Available online at www.sciencedirect.com

MYCOSCIENCE

ISSN 1340-3540 (print), 1618-2545 (online)

journal homepage: www.elsevier.com/locate/myc

Short communication

Bojamyces repens (Harpellales) from exuviae of mayfly, a new record from Japan

*Hiroki Sato**

Department of Forest Entomology, Forestry and Forest Products Research Institute, Matsunosato 1, Tsukuba, Ibaraki 305-8687, Japan

ARTICLE INFO

Article history:
Received 11 February 2012
Received in revised form
5 September 2012
Accepted 6 September 2012
Available online 10 January 2013

Keywords:
Aquatic insects
Gut fungi
Legeriomycetaceae
Molting
Trichomycetes

ABSTRACT

Bojamyces repens (Harpellales, Legeiomycetaceae) was discovered from exuviae of *Paraleptophrebia* sp. (Ephemeroptera: Leptophlebiidae). Trichosporogenesis required at least 2 days from the accumulation of cytoplasm in generative cells to mature trichospores at 5 °C in exuviae, which were kept in a drop of water on glass slides. Each mature trichospore possessed a structure with different refraction than the cytoplasm and was considered as a spore body, including a holdfast substance for the next infection. This is the first record of this genus in Japan.

Harpellales is a fungal order, which have been recorded in the gut of mainly aquatic insects. Formerly, this order had belonged to one of four orders of the class Trichomycetes (subphylum Zygomycotina; phylum Eumycota) which had been ecologically characterized by inhabiting in guts of Arthropod. Two orders, Amoebidiales and Eccrinales, have been moved to Protozoa (Benny and O'Donnell 2000; Cafaro 2005), and the other Harpellales and Asellariales have been moved to subphylum Kicksellomycotina. Class for Harpellales and Asellariales is now not clear (Hibbett et al. 2007).

Harpellales has more than 200 species and is composed of Harpellaceae (5 genera including *Harpella*) and Legeriomycetaceae (36 genera including *Legeriomyces*) (http://www.nhm.ku.edu/~fungi/Monograph/Text/Mono.htm). Species in Harpellales attach their thalli to the lining of the midgut or hindgut of host insects by a special structure called a holdfast. Nymphs of Ephemeroptera (mayfly) and Plecoptera (stonefly) or larvae of Diptera (blackfly, mosquito, chironomid, etc.) are known as major hosts (Lichtwardt 1986). Generally, to observe harpellalean fungi, the basic procedure involves the collection and dissection of insects (Lichtwardt 1986; Sato 2002b). Conversely, insects molt as part of metamorphosis, whereupon the lining of the hindgut would be shed with thalli of Harpellales. Therefore, observation of cast exuviae leads to Harpellales being found without dissection. Research of Harpellales has been limited in Japan to larval dipteran hosts (Lichtwardt et al. 1987; Sato et al. 1989), and no research has been conducted on species inhabiting in Ephemeroptera. Preliminary observation of ephemeropteran exuviae, including a species new to Japan, is reported.

Collections were conducted in a small stream of Mt. Tsukuba, Ibaraki Pref., Japan, from April to June 2011 (N36°12′37.11″,

* *Corresponding author*. Tel.: +81 29 829 8254; fax: +81 29 873 1543.
E-mail address: hirokis@ffpri.affrc.go.jp.

1340-3540/$ – see front matter
http://dx.doi.org/10.1016/j.myc.2012.10.001

E140°06′32.38″ ca. 200 m above sea level). The temperature of the stream was measured once on May 7th at 2 p.m. Decaying leaves submerged at the bottom of the stream and leaves accumulating between small rocks were collected; containing nymphs of Ephemeroptera and their exuviae. Samples were placed in plastic bags, kept cool in a box with ice, and carried back to the laboratory. The leaves were then spread in a container (30 × 40 × 5 cm) with tap water. Exuviae of Ephemeroptera were carefully picked up with sharp forceps and transferred to distilled water in glass vials. Each exuvia was transferred onto a drop of distilled water on a glass slide and observed with a compound microscope (Optiphoto; Nikon, Tokyo) with Nomarsky's interference apparatus. Several glass slides with exuvia were put in a plastic box with wet tissue paper to prevent them drying, and the box was kept at 5 °C, for 24 h in darkness. These specimens were used for observation of sporogenesis. After the observation with water mounting, all specimens were mounted with lactophenol and compared with the holotype slide of *Bojamyces repens* Longcore (JL01V88, FH) loaned from Farlow Herbarium, Harvard University, U. S. A.

***Bojamyces repens* Longcore, Mycologia 81: 482, 1989** (Fig. 1).

Morphology: thalli extending in the body cavity of exuvia from hindgut (Fig. 1A), without main axis, slightly branched 8–12 μm in width, randomly disarticulated at septa (Fig. 1F-b), consisted of vegetative cells and generative cells (Fig. 1E). Vegetative cells about three to four times longer than the adjacent generative cells (Fig. 1E). Generative cells interspersed with vegetative cells in a line, producing a trichospore from peg-like structure (Fig. 1C–E, F-b). Trichospores elongate-ellipsoidal in shape, 39.2–55.9–68.5 × 6.8–8.0–9.2 μm, collar without appendage at the base, 2.8–4.2–5.9 × 2.9–4.0–5.3 μm, (Fig. 1B, D, E, F-b, G-a, b). Holdfast attaching the base of thallus to host hindgut lining, inconspicuous, round from top view (Fig. 1H).

Specimen examined: collection site: a small stream (close to Fureai-no-Sato Park) of Mt. Tsukuba, Ibaraki Pref., Japan. Host insect: *Paraleptophlebia* sp. (Ephemeroptera: Leptophlebidae). Collector: H. Sato. Voucher slides: deposited in National Museum of Nature and Science, TNS-F-47301, 47302.

Notes: nineteen exuviae of *Paraleptophlebia* sp. were observed, eighteen of which had slightly branched thalli were detected. Trichosporogenesis required about 2 days at 5 °C from the accumulation of cytoplasm around a peg-like structure of generative cells (Fig. 1C). When generative cells were producing trichospores, the adjacent vegetative cells were already empty (Fig. 1D, E). Subsequently, after sporulation, all parts of the thalli became empty. The orientation of trichospores on the thallus was random, while adjacent spores were sometimes seen pointing in opposite directions (Fig. 1D). Disarticulation of the thalli occurred irregularly at septa during both the thalloidal growing period and sporogenesis (Fig. 1F-a, b). A cylindrical structure with different

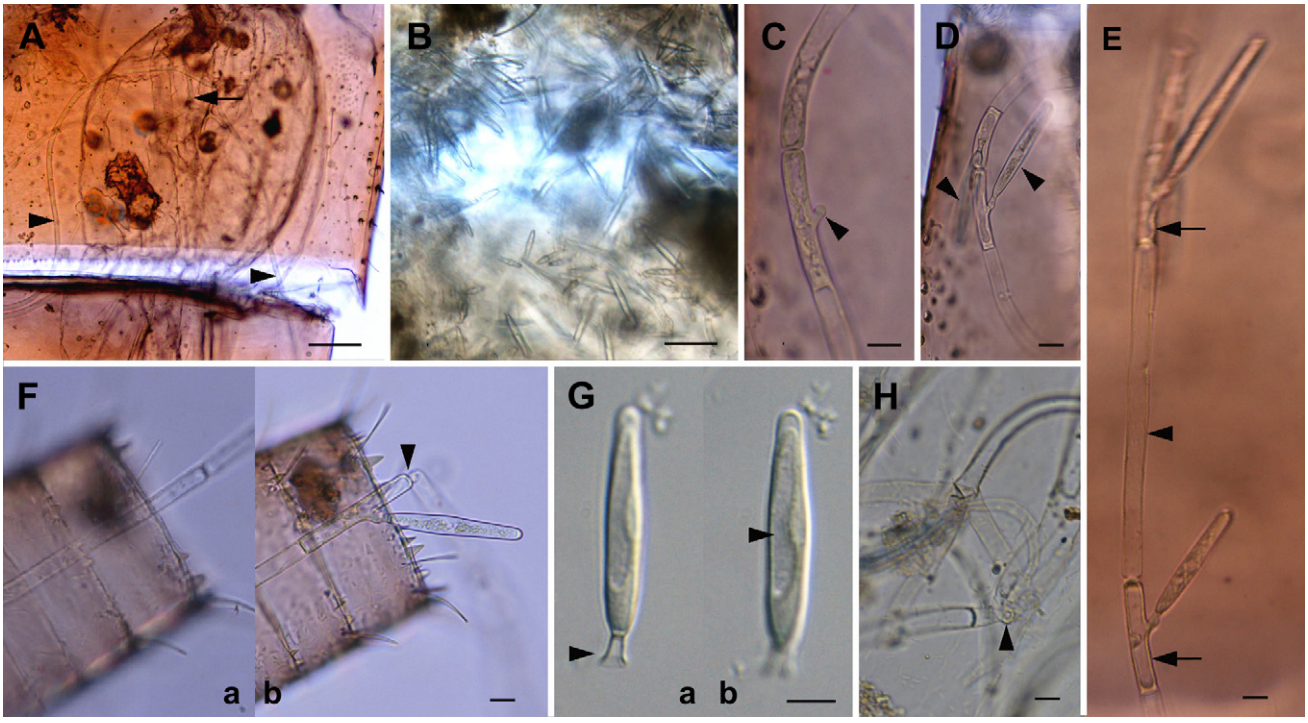

Fig. 1 – *Bojamyces repens*. A: Thalli growing in both hindgut (arrow) and body cavity (arrowheads) of an exuvia. B: Scattered trichospores in the cavity of an exuvia. C: Initial formation of trichospore as a peg-like structure at lateral side of a generative cell (arrowhead). D: Trichospores pointing in opposite directions (arrowheads). One is out of focus. E: Generative cell (arrows) and long empty vegetative cell (arrowhead). F: Trichosporogenesis. a, 0 h; b, 48 h after incubation at 5 °C. Disarticulation of thallus (arrowhead) and a mature trichospore. G: Trichospore. a, collar without appendage (arrowhead); b, spore body in the trichospore (arrowhead). H: Holdfast observed in young thallus (arrow head). A–F, H: water mounted. G: lactophenol mounted. *Bars* A, B: 50 μm; C–H: 10 μm.

Table 1 – Comparison of trichospore sizes between *Bojamyces spp.*

Species	Source	Size of trichospore	N	Size of collar	N	Appendage	Literature
Bojamyces repens	Tsukuba	39.2–55.9–68.5 × 6.8–8.0–9.2 μm	30	2.8–4.2–5.9 × 2.9–4.0–5.3	30	–	
B. repens	Holotype slide	42.0–47.4–53.4 × 7.4–8.5–9.6 μm	23	2.3–3.1–4.3 × 2.2–3.0–4.2	20	–	
B. repens	Article	30–45–77 × 6–8		3–5 × 3–5		–	Longcore (1989)
B. repens	Article	43–47–51 × 7–8		2–3.5 × 2–3		–	Valle and Santamaria (2004)
B. olmecensis	Article	30–35 × 3.5–5		3.5–5 × 2–2.5		+	Valle et al. (2008)
B. tramsfuga	Article	24–30–36 × 5–6		1.5–2.5 × 2–2.5		+	Valle and Santamaria (2004)

refraction was observed longitudinally in the cytoplasm of mature trichospores (Fig. 1G-b).

In the holotype slide an exuvia was mounted, around which the mounting fluid had almost vaporized. Fortunately, the exuvia still contained mounting fluid and trichospores could be observed. The length and width of trichospores were 42.0–47.4–53.4 × 7.4–8.5–9.6 μm ($N = 23$) (Table 1), and the collar sizes were 2.3–3.1–4.3 × 2.2–3.0–4.2 μm ($N = 20$) (Table 1). Collars had no appendages.

The specimens collected in Mt. Tsukuba showed that generative cells were interspersed with vegetative cells, which is the key characteristic distinguishing the genus *Bojamyces* from other harpellalean fungi. Three species have been described in *Bojamyces*: *B. olmecensis* M.M. White, L.G. Valle & Cafaro (Valle et al. 2008), *B. repens* (Longcore 1989) and *B. transfuga* L.G. Valle & Santam (Valle and Santamaria 2004), all of which were derived from exuviae and hindgut of aquatic insects. *B. repens* was also reported from Spain (Valle and Santamaria 2004) after the first record in the U.S.A. (Longcore 1989). The morphological features of trichospores and the host information of the three species were summarized in Table 1. The species under consideration was distinguished from both *B. transfuga* and *B. olmecensis* by non-appendage collar and size ranges of trichospores corresponding to *B. repens*. Moreover, the species under consideration was taken from the same insect host family (Leptophlebiidae) as in previous reports, including the original paper of *B. repens* (Table 1) (Longcore 1989; Valle and Santamaria 2004). Hence this species was identified as *B. repens*. The smaller sizes of trichospores observed in the holotype specimen might be caused by vaporization of mounting fluid. To date, fourteen species belonging to six genera (*Harpella*, *Harpellomyces*, *Stachylina*, *Caudomyces*, *Pennella*, and *Smittium*) of Harpellales have been recorded in Japan (Lichtwardt et al. 1987; Sato et al. 1989). This genus is the first record from Japan.

Harpellales has two families. One is Harpellaceae, which has a simple holocarpic thallus (all the cytoplasm of thallus will be used for sporulation) except *Stachylina reflexa* Lichtwardt & Williams (Lichtwardt and Williams 1988). Conversely, Legeriomycetaceae has branched thallus and produces a series of generative cells at the distal end of the thalli. *Bojamyces* belongs to Legeriomycetaceae because of branched thalli. However, Longcore (1989) speculated that *Bojamyces* is potentially holocarpic based on observation from the incubation of this fungus on a nutrient agar medium PmTG (Longcore 1989). The empty thalli observed in this study, both vegetative and generative, support her speculation.

Trichospores in several species have been reported having a special structure called a “spore body” in the cytoplasm (Horn 1989), which contains an adhesive to attach the juvenile thalli to the gut cuticle. This structure has been observed in *Genistellospora homothallica* Lichtwardt, *Pennella angustispora* Lichtwardt, *Smittium culisetae* Lichtwardt and *S. culicis* Manier by electron microscopy (Moss and Lichtwardt 1976; Horn 1989; Sato 2002a). The structure is also sometimes recognized by light microscopy as one with different refraction (Williams 1983; Sato 2002a), but was not observed in *Bojamyces* (Longcore 1989). The cylindrical structure in trichospores appeared to be a spore body and implies that *Bojamyces* has the same infection strategy as other harpellalean fungi.

Longcore (1989) observed exuviae incubated at 10 °C. Though the temperature of the stream was 12.2 °C in May 7th at 2 p.m. at the Tsukuba collection site, incubation of exuviae was conducted at 5 °C. At this lower temperature, *B. repens* grew and showed both trichosporogenesis and disarticulation of thalli, as were observed by Longcore (1989). Generally, April to May is the main emergence season for Ephemeroptera in Japan (Gose 1985), while winter to spring is the growing (including molting) season for Ephemeroptera. Spore production at lower temperatures may suggest adaptation for expanding distribution in a cold environment in Japan. From a pragmatic perspective, controlling morphogenesis at a lower temperature aids preparation of specimens for both compound and electron microscopy fixation.

Acknowledgments

The author is grateful to Dr. Takashi Kagaya for the identification of host insects. The author thanks Dr. Y. Hirooka for his valuable comments on this manuscript. The author expresses thanks to both Dr. Donald Pfister and curator at the Farlow Herbarium (FH) for loaning the type specimen of *Bojamyces repens*.

REFERENCES

Benny GL, O’Donnell K, 2000. *Amoebidium parasiticumis* a protozoan, not a Trichomycete. *Mycologia* 92: 1133–1137.

Cafaro M, 2005. Eccrinales (Trichomycetes) are not fungi, but a clade of protists at the early divergence of animals and fungi. *Molecular Phylogenetics and Evolution* 35: 21–34.

Gose K, 1985. Ephemeroptera. In: Kawai T (ed) *An illustrated book of aquatic insects of Japan*. Tokai University Press, Tokyo, pp 7–32 (In Japanese).

Hibbett DS, Binder M, Bischoff JF, Blackwell M, Cannon PF, Eriksson OE, Huhndorf S, James T, Kirk PM, Lücking R, Thorsten Lumbsch H, Lutzoni F, Matheny PB, McLaughlin DJ, Powell MJ, Redhead S, Schoch CL, Spatafora JW, Stalpers JA, Vilgalys R, Aime MC, Aptroot A, Bauer R, Begerow D, Benny GL, Castlebury LA, Crous PW, Dai Y-C, Gams W, Geiser DM, Griffith GW, Gueidan C, Hawksworth DL, Hestmark G, Hosaka K, Humber RA, Hyde KD, Ironside JE, Köljalg U, Kurtzman CP, Larsson K-H, Lichtwardt RW, Longcore J, Miadlikowska J, Miller A, Moncalvo J-M, Mozley-Standridge S, Oberwinkler F, Parmasto E, Reeb V, Rogers JD, Roux C, Ryvarden L, Sampaio JP, Schüjler A, Sugiyama J, Thorn RG, Tibell L, Untereiner WA, Walker C, Wang Z, Weir A, Weiss M, White MM, Winka K, Yao Y-J, Zhang N, 2007. A higher-level phylogenetic classification of the Fungi. *Mycological Research* 122: 509–547.

Horn BW, 1989. Ultrastructural changes in trichospores of *Smittium culisetae* and *S. culicis* during in vitro sporangiospore extrusion and holdfast formation. *Mycologia* 81: 724–740.

Lichtwardt RW, 1986. *The Trichomycetes: fungal associates of arthropods*. Springer-Verlag, New York.

Lichtwardt RW, Kobayasi Y, Indoh H, 1987. Trichomycetes of Japan. *Transactions of the Mycological Society of Japan* 28: 359–412.

Lichtwardt RW, Williams MC, 1988. Discovery of sexual reproduction in an unusual new species of *Stachylina* (Trichomycetes). *Mycologia* 80: 400–405.

Longcore JE, 1989. *Bojamyces repens*: a new genus and species of Harpellales (Trichomycetes) from a lentic mayfly. *Mycologia* 81: 482–486.

Moss ST, Lichtwardt RW, 1976. Development of trichospores and their appendages in *Genistellospora homothallica* and other Harpellales and fine-structural evidence for the sporangial nature of trichospores. *Canadian Journal of Botany* 54: 2346–2364.

Sato H, Shimada N, Aoki J, 1989. Light and electron microscopy of *Smittium morbosum* (Trichomycetes), newly recorded from Japan. *Transactions of the Mycological Society of Japan* 30: 51–59.

Sato H, 2002a. Two ultrastructural aspects of the trichospore of *Pennella angustispora* (Harpellales): canals in the sporangiospore cell wall and appendage formation. *Mycoscience* 43: 33–36.

Sato H, 2002b. Collection and culture establishment of the genus *Smittium* (Trichomycetes). *Nippon Kingakukai Kaiho* 43: 79–82 (In Japanese).

Valle LG, Santamaria S, 2004. *Bojamyces transfuga* sp. nov. and new records of Trichomycetes from mayfly larvae in Spain. *Mycologia* 96: 1386–1392.

Valle LG, White MM, Cafaro MJ, 2008. Harpellales in the digestive tracts of Ephemeroptera and Plecoptera nymphs from Veracruz, Mexico. *Mycologia* 100: 149–163.

Williams MC, 1983. Zygospores in *Smittium culisetae* (Trichomycetes) and observations on trichospore germination. *Mycologia* 75: 251–256.