Harpellales (*Zygomycota*: *Trichomycetes*) associated with black flies (*Diptera*: *Simuliidae*): world review and synthesis of their ecology and taxonomy

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The *Harpellales* are obligate, symbiotic trichomycete fungi that colonize the digestive tracts of arthropods, including black flies (simuliids). Worldwide, black flies are hosts for 9 genera and 36 species of *Harpellales*. Black flies are ideal organisms for the study of trichomycete ecology because they are taxonomically well known, allowing precise identifications of trichomycete hosts. Trichomycete fungi are among the few symbiotes known to encompass the entire spectrum of symbiotic relationships: commensalism, mutualism, and parasitism. To provide an enhanced framework for investigating trichomycete-host symbioses, we review and synthesize the biology of *Harpellales* colonizing black flies, suggest areas requiring further study, and present a key to the species of *Harpellales* that colonize black flies worldwide.

Key words: aquatic insects, black flies, fungi, *Harpella*, identification key, Nematocera, *Pennella*, *Smittium*, streams, symbiosis

Introduction

The *Harpellales* are obligate, fungal associates of the guts of isopods (Isopoda) and larval insects, including beetles (Coleoptera), true flies (Diptera), mayflies (Ephemeroptera), stoneflies (Plecoptera), and caddisflies (Trichoptera) (Misra and Lichtwardt, 2000; Lichtwardt *et al*., 2003). *Harpellales*, the most species-rich order in the class Trichomycetes, contains 2 families (i.e., *Harpellaceae* and *Legeriomycetaceae*), 36 genera, and more than 170 species worldwide (Lichtwardt *et al*., 2001a). The name "trichomycetes" is derived from the Greek word "tricho," meaning hair, in reference to the hair-

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like appearance of thalli in the host gut, and "mycetes," meaning fungi (Duboscq *et al*., 1948). *Harpellales* is probably derived from the Latin word "harpe" for a curved sword, a reference to certain trichomycetes (i.e., *Harpella* spp.) that have curved propagules.

Larval black flies provide a model host system for the study of *Harpellales* because of their ease of collection, ubiquity, and status as one of the taxonomically best-known groups of aquatic insects at the species level (Adler *et al*., 2004). These attributes have contributed to a rich investigative history of trichomycetes in black flies compared to other host groups. Symbioses in lotic habitats are poorly understood (e.g., Brönmark and Hansson, 2005). The trichomycete-simuliid relationship, therefore, can be particularly instructive.

Species richness of *Harpellales* in larval black flies is high compared with that of other aquatic insects such as biting midges (Diptera: Ceratopogonidae), mosquitoes (Diptera: Culicidae), mayflies (Ephemeroptera), and stoneflies (Plecoptera), and is exceeded only by larval midges (Diptera: Chironomidae). Thirty-six species of *Harpellales* are known from black flies (Nelder *et al*., 2005a). By the end of 2004, 1907 valid species of extant black flies had been described worldwide (Crosskey and Howard, 1997; Adler, unpublished data) – about 53 species of black flies per species of simuliid-associated trichomycete.

We review the existing knowledge of the trichomycete-simuliid symbiosis, synthesize the scattered literature, and suggest areas of the trichomycete-simuliid relationship needing further study. We also present a key to the described *Harpellales* in black flies worldwide.

History of trichomycete study in black flies

Trichomycetes were originally described by Leidy (1849) as "entophyta," or colorless algae (*Confervaceae*), from beetles and millipeds (Diplopoda). The study of *Harpellales* of black flies began in France, with the description of *Harpella melusinae* Léger & Duboscq from larvae of *Simulium ornatum* Meigen (Léger and Duboscq, 1929). From 1929 to 1959, descriptions of *Harpellales* associated with black flies increased at the rate of approximately two species every 10 years (Fig. 1). Since 1959, descriptions of new species of *Harpellales* in black flies have appeared at the rate of six species every 10 years. The increased rate of descriptions since 1959 can be attributed partly to an interest in black flies as fungal hosts by workers such as Robert W. Lichtwardt, Jehanne-Françoise Manier, Odette Tuzet, and Marvin C. Williams. The increase also coincides with a period of expanding

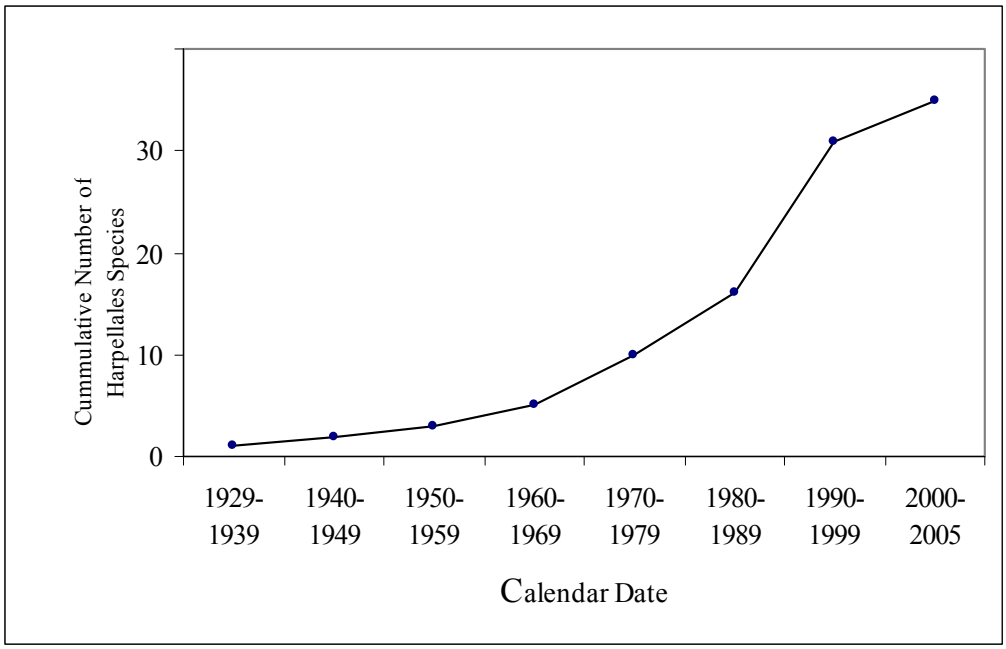


Fig. 1. Species accumulation curve for simuliid-associated *Harpellales* described from 1929 to present. The first species (*Harpella melusinae* Léger & Duboscq) associated with black flies was described in 1929.

knowledge of simuliid taxonomy and appreciation of black flies as vectors of disease agents. The rapid increase in descriptions of *Harpellales* since 1990 is attributable to the study of trichomycetes in black flies in areas such as Australia (e.g., Lichtwardt and Williams, 1992a), Central America (Lichtwardt, 1997), and South America (e.g., Alencar *et al*., 2003). The absence of a plateau in the species accumulation curve suggests that more species remain to be discovered (Fig. 1).

The higher classification of gut fungi has developed over about 150 years and, in light of recent advances in molecular biology, continues to be reworked (Table 1). Two independent groups of entophyta (Eccrindides and *Amoebidium*) were recognized in the 1800s. Léger and Duboscq (1929) added another group, the "Harpellacées," which they believed were related to the *Entomophthorales*. Duboscq *et al*. (1948) combined these three groups of entophyta into the new fungal taxon Trichomycetes, which for about 50 years would include four orders: *Amoebidiales*, *Asellariales*, *Eccrinales*, and *Harpellales* (Benny, 2001). Some researchers (e.g., Lichtwardt, 1986), however, suggested that the *Amoebidiales* might not be fungal.

Table 1. Time line for investigations of trichomycetes associated with black flies (1850-present). Aff. = affinity. Desc. = described. Cl. = classified

	1850	**1860**	**1870**	**1880**	**1890**	**1900**	**1910**	**1920**
Affinities and higher taxa described	1849, 1850-Leidy described first trichomycetes. Aff. Confervaceae (algae). "Eccrinides" 1853-Robin. Aff. Saprolegniales	1861-Ciekowski. Aff. Algae and lower mushrooms		1882-Bütschli 1883-Balbiani. Aff. "Sporozoaires" (Butschli, similar to gregarines)	1895-Hauptfleisch. Aff. Saprolegniales 1897-Schröter. Aff. Myxomycetes 1892-Perrier 1896-Delage et Hérouard 1899-Labbé 1899-Mesnil. Aff. "Sporozoaires"	1906-Chatton. Aff. Lower fungi near myxomycetes or chytrids, not algae	1914-Mercier. Aff. "Protophytes" 1917-Lichtenstein. Aff. "Protoascomycetes"	1929a-Léger & Duboscq. Desc. Eccrinales and Amoebidiales. 1929-Léger & Duboscq. Desc. & Cl. Phycomycetes Entomophthorales Harpellacées 1929-Poisson. Aff. Thallophytes
Genera described	1849-Leidy. *Enterobrus* (sic)	1861-Cienkowski. *Amoebidium*						1929-Léger & Duboscq. *Harpella*[2] & *Paramoebidium*
Other events	1856-Lieberkühn 1858-Schenk. Independently discovered *Amoebidium* but did not name it (Lieberkühn, Aff. Sporozaires)				1895-Hauptfleisch suggested aff. with Saprolegniales, based on his discovery of *Astreptonemia*	1909-Chatton & Roubaud. Amoebidiales from black fly[1]		

[1]First trichomycete in black flies (Chatton and Roubaud 1909).
[2]First fungal trichomycete in black flies (Léger and Dubosq 1929).

Table 1, continued.

	1930	1940	1950	1960	1970	1980	1990	2000
Affinities and higher taxa described	1932-Léger & Gauthier. Desc. Génistellacées	1948-Duboscq *et al*. Cl. Trichomycètes Eccrinides Amoebidiales Eccrinales Harpellides Harpellales Genistellales	1950(1951)-Manier. Cl. Trichomyctes Eccrinides Amoebidiales Eccrinales Palavascides Palavasciales Harpellides Harpellales Génistellides Génistellales Spartiellales Asellariales 1955-Manier. Cl. Prototrichomyceta Amoebida Eutrichomyceta Eccrinida Harpellida	1960-Lichtwardt. Cl. Trichomycetes Amoebidiales Eccrinales Harpellales Asellariales Genistellales 1968- Manier & Lichtwardt. Cl. Trichomycetes Amoebidiales Eccrinales Asellariales Harpellales		1986-Lichtwardt. Cl. Zygomycota Trichomycetes Harpellales Asellariales Eccrinales Amoebidiales		2005-Cafaro. Cl. Mesomycetozoa Ichthyophonida Amoebidiales Eccrinales
Genera described	1932-Léger & Gauthier. *Stachylina* & *Stipella* 1936-Poisson. *Smittium*			1961-Tuzet, Rioux, & Manier. *Rubetella* (syn *Smittium*) 1963-Manier. *Pennella* (nom. nud.) 1968-Manier & Lichtwardt. *Pennella* (validated)	1972-Lichtwardt. *Genistellospora* & *Simuliomyces*		1997-Lichtwardt. *Genistelloides*	2005-New genus from Great Smoky Mountains National Park (White, Siri, & Lichtwardt, in press)
Other events		1948-Duboscq *et al*. Coined the name Trichomycete which grouped the various taxa of "entophyta"		1960-Whisler cultured *Amoebidium* 1963-Clark, Kellen, & Lindegren cultured *Smittium*	1972-Pouzar. Legeriomycetaceae replaces Genistellaceae of Léger & Gauthier 1932. Type genus had been used for a legume in 1773. Sanger. Serology studies	1987-Peterson and Lichtwardt. Serology studies.	1996-Grigg & Lichtwardt. Isozyme studies.	2000-Benny & O'Donnell. Confirmed *Amoebidium* is a protozoan in Ichthyosporea 2000-2003-PEET grant: molecular studies by Cafaro, Gottlieb, Lichtwardt, & White (University of Kansas)
Theses			1951-Lichtwardt (MS) 1954-Lichtwardt (PhD)	1961-Whisler (PhD) 1965-Farr (MA) 1966-Chapman (MA) 1969-Sangar (PhD)	1970- Coste-Mathiez (PhD) 1971-Galt (MS), Grizel (PhD), Williams (PhD) 1972-El-Buni (MA), Moss (PhD) 1973-Preisner (PhD) 1975-El-Buni (PhD), El-Sherif (PhD) 1976-Starr (MA) 1978-Hollingsworth (MS) 1979-Dang (MS)	1980-Horn (MS), Yeboah (MS) 1984-Peterson (PhD) 1988-Grigg (MS) 1989-Horn (PhD)	1992-Taylor (PhD) 1994-Grigg (Ph.D) 1996-Frost (BS Honors)	2002-Beard (PhD), White (PhD) 2003-Alencar (PhD), Cafaro (PhD), Nelder (MS) 2004-Valle (PhD) 2005-Kim (PhD)

In the late 1980s and the 1990s, serology and molecular techniques, respectively, demonstrated that *Amoebidiales* do not belong with the trichomycetes. Cavalier-Smith (1998) placed *Amoebidiales* and *Eccrinales* in a new class, Enteromycetes, because they share Golgi dictyosomes. Within the *Amoebidiales*, the genera *Paramoebidium* and *Amoebidium* include at least four species associated with black flies: *Paramoebidium curvum* Lichtwardt, *Paramoebidium grande* Lichtwardt, and *Paramoebidium chattoni* Léger & Duboscq (*nomen nudum*), which inhabit the hindgut, and *Amoebidium colluviei* Lichtwardt, which lives on the external cuticle. The *Amoebidiales* are now considered protists (Benny and O'Donnell, 2000; Ustinova *et al*., 2000; Benny and White, 2001; Reeves, 2003a) in a clade with the *Eccrinales*, leaving the class Trichomycetes with two fungal orders, the *Asellariales* and the *Harpellales* (Cafaro, 2005).

The symbiotes: *Harpellales*

The generalized life cycle of a harpellid in a black fly host is presented in Fig. 2. *Harpellales* consist of a holdfast and a main thallus with terminal or subterminal propagules that can be asexual (trichospores) or sexual (zygospores). They colonize the guts of larval black flies, attaching to the peritrophic matrix of the midgut or the cuticle of the hindgut by means of a permanent holdfast produced by the basal cell(s) of the thallus (Lichtwardt, 1986; Moss, 1998). The structure of the holdfast varies among and within genera of *Harpellales*. The holdfast is tapered or rounded in *Harpella* species, often bifurcated and covered by a mucilaginous sheath in *Pennella* species, and typically small and sometimes limuloid in *Smittium* species. The hindgut cuticle of a black fly folds around the holdfast of species such as *Genistellospora homothallica* Lichtwardt (Mayfield and Lichtwardt, 1980). The thalli of *Harpellales* exhibit one of two growth forms: branched, typically with a thallus giving rise to lateral branches (*Legeriomycetaceae*) (Fig. 3) or unbranched (*Harpellaceae*). The ultrastructure of trichomycetes colonizing black flies has been treated by Reichle and Lichtwardt (1972), Moss and Lichtwardt (1976, 1977), Mayfield and Lichtwardt (1980), Horn (1989b), and Sato (2002).

The production of reproductive propagules in higher fungi is related to physiological and abiotic factors. Hence, the biomass of higher fungi increases as nutrients become available and biomass is converted to reproductive propagules as nutrients diminish (Griffin, 1994). Trichomycetes might respond similarly. For example, *Smittium* spp. cultured in high-nutrient broth produce predominantly vegetative growth, whereas low-nutrient broth results in high

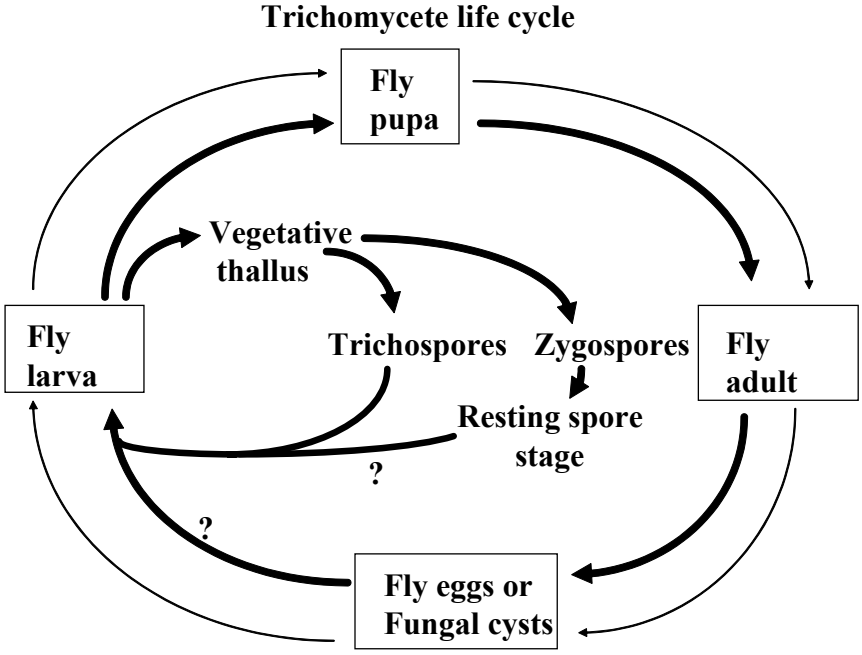


Fig. 2. Generalized life cycle of *Harpellales*, with a simuliid host. Bold line represents fungal life cycle; thin line represents fly life cycle. Question marks indicate hypothesized routes.

production of trichospores (El-Buni and Lichtwardt, 1976a). Some workers have suggested that trichospore production increases prior to host molting (Lichtwardt *et al*., 2001a) and responds to host-moulting hormones. As black flies prepare to molt, they cease feeding (Hinton, 1958). Thus, nutrients available to the fungus might decrease at this time.

Harpellales reproduce asexually by trichospores (El-Buni and Lichtwardt, 1976a,b) (Fig. 4), which are basipetal, monosporous sporangia (housing a single sporangiospore); they are produced terminally or laterally from generative cells on fertile branches (Lichtwardt, 1986). Although trichospores have been found in the shed exuviae of the host (Lichtwardt *et al*., 2003), they are observed most often in the arthropod gut where the fungus is growing. Trichospores often have various numbers of nonmotile, basal appendages, depending on the genus (Fig. 4) (Moss and Lichtwardt, 1976). The appendages can be coiled or spiralled within the thallus (but outside the plasma membrane) before the trichospore is released (Fig. 5), and in *Genistellospora homothallica* they are directed downward along the inner wall (Moss and Lichtwardt, 1976). Appendages are believed to entangle with food consumed by the host (Lichtwardt, 1996). They also allow for direct entanglement with

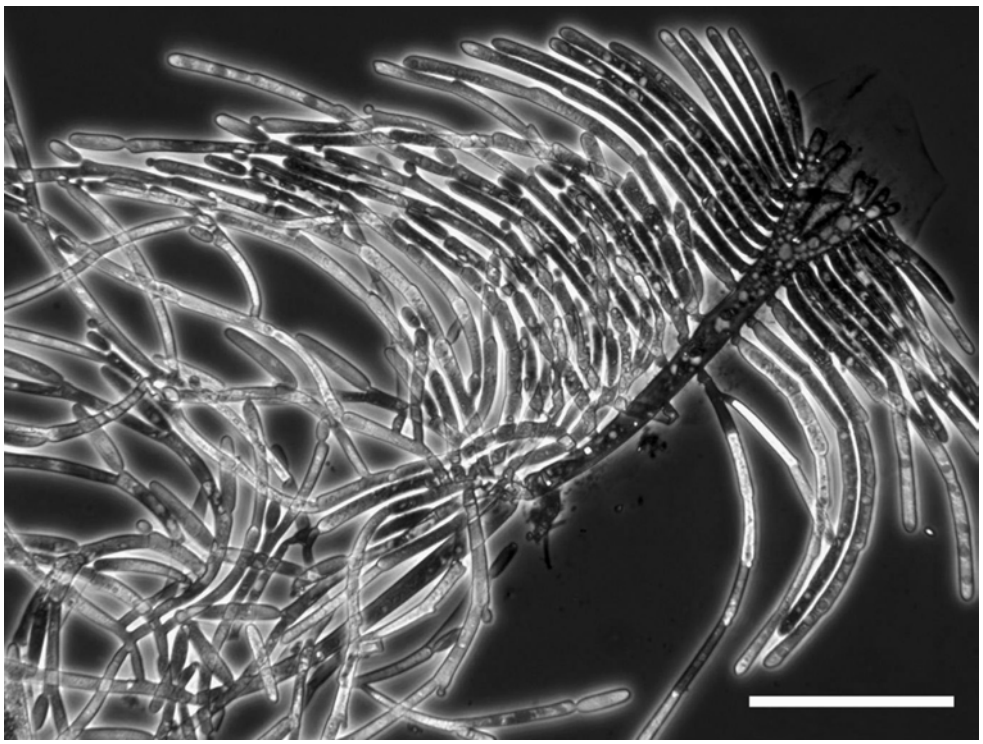
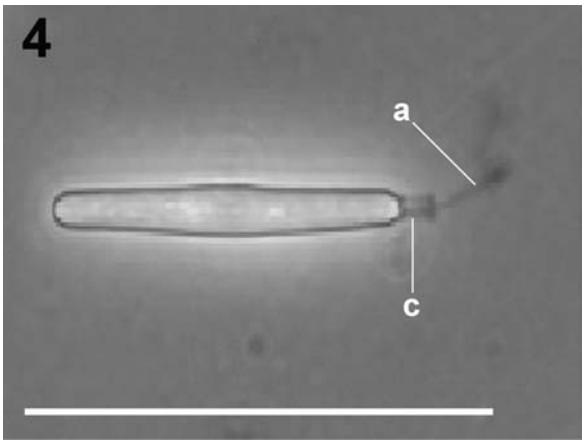
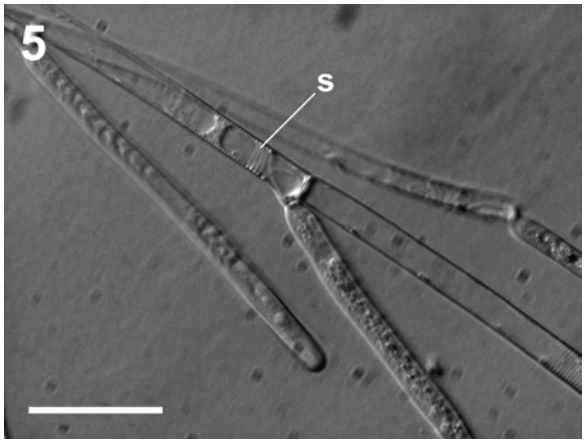


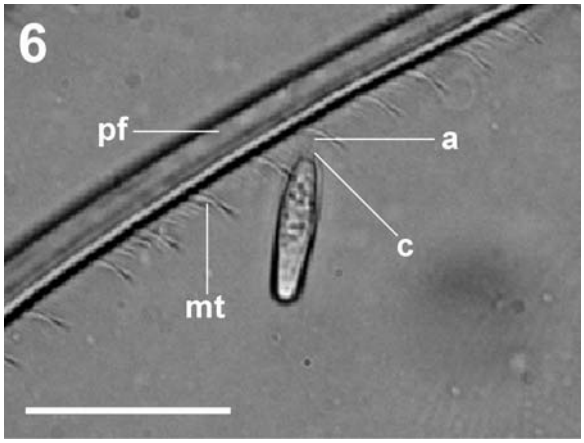
Fig. 3. *Pennella* sp. attached to the hindgut cuticle of *Simulium tribulatum*. Bar = 100 µm.

the microtrichia on the labral fans of black flies during filter feeding (Fig. 6). Once in the gut, appendages might snag on the thalli of existing fungi and other materials, keeping them in the appropriate microhabitat for a longer period of time.

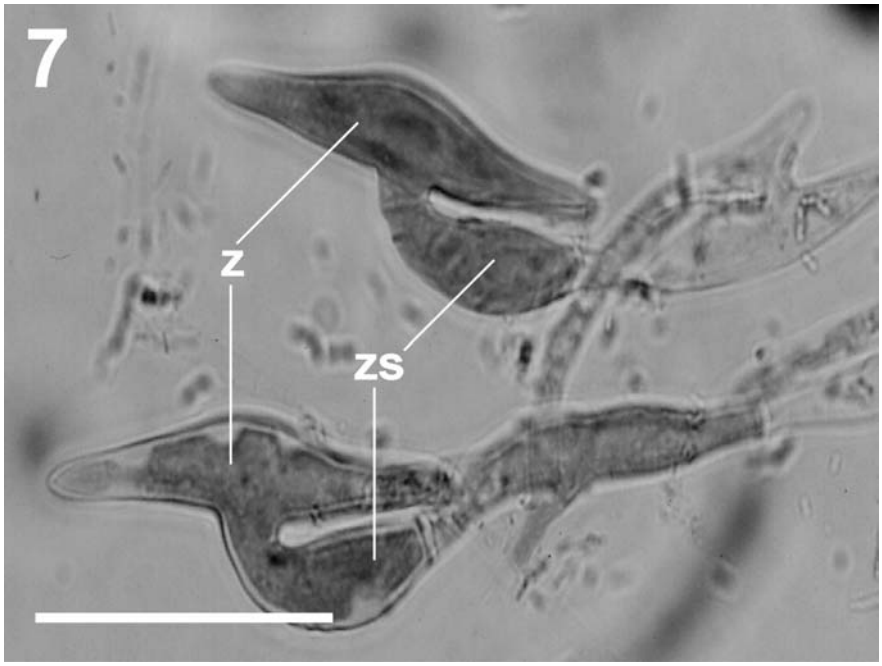
The *Harpellales* reproduce sexually by zygospores, which are either pointed at one terminus or, more commonly, biconical (Fig. 7) (Lichtwardt, 1986). The production of pointed zygospores is a distinguishing feature of the order *Harpellales*. The shape of the zygospore possibly is conducive to rapid extrusion in the host gut (Lichtwardt, 1996). Production of zygospores requires thallial conjugation in some species (e.g., *Pennella*) but not in others (e.g., *Genistellospora*). Zygospores are produced on zygosporophores, probably after karyogamy and, unlike other *Zygomycota*, after meiosis (Moss and Lichtwardt, 1977). Reproduction by means of zygospores is rare in many *Harpellales* and might have been essentially lost in some species (e.g., *Smittium culisetae* Lichtwardt). Zygospores are classified according to their orientation with the zygosporophore: perpendicular = type I, median attachment to zygosporophore (Fig. 8); oblique = type II, submedian attachment; parallel = type III, median attachment (Fig. 7); and polar = type IV, coaxial attachment (type IV zygospores are not found in black flies) (Lichtwardt, 1986).

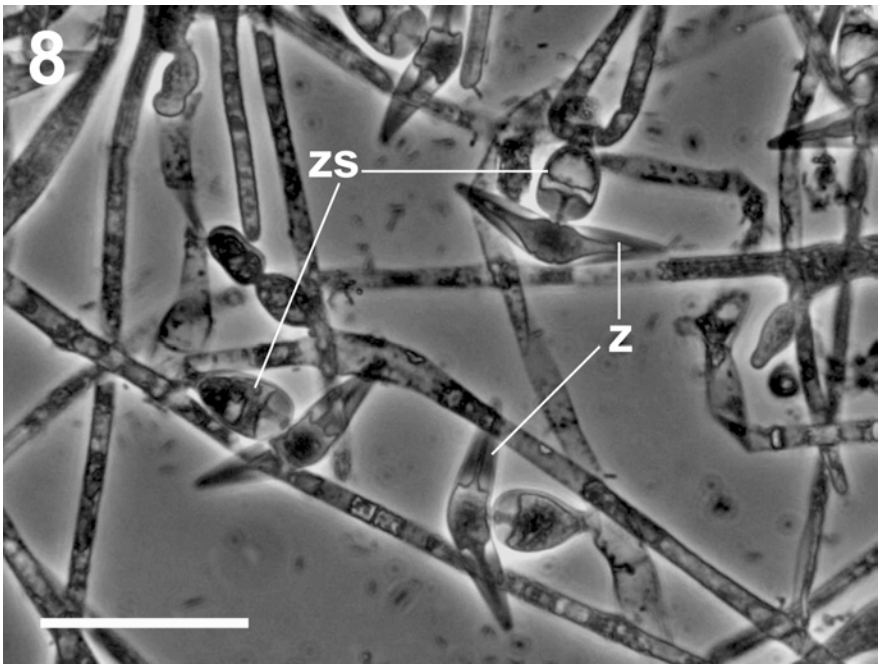






Figs. 4-6. Trichospopres of *Harpellales*. **4.** *Smittium brasiliense* in axenic culture; a = appendage, c = collar. **5.** *Harpella* sp. from the gut of *Simulium innoxium*; s = spiraled appendages. **6.** Trichospore of *Smittium culisetae* captured by microtrichia of the labral fan of *Simulium tribulatum*; a = appendage, c = collar, m = microtrichia, pf = primary fan ray. Bars = 25 µm, except Fig. 5 = 30 µm.





Figs. 7-8. Zygospores of *Harpellales*. **7.** *Pennella* sp. (immature stage) in hindgut of *Simulium* sp. **8.** *Simuliomyces microsporus* in hindgut of *Simulium tribulatum*. z = zygospores, zs = zygosporophores. Bars = 30 µm.

Moss (1998) suggested that zygospores probably are not required for colonization of other hosts. Lichtwardt *et al*. (2003), however, suggested that zygospores are transmitted from larva to larva. The latter scenario has not been tested and the role of zygospores in the life history of simuliid-gut fungi remains unknown (Beard and Adler, 2003). Zygospores might be part of a parasitic stage of some *Harpellales* (Horn, 2001) or a resistant stage during unfavorable conditions in the life cycle of the *Harpellales* (Lichtwardt *et al*., 2001a). These hypotheses, however, are difficult to test because zygospores have not been produced in axenic cultures of *Harpellales* and, in field collections, they are unpredictable.

The hosts: black flies (*Simuliidae*)

Larval black flies are obligate stream-dwelling insects that inhabit the full range of flowing waters, from the smallest rivulets to the largest rivers. Black flies are found from Bear Island, Norway, at 74°30' N, to Isla Navaro, Chile, at 54°56' S. Eggs are deposited directly in the water or in nearby wetted areas, or they are glued onto trailing vegetation and wet stones. After hatching, the larva spins a silk pad on an object (e.g., a stone or trailing vegetation) in the current and attaches the microhooks of its posterior proleg to the silk pad. Thus anchored, the larva filters particulate matter from the current by using a pair of rake-like labral fans on its head, or it scrapes periphyton and associated debris from the substrate. Either mode of feeding presumably brings trichospores into the gut.

The larval stage lasts about a week to nine months or more, depending largely on water temperature. Pupation takes place in a silken cocoon spun on an object in the current. Within a few days to several weeks after pupation, the adult black fly emerges from the pupa and quickly rises to the surface of the water. Some species complete a single generation annually, undergoing an obligatory diapause, whereas other species, particularly those in warmer climes, can complete up to 20 or more generations per year. Mating and sugar feeding (for energy) take place shortly after adult emergence. Most females then acquire a blood meal (necessary for egg maturation) from a bird or mammal, and return to flowing water, often flying upstream to deposit their eggs. The quest for blood is responsible for the status of black flies as pests and vectors of disease agents of humans and domestic animals (Crosskey, 1990; Adler *et al*., 2004).

Techniques for study of *Harpellales*

Host rearing and collection

Laboratory studies of the trichomycete-simuliid symbiosis have been facilitated by the relative ease of rearing larval black flies. Eggs of species such as *Simulium vittatum* Zetterstedt can be collected from the field by anchoring yellow strips of plastic (resembling trailing grasses) in the current and retrieving the strips a few days later. We have routinely obtained eggs of *Simulium vittatum* from a parasite-free colony at the University of Georgia, Athens, Georgia, USA (Gray and Noblet, 1999). Eggs can be placed in aerated containers of water at room temperature. When the larvae hatch, they can be fed pulverized fish food. Continuous-generation rearing of black flies is possible but time intensive (Edman and Simmons, 1985a,b; Cupp and Ramberg, 1997; Gray and Noblet, 1999)

Larval black flies in the field are best collected by hand, using forceps to remove them from in-stream objects such as trailing vegetation, artificial substrates (e.g., plastic), and stones (Adler *et al*., 2004). Although most larval instars can be colonized by trichomycetes, older instars generally contain more fungi, often with zygospores (Lichtwardt *et al*., 2003). Larvae in large rivers can be collected by boat or by anchoring monofilament fishing line or plastic tubing to a bank or bridge, allowing it to trail in the current, and removing it several days later. Accessible watercourses are sampled by hand collecting larvae from all available substrates while walking swaths from bank to bank. Live larvae are refrigerated on moist filter paper in a Petri dish until dissection. Larvae can be held for several days at 4°C for evacuation of food from the gut, facilitating fungal detection. Ecological data can be obtained at the time of collection by recording selected stream characteristics such as stream conductivity, discharge, pH, temperature, and width (McCreadie and Adler, 1998). These data, particularly if used in multivariate analyses, can be useful predictors of both host and symbiote distributions among streams (Beard, 2002; Beard *et al*., 2003; Nelder, 2003).

Larval black flies can be identified using the illustrated keys of Yankovsky (2002) for the Palearctic Region, Adler *et al*. (2004) for the Nearctic Region, and Takaoka (2003) for the Indonesian portion of the Oriental Region. Keys to other areas of the world are listed by Crosskey and Howard (1997).

Detection of Harpellales in the host

To assay black flies for trichomycetes, live larvae are slit ventromedially in a drop of water on a glass slide, using fine needles or a scalpel. The larva is

cut transversely just posterior to the thoracic proleg and a second cut is made immediately anterior to the anal proleg. (The larval host morphology is reviewed by Adler *et al*. (2004)). The midgut is grasped with forceps and pulled anteriorly from the body. The remainder of the gut is pulled posteriorly from the body. With the gut removed, the larval carcass is placed in acetic ethanol (1:3) or 80% ethanol for subsequent identification. The midgut musculature is stripped from the cellophane-like peritrophic matrix. The hindgut musculature requires more care to tease it from the cuticle.

The peritrophic matrix and hindgut cuticle are then slide-mounted in a drop of water, a coverslip is applied, and the preparation is examined with phase-contrast microscopy. Morphological features of trichomycetes are measured using an ocular micrometer. A drop of lactophenol with cotton blue (a fixative and stain) is added to the edge of the coverslip and the preparation is sealed with clear nail polish. We use lactophenol containing1 part phenol liquid (e.g., 20 ml for a final volume of 100 ml), 1 part lactic acid (20 ml), 2 parts glycerol (40 ml), 0.5 g cotton blue, and 1 part distilled water (20 ml). The solution is kept in a tightly closed glass bottle.

Harpellales also can be recovered from larvae fixed in ethanol (> 70%) or acetic ethanol (Carnoy's solution) (Adler *et al*., 1996; Nelder *et al*., 2004, 2005a). Larvae stored in acetic ethanol (1: 3) contain identifiable fungi after at least 15 years of storage at 4°C (Nelder, unpublished data). Trichomycetes are more difficult to remove from fixed larvae (e.g., in museum collections) than from live larvae (Lichtwardt *et al*., 2003) but are a valuable source of trichomycetes.

The relative abundance of hyphae among hosts can be assessed by viewing the gut at 400× through a 10-mm × 10-mm ocular grid (McCreadie and Beard, 2003). The number of grid squares that contain one or more hyphae is counted and relative abundance expressed as the percentage of grid squares containing hyphae, relative to the total number of grid squares over the gut surface.

Culturing of Harpellales

Most modern culturing techniques are modifications of those presented by Lichtwardt (1964) and Lichtwardt *et al*. (2001a). Cultures can be derived from trichomycetes taken from live or freshly killed larvae. A slide with the larval hindgut – midgut fungi cannot yet be cultured – is viewed under low magnification (*ca*. 40×), without a coverslip, and the mycelia removed with fine forceps. On occasion, we have placed the entire slide with fungus in a Petri dish (100 mm × 15 mm) with sufficient broth to cover the slide.

Media preparation before isolation

Standard culture dishes consist of 15-20 ml of 3.7 g/l Brain Heart Infusion (BHI, Difco 237400), and 1.5-2% agar in a standard Petri dish (100 mm × 15 mm). We find a harder agar (1.75-2%) preferable to the standard bacterial formulation (1.5%) because it resists gouging if aggressively scraped with an inoculating loop.

Although these pre-made dishes of media can be used for initial isolation, we alternatively use a broth method, which facilitates the addition of antibiotics. A small amount (*ca*. 100 ml) of concentrate of BHI broth is prepared at 15 times (i.e., 55.5 g/l) the final concentration. This concentrate is dispensed in single-use units by allocating 0.7 ml (sufficient for 10 ml final volume) to small cryovials (*ca*. 2 ml capacity) or microfuge tubes. These small vials are stored at about -80°C until needed. We do not store unused, diluted broth media for more than 24 hours. Standard culture dishes of agar can be sealed with Parafilm and stored for several months at room temperature.

Single-use aliquots of antibiotics and vitamins are prepared in a similar manner. Antibiotic concentration for the final solution is about 8,000 units/ml streptomycin and 4,000 units/ml penicillin G. Concentrated antibiotics are made up in a stock solution with 80,000 units/ml streptomycin and 40,000 units/ml penicillin G. (The exact amounts of powdered antibiotics will depend on the assay for the production batch). Aliquots (1 ml) of concentrated antibiotics are stored at -80ºC. When bacteria seem resistant to control by these antibiotics, tetracycline HCl can be used as an alternative antibiotic. A concentration of 3 ppt in an overlay is beneficial.

If vitamins are to be used in fungal isolations, a diluted vitamin solution, rather than water, is used to prepare the isolation medium. The vitamin concentrate, which is made using 5 mg biotin/ml water and 20 mg thiamine/ml water, is 100× the final concentration, yielding 50 µg/ml biotin and 200 µg thiamine. The final solution is made by adding 1 ml of vitamin concentrate to 99 ml of distilled water.

When a suitable mass of hyphae is found in a dissected larva, one tube each of BHI and antibiotics is defrosted and placed in a small beaker. The volume is brought up to 10 ml with water (or vitamin solution) and loaded into a 10-ml syringe. A sterile syringe filter (0.4 µm or smaller) is added to the syringe and the solution is sterile filtered into a sterile Petri dish, typically 35 or 60 mm × 15 mm; we dispense only enough solution to cover the bottom of the dish. The fungus is then placed in the dish.

Maintenance

Dishes should be examined daily for growth and for bacterial or fungal contaminants. If trichomycete growth is evident after 4-5 days, the hyphae can be moved aseptically to a BHI-agar plate or another broth dish without antibiotics. Antibiotics can slow the growth of freshly extracted trichomycetes. If competing fungi develop, moving the trichomycete to a BHI-agar plate and smearing it around (without overlays) can provide isolated colonies. If bacteria develop, a transfer of the mycelial mass to a dish with fresh broth and antibiotics is beneficial.

Once an axenic isolate is growing, it can be maintained by monthly transfers of mycelia, using an inoculating loop. The Petri dishes with trichomycetes are sealed with Parafilm. Backup cultures can be refrigerated for months in sealed dishes or culture tubes, and maintenance cultures can be kept at room temperature (*ca*. 25°C). For backup cultures, we use screw-cap tubes 20 mm × 125 mm, with 8 ml of media (BHI agar) solidified at a slant. We seldomly use a water overlay for maintenance cultures, although it is commonly used by other workers (e.g., Lichtwardt, 1986). However, cultures grow faster and produce more trichospores with water. We have had month-old cultures produce many trichospores within about 48 hours after a water overlay is added to a previously non-overlay dish. Trichomycetes in culture grow slowly relative to some common contaminants such as *Penicillium* and they need to be monitored. If a contaminant appears, the trichomycete can be transferred to another dish before it is overgrown.

Trichomycetes isolated from black flies can be archived at the United States Department of Agriculture-Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, Ithaca, New York (http://www.ppru.cornell.edu/mycology/Insect_Mycology.htm). We usually ship specimens on slants in screw-cap vials, as in backup cultures.

The host gut as habitat

The vegetative stage of trichomycetes is restricted to the larval host gut and, therefore, depends on the host for protection. Hosts must select appropriate microhabitats. Larval black flies, for example, can relocate in inchworm fashion or on silk life lines to optimize the flow regime for feeding (Eymann, 1991; Poff and Ward, 1991) or to minimize exposure to ultraviolet light (Donahue and Schindler, 1998).

Gut structure

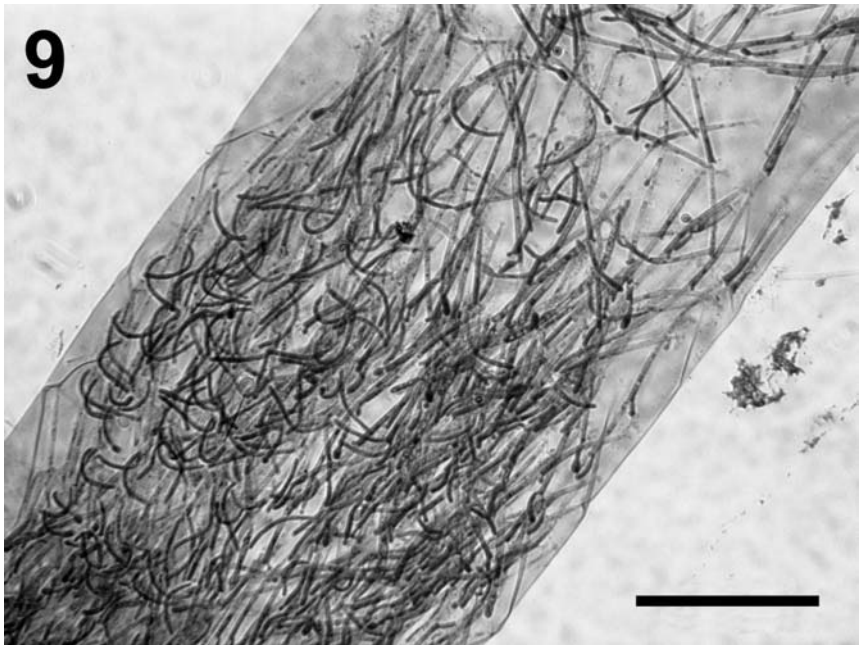
The gut of the larval black fly, roughly 3-15 mm long in mature larvae, provides the developmental milieu for *Harpellales*. It is divided into three sections: foregut, midgut, and hindgut (Crosskey, 1990). The cellophane-like envelope of the midgut (i.e., the peritrophic matrix, Figs. 9-10) and the cuticle-lined hindgut provide attachment sites for trichomycete holdfasts. No *Harpellales* have been found attached to the cuticular lining of the foregut of black flies.

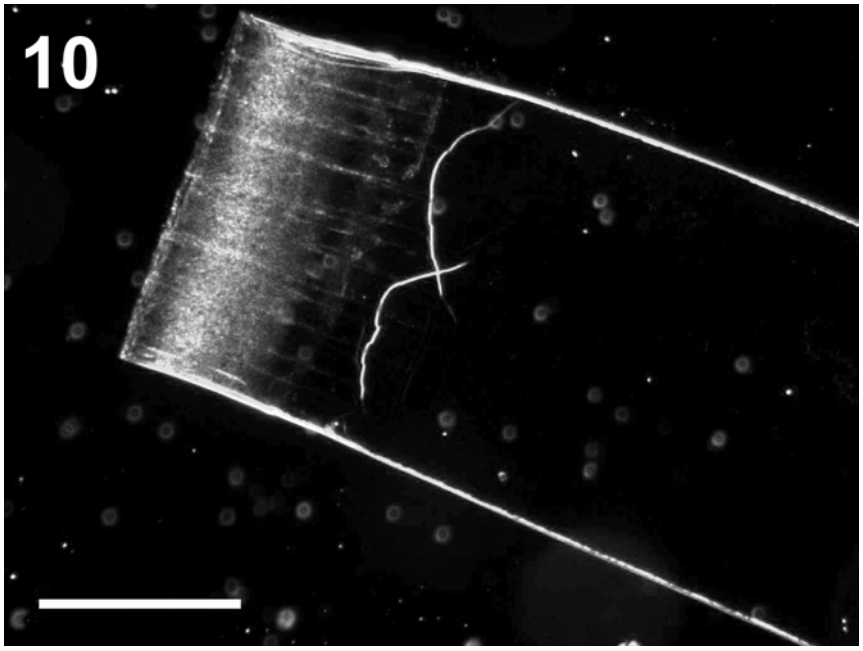
The peritrophic matrix of larval black flies is secreted by the anteriormost section of the midgut (i.e., the cardia) and is termed a Type II peritrophic matrix. The matrix consists of proteins, proteoglycans, and chitin, and is permeable to dissolved materials (Peters, 1992). The peritrophic matrix acts as a mechanical barrier between the food and the gut epithelium (Lehane, 1997). The Type II peritrophic matrix moves posteriorly, eventually breaking up in the hindgut (Shao *et al*., 2001). *Harpellales*, such as *Harpella* spp., must attach and grow before the matrix travels to the end of the midgut. The *Harpellales* that colonize the midgut are unbranched and have relatively determinate growth, which might be an adaptation to the continuous posterior advancement of the matrix.

The hindgut is a looped structure with three distinct regions: pylorus, colon, and rectum. The cuticular lining of the pylorus is often beset with posteriorly directed spines that might help move the peritrophic matrix posteriorly. Although the pylorus and rectum are colonized by trichomycetes, maximum trichomycete abundance occurs in the posterior colon (McCreadie and Beard, 2003) (Fig. 11). The fungi in the hindgut are generally branched and of indeterminate growth, limited only by the periodic moulting of the hindgut cuticle.

The lumen of the larval gut is filled with ingested materials that range in size from colloids to particles up to 350 μm in diameter, including algae, bacteria, detritus, and fungi (Wallace and Merritt, 1980; Adler *et al*., 2004). The food bolus typically contains sand grains, mica flecks, and similar inorganic matter that might act as roughage, pushing organic material through the gut (Colbo and Wotton, 1981). Although the inorganic matter might have abrasive properties, it evidently does not damage attached trichomycetes. Movement of food through the gut is temperature dependent, requiring about 20 minutes to 2 hours (Colbo and Wotton, 1981).

Because trichomycetes are attached to the hindgut cuticle, they are sloughed with the cuticular exuviae during larval molting. The thalli of *Smittium* species convert most of their biomass to trichospores during host molting (McCreadie, unpublished data). Under laboratory conditions, thalli of





Figs. 9-10. Peritrophic matrix of the black fly midgut. **9.** *Harpella melusinae* colonizing the peritrophic matrix of *Cnephia ornithophilia*. Bar = 200 µm. **10.** Uncolonized peritrophic matrix of *Simulium innoxium*; the somewhat thickened anterior end is to the left. Bar = 300 µm.

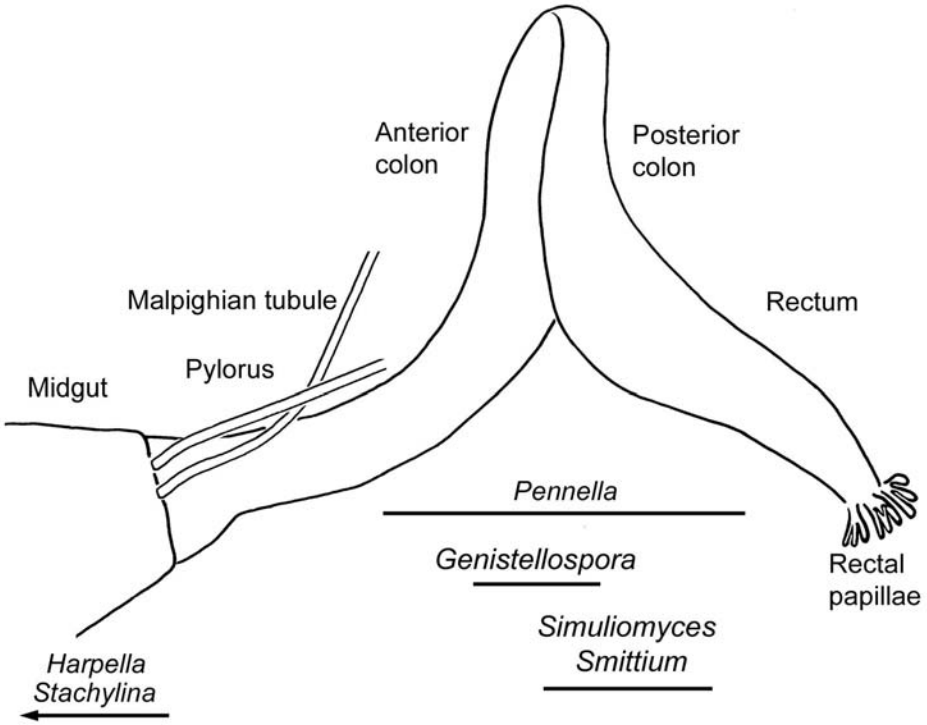


Fig. 11. Generalized location of *Harpellales* in the gut of *Simulium innoxium* (diagrammatic representation). Lines under genus names represent range of colonization in the gut.

Smittium culisetae produce trichospores after the hindgut is removed from the host (S. Vojvodic, University of South Alabama, pers. comm.). Conjugating *Harpella melusinae* and perhaps *Stachylina* species can form zygospores after larval molting (Beard and Adler, 2003). Reproduction apart from the living host might be a means of reducing the need for synchrony between host development and symbiote reproduction.

Gut physiology

The source of nutrition for trichomycetes in the host gut is unknown, although two sources are possible: 1) nutrients released from the food by enzymatic action of the larva, the fungus, or other entities (e.g., bacteria) in the food or gut, and 2) natural materials of the host (e.g., secretions, tissues, and wastes). The most likely source of nutrition is the material ingested and acted on by larval enzymes, which might provide absorbable elements for the trichomycetes. The best-documented enzymes used by larvae are proteases

released in the midgut (Martin *et al*., 1985). The action of the high pH derived from carbonates (Boudko *et al*., 2001) might play a part in digestion by hydrolyzing materials and releasing nutrients. Bacteria inhabiting the gut (Malone and Nolan, 1978) also might aid release of nutrients. The enzymes and chemicals secreted by the larva might be used by the fungus, possibly reducing the fitness of the host. If trichomycetes assist digestion by secreting enzymes, they might provide some benefit to host larvae by making more nutrients available, as demonstrated in mosquitoes (Horn, 1980). Starved larval black flies with trichomycetes have significantly higher survival than do trichomycete-free larvae (McCreadie *et al*., 2005). The basis of this mutualistic relationship is unknown, but might be nutritional.

Gas exchange is probably not a limiting factor for *Harpellales*. Larval black flies live in running water that is usually high in dissolved oxygen. Aerobic bacteria inhabit the gut (Taylor *et al*., 1996), suggesting that oxygen is available to trichomycetes in the gut. Other gases, such as carbon dioxide and ammonia, probably diffuse into the water through the body of the host.

Living in a host protects the trichomycetes from the vagaries of pH and osmotic changes in the host's external environment. The larval midgut of a black fly varies in pH from 8.2 anteriorly to 11.4 near the middle (Lacey and Federici, 1979). This high pH would be suboptimal for most fungi, but the *Harpellaceae* thrive in this environment. The available nutrients in the midgut have not been documented, but probably include carbonate complexes, which have been implicated in the maintenance of high pH in mosquitoes (Boudko *et al*., 2001). The hindgut probably has a near-neutral pH, based on data available for mosquitoes (Bradley, 1985).

Ecology of the symbiosis

Symbiotic nature of the relationship

The relationship between *Harpellales* and their hosts generally has been considered commensalistic (Lichtwardt, 1996; Moss, 1998), although in mosquitoes, examples of parasitism (Williams and Lichtwardt, 1972; Sweeney, 1981) and mutualism (Horn and Lichtwardt, 1981) have been suggested. The symbiotic association between *Smittium culisetae* and black flies is dynamic: commensalistic when larvae are well fed, but mutualistic when larvae are starved (McCreadie *et al*., 2005). The basis of this mutualistic relationship is believed to be nutritional; hyphae in stressed larvae might produce sufficient vital nutrients to increase survival. In the female black fly, the symbiotic association can be parasitic, the trichomycetes replacing eggs with fungal cysts

(Undeen and Nolan, 1977; Taylor, 1992; Lichtwardt, 1996; White, 2002). The symbiosis between *Harpellales* and black flies thus represents one of the few examples of a relationship shifting among the three states of symbiosis (commensalism, mutualism, parasitism) in a single pair of associated species.

Dispersal of Harpellales

The propagules of the *Harpellales*, once released from the host, are subject to downstream displacement in flowing water. Upstream dispersal by the fungus is, therefore, essential. Dispersal of gut fungi by black flies is poorly understood and generally assumed to be via females flying upstream to deposit their eggs, thereby releasing cysts (i.e., chlamydospores of Taylor, 1992) into the water and maintaining the fungal population locally (Lichtwardt *et al*., 2003). In this manner, *Harpellales* are parasitic, although the role that ovarian cysts play in populations of black flies remains poorly known. *Harpellales* might also be dispersed on the feathers of birds (Williams, 1983; Misra, 1998), through human activities (Lichtwardt, 1986, 1996), on alternative host species carrying the fungi in their guts or on their body surface (Lichtwardt, 1996), and on or in lotic organisms moving along the stream course.

The production of ovarian cysts by species of *Harpellales* (*Genistellospora, Harpella*, *Pennella*, and *Smittium*) has been observed in black flies on the Avalon Peninsula of Newfoundland, Canada (Undeen and Nolan, 1977; Undeen, 1978; Yeboah, 1980; Yeboah *et al*., 1984); in New York State, USA (Tarrant, 1984; Labeyrie *et al*., 1996); in Liberia (Steinke and Garms, 1990; Taylor, 1992); and in the United Kingdom (Moss and Descals, 1986; Taylor, 1992; Rizzo and Pang, 2005a). Possible trichomycete cysts in female black flies have been recorded from Cameroon (Lewis, 1960a, 1965), Guatemala (Garms, 1975), Ivory Coast (Walsh *et al*., 1981), Liberia (Lewis, 1960a; Garms, 1973), Russia (Shipitsina, 1963), Sudan (Lewis, 1953), and Tanganyika (Lewis, 1960b). Trichomycete infection of female black flies is common in some populations in Newfoundland, Canada (M.H. Colbo, Memorial University of Newfoundland, pers. comm.). However, our searches for ovarian cysts in female black flies in South Carolina, USA, have been unsuccessful, suggesting that it is not a universal means of dispersal.

Spatial-temporal ecology and host preferences of Harpellales

Few established laws exist in community ecology but the abundance, biodiversity, distribution, and species composition of organisms are almost universally recognized as uneven across any landscape and over time

(Rosenzweig, 1995). Trichomycetes are no exception to this rule. Both the patterns of distribution and the causal factors of these patterns vary with scale. The scales of distribution considered here are the gut, the host, and the stream habitat.

At the smallest scale of distribution (the gut), the distribution of hyphae varies both across and within the fore-, mid-, and hindgut. Members of the families *Harpellaceae* and *Legeriomycetaceae* attach to the lining of the midgut or hindgut, respectively (Lichtwardt, 1986, 1996). Physiological differences in the midgut and hindgut are believed to be responsible for this difference in location of *Harpellaceae* and *Legeriomycetaceae* (e.g., Horn, 1989a). Although the restriction of trichomycetes at the family level to either the midgut or hindgut of arthropods is well documented, we know much less about the distribution and abundance of thalli in each region of the gut. For example, *Simuliomyces microsporus* Lichtwardt attaches to *Paramoebidium chattoni*, which in turn attaches to the anterior section of the hindgut in black flies (Beard and Adler, 2002). *Smittium culisetae* in mosquitoes is restricted to the rectum (Horn, 1989a, 1989b). In contrast, this species is most abundant in the posterior colon of black flies. The distribution of *Harpellales* in the gut, therefore, can be related to the host taxon.

The next scale of spatial distribution is the host, which is exhibited (or measured) as differences in host specificity and preference. Genera of most *Harpellales* are specific to a single family of hosts. Apparent host specificity at some taxonomic levels of trichomycetes, however, might be the result of insufficient sampling of hosts. For example, the genus *Stachylina*, previously known only from chironomids, recently has been found in black flies (Labeyrie *et al*., 1996; Reeves *et al*., 2004). As more black flies are screened for trichomycetes, more genera of trichomycetes currently known only from other host families possibly could be found in black flies.

Some species of *Smittium* can colonize several families of Diptera. *Smittium culisetae*, for example, colonizes larvae of the families Chironomidae, Culicidae, and Simuliidae. However, some species such as *Smittium orthocladii* Manier colonize only chironomid midges (Lichtwardt *et al*., 2001a). The wide host range of certain members of *Smittium* might reflect unresolved cryptic species or broader physiological tolerances. Certain species of *Smittium* differentially colonize particular species of black flies under controlled laboratory conditions (Nelder *et al*., 2005b), suggesting host preferences or differential survival in the different host species. Similarly, the prevalence of *Harpella melusinae* in the field is significantly higher in *Simulium venustum* Say than in *Prosimulium magnum* Dyar & Shannon in the same stream (Beard *et al*., 2003).

Relatively little is known about the distribution of trichomycetes within and among streams. Based on other symbiotic relationships (e.g., Sapp, 1994) several suppositions can be offered. First, symbiote distribution is influenced by both host and environment; hence, symbiotes will occur within a subset of the sites occupied by their hosts. For example, *Harpella melusinae* readily colonizes *Simulium vandalicum* Dyar & Shannon, *Simulium tuberosum* (Lundström), and *Simulium verecundum* Stone & Jamnback, although not all sites where these hosts occur harbor *Harpella melusinae* (Beard *et al*., 2003).

Because all trichomycetes have a "free-living" stage (or at least spend time outside the host), stream conditions have the potential to influence the distribution of this stage in much the same way that stream conditions influence the distributions of their hosts (Ross and Merritt, 1988; Adler and McCreadie, 1997; McCreadie and Adler, 1998). In larvae of *Simulium tuberosum*, *Harpella melusinae* is most prevalent in acidic streams with low conductivity, whereas in larvae of *Simulium verecundum* it is most prevalent in streams of lower velocity (Beard *et al*., 2003). In contrast, the prevalence of *Harpella melusinae* in black flies is not correlated with location of the hosts along streams in the Rocky Mountains of Colorado, USA (Lichtwardt and Williams, 1988).

The temporal distribution of organisms can be examined on scales from the longevity of the organism to its evolutionary history. Other than several studies on the phenology of trichomycetes, little is known about the temporal ecology of these organisms. The monthly prevalence of *Harpella melusinae* and *Stipella vigilans* Léger & Gauthier shows little change over a year in some streams in North Wales (El-Sherif, 1975 in Taylor, 1992). In contrast, the prevalence of *Harpella melusinae* in *Simulium ornatum* in another English stream is low in February, March, and April (5-42%) but high (>75%) in most other months (Taylor *et al*., 1996). Trichomycete prevalence varies with season in New York State (Labeyrie *et al*., 1996), and *Harpella melusinae* and *Simuliomyces microsporus* show significant seasonality in black flies in South Carolina (Beard and Adler, 2002). *Harpella melusinae* quickly spreads through a black fly population from an initial low inoculum to a maintained high prevalence (Lichtwardt and Williams, 1988; Taylor *et al*., 1996).

Distribution and biogeography of Harpellales (Tables 2 and 3)

Some species of *Harpellales*, such as *Harpella melusinae* and *Simuliomyces microsporus*, have a cosmospolitan distribution, whereas other species, such as *Harpella leptosa* Moss & Lichtwardt (western USA) and *Pennella asymetrica* Williams & Lictwardt (New Zealand), are apparently more restricted in distribution. Biogeographic information is constrained by

Table 2. Geographical distribution of *Harpellales* colonizing larval black flies worldwide.

Harpellid fungus[1]	Distribution[2]	Reference
Undescribed genus and species	USA: NC	White *et al*., in press
Genistellospora guanacastensis Lichtwardt, 1997	Costa Rica	Lichtwardt, 1997
Genistellospora homothallica Lichtwardt, 1972	Argentina	Lichtwardt *et al*., 1999, 2000
	Armenia	Nelder *et al*., 2005a
	Chile	Lichtwardt and Arenas, 1996
	Costa Rica	Lichtwardt, 1997, 2000
	Spain	Gibral and Santamaria, 1998
	UK	Moss and Descals, 1986; Taylor *et al*., 1995
	USA: AL, AR, CA, CO, FL, KS, MO, NE, NY, OK, Puerto Rico, SC, TN, UT, VT, WY	Lichtwardt, 1972; Preisner, 1973; Moss and Lichtwardt, 1976, 1977; Mayfield and Lichtwardt, 1980; Grigg, 1988; Labeyrie *et al*., 1996; Slaymaker, 1998; Beard and Adler, 2000, 2002; White *et al*., 2000, Beard, 2002; Cafaro, 2002[3], 2003[3]; White, 2002; Nelder, 2003; Reeves, 2003b; Adler *et al*., 2004; Kim and Adler, 2005
Genistellospora nubila Lichtwardt, 1997	Costa Rica	Lichtwardt, 1997
Genistellospora tepidaria Lichtwardt, 1997	Costa Rica	Lichtwardt, 1997
Genistellospora tropicalis Ríos-Velásquez, Alencar, Lichtwardt & Hamada, 2003	Brazil	Alencar *et al*., 2003
Graminelloides biconica Lichtwardt, 1997	Costa Rica	Lichtwardt, 1997
Harpella amazonica Ríos-Velásquez, Lichtwardt, Hamada & Alencar, 2003	Brazil	Alencar *et al*., 2003
Harpella leptosa Moss & Lichtwardt, 1980	USA: AZ, MT, TX, UT	Moss and Lichtwardt, 1980, Alencar *et al*., 2003, Adler *et al*., 2004 (*Harpella* near *leptosa*), Reeves and Adler, 2004

Table 2 continued. Geographical distribution of *Harpellales* colonizing larval black flies worldwide.

Harpellid fungus[1]	Distribution[2]	Reference
Harpella melusinae Léger & Duboscq, 1929	Armenia	Nelder *et al*., 2005a
	Australia	Lichtwardt and Williams, 1990, 1992a, 1992b
	Canada	Brassard *et al*., 1971; Frost and Manier, 1971; Lichtwardt *et al*., 2001b; Adler *et al*., 2004, 2005; Kim and Adler, 2005
	China	Adler *et al*., 1996
	France	Léger and Duboscq, 1929; Grenier, 1944; Manier, 1950; Tuzet and Manier, 1955 (= *Harpella melusinae var. eyziesi nom. nud.*); Manier, 1963, 1969/1970
	Japan	Lichtwardt, 1967; Lichtwardt *et al*., 1987
	Malaysia	Takaoka and Adler, 1997
	New Zealand (Campbell Islands)	Crosby, 1974, 1980; Williams and Lichtwardt, 1990; Lichtwardt and Williams, 1992b
	Norway	White and Lichtwardt, 2004
	UK	Moss, 1970; El-Sherif, 1975 (in Taylor, 1992); Moss and Descals, 1986; Taylor *et al*., 1995, 1996; Rizzo and Pang, 2005
	Spain	Gibral and Santamaria, 1998
	Thailand	Takaoka and Adler, 1997
	USA: AK, AR, AZ, CA, CO, GA, KS, MN, MT, NC, NV, NY, SC, TN, UT, WY	Chapman, 1966; Lichtwardt, 1967, 1972, 1984; Williams and Lichtwardt, 1971; Reichle and Lichtwardt, 1972; Moss and Lichtwardt, 1977; Lichtwardt and Williams, 1988; Labeyrie *et al*., 1996; Slaymaker, 1998; Beard and Adler, 2000, 2002, 2003; Beard, 2002; Cafaro, 2002[3], 2003[3]; White, 2002; Beard *et al*., 2003; Reeves, 2003b; Adler *et al*., 2004; Kim and Adler, 2005; White *et al*., 2006
Harpella meridianalis Lichtwardt & Arenas 1996	Argentina	Lichtwardt *et al*., 1999, 2000; White, 2002
	Chile	Lichtwardt and Arenas, 1996
Harpella tica Lichtwardt, 1997	Argentina	Lichtwardt *et al*., 2000
	Costa Rica	Lichtwardt, 1997
	USA: Puerto Rico	White *et al*., 2000, White, 2002

Table 2 continued. Geographical distribution of *Harpellales* colonizing larval black flies worldwide.

Harpellid fungus[1]	Distribution[2]	Reference
Pennella angustispora Lichtwardt 1972	Armenia	Nelder *et al*., 2005a
	Japan	Lichtwardt *et al*., 1987, Sato, 2002
	Spain	Valle, 2004
	USA: CA, CO, UT, WY	Lichtwardt, 1972; Mayfield and Lichtwardt, 1980; Cafaro, 2002[3], 2003[3]
Pennella arctica Williams & Lichtwardt, 1984	Norway	White and Lichtwardt, 2004
	Sweden	Lichtwardt, 1984
	USA: MT	Lichtwardt, 1984
Pennella asymetrica Williams & Lichtwardt, 1990	New Zealand	Williams and Lichtwardt, 1990, Lichtwardt and Williams, 1992b
Pennella grassei Manier, 1968	France	Tuzet and Manier, 1955; Manier, 1963, 1968, 1969/1970
Pennella hovassi Manier, 1968	Armenia	Nelder *et al*., 2005a
	Canada	Frost and Manier, 1971
	France	Manier, 1963, 1968; Peterson and Lichtwardt, 1987
	USA: SC	Beard and Adler, 2002 (*Pennella* near *hovassi*)
Pennella montana Lichtwardt, 1997	Argentina	Lichtwardt *et al*., 2000
	Costa Rica	Lichtwardt, 1997
Pennella simulii Williams & Lichtwardt, 1971	Canada	Lichtwardt *et al*., 2001b, White, 2002, Kim and Adler, 2005
	Costa Rica	Lichtwardt, 1997
	UK	Moss and Descals, 1986
	USA: SC, NC, TN, WY	Williams and Lichtwardt, 1971, Beard and Adler, 2000, Reeves, 2003b, Kim and Adler, 2005, White *et al*., 2006
Simuliomyces microsporus Lichtwardt, 1972	Argentina	Lichtwardt *et al*., 1999, 2000
	Armenia	Nelder *et al*., 2005a
	Australia	Lichtwardt and Williams, 1990; Lichtwardt and Williams, 1992a, 1992c
	Canada	Lichtwardt *et al*., 2001b, Kim and Adler, 2005
	Chile	Lichtwardt and Arenas, 1996
	Costa Rica	Lichtwardt, 1997, 2000
	France	Manier, 1955 (= *Stipella vigilans,* misidentified)
	Norway	White and Lichtwardt, 2004

Table 2 continued. Geographical distribution of *Harpellales* colonizing larval black flies worldwide.

Harpellid fungus[1]	Distribution[2]	Reference
	Spain	Gibral and Santamaria, 1998
	UK	Ingold, 1967; Moss, 1970 (= *Smittium* sp. ?), Moss and Descals, 1986, Taylor *et al*., 1995
	USA: AL, AR, CA, CO, KS, MO, NC, NY, OK, SC, TN, UT, WY	Lichtwardt 1972, 1984; Lichtwardt and Williams 1988; Labeyrie *et al*. 1996; Beard and Adler, 2002; Cafaro, 2002[3], 2003[3]; Nelder, 2003; Reeves, 2003b; Kim and Adler, 2005; White *et al*., 2006
Smittium aciculare Lichtwardt, 1990	Australia	Lichtwardt and Williams, 1990
	Brazil	Alencar *et al*. 2003
Smittium annulatum Lichtwardt, 1997	Costa Rica	Lichtwardt, 1997, Gottlieb and Lichtwardt, 2001
Smittium brasiliense Alencar, Lichtwardt, Ríos-Velásquez & Hamada, 2003	Brazil	Alencar *et al*., 2003
Smittium colaradense Lichtwardt & Williams, 1987	USA: CO	Williams and Lichtwardt, 1987, Lichtwardt and Williams, 1988
Smittium culicis Manier, 1970	Australia	Lichtwardt and Williams, 1990, 1992c
	Canada	Preisner, 1973; El-Buni and Lichtwardt, 1976a,b; Starr *et al*., 1979; Horn, 1989a; Grigg, 1994; Gottlieb and Lichtwardt, 2001
	Costa Rica	Grigg and Lichtwardt, 1996
	France	Manier, 1969/1970
	New Zealand	Williams and Lichtwardt, 1990
	USA: AR, KS, MO, NY, OK	Williams *et al*., 1982 (state not given); Horn, 1989a; Labeyrie *et al*., 1996; Cafaro, 2002[3], 2003[3]
Smittium culicisoides Lichtwardt, 1997	Costa Rica	Lichtwardt, 1997
	Crozet Islands	Reeves *et al*., 2004
Smittium culisetae Lichtwardt, 1964	USA: AR, KS, MO, NC, OK, SC, TN	Williams *et al*., 1982 (state not given); Horn, 1989a,b; Grigg, 1994; Grigg and Lichtwardt 1996; Beard and Adler, 2000, 2002; Gottlieb and Lichtwardt, 2001; Cafaro, 2002[3], 2003[3]; White, 2002; McCreadie and Beard, 2003; Reeves, 2003b; McCreadie *et al*., 2005; Nelder *et al*., 2005b

Table 2 continued. Geographical distribution of *Harpellales* colonizing larval black flies worldwide.

Harpellid fungus[1]	Distribution[2]	Reference
Smittium dipterorum Lichtwardt, 1997	Costa Rica	Lichtwardt, 1997, White, 2002, Gottlieb and Lichtwardt, 2001
	Spain	Valle and Santamaria, 2004
Smittium imitatum Lichtwardt & Arenas, 1996	Chile	Lichtwardt and Arenas, 1996, Gottlieb and Lichtwardt, 2001
Smittium megazygosporum Manier & Coste, 1971	Canada	Kim and Adler, 2005
	USA: SC	Beard and Adler, 2000, 2002; Gottlieb and Lichtwardt, 2001; Nelder and McCreadie, 2003; Nelder *et al*., 2005b
Smittium morbosum Sweeney	Armenia	Nelder *et al*., 2005a
*Smittium pennelli*Lichtwardt, 1984	USA: CO, MT	Lichtwardt, 1984, Lichtwardt and Williams, 1988
Smittium simulii Lichtwardt, 1964	Australia	Lichtwardt and Williams, 1990
	Argentina	Lichtwardt *et al*., 2000
	Chile	Lichtwardt and Arenas, 1996
	France	Manier, 1963
	Japan	Preisner, 1973; El-Buni and Lichtwardt, 1976a,b; Moss and Lichtwardt, 1976; Starr *et al*., 1979; Lichtwardt *et al*., 1987; Horn, 1989a; Grigg, 1994; Grigg and Lichtwardt, 1996; Gottlieb and Lichtwardt, 2001
	New Zealand	Williams and Lichtwardt, 1990; Lichtwardt and Williams, 1992b
	Norway	White and Lichtwardt, 2004
	Spain	Gibral and Santamaria 1998, Valle and Santamaria, 2004
	UK	Moss in Crosskey, 1990
	USA: AL, AR, CA, CO, KS, MO, NY, OK	Lichtwardt, 1964; Sanger *et al*., 1972; El-Buni and Lichtwardt, 1976a,b; Lichtwardt *et al*., 1987; Peterson and Lichtwardt 1987; Lichtwardt and Williams 1988; Horn 1989a; Grigg 1994; Grigg and Lichtwardt 1996; Horn and Lichtwardt 1996; Labeyrie *et al*. 1996; Gottlieb and Lichtwardt 2001; Cafaro 2002[3], 2003[3]; Nelder 2003
Smittium tronadorium Lichtwardt, Ferrington & López Lastra	Armenia	Nelder *et al*., 2005a
Stachylina litoralis Lichtwardt, White & Colbo	Crozet Islands	Reeves *et al*., 2004

Table 2 continued. Geographical distribution of *Harpellales* colonizing larval black flies worldwide.

Harpellid fungus[1]	Distribution[2]	Reference
Stipella vigilans Léger & Gauthier, 1932	Armenia	Nelder *et al*., 2005a
	France	Léger and Gauthier, 1932; Manier, 1950; Manier, 1955b (identified as *Simuliomyces* sp.?); Tuzet and Manier, 1955; Chadefaud and Emberger, 1960; Manier 1963, 1969/1970
	Spain	Valle, 2004
	UK	Moss, 1970, El-Sherif, 1975 (in Taylor, 1992)

[1]Only those *Harpellales* with valid published names and identified to species are included. Published works mentioning isolates of *Harpellales* are included if they reference the isolation from a simuliid host. Dogma (1975) reported the presence of *Enterobryus* sp. (Trichomycetes: *Eccrinales*: *Eccinaceae*) from *Simulium* sp. larvae in the Philippines; however, the fungus is likely a member of the *Harpellales*, based on the presence of "diamond-shaped" zygospores, which are found only in the *Harpellales*.

[2] Reports from USA indicated by states; all others reported by country.

[3] The author did not specifically mention from which state(s) (AR, KS, MO, OK) the material was collected.

Table 3. Zoogeographical distribution of *Harpellales* (arranged alphabetically) colonizing larval black flies; the Afrotropical Region was excluded because no published reports are available for the region.

Harpellales[1]	**Austral-asian**	**Nearctic**	**Neo-tropical**	**Oceanic**	**Oriental**	**Pale-arctic**
Undescribed species[2]		+				
Genistellospora guanacastensis			+[3]			
Genistellospora homothallica		+	+			+
Genistellspora nubila			+			
Genistellospora tepidaria			+			
Genistellopsora tropicalis			+			
Graminelloidae biconica			+			
Harpella amazonica			+			
Harpella leptosa		+				
Harpella melusinae	+	+			+	+
Harpella meridianalis			+			
Harpella tica			+			
Pennella angustispora		+				+
Pennella arctica		+				+
Pennella asymetrica	+					
Pennella grassei						+
Pennella hovassi		+				+
Pennella montana			+			
Pennella simulii		+	+			+
Simuliomyces microsporus	+	+	+			+
Smittium aciculare	+		+			
Smittium annulatum			+			
Smittium brasiliense		+	+			
Smittium colaradense		+				
Smittium culicis	+	+	+			+
Smittium culicisoides			+	+		
Smittium culisetae		+				
Smittium dipterorum			+			
Smittium imitatum			+			
Smittium megazygosporum		+				
Smittium morbosum						+
Smittium pennelli		+				
Smittium simulii	+	+	+			+
Smittium tronadorium						+
Stachylina litoralis				+		
Stipella vigilans						+
Total Species Richness	**6**	**16**	**20**	**2**	**1**	**13**

[1] Only simuliid-associated Harpellales are included; Harpellales from other host taxa from other regions are not included, [2] White *et al*., in press, [3] + = present.

Fig. 12. Areas of the world where black flies have been examined for *Harpellales*. Solid squares = published records; open squares = unpublished records of the authors; some squares represent multiple studies and are not representative of exact locations.

sampling efforts, which have been largely in the USA, Central America (Costa Rica, Lichtwardt, 1997), South America (Argentina, Lichtwardt *et al*., 2000; Brazil, Alencar *et al*., 2003; Chile, Lichtwardt and Arenas, 1996), and western Europe (England, Taylor *et al*., 1995; France, Manier, 1963) (Fig. 12). Large geographic areas, including Africa, Canada, many oceanic islands, and the Oriental and Palearctic Regions, remain poorly explored for *Harpellales*.

Areas of similar size with high species richness of *Harpellales* in black flies include Costa Rica (13 species, Lichtwardt, 1997); Alabama, USA (9 species, Nelder, 2003); and Armenia (8 species, Nelder *et al*., 2005a). Areas with apparently low richness are Japan (3 species, Lichtwardt *et al*., 2001a) and Norway (4 species, White and Lichtwardt, 2004). Although the Caucasus Region of Armenia is noted for high levels of endemic black flies, the gut symbiotes are typically widespread species (Nelder *et al*., 2005a). Australia and New Zealand have high endemicity of black flies but low endemicity of *Harpellales* (Lichtwardt and Williams, 1990; Williams and Lichtwardt, 1990). The lack of endemic gut symbiotes in larval black flies might reflect their lack of host specificity; an absence of host specificity would not constrain the geographic distribution of these symbiotes (Nelder *et al*., 2005a).

The distributions of widespread trichomycetes such as *Harpella melusinae* and *Simuliomyces microsporus* likely are limited only by the dispersal of adult black flies and the presence of suitable larval host habitats. However, *Simuliomyces microsporus* is closely associated with other symbiotes (i.e., *Paramoebidium* spp., *Pennella* spp., and *Genistellospora* spp.) to which they often attach their holdfast-like structures. Because some species of *Smittium* can colonize several host families in various habitat types, their dispersal does not depend on a single host family; the geographic range of these species, therefore, is potentially greater (Nelder *et al*., 2005b).

Taxonomy, classification, and evolutionary relationships

Classification and phylogeny of Harpellales

The class Trichomycetes is a member of a larger clade of *Zygomycota* that includes the *Dimargaritales*, *Kickxellales*, and possibly *Zoopagales* (Benny and White, 2001; Tanabe *et al*., 2004). The class Trichomycetes contains two fungal orders, the *Asellariales* and *Harpellales* (Benny and White, 2001; Cafaro, 2005). The *Asellariales* inhabit isopods (Isopoda) and springtails (Collembola) in terrestrial, freshwater, and intertidal habitats (Lichtwardt, 1986). They are not known from black flies or other aquatic insects, with the exception of a possible *Asellariales* record from one larva of *Simulium ubiquitum* Adler, Currie & Wood in Alabama (Nelder, 2003). The

order *Harpellales* comprises two families, the *Harpellaceae* and *Legeriomycetaceae*; however, molecular evidence suggests that these two families are not in separate clades (White, 2002). The *Harpellaceae* contain five genera, two of which (*Harpella* and rarely *Stachylina*) are found in black flies. The *Legeriomycetaceae* comprises 30 genera, seven of which (*Genistellospora, Graminelloides*, *Pennella*, *Simuliomyces*, *Smittium*, *Stipella*, and one undescribed genus (White *et al*., in press)) are found in black flies. The genera *Genistellospora*, *Graminelloides*, *Harpella*, *Pennella*, *Simuliomyces, Stipella*, and the undescribed genus are found exclusively in larval black flies, whereas *Smittium* and *Stachylina* are found in other host taxa.

Morphological and molecular phylogenies within and among genera of the *Harpellales* are not congruent (Gottlieb and Lichtwardt, 2001; White, 2002). Phylogenetic relationships within the *Harpellales* have yet to be fully elucidated (Lichtwardt *et al*., 2003). The genus *Smittium*, for example, appears to be polyphyletic. More molecular work, such as that of White (2002), who used the gene sequences of 18S and 28S ribosomal DNA (rDNA) to infer a phylogeny of the *Harpellales*, is needed to resolve the evolutionary relationships within the *Harpellales*.

Taxonomy of the Harpellales

Morphological characters used in trichomycete taxonomy include those of the trichospore, zygospore, generative cell, and holdfast, along with thallial growth form. Some characters routinely used to define species of *Harpellales* are subject to environmental influence. For example, the size of trichospores of species such as *Smittium megazygosporum* Manier & Coste varies with host and medium (Beard and Adler, 2000); trichospores are significantly longer and wider in broth culture and in larval chironomids than in larvae of *Simulium innoxium* Comstock & Comstock. This finding is significant because species descriptions of *Harpellales* often rely on trichospore sizes from a single species, genus, or family of host. Trichospore dimensions of *Harpella* species, for example, change dramatically with the method of fixation (Kim, 2005). We suggest that when new species are described, descriptions and, especially measurements, be made of both fresh and fixed (or stained) material.

Preliminary efforts to distinguish species of *Harpellales* molecularly have used 5S rRNA (Walker, 1984), 18S rDNA (O'Donnell *et al*., 1998; Rizzo and Pang, 2005b), ITS and 18S rDNA (Gottlieb and Lichtwardt, 2001), and both 18S and 28S rDNA (White, 2002). Molecular techniques also offer promise for revealing hidden biodiversity (i.e., cryptic species) of *Harpellales* (White, 2002). Some species analyzed molecularly (e.g., *Smittium culisetae*) are composed of geographically and ecologically distinct populations,

suggesting cryptic species (Peterson and Lichtwardt, 1987; Gottlieb and Lichtwardt, 2001). *Harpella melusinae* is probably a complex of cryptic species (Adler *et al*., 1996).

All diagnostic and developmental stages of a fungus are seldom present in a single dissected host and identification of *Harpellales* by host association alone is poor practice (Lichtwardt, 1986). Therefore, trichomycetes from other hosts should be considered when identifying specimens from black flies.

Axenic culturing is not possible for most species of *Harpellales*, making identification and description challenging. The investigator must rely on slide preparations, photomicrographs, and detailed notes and drawings of representative fungal specimens. Slides treated with lactophenol and cotton blue are not permanent under most situations and do not provide long-term voucher or type specimens. Photomicrographs on high-quality (i.e., acid-free) paper and digital images on compact discs can be used in conjunction with semipermanent slides as a means of documenting specimens.

Key to Harpellales colonizing larval black flies

A morphologically based dichotomous key to the *Harpellales* that colonize larval black flies worldwide is presented below. Moss (1981) published the first key to simuliid trichomycetes, including only 9 of the 36 *Harpellales* species now known from black flies. The following key, compiled from original descriptions and the authors' experience, follows the terminology of Lichtwardt (1986) and Lichtwardt *et al*. (2001a).

The key pertains to fungal material from freshly killed larval black flies. It is useful only for sporulating thalli with mature trichospores (i.e., recently detached propagules or those near ready to detach) or zygospores. Thalli lacking trichospores are generally insufficient for species identification, even when thallial form or holdfast shape matches a described taxon. In these cases, identifications often can be made to the family or generic level. Only trichomycete species occurring naturally in larval black flies (i.e., not laboratory induced) are included in the key. In the laboratory, several species of *Smittium* isolated from non-simuliid hosts colonize and sporulate in larval black flies (Beard and Adler, 2003; Nelder *et al*., 2005b). All *Smittium* species, therefore, should be considered in making identifications. Other keys for identifying *Harpellales* are those of Lichtwardt (1973, 1986, 1997), Moss (1981), Lichtwardt and Arenas (1996), Lichtwardt *et al*. (1999), Misra and Lichtwardt (2000), Lichtwardt *et al*. (2001a), Valle and Santamaria (2004), and Ferrington *et al*. (2005).

Key to *Harpellales*

1. Thallus unbranched, attached to peritrophic matrix or rarely to anterior of hindgut cuticle (Family *Harpellaceae*) 2
1. Thallus branched, attached to hindgut cuticle, to other *Harpellales* (i.e., *Pennella* spp. or *Genistellospora* spp.), or to *Paramoebidium* spp. (Family *Legeriomycetaceae*).....7

2. Trichospore cylindrical, often curled or coiled (Fig. 13), sometimes straight (Fig. 14), with 4 (rarely 2 or 3) appendages (Genus *Harpella*) 3
2. Trichospore ovoid, straight, with 1 appendage (Genus *Stachylina*) .....***Stachylina litoralis***

3. Thallus twice as wide as trichospore .....***Harpella tica***
3. Thallus as wide as or narrower than trichospore 4

4. Basal cell tapered. Trichospore greater than or equal to 110 µm long 5
4. Basal cell rounded. Trichospore less than or equal to 100 µm long 6

5. Thallus 4-6 µm wide. Trichospore 4.5 µm wide. Basal cell tapered immediately above holdfast ..... ***Harpella leptosa***
5. Thallus 6-10 µm wide. Trichospore 6-10 µm wide. Basal cell tapered gradually above holdfast (Fig. 15) ..... ***Harpella melusinae***

6. Trichospore 80-100 µm long by 4-8 µm wide. Holdfast wider than thallus..... ..... ***Harpella meridianalis***
6. Trichospore 33-52 µm long by 3-4 µm wide. Holdfast narrower than thallus .... ***Harpella amazonica***

7. Trichospore with collar (Fig. 4) (Genus *Smittium*).....8
7. Trichospore without collar (Fig. 16).....21

8. Trichospore greater than 24 µm long.....9
8. Trichospore less than or equal to 24 µm long.....12

9. Trichospore greater than or equal to 6 µm wide.....10
9. Trichospore less than or equal to 5 µm wide.....11

10. Holdfast and basal thallus region covered by mucilaginous sheath..... ***Smittium pennelli***
10. Holdfast and basal thallus region not covered by mucilaginous sheath..... .....***Smittium coloradense***

11. Trichospore less than or equal to 30 µm long.....12
11. Trichospore greater than or equal to 36 µm long.....***Smittium megazygosporum***

12. Trichospore with medial bulge, averaging more than 28 µm long ..... ***Smittium acicular***
12. Trichospore without medial bulge, averaging less than 28 µm long ***Smittium tronadorium***

13. Trichospore 3 µm or less wide 14
13. Trichospore greater than 3 µm wide.....15

14. Holdfast limuloid. Trichospore 1.6 µm wide ..... ***Smittium brasiliense***
14. Holdfast not limuloid. Trichospore 2-3 µm wide ..... ***Smittium dipterorum***

15. Collar less than or equal to 2.5 µm long 16
15. Collar 3 µm or more long 18

16. Trichospore widest proximal to midregion ..... ***Smittium culisetae***
16. Trichospore widest at midregion 17

17. Thalli with compact branching. Known from Chile ..... ***Smittium imitatum***
17. Thalli with branching less compact. Widespread ..... ***Smittium morbosum***

18. Basal cell as wide as or narrower than thallus 19
18. Basal cell wider than thallus 20

19. Generative cells 1-4 ..... ***Smittium culicisoides***
19. Generative cells 4-6 ..... ***Smittium culicis***

20. Holdfast and basal region composed of 6 cells arranged in ring. Trichospore elongate, oval ..... ***Smittium annulatum***
20. Holdfast and basal region arched, wrench-like. Trichospore cylindrical with medial swelling ..... ***Smittium simulii***

21. Holdfast surrounded by mucilaginous sheath (Fig. 17) 22
21. Holdfast not surrounded by mucilaginous sheath 29

22. Trichospore ovoid or obpyriform, with thin appendages. Zygospore parallel to zygosporophore. Widespread (Genus *Pennella*) 23
22. Trichospore cylindrical, with petiolate appendages. Zygospore perpendicular to zygosporophore. Known only from Europe ..... ***Stipella vigilans***

23. Trichospore less than 41 µm long 24
23. Trichospore about 41 µm or more long 25

24. Trichospore obpyriform (Fig. 18), less than 33 µm long, less than or equal to 7 µm wide. Zygospore less than or equal to 80 µm long, 13.6 µm wide ..... ***Pennella hovassi***
24. Trichospore ovoid, greater than or equal to 28 µm long, 7 µm wide. Zygospore greater than or equal to 74 µm long, 16 µm wide ..... ***Pennella simulii***

25. Trichospore ovoid or obpyriform 26
25. Trichospore cylindrical (Fig. 19) 28

26. Trichospore ovoid 27
26. Trichospore obpyriform ..... ***Pennella arctica***

27. Holdfast pointed. Trichospore about 42 µm long by 14 µm wide ..... ***Pennella asymetrica***
27. Holdfast bifurcated (Fig. 20). Trichospore about 60 µm long by 8-19 µm wide (Fig. 21) ..... ***Pennella montana***

28. Trichospore about 80 µm long, 4 µm wide.....***Pennella angustispora***
28. Trichospore less than or equal to 59 µm long, greater than 4.5 µm wide.. ***Pennella grassei***

29. Holdfast C shaped, with refractive material (Fig. 22). Trichospore ovoid or obpyriform (Genus *Genistellospora*).....30
29. Holdfast pointed or not C shaped; if C shaped, without refractive material. Trichospore biconical or cylindrical.....34

30. Trichospore about 38 µm or less long 31
30. Trichospore greater than or equal to 39 µm long.....33

31. Zygospore 50-70 µm long, less than or equal to 15 µm wide.....32
31. Zygospore about 100 µm long, 20 µm wide (Fig. 23)..... ***Genistellospora homothallica***

32. Trichospore greater than or equal to 9 µm wide..... ***Genistellospora tepidaria***
32. Trichospore less than or equal to 8 µm wide.....***Genistellospora tropicalis***

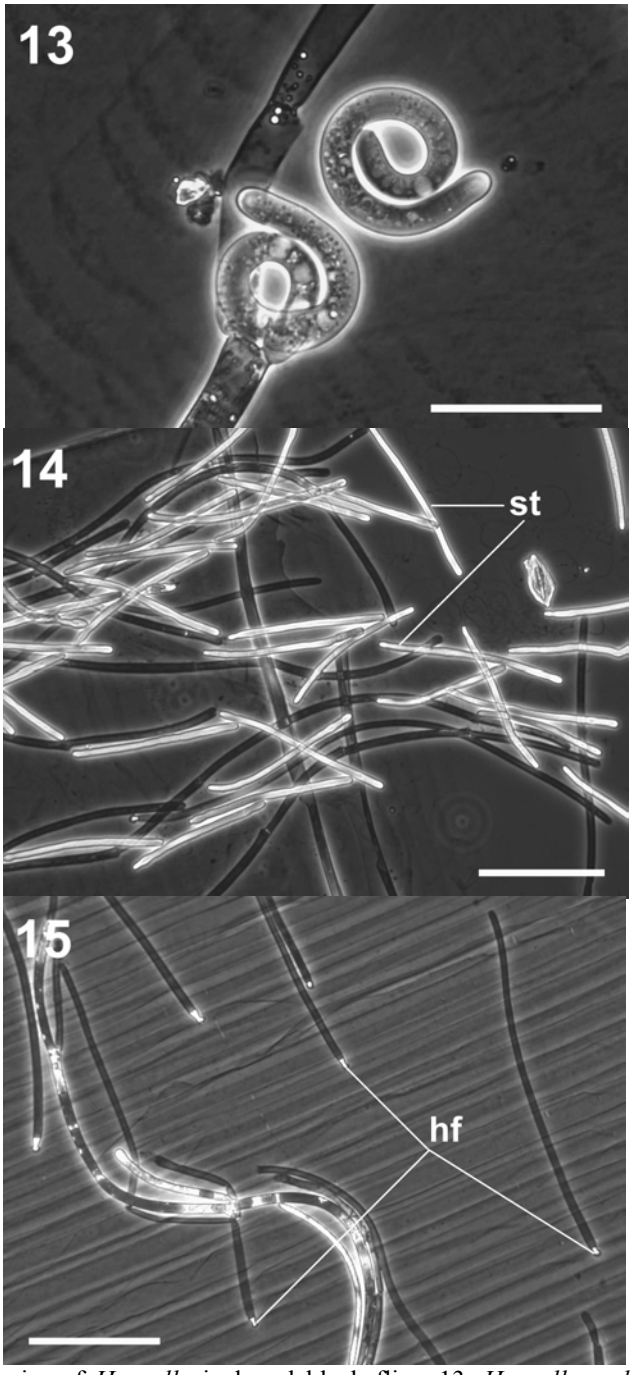
33. Trichospore greater than 50 µm long. Thallus less than 250 µm long..... ***Genistellospora guanacastensis***
33. Trichospore less than 41 µm long. Thallus greater than 400 µm long..... ***Genistellospora nubila***

34. Trichospore typically with terminal cap or fine filaments..... ***Undescribed genus and species* (White *et al*., in press)**
34. Trichospore without terminal cap or fine filaments.....35

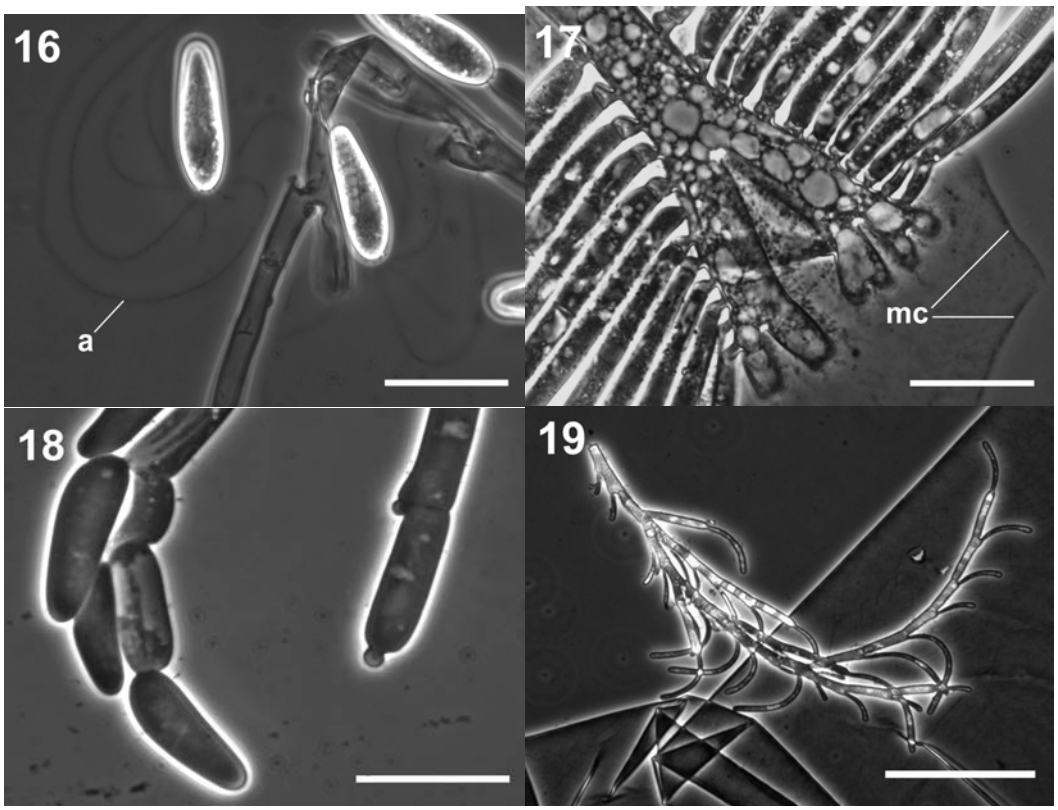
35. Trichospore biconical. Thallus attached to hindgut cuticle ..... ***Graminelloides biconica***
35. Trichospore cylindrical (Fig. 24). Thallus typically attached to *Paramoebidium* spp. (Fig. 25), *Genistellospora* spp., *Pennella* spp. (rarely hindgut cuticle)..... ***Simuliomyces microsporus***

Future directions and questions

Since the discovery of trichomycetes in black flies in 1929, this relationship has fascinated scientists and led to a better understanding of symbioses in aquatic systems. Most studies of trichomycetes have focused on taxonomy, culturing, host records, life cycles, and geographic distributions. Little is known of the community structure of trichomycetes in black flies or other hosts. A goal of future studies of trichomycete ecology, therefore, should be the estimation of basic community parameters such as species richness, diversity, dominance, species turnover, and rank abundance distributions. Characterizing ecological patterns can illuminate the underlying mechanisms responsible for these patterns. Future research also should consider how these parameters vary across both temporal and spatial scales. For example, what is the relationship between local trichomycete species richness (e.g., in a stream)



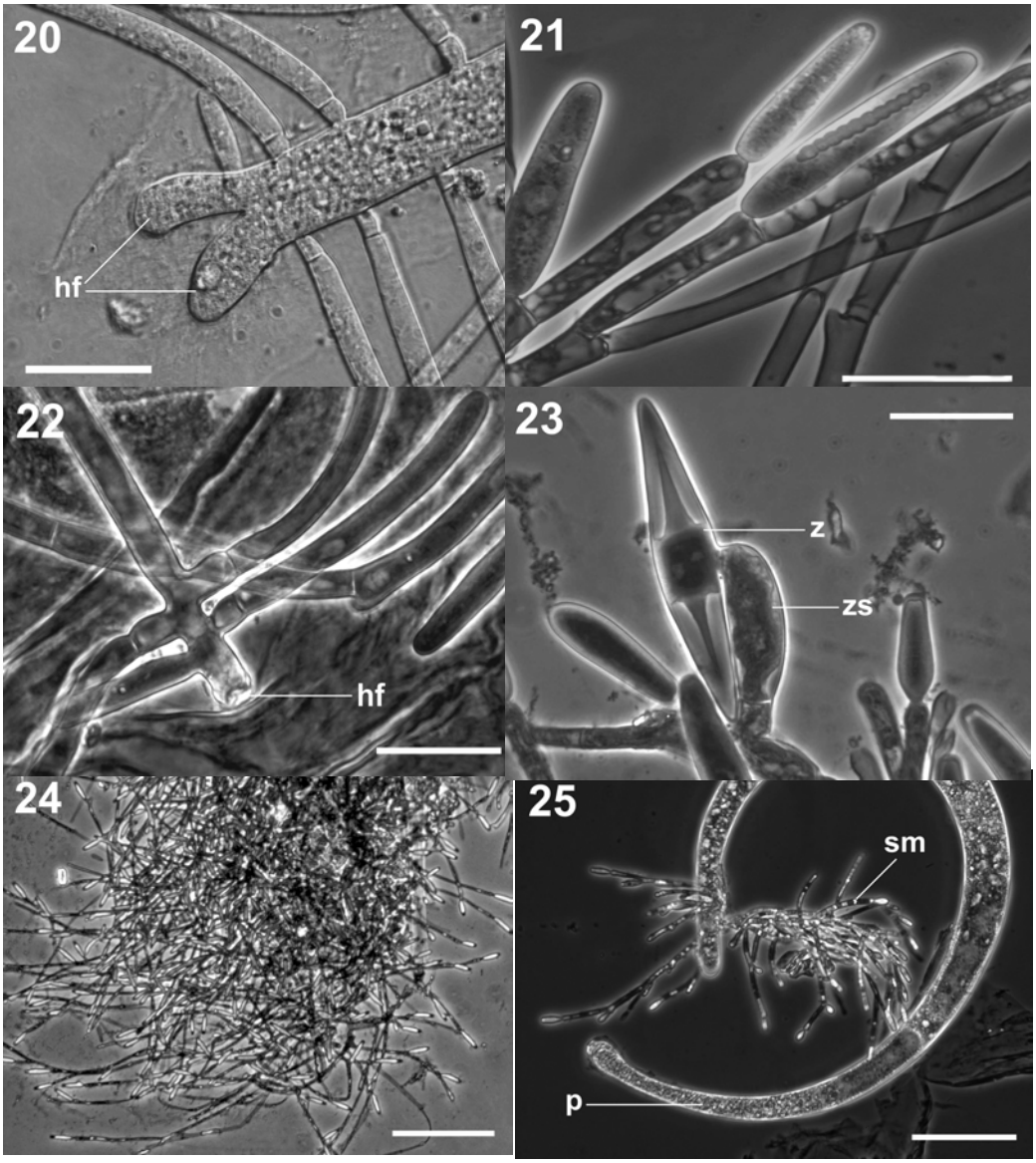
Figs. 13-15. Species of *Harpella* in larval black flies. 13. *Harpella melusinae* in *Simulium tuberosum*. Bar = 30 µm. 14. *Harpella* sp. in *Simulium innoxium*; st = straight trichospores. Bar = 100 µm. 15. *Harpella melusinae* in *Simulium innoxium*; hf = holdfasts. Bar = 100 µm.



Figs. 16-19. *Genistellospora* and *Pennella* species in larval black flies. 16. *Genistellospora homthallica* trichospores in hindgut of *Simulium tuberosum*; a = appendage. Bar = 30 µm. 17. *Pennella hovassi* holdfast in hindgut of *Simulium tuberosum*; mc = mucilaginous covering. Bar = 30 µm. 18. *Pennella hovassi* trichospores in hindgut of *Simulium tuberosum*. Bar = 30 µm. 19. *Pennella* sp. in hindgut of *Simulium tribulatum*. Bar = 100 µm.

and regional richness (e.g., in an ecoregion)? Does local richness depend on regional species richness in a simple linear relationship? Does local richness reach an asymptote in which further increases in regional richness are not matched by increases in local richness?

Ecological studies of trichomycetes not only will increase our knowledge about a cosmopolitan group of fungi, but also will add to our understanding of symbiotic relationships in aquatic habitats. Few general theories exist concerning mutualism (Bronstein, 1998), and May's (1981) plea for increased development of theory has largely been ignored (Ives, 1998). Most studies on mutualism and commensalism have focused on natural history and anecdotal descriptions (Begon *et al*., 1996; Ives, 1998). Given the importance of symbiotic relationships (Sapp, 1994), neglect of the ecological ramifications of these associations will perpetuate a meager understanding of community dynamics.



Figs. 20-25. *Genistellospora, Pennella,* and *Simuliomyces* species in black fly larvae. **20.** *Pennella* sp. in hindgut *of Simulium innoxium*; hf = holdfast. Scale bar = 30 µm. **21.** Trichospores of *Pennella* sp. in hindgut of *Simulium tribulatum*. Scale bar = 30 µm. **22.** *Genistellospora homothallica* in hindgut of *Simulium notiale*, hf = holdfast. Scale bar = 30 µm. **23.** *Genistellospora homothallica* zygospores in the hindgut of *Simulium notiale*, z = zygospores, zs = zygosporophore. Scale bar = 30 µm. **24.** *Simuliomyces microsporus* in the hindgut of *Simulium tuberosum*. Scale bar = 100 µm. **25.** *Simuliomyces microsporus* (sm) attached to *Paramoebidium* sp. (p) in hindgut of *Simulium tuberosum*. Bars = 100 µm.

Reasons for the production of zygospores and the mechanism by which ovarian cysts are produced remain unknown. Why are zygospores produced in some *Harpellales* and not in others? What are the biological cues that induce zygospore production? Does the zygospore represent an environmentally resistant stage of the *Harpellales* life cycle? A deeper understanding of the production of ovarian cysts might explain why some areas of the world have populations of cyst-infected black flies while others apparently do not. From a practical perspective, an increase in our knowledge of ovarian cysts will help determine if they might be used as a possible means of biological control of pestiferous black flies.

An understanding of trichomycete fitness, including spore production, relies partly on a better understanding of the physiology of their habitat (i.e., the host gut). Although the physiology of the mosquito gut is well understood, the same cannot be said for black flies. We do not fully understand the essential elements (e.g., nutritional) that are provided to the simuliid host by the fungi or vice-versa.

A more complete picture of the biodiversity of the gut mycota associated with black flies is needed. Global prospecting for simuliid-associated *Harpellales* should pay dividends. Areas such as Africa and Asia are virtually unexplored. Parallel ecological work in these areas of the world will provide a test of the ecological patterns and hypotheses that are based largely on North American simuliid-trichomycete relationships.

Because the species is the ecological unit of study, the detection of all species in any investigation is critical. New methods for detecting the presence of cryptic (sibling) species of *Harpellales*, therefore, are needed. The rapidly expanding field of molecular biology is likely to afford multiple approaches for discovering and resolving complexes of morphologically similar trichomycete species. Similarly, phylogenetic studies of the *Harpellales* will continue to benefit from molecular investigations, particularly as molecular studies are integrated with other approaches, including classical morphological studies.

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