FULL PAPER

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Secondary spore formation in *Orchesellaria mauguioi* (Asellariales, Trichomycetes) and its taxonomic and ecological implications

Abstract *Orchesellaria (Orc.) mauguioi* (Asellariales) was detected from the hindguts of *Isotomurus palustris* (Collembola, Hexapoda) collected in Japan. The secondary spore formation on the exuviae of its host is described. When the host molted, its exoskelton including the ectoderm-originated hindgut cuticle to which *Orc. mauguioi* mycelia attaches was shed. Arthrospores of *Orc. mauguioi* germinated and penetrated the molt skin and produced secondary spores terminally. The secondary spore is a narrowly ovoid monosporangious sporangium accompanied by a capitate-lageniform terminal cell; these fall off together as a dispersive unit. The terminal cell is sterile and bears one to three fine filamentous extensions seven times as long as the length of the cell. In contrast to the appendages produced endogenously by other harpellids, the filament is produced exogenously and is similar to those of the genus *Orphella* (Harpellales). The taxonomic and ecological implications of the secondary spore formation of *Orc. mauguioi* are discussed by comparison with those of other Trichomycetes.

Key words Asellariales · Collembola · *Orchesellaria* · *Orphella* · Trichomycetes

Introduction

The class Trichomycetes consists of two orders, Harpellales Lichtw. & Manier and Asellariales Manier ex Manier & Lichtw. All members of this class are endosymbionts of arthropods and produce trichospores or arthrospores, and

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zygospores as asexual and sexual propagules. The trichospore is a unique structure of Harpellales, defined as "an exogenous, deciduous, dehiscent sporangium containing a single sporangispore normally having one to several basally attached filamentous appendages" (Lichtwardt 1986; Kirk et al. 2008). The appendages develop between the plasmalemma and cell walls in the generative cells and extend when the trichospores are released from the generative cells. They easily entangle with various external objects and help positive dispersal of trichospores. When the trichospores attaching to debris are fed on by the host arthropods, they can recolonize to the host gut. Asellariales, the other order of the class, is distinguished from Harpellales by producing arthrospores instead of trichospores. The order had been regarded as a kind of an "anamorphic taxon" in Trichomycetes until the recent discovery of the zygospores (Valle and Cafaro 2008).

The phylogenetic position of Asellariales, including its relationship to Harpellales, has been discussed for a long time. Ultrastructural studies revealed that Asellariales has the characteristic septal pores with plugs that are common in Harpellales, Kickxellales, and Dimargaritales (Manier 1973; Moss 1975; Moss and Young 1978; Lichtwardt 1986; Saikawa et al. 1997). A molecular analysis showed the monophyly of this group of fungi, except for Asellariales, for which no molecular data were available at that time (White et al. 2006). Hibbet et al. (2007) placed Asellariales in the subphylum Kickxellomycotina with the aforementioned three orders on the basis of unpublished molecular data. However, no definitive evidence showing the relationship between Asellariales and Harpellales has been found.

The Asellariales consists of the family Asellariaceae Manier ex Manier & Lichtw. including 3 genera (*Orchesellaria* Manier ex Manier & Lichtw., *Asellaria* R.A. Poiss. and *Baltomyces* Cafaro) and 14 species that are fundamentally delimited by host arthropods and the morphology of holdfast and arthrospore (Lichtwardt 1986). *Orchesellaria* lives in the hindguts of terrestrial springtails, while *Asellaria* and *Baltomyces* inhabit those of aquatic or terrestrial isopods. Four currently known *Orchesellaria (Orc.)* species have been found from collembolans inhabiting humid places

(*Agrenia*, *Isotoma*, *Isotomurus*, *Hydroisotomurus*, and *Podura*). In the course of a floristic survey of Japanese zygomycotan flora, *Orc. mauguioi* Manier and its secondary spore was discovered. Here I describe the secondary spore formation and discuss its taxonomic and ecological implications.

Materials and methods

Host collembolans (*Isotomurus palustris*) and their exuviae were collected at five riverside grasslands or marshes in Kanagawa Prefecture, central Japan, in winter 2004. Living individuals were kept in moist chambers with soil or water collected at the sampling sites, using 2×9 cm petri dishes at room temperature (~23°C). Most collembolans were dissected to detect fungi under a stereomicroscope. When a mycelium was detected, the living host collembolans collected from the same population were transferred to a new moist chamber and kept for several weeks for further observation and experiments. When their exuviae were found, these were transferred to a plain agar plate (15 g agar/1 l DW) to observe the secondary growth of the fungus possibly inhabiting there. Slide materials were mounted in 99% lactic acid and stained by aniline blue when needed. Stereo- and differential interference contrast (DIC) microscopy were conducted using an Olympus SZX90 and BX60, and all micrographs were recorded on digital image files (jpeg) using a DP12.

Results

Identification of the fungus

The fungal materials were observed in the hindguts of *I. palustris* (Fig. 1) collected at two localities in Kanagawa Prefecture; at the Sagami River, Hakkei, Shimomizo, Sagamihara (35°31′5″ N, 139°22′32″ E), 3 Jan. 2004, and at a small stream branched from the Hayakawa River, Iryuda, Odawara (35°14′16″ N, 139°7′13″ E), 13 Jan. 2004. More than five in ten collembolans were infected by the fungus at each sampling site. Additional materials were collected repeatedly at the latter site from January to March in 2004.

In the observed materials, thalli made radiating clusters of repeatedly branching branchlets that disarticulate into arthrospores, and the basal cells were bulbous with cap-like holdfasts. The arthrospores were clavate, slightly tapering toward the proximal ends, and $18.0–28.0 \times 4.0–8.0$ μm on average (Fig. 2). All materials were identified as *Orc. mauguioi* based on morphology.

When the dead bodies that floated on the water surface were dissected within a day after their death, some arthrospores attaching to the thallus germinated apically (Fig. 3). Lateral germination from the distal end was also observed on a detached arthrospore (Fig. 4). In spite of various trials, further growth of the germinating arthrospores could not be induced, as described by Lichtwardt and Moss (1984); the germ tubes did not grow in water or on various agar media such as 1/10 brain heart infusion agar, corn meal agar, and 0.3% shrimp agar.

In a field-collected exuvia from Iryuda, spherical spores ~10 μm in diameter were observed among the thallus (Fig. 5). Although their connections to the thallial hyphae could not be traced precisely, the shape and size was almost identical with the chlamydospores of *Orc. mauguioi* illustrated by Lichtwardt and Moss (1984).

Secondary spore formation on exuviae

When the host collembolans molted on the agar surface, the exuviae shrank. However, when they shed their skins on the water surface, the exuviae floated and spread because of the hydrophilic/hydrophobic nature of the inner and outer surfaces of exuviae. Fine straight hyphae developed on several exuviae within 48 h after molting (Figs. 6–8). The hyphae radiate from the cells produced on the hindgut cuticle of the molt (Figs. 6, 7). On the water surface, the hyphae adhered to the setae of the stretched molt all around (Fig. 7). When the host molts, the hindgut-lining cuticle is also shed and cast off with other parts of the exoskeleton. The cells bearing filamentous hyphae were connected to the hyphae from the thallus of *Orc. mauguioi* tightly attached to the hindgut cuticle of the host exuvia.

In a lactic acid-aniline blue-mounted preparation, spore-like cells were entangled with the setae of the exuvia (Fig. 9). Two cells were produced on the tip of a hypha from the thallus (Fig. 10), and in the following section I call these a "secondary spore" and a "terminal cell," respectively. The secondary spore was a thick-walled sporangium, narrowly oval, slightly swollen at the base, and $23.0–25.0 \times 4.0–5.0$ μm. It contained protoplasm that was strongly stained in aniline blue when young and lost stainability and became refractive under DIC when matured. The terminal cell was sterile, capitate-lageniform, tapering toward the apex, $25–37 \times 5–$

Figs. 1–15. *Orchesellaria mauguioi* on a host collembolan, *Isotomurus palustris*. **1** A host collembolan, *I. palustris*. **2** A mycelium detected from a living collembolan. *Arrowhead* indicates a bulbous holdfast. **3** Germinating arthrospores detected from a dead body of a collembolan. **4** A germinating arthrospore. **5** Chlamydospores. **6** An exuvia of a collembolan floated on a water drop. *Arrowhead* indicates a part of hindgut. **7** Magnified micrograph of a hindgut part of Fig. 6, showing filaments of terminal cells sticking to the setae on an exuvia. **8** Exuvia of a collembolan incubated on a plain agar plate, showing secondary spores and undisturbed erected terminal cells. **9** A mass of secondary spores around a part of the hindgut. **10** Magnified micrograph of Fig. 9, showing immature secondary spores with terminal cells produced on hyphal tips. **11** Two filamentous extensions from a terminal cell. **12** A separated disseminating unit consisting of an immature secondary spore (*S*), a terminal cell (*T*) and a filament (*F*). **13** Five disseminated units after separation from hyphal tips. **14** Magnified micrograph of **13** showing mature secondary spores (*S*) in sporangial walls (*W*, *arrowhead*), terminal cells (*T*) and a filament (*F*). **15** Secondary spores detached from sporangial wall (*W*, *arrowhead*) with terminal cells and a filament (*F*). **2, 4** Mounted in lactic acid; **3, 5, 9–15** mounted in lactic acid with aniline blue. *Bars* **1** 1 mm; **2–5, 7, 9–15** 50 μm; **6** 0.5 mm; **8** 100 μm

hindgut

furcula

ventral tube

legs

F   T   S

S

W

W

F

T

S

W

F

T
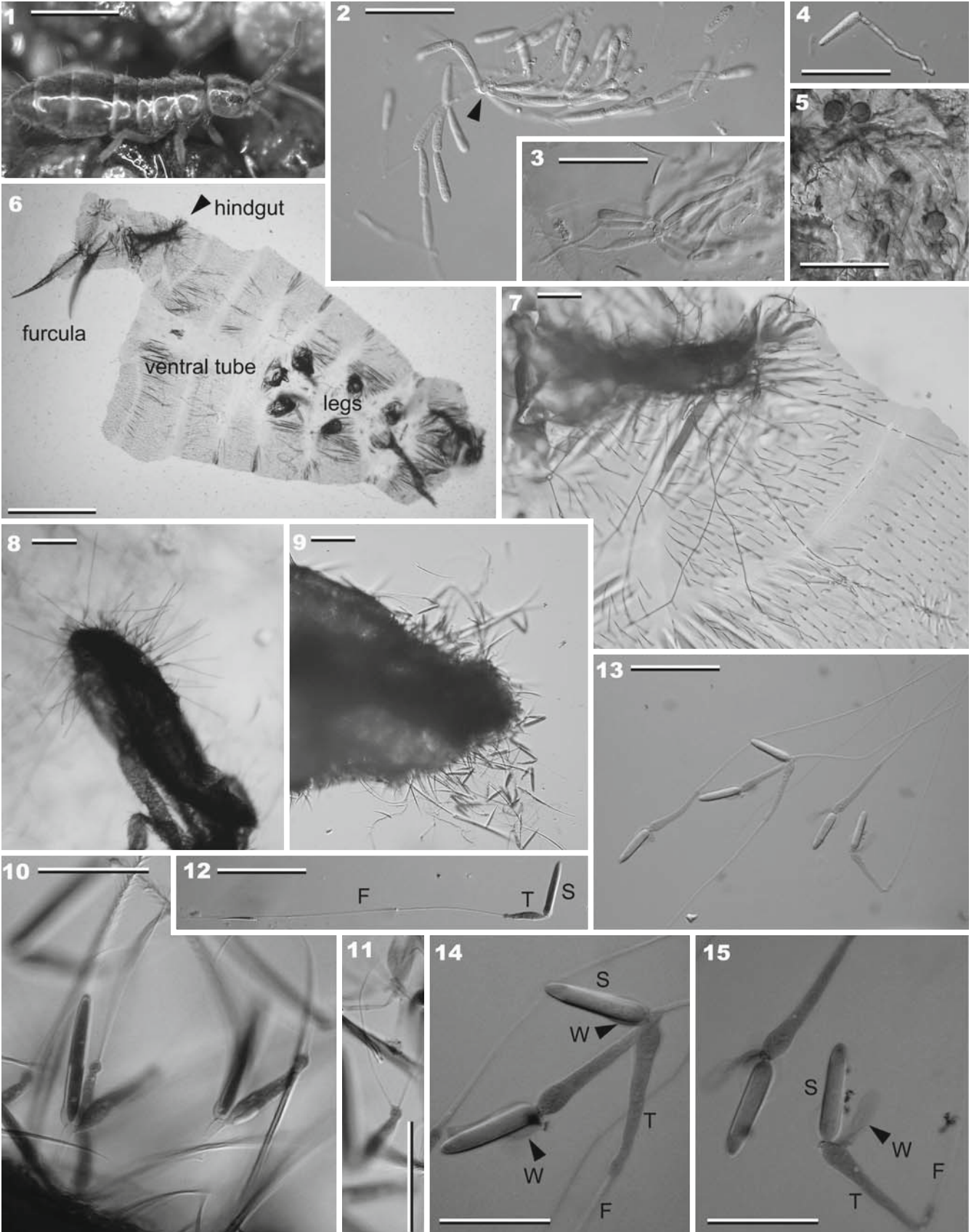
6 μm at the base, 3–4 μm at the tip, with a little spherical head bearing one to three long and filamentous extensions, and not exceeding 200 × 1.0–1.5 μm. (Figs. 11, 12). It was stained blue but lost protoplasm when mature. The secondary spore and the terminal cell fall off as a disseminating unit (Figs. 13–15), from a short basal hypha born on the surface of the exuvia.

Discussion

This is the first record of *Orchesellaria* including *Orc. mauguioi* from Asia. *Orchesellaria mauguioi* is the most common and the most widely distributed species of the genus (Lichtwardt and Moss 1984; Valle 2006) and has been found from Europe (France, Spain) and the United States (Washington, Montana) (Manier 1964, 1969). Other species of the genus have been found from France (*Orc. lattesi* Manier ex Manier & Lichtw. and *Orc. podurae* Manier) or the United States (*Orc. pelta* Lichtw.) (Manier 1958, 1979; Manier and Lichtwardt 1968; Lichtwardt and Moss 1984), except for an undetermined species found from the Dominican Republic (Valle and Cafaro 2008).

Although Asellariales including *Orchesellaria* essentially reproduce by arthrospores, some produce other kinds of spores. *Orchesellaria pelta* exceptionally produces "specialized cells" or "single filiform refractive deciduous cells" found only in matured thalli (Lichtwardt and Moss 1984); this may be a kind of "spore," as it is produced singly and is terminal and morphologically distinct from vegetative cells. *Baltomyces* has a "cylindrical spore" in a long fusiform sporangium (Cafaro 1999), and this unusual sporangium and the absence of holdfast suggest that *Baltomyces* is quite different from other members in Asellariales (Cafaro 1999). Actually, the affiliation of the genus in Asellariales is not fully established (Misra and Lichtwardt 2000; Lichtwardt et al. 2001; White et al. 2006; Kirk et al. 2008).

The "secondary spores" of *Orc. mauguioi* are different from these spores of other Asellariales in overall morphology and formation process. This spore rather reminds us of the germinated arthrospores of *A. ligiae* reported by Manier (1963), Lichtwardt (1973), and Valle (2006). The arthrospore of *A. ligiae* germinates in vitro (in water or on media) to produce an exogenous determinate-sized cell. This elongate cell appears to be thick walled (fig. 6 in Lichtwardt 1973) and has a similar appearance to the secondary spore of *Orc. mauguioi*. However, the germinated cell of *A. ligiae* does not detach from the vacuolated arthrospore. Lichtwardt (1973) noted its close resemblance to the dispersive unit (a generative cell and a trichospore) in *Carouxella scalaris* (Harpellales), whose position is warranted by its zygospores.

In addition to *Carouxella*, several other genera of Harpellales (*Bojamyces*, *Caudomyces*, *Gauthieromyces*, *Orphella*, and *Zygopolaris*) produce "trichospores," which lack obvious appendages (Lichtwardt et al. 2001) (Table 1). Among them, the trichospores of three *Orphella* species (*Orp. hiemalis* Peterson, Lichtw. & Huss, *Orp. catalaunica* Santamaria & Girbal, and *Orp. avalonensis* M. M. White, Lichtw. & Colbo) resemble the "secondary spore" of *Orc. mauguioi* in having accompanying exogenously produced long filamentous cells, which are called "terminal cells" in *Orphella*. Zygospores also accompany the long filamentous

Table 1. Hosts, habitats, and spore morphologies of the representative genera of Trichomycetes

Genus	Host arthropod	Habit of host arthropod	Asexual spore:		Sexual spore
			Existence of spore modification	Morphology of spore modification (appendage or filament)	Morphology of zygospore
Asellariales			Arthrospore		
Asellaria	Isopoda	Terrestrial/aquatic	–		Spherical
Baltomyces	Isopoda	Aquatic	–		?
Orchesellaria	Collembola	Terrestrial	(+)[a]	Exogenous filament on terminal cell	? (Spherical chlamydospore[c])
Harpellales			Trichospore		
Orphella	Plecoptera	Aquatic	+	Exogenous filamentous terminal cell	Hericoidal (with exogenous filamentous terminal cell)
Caudomyces	Diptera	Aquatic	+	Exogenous extension of generative cell	?
Carouxella	Diptera	Aquatic	–		Hydrodynamic conical
Bojamyces	Ephemeroptera	Aquatic	–/+[b]	Endogenous appendage	Hydrodynamic biconical
Gauthieromyces	Ephemeroptera	Aquatic	–/+	Endogenous appendage	?
Zygopolaris	Ephemeroptera	Aquatic	–/+	Endogenous appendage	Hydrodynamic conical
Trichozygospora	Diptera	Aquatic	+	Endogenous appendage	Hydrodynamic biconical (with endogenous appendage)
Legerioides	Isopoda	Aquatic	+	Endogenous appendage	Hydrodynamic biconical
Other genera	Ephemeroptera Plecoptera Diptera	Aquatic	+	Endogenous appendage	Hydrodynamic biconical

[a] Secondary spores with modification
[b] Within the genus, some species have and some species lack modification on trichospores
[c] At the present, regarded as an asexual spore

terminal cells in some species (*Orp. coronata* Léger & Gauthier, *Orp. helicospora* Santamaria & Girbal, *Orp. dalhousiensis* Strongman & M. M. White) (Valle and Santamaria 2005; Strongman and White 2006). The general appendages in Harpellales are endogenously produced between the cell wall and plasmalemma in the generative cell (Lichtwardt 1986), and they are much more delicate than the "filaments" of *Orphella* and *Orchesellaria* (~1–2 µm in diameter). The exogenous "filaments" of the terminal cells and the endogenous typical harpellalean appendages might have similar functions for dispersal, although their formation processes are distinctly different. In *Orc. mauguioi*, the "secondary spore" detaches its surrounding wall connected to the wall of the terminal cell. These two cells are produced at the tip of a hypha and disarticulated as a unit. In *Orphella*, however, a generative cell, a trichospore, and a filamentous terminal cell fall off together as a dissemination unit. In another harpellid, *Caudomyces*, a generative cell itself elongates to be a long extension attaching to the trichospore, which also function as an appendage (Strongman 2007).

The "secondary spore" of *Orc. mauguioi* was produced on the exuvia of the host. Trichomycetous fungi have to adapt themselves to their hosts' behaviors to live in their guts. As the hindgut lining originates from the ectoderm, it is shed with the exoskeleton cuticle. Thus, the fungus on the hindgut cuticle must overcome the periodic expulsion (Lichtwardt 1986). Many trichomycete species start their sexual reproduction just before confronting the ecdysis of the hosts. An unusual harpellid, *Bojamyces repens* Longcore, produces trichospores only on the exuviae of host ephemeropterans. The absence of appendages is regarded as an adaptation to the host living in a lentic habitat (Longcore 1989). *Isotomurus*, the host of *Orc. mauguioi*, is terrestrial although it prefers humid places. They can skate on the water surface but cannot dive into the water. When their molts are shed on the water surface, the hindgut cuticle turns inside out and the inner side of the gut lining is directed toward the water. The submerged fungus must produce their spores upward. The secondary spore formation in this species can be an adaptation to the terrestrial host. Filaments of the terminal cells help secondary spores sticking on the exuviae or terrestrial organic material on which the host may feed.

The zygospore of *Asellaria jatiboticua* L. G. Valle & Cafaro, which was the first instance of zygospore formation in Asellariales, is spherical, whereas those of Harpellales are hydrodynamic – fusiform or conical (Valle and Cafaro 2008) (see Table 1). The hydrodynamic morphology of zygospores could have no usefulness for the fungus living in terrestrial host guts (Valle and Cafaro 2008). Two species of *Orchesellaria* produce similar spherical spores on thalli, which are regarded as chlamydospores; these are formed intercalarily or terminally in *Orc. mauguioi* and laterally in *Orc. pelta* (Lichtwardt and Moss 1984). Valle and Cafaro (2008) clearly demonstrated the conjugations of the thalli in zygosporogenesis of *A. jatibotiboticua* as proof for the sexual reproduction. Unfortunately, in the present observation on the spherical spores of *Orc. mauguioi*, the precise

formation process could not be observed because of the inadequate condition of the material. Although the spherical spores might be parthenogenetically produced azygospores, we call them chlamydospores until further information on sexual reproduction of the *Orchesellaria* becomes available (Lichtwardt and Moss 1984; Valle and Cafaro 2008).

Recent molecular analysis supports the monophyly of the Pancrustacean, including Hexapoda and Crustacea (Glenner et al. 2006), in which the trichomycetous fungi are distributed. Asellarialean species inhabit in the guts of Isopoda (Malacostraca, Crustacea) and Collembola (Entognatha, Hexapoda). All harpellalean species live in several orders of Insecta (Ectognatha, Hexapoda), except a monotypic genus, *Legerioides*, that lives in Isopoda (White 1999) (see Table 1). The Collembola, the host of *Orchesellara*, is one of the most primitive groups of Hexapoda, positioned between Insecta and the other crustaceans (Regier et al. 2005). If the biconical zygospores and endogenous appendages of Harpellales are derived characters, the Asellariales may be primitive for reasons of their kickxellid-like spherical zygospores and the absence of appendages. The exogenous filaments on the terminal cell of *Orchesellaria* may be a transitional state before the establishment of further sophisticated endogenous appendages in the harpellids. The genus *Orphella*, possessing similar terminal cells, is in a sister relationship with kickxellaleans and distinct from the monophyletic clade of other harpellalean taxa (White 2006; White et al. 2006). The unusual helicoidal zygosporogenesis of *Orphella* is also distinct from that of other harpellalean taxa (Valle and Santamaria 2005). The molecular analysis of Asellariales, including *Orchesellaria*, would reveal the phylogenetic relationships with other taxa, including *Orphella*, and determine whether the exogenous appendage-like filaments of *Orchesellaria* and *Orphella* are homologous.

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