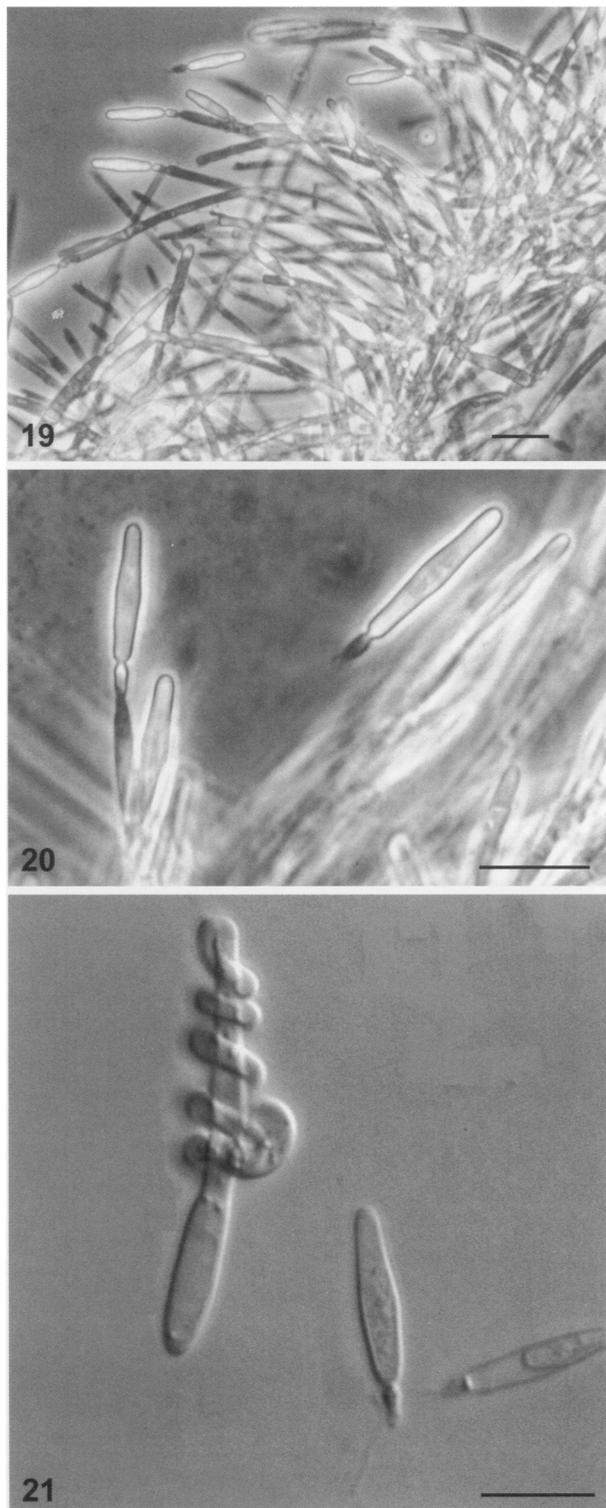
In the hindgut of four Chironomidae larvae from Goat Cove Brook (Site NF-26) we found a species of *Smittium* that is probably new, but we are not naming it at this time because of insufficient specimens to fully describe it and clearly distinguish it from the other 55 species of the genus. The ellipsoidal trichospores [(14–)19–32 × 3–3.5 μm] with a median bulge and a collar 3–5 μm long do not fit descriptions of other *Smittium* spp. In the midgut of most of the chironomid larvae we also found thalli of *Stachylina robusta* Lichtw. & M.C. Williams (see below).

An axenic culture (NF-26-3) of this *Smittium* sp. produces abundant trichospores, a small number of which extrude their sporangiospores from the sporangium wall (FIG. 21). An unusual feature is that sometimes the extruded sporangiospore produces a germ tube that twists and at times wraps around itself (FIG. 21). There is no evidence that these extruded spores successfully establish new colonies in culture.

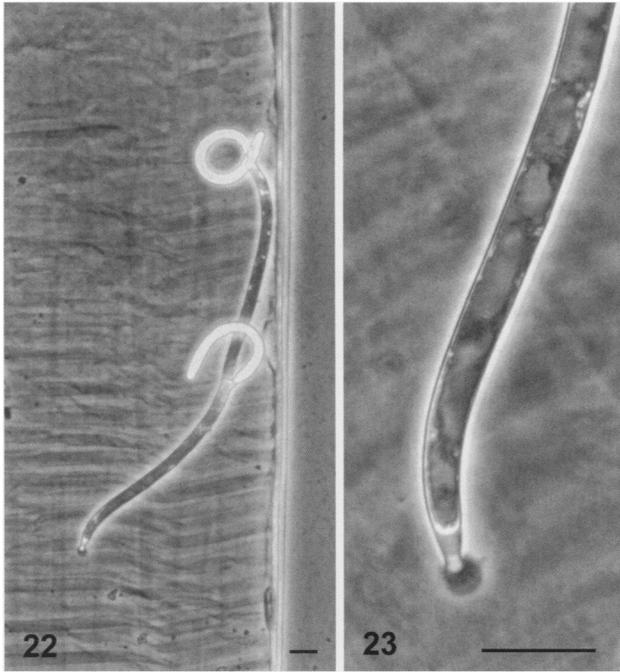
#### HOSTS AND DISTRIBUTION OF PREVIOUSLY KNOWN SPECIES

#### *Harpella melusinae* Léger & Duboscq FIGS. 22, 23

*Harpella melusinae* grows attached to the larval peritrophic membrane of a wide range of blackfly species. It is one of the most widespread species of Harpellales, though it has not yet been reported from Neotropical regions nor from southern South America where two other species of *Harpella*, namely *H. tica* and *H. meridionalis*, are common (Lichtwardt and Arenas 1996, Lichtwardt 1997, Lichtwardt et al 1999, 2000, White et al 2000). Its unbranched thalli produce two to eight or more curved to coiled trichospores (FIG. 22), and it is most easily distinguished from the other three species of *Harpella* by its tapered holdfast clearly seen in FIG. 23. We found *H. melusinae* at Sites NF-3, 4, 5, 13, 19, 30, 31, 32, 33, 34, where hosts included *Prosimulium mixtum* Syme & Davies, *Simulium venustum/verecundum* complex, *Stegopterna mutata* (Malloch), *Simulium vittatum* complex, and possibly other simuliid species whose identity we did not record. Frost and Manier (1971) reported finding *H. melusinae* in more than four spe-



FIGS. 19–21. *Smittium* sp. from the hindgut of chironomid larvae. 19. Part of a densely branched sporulating thallus. 20. Trichospores. 21. Axenic culture NF-26-3 showing unusual development in culture; in middle an unextruded trichospore, on right a sporangiospore extruding from its sporangium, and on left a germinated sporangiospore whose germ tube has wrapped around itself. Scale bars = 20 μm.



FIGS. 22, 23. *Harpella melusinae* within a simuliid peritrophic membrane. 22. A thallus producing two trichospores. 23. Typical tapered holdfast attached to the peritrophic membrane. Scale bars = 20  $\mu\text{m}$ .

cies of simuliid larvae from many stream sites in Newfoundland.

*Pennella simulii* M.C. Williams & Lichtw. FIGS. 24–30

This common inhabitant of blackfly hindguts in Newfoundland was found at Sites NF-1, 3, 4, 5, 6, 10, 13, 14, 19, 30, and 31. Hosts included *Prosimulium mixtum* and *Stegopterna mutata* among other species not identified. *Pennella simulii* was originally reported from the Rocky Mountains of Colorado (Williams and Lichtwardt 1971) and is illustrated here because there are few adequate photomicrographs of the species. The trichospores (FIGS. 24, 25) and zygospores (FIGS. 26–28) of *Pennella* are very similar to the relatively common and more widespread species *Genistellospora homothallica*, also a blackfly gut inhabitant, with the primary distinction being the nature of the holdfast. *Genistellospora* spp. have a well-defined, solid holdfast whereas *Pennella* spp. have simple to bifurcate basal cells attached to the hindgut cuticle by means of a mucilaginous secretion from the basal cells (FIGS. 29, 30).

Frost and Manier (1971) reported finding *P. hovassi* Manier in Newfoundland blackflies, a species previously known only in France. We cannot discount the presence of *P. hovassi* in Newfoundland, but the identification appears to have been made by Manier from preserved material, which may have distorted some of the measurements.

Our Newfoundland material fits the description of *P. simulii*, allowing for a slightly broader range of spore measurements than the original description. Trichospores were 28–38  $\times$  7–10  $\mu\text{m}$ , zygospores 74–89  $\times$  16–19  $\mu\text{m}$ , and zygosporephores 26–45  $\times$  13–19  $\mu\text{m}$ .

*Stachylina robusta* Lichtw. & M.C. Williams

FIGS. 31–33

This species was recently described (Lichtwardt and Williams 1999) from *Psectrocladius* sp. (Chironomidae) in the Rocky Mountains of Colorado. Our Newfoundland collection came from an unidentified chironomid larva from Site NF-3. Trichospores measured 30–40  $\times$  6–8  $\mu\text{m}$ , and the robust thallus tapered to a narrow holdfast (FIG. 32) as in the Colorado specimens.

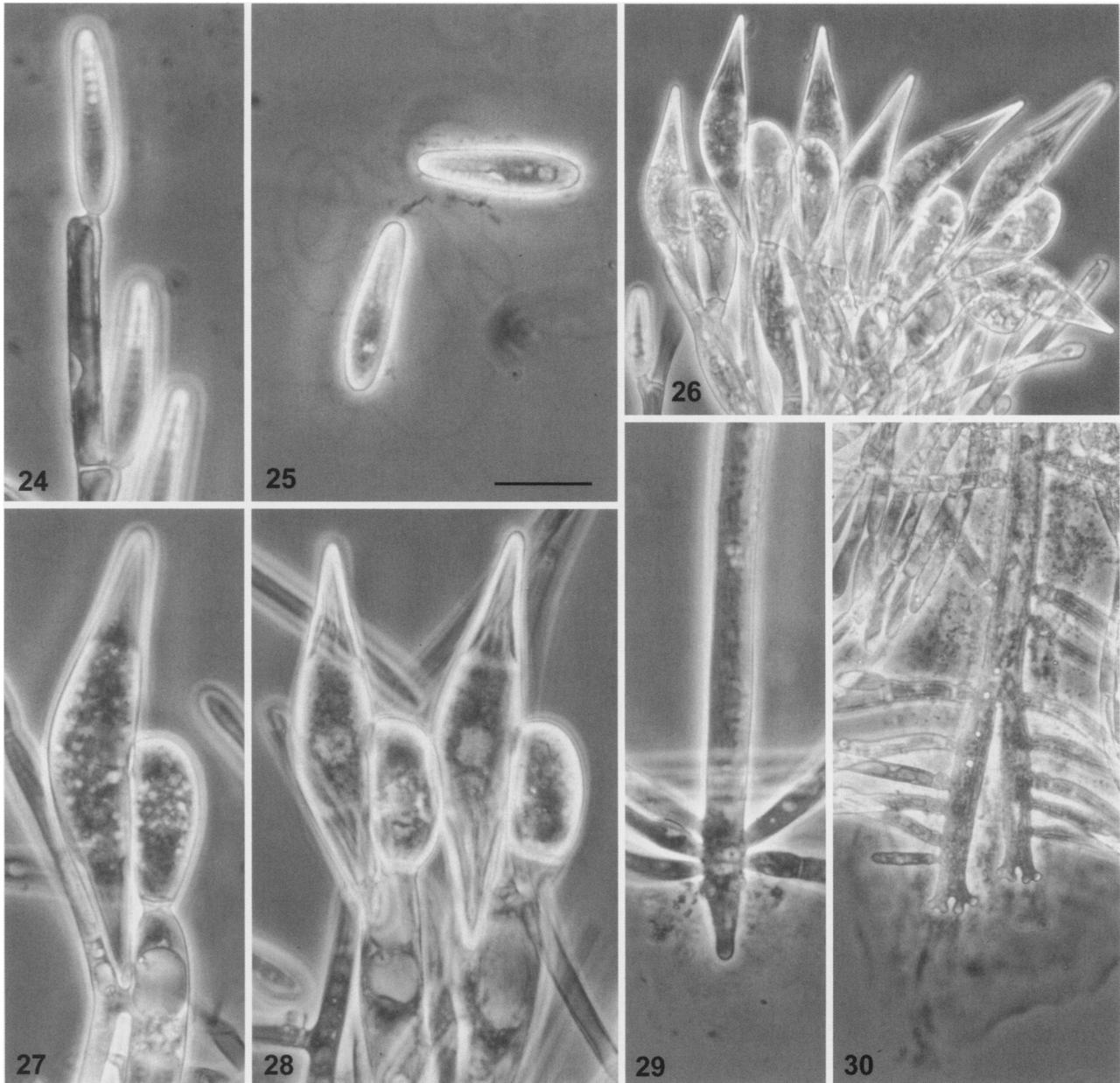
#### OTHER COLLECTIONS

Among the identifiable trichomycetes not reported above, we include the following record: *Simuliomyces microsporus* Lichtw. in a *Prosimulium mixtum* larva from Site NF-13. Various unidentifiable species of *Smittium* were found in chironomids and simuliids (*Stegopterna mutata*, *Prosimulium mixtum*, *Simulium vittatum* complex), as were *Paramoebidium* spp. in various simuliid species from several sites (including *P. curvum* Lichtwardt in *Simulium venustum/verecundum* complex from Site NF-34, and in another simuliid from Site NF-14), and in *Ephemerella aurivillii* Bengtsson from Site NF-3. An unidentified genus was also found in caddis fly larvae (Trichoptera) belonging to the Limnephiloidea, a recently discovered new order of hosts for Harpellales (White 1999).

#### DISCUSSION

The relatively cool water temperatures of streams during our major collections in May, 1999 (10.5–18.5 C, av. 15.4 C) provided a diversity of aquatic insect larvae. In turn, this led to our finding more than eight species of Harpellales, four of them new, and several species of *Paramoebidium* (Amoebidiales). Members of the Amoebidiales, formerly considered to be Trichomycetes, have now been shown convincingly to be protozoans rather than fungi (Benny and O'Donnell 2000).

Only two previous papers reported Trichomycetes in Newfoundland. Frost and Manier (1971) found *Harpella melusinae* and a *Pennella* that they identified as *P. hovassi*. Yeboah et al (1984) investigated several kinds of fungal cysts produced by "Phycomycetes" in the ovaries of adult blackflies in the area where we conducted the studies reported here. Moss and Des-

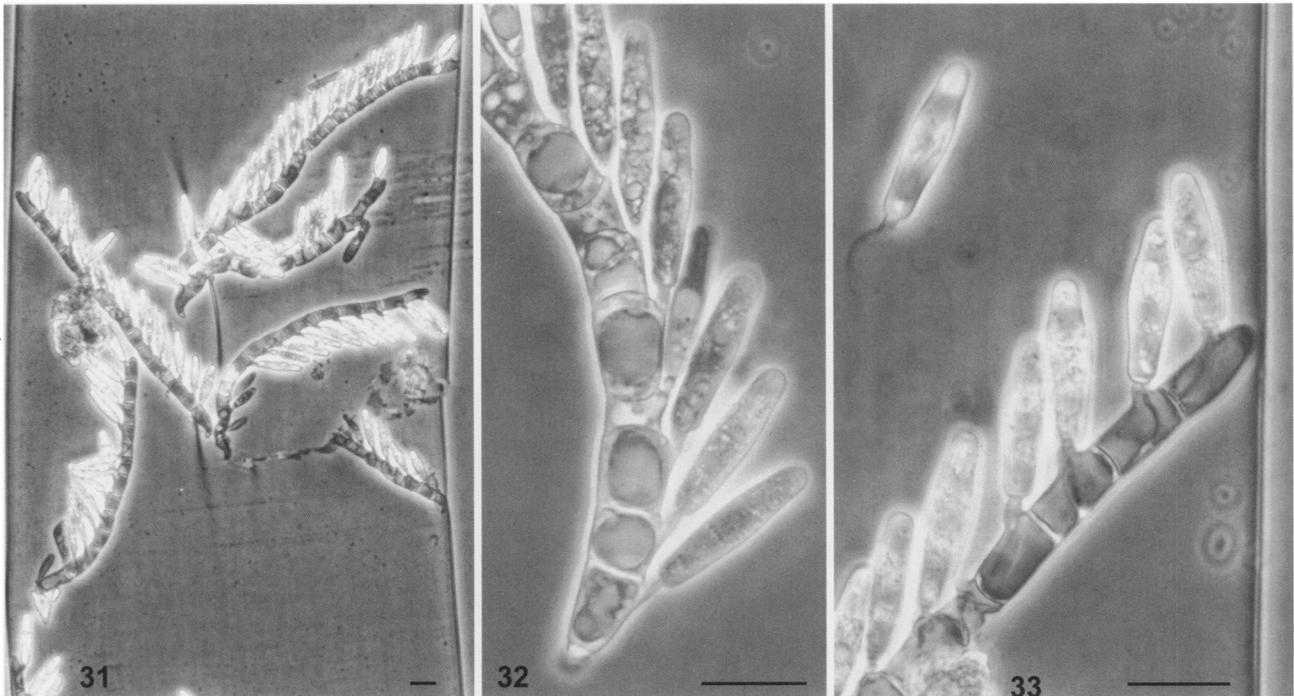


FIGS. 24–30. *Pennella simulii* from simuliid hindguts. 24, 25. Trichospores, the released ones with barely visible multiple appendages. 26–28. Zygospores. 29, 30. Two variants of holdfast cells that have secreted a mucilaginous substance. Scale bar = 20  $\mu\text{m}$ .

icals (1986) in Great Britain showed conclusively that such cysts were a stage of Harpellales development. In their study the cysts belonged to *Harpella melusinae*. Our own investigations on harpellid ovarian cysts in Newfoundland will be reported in another article.

Of special interest is the distribution of species of *Orphella* and *Harpellomyces*. *Orphella* spp. are known to occur only in nymphs of three sister families of Plecoptera: Nemouridae, Leuctridae, and Capniidae. The families belong to the Suborder Arctoperlaria whose species are restricted to Nearctic and Palearctic re-

gions. These stoneflies are among the least vagile of aquatic insects. Three *Orphella*s in southern Europe include the type species, *O. coronata* Léger & Gauthier in France (in Nemouridae), and two species in Spain recently described by Santamaria and Girbal (1998), *O. catalaunica* and *O. helicospora* (in Leuctridae). In North America, Williams and Lichtwardt (1987) found *Orphella haysii* Lichtw. & M.C. Williams in Nemouridae from a Colorado Rocky Mountain stream. A midwestern species, *O. hiemalis* Peterson, Lichtw. & Huss, was described from Capniidae (Lichtwardt et al



FIGS. 31–33. *Stachylina robusta* thalli sporulating within the peritrophic membrane of a chironomid larva. Scale bars = 20  $\mu\text{m}$ .

1991, 1993). The new species, *O. avalonensis* in Leucitridae from Newfoundland, is the third North American species. White (unpubl) has found what may be the same *Orphella* species in Nova Scotia, Canada. Thus, there exist six different species of *Orphella*, three from southern Europe and three from North America. The low vagility of the host stoneflies suggests that the genus *Orphella* existed in Arctoperlarian hosts prior to severance of land connections across the North Atlantic during the Lower Eocene (Brown and Lomolino 1998) and was followed by vicariant speciation on the two continents.

The thaumaleid hosts of *Harpellomyces* perhaps are more vagile than stoneflies, but vicariance may also have contributed to speciation of their gut fungi. Many Thaumaleidae are ecologically constrained and often occur in seepage habitats near waterfalls and roadcuts (Sinclair 1996). The type specimen of *Harpellomyces*, *H. eccentricus*, was producing zygospores in addition to trichospores when first found in northern Sweden in an unknown species of *Thaumalea* larva. Subsequently, *H. eccentricus* was discovered in larvae of another *Thaumalea* sp. in Wales (Lichtwardt and Moss 1984), an allopatric distribution that no doubt is attributable to collecting bias. *Thaumalea verralli*, host of *H. abruptus* in Newfoundland, is a species that has successfully dispersed and colonized areas across the North Atlantic (Sinclair 1996). In North America, Ferrington (unpubl) has found in

Thaumaleidae larvae another *Harpellomyces* sp. in two seepage sites in Pennsylvania, USA, that is similar to the species in Newfoundland. None of the North American collections has included zygospores that would provide additional evidence of differences or similarity between species on the two continents. To complicate distribution patterns, Lichtwardt et al (1987) reported finding a species of *Harpellomyces* similar to *H. eccentricus* from *Thaumalea* sp. larvae at a waterfall in Japan.

A better understanding of the evolution and distribution of such gut fungi and their insect hosts will depend upon a focused attempt to obtain specimens in their particular habitats from different geographic regions.

#### ACKNOWLEDGMENTS

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