Journal of Invertebrate Pathology 186 (2021) 107677

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

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

Trichoderma infection of limno-terrestrial tardigrades

Jacob Loeffelholz, Leigha Stahl, Sogol Momeni, Caleb Turberville, Jason Pienaar *

Department of Biological Sciences, The University of Alabama, Tuscaloosa, AL 35475, United States

ARTICLE INFO

Keywords:
Tardigrades
Trichoderma
Infection
Diaforobiotus
Milnesium
Viridiscus

ABSTRACT

Interactions between fungi and tardigrades have scarcely been described. The few studies that address such relationships suggest a primarily parasitic nature for various fungal taxa, including the infectious chytridiomycetes. The aim of this study was to determine the identity of a fungus growing on a tardigrade of the genus *Diaforobiotus* and if it could infect other tardigrade genera. Using morphological analysis and ITS barcoding, we identified a mold isolate belonging to the *Trichoderma harzianum* species complex and found that it infected *Diaforobiotus* tardigrades, as well as animals in the eutardigrade genus *Milnesium*, and heterotardigrade genus *Viridiscus*.

1. Introduction

Although a few tardigrade-specific fungal infections are described, such relationships remain mostly undocumented. Earlier studies mention tardigrade fungal infections (Murray, 1907; Marcus, 1929; Morgan, 1977; Nelson, 1977), but the fungal taxa were not identified. More recently Nelson et al. (2018) report that unidentified fungal infections are common across the *Eutardigrada* class. The first tardigrade fungal parasites identified belong to the order Enthomophthorales. Reukauf (1912) described *Macrobiotopthora vimariensis* found in *Macrobiotus lacustris* (=*Hypsibius dujardini*) tardigrades, erecting the fungal genus *Macrobiotophthora*. Drechsler (1951) then described *Ballocephala sphaerospora* that infects tardigrades in the genus *Macrobiotus*. A second *Ballocephala* species infecting *Macrobiotus* sp. tardigrades was described by Richardson (1970), named *B. verrucospora,* that was later confirmed by Hallas (1977) to infect *Macrobiotus hufelandi*. A third species, *B. pedicellata*, was found parasitizing *Diphascon pinguis* and *Hypsibius dujardini* tardigrades (Pohlad & Bernard, 1978). Dewel et al. (1985) discovered and described a chytridiomycete in the order Blastocladiales, exclusively parasitic to the tardigrade *Milnesium tardigradum,* known as *Sorochytrium milnesiopthora.* The life cycle and zoospore structure were described later (Dewel & Dewel, 1990). Saikawa et al. (1991) identified a fungus in the order Hypocreales, *Harposporium anguillulae*, and an Oomycete, *Haptoglossa intermedia* that infect *Macrobiotus* sp. tardigrades. One of the most recent records in the order Orbiliales, *Lecophagus antarcticus*, was found infecting *Acutuncus antarcticus* tardigrades and *Philodina sp*. rotifers (McInnes, 2003). These studies suggest that fungal infections are common amongst tardigrades.

The aim of this study was to identify a fungus infecting a tardigrade of the genus *Diaforobiotus* and to test if it would infect other *Diaforobiotus* individuals as well as tardigrades in the genera *Viridiscus* and *Milnesium*, respectively representing both tardigrade classes the Heterotardigrada and Eutardigrada.

2. Methods

2.1. Initial observations

Tardigrades were extracted from a *Platydictya confervoides* moss growing on a concrete post by Lake Harris in Tuscaloosa, Alabama, U.S.A. (33°16′12″ N, 87°28′22″ W), using a custom Bauerman pan and filtration system (Davison, 2015). Some tardigrades were mounted for identification, and others dried on sterile Petri dishes. The dehydrated tardigrades were examined under a surface-sterilized dissecting microscope in a laminar flow hood, and fungal growth was observed on a tardigrade belonging to the *Diaforobiotus* genus. A drop of deionized water was placed on the tardigrade, and a surface-sterilized Irwin loop used to transfer the tardigrade to a sterile Sabouraud dextrose agar plate. After 4–5 days of incubation at room temperature and ambient light/dark conditions, the mycelium of the fungus grew to the edges of the plate, and conidiophores formed near the original inoculation site.

* Corresponding author at: Science & Engineering Complex 2312, Department of Biological Sciences, The University of Alabama, Tuscaloosa, AL 35475, United States.
E-mail address: jpienaar@ua.edu (J. Pienaar).

https://doi.org/10.1016/j.jip.2021.107677
Received 30 May 2021; Received in revised form 27 September 2021; Accepted 1 October 2021
Available online 7 October 2021

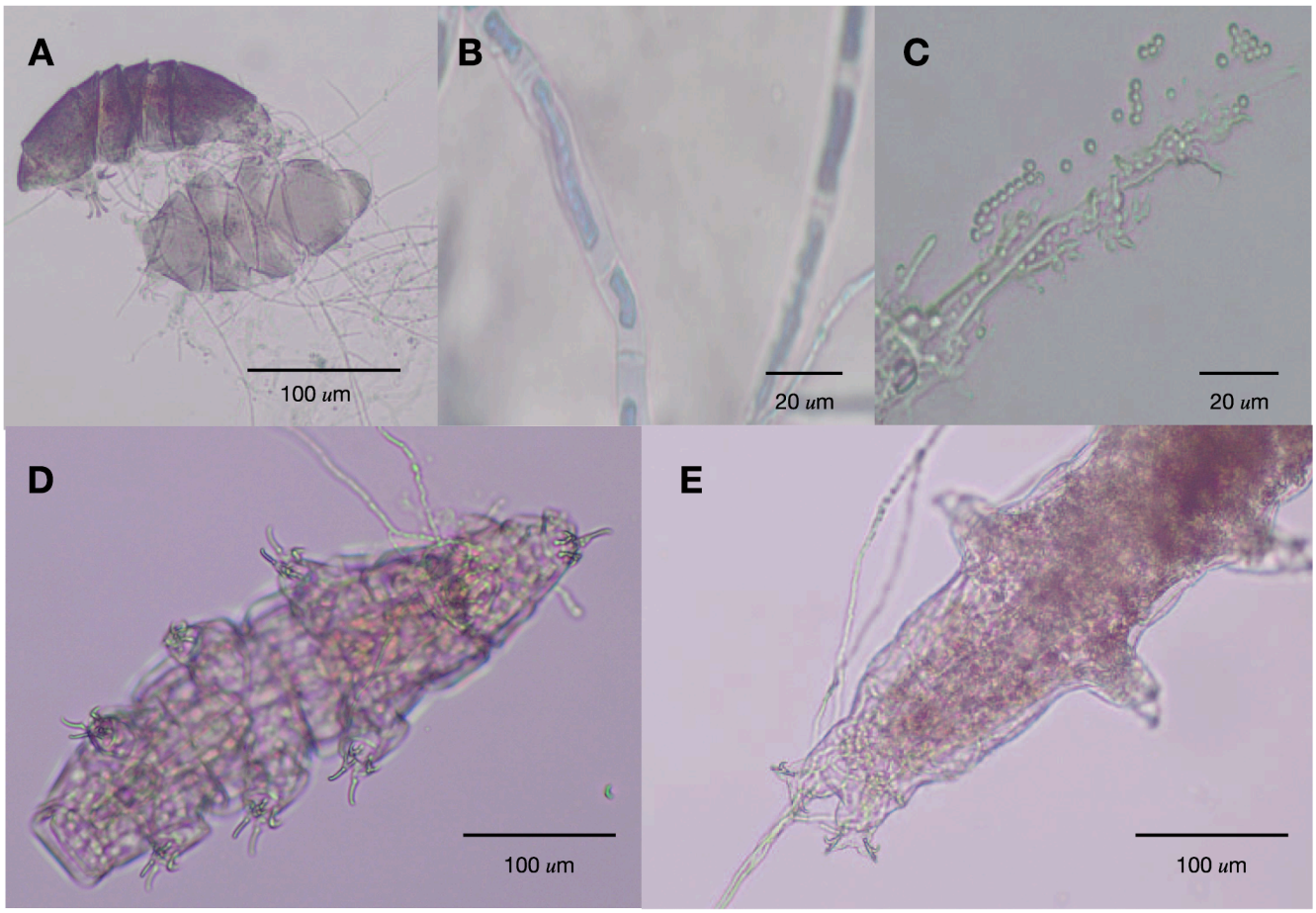

Fig. 1. A) Two *Viridiscus* sp. tardigrades infected by mold at 10×. B) Septate hyphae stained with lactophenol cotton blue at 40×. C) Conidia and conidiophores at 40×. D) *Milnesium* sp. tardigrade infected by mold at 10×. E) *Diaforobiotus* sp. tardigrade infected by mold at 10×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Fungal isolation

Pure cultures of the mold were isolated by serial mycelial plug transfers. Edges of the mold mycelium were transferred to four other growth plates under sterile conditions. After 4–5 days of growth at room temperature and ambient light/dark, hyphal tips from each plate were transferred to new growth plates and this process was repeated twice more.

2.3. Infectivity assays

Twenty-four *Diaforobiotus* sp. tardigrades were extracted from a moss sample (*Pylaisia polyantha)*, and twenty-four each of *Milnesium* sp. and *Viridiscus* sp. tardigrades were extracted from a lichen sample (*Xanthoparmelia conspersa*), near the original sampling site. Tardigrades, moss, and the lichen were identified to genus, or species level using dichotomous keys and taxon-specific literature (Pilato & Binda, 2010; Guidetti et al., 2016; Gąsiorek et al., 2019; Vitt & Buck, 2019; Tripp & Lendemer, 2020). *Viridiscus* sp. tardigrades were transferred to a Petri dish with deionized water and immobilized by inducing a state of anoxybiosis (an immobilized but still alive state due to low oxygen) by heating their environment to 55 °C for 45 min (the other two tardigrade species did not survive these conditions). For each of the three tardigrade genera (*Diaforobiotus, Milnesium, Viridiscus*), a sterile Irwin loop was used to place twenty-four active tardigrades of each species into three separate petri dishes containing the growing fungus. Observations were made after 1, 8 and 24 h of fungal growth at room temperature and ambient light / dark conditions.

2.4. Fungal taxon identification

Once conidiophores appeared on fungal isolates, a slide mount of the mycelium stained with lactophenol cotton blue was prepared, and septate hyphae (Fig. 1B) were observed, suggesting a *Trichoderma* sp. mold (Munoz et al. 1997). A wet mount of the conidiophores was prepared and the morphological characteristics of the conidiophores and conidia were consistent with those of *Trichoderma* spp. (Fig. 1C). We also used standard ITS barcoding. Genomic DNA was extracted using 5% Chelex® 100 resin (Bio-Rad Laboratories) with freeze/thaw cycles. DNA extracts were standardized to 40 ng/μl, and 1 μl was used in polymerase chain reaction (PCR) to amplify the ITS1, 5.8S, and ITS2 gene regions using fungal primers (White et al. 1990). Each 50 μl PCR mixture consisted of 1 μl DNA template, 10 μl 5X Green GoTaq Buffer (Promega), 1 μl each of 25 pmol of ITS1F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC-3′) primers, 0.4 μl GoTaq DNA polymerase (Promega), 0.3 μl of 10 mM deoxynucleoside triphosphates, 4 μl of 5% DMSO, and 32.3 μl of sterile water. Amplification was obtained through an initial hold at 95 °C for 3 min followed by 30 cycles of 95 °C for 45 s, 53 °C for 60 s, and 72 °C for 90 s followed by a final 5 min extension at 72 °C. A positive control of *Candida albicans* and negative control without DNA template was included. Amplification products were separated and visualized by gel electrophoresis, purified with the E.Z.N.A Cycle Pure kit (Omega) and submitted for bidirectional sequencing (Eurofins Genomics). Sequences were trimmed and aligned, and BLAST (Altschul et al., 1990) was used to analyze sequence identity from the consensus sequences compiled by Geneious Prime® (v.2021.1.1. Biomatters Ltd.). Several BLAST parameters including Bit-

Table 1
BLAST results of bidirectional sequence consensus assemblies. Shown in the table are the BLAST hit data for T. *simmonsii* and T. *harzianum* for each of the four sub cultures to show that we cannot distinguish between the two.

Subculture	Hit number	Bit score	E value	Grade	Accession number	Shortened Identity Description
1	2	1098.0	0.0	99.9%	NR_137297	T. *simmonsii*
1	5	1098.0	0.0	99.9%	MH865865	T. *harzianum*
2	2	1088.8	0.0	100%	MN396598	T. *simmonsii*
2	4	1088.8	0.0	100%	MK322687	T. *harzianum*
3	3	1090.6	0.0	99.8%	NR_137297	T. *simmonsii*
3	6	1090.6	0.0	99.8%	MK322687	T. *harzianum*
4	2	1092.5	0.0	99.9%	NR_137297	T. *simmonsii*
4	5	1092.5	0.0	99.9%	MK322687	T. *harzianum*

score, E Value and Grade were used to narrow down fungal identity.

3. Results

The *Viridiscus* tardigrades immobilized by anoxybiosis all reverted to an active state within an hour, and all were either infected by the *Trichoderma* mold or the mycelium was observed to be growing towards them. In all cases for the active tardigrades, the water droplet they were transferred in dried-up within an hour, resulting in desiccation-resistant tun formation. Twenty-four hours later, at least one individual from each genus was revived upon addition of water. Each revived individual had fungal hyphae growing inside of it. Within 48 h, the previously revived tardigrades died from fungal infection despite their continued presence in water (see Fig. 1 A, D, E).

The barcode sequences for the fungal isolates most closely matched *Trichoderma simmonsii* or *Trichoderma harzianum*. Fungal identities are similar across the replicates, indicating appropriate fungal isolation and *Trichoderma* as the agent of infection (Table 1). While Table 1 suggests *T. simmonsii*, *T. harzianum* cannot be excluded because Bit-scores, E-values, and Grades were similar, 0, or above 97% respectively. These results suggest that it is difficult to discern between *T. simmonsii* and *T. harzianum* using the above-mentioned primers, despite coverage of the entire ITS region, where amplicons were approximately 589 bp to 595 bp in length after bidirectional sequencing trimming and consensus sequence assembly.

4. Discussion and conclusions

The ITS1, 5.8S, and ITS2 barcoding results point to either *T. simmonsii* or *T. harzianum*. Chaverri et al. (2015) proposed that ITS barcoding is not reliable in genetically identifying specific species within the *T. harzianum* species complex and erected the *T. simmonsii* species from the *T. harzianum* species complex using a secondary barcode, nuc translation elongation factor 1-α (TEF1). As we did not perform TEF1 barcoding, we conclude that this mold is within the *T. harzianum* species complex, but cannot determine which species exactly.

This is the third report of a fungus in the Ascomycota phylum infecting tardigrades, following the report of *Harposporium anguillulae* by Saikawa et al. (1991) and *Lecophagus antarcticus* by McInnes (2003). The Ascomycota phylum of fungi currently comprises over 64,000 species (Kirk et al. 2008), indicating that the potential for interactions between the Ascomycota and Tardigrada phyla is immense. One *Diaforobiotus* tardigrade in this study was observed having hyphae growing out of the back of its head, resembling a recurring phenomenon of fungal-invertebrate interactions, such as the *Ophiocordyceps unilateralis* fungus that infects ants of the *Camponotus* and *Polyrhachis* genera and grows out of the back of their heads (Mongkolsamrit et al. 2012). It appears that this fungus takes advantage of tardigrades when immobilized, either in a tun or anoxybiotic state, for initial infection. Once inside the tardigrades, it rapidly grows, even when the tardigrade is revived to an active state.

Trichoderma harzianum has been used as a biocontrol agent against root-knot nematodes that are damaging to a multitude of crops (Khan et al., 1997; Sharon et al., 2001; Khosravi et al., 2014), but has never been reported to infect tardigrades that occur in similar habitats. Our study also demonstrated that this *Trichoderma* species isolate can infect three different genera of tardigrades, suggesting *Trichoderma* may infect tardigrades as a generalist rather than infecting specific taxa. Further studies are needed to understand infection dynamics and whether *Trichoderma* infects tardigrades as a pathogen or opportunistically while the tardigrades are in a tun state or state of anoxybiosis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Diane Nelson of East Tennessee State University and Daniel Stec of the Jagiellonian University for assistance with identification of the tardigrades in the *Diaforobiotus* genus. We also thank Martha Powell for providing advice and resources for fungal identification. Lastly, we thank Julie Olson for laboratory accommodations and insight into fungal cultivation and amplification. This material is based upon work supported by the National Science Foundation under grant number 2045035.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215 (3), 403–410.
Chaverri, P., Branco-Rocha, F., Jaklitsch, W., Gazis, R., Degenkolb, T., Samuels, G.J., 2015. Systematics of the *Trichoderma harzianum* species complex and the re-identification of commercial biocontrol strains. Mycologia 107 (3), 558–590.
Davison, P.G., 2015. Baermann Pans. The International Society of Tardigrade Hunters. https://tardigradehunters.weebly.com/uploads/2/5/9/3/25930056/baermann_pan_instructions2014breif.pdf.
Drechsler, C., 1951. An entomophthoraceous tardigrade parasite producing small conidia on propulsive cells in spicate heads. Bull. Torrey Bot. Club 78 (3), 183–200.
Dewel, R.A., Dewel, W.C., 1990. The fine structure of the zoospore of *Sorochytrium milnesiophthora*. Can. J. Bot. 68 (9), 1968–1977.
Dewel, R.A., Joines, J.D., Bond, J.J., 1985. A new chytridiomycete parasitizing the tardigrade *Milnesium tardigradum*. Can. J. Bot. 63 (9), 1525–1534.
Gąsiorek, P., Morek, W., Stec, D., Michalczyk, Ł., 2019. Untangling the *Echiniscus* Gordian knot: paraphyly of the "arctomys group" (Heterotardigrada: Echiniscidae). Cladistics 35 (6), 633–653.
Guidetti, R., Rebecchi, L., Bertolani, R., Jönsson, K.I., Møbjerg Kristensen, R., Cesari, M., 2016. Morphological and molecular analyses on *Richtersius* (Eutardigrada) diversity reveal its new systematic position and lead to the establishment of a new genus and a new family within Macrobiotoidea. Zool. J. Linn. Soc. 178 (4), 834–845.
Hallas, T.E., 1977, January. Survey of the Tardigrada of Finland. In: Annales Zoologici Fennici, pp. 173-183.
Khan, T.A., Khan, S.T., Fazal, M., Siddiqi, Z.A., 1997. Biocontrol of *Meloidogyne incognita* and *Fusarium solani* disease complex in papaya using *Paecilomyces lilacinus* and *Trichoderma harzianum*. Int. J. Nematol. 7, 127–131.
Khosravi, M., Abdollahi, M., Sadravi, M., 2014. Effect of *Metarhizium anisopliae* and *Trichoderma harzianum* on root knot nematode, *Meloidogyne javanica*. Biol. Control Pests Plant Dis. 3 (1), 67–76.
Kirk, P.M., Cannon, P.F., Minter, D.W., Stalpers, J.A., 2008. Ainsworth and Bisby's Dictionary of the Fungi, tenth ed. Centre for Agriculture and Bioscience International. Wallingford, United Kingdom.

Marcus, E., 1929. Tardigrada. Dr. HG Bronns Klassen und Ordnungen des Tier-Reichs. IV. Abteilung, Buch, 3, p.608.

McInnes, S.J., 2003. A predatory fungus (Hyphomycetes: *Lecophagus*) attacking Rotifera and Tardigrada in maritime Antarctic lakes. Polar Biol. 26 (2), 79–82.

Mongkolsamrit, S., Kobmoo, N., Tasanathai, K., Khonsanit, A., Noisripoom, W., Srikitikulchai, P., Somnuk, R., Luangsa-ard, J.J., 2012. Life cycle, host range and temporal variation of *Ophiocordyceps unilateralis/Hirsutella formicarum* on formicine ants. J. Invertebr. Pathol. 111 (3), 217–224.

Morgan, C.I., 1977. Population dynamics of two species of Tardigrada, *Macrobiotus hufelandii* (Schultze) and *Echiniscus (Echiniscus) testudo* (Doyere), in roof moss from Swansea. J. Animal Ecol. 46 (1), 263. https://doi.org/10.2307/3960.

Munoz, F.M., Demmler, G.J., Travis, W.R., Ogden, A.K., Rossmann, S.N., Rinaldi, M.G., 1997. *Trichoderma longibrachiatum* infection in a pediatric patient with aplastic anemia. J. Clin. Microbiol. 35 (2), 499–503.

Murray, J., 1907. XXIV.—Scottish Tardigrada, collected by the Lake Survey. Earth Environ. Sci. Trans. Roy. Soc. Edinburgh 45 (3), 641–668.

Nelson, D.R., 1977. Fungal parasites of New Zealand tardigrades (phylum: Tardigrada). Bull. Assoc. Southeastern Biol. 24, 73.

Nelson, D.R., Bartels, P.J., Guil, N., 2018. Tardigrade ecology. In: Schill, R. (Eds.) Water Bears: The Biology of Tardigrades. Zoological Monographs, vol 2. Springer. Cham, Switzerland. pp. 163-210.

Pilato, G., Binda, M.G., 2010. Definition of families, subfamilies, genera and subgenera of the Eutardigrada, and keys to their identification. Zootaxa 2404 (1), 1. https://doi.org/10.11646/zootaxa.2404.110.11646/zootaxa.2404.1.1.

Pohlad, B.R., Bernard, E.C., 1978. A new species of Entomophthorales parasitizing tardigrades. Mycologia 70 (1), 130–139.

Reukauf, E., 1912. Ein Verderber des Wasserbaren *Macrobiotus lacustris* Doy., *Macrobiotophthora vimariensis* (Reukauf). Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten 63, 390–393.

Richardson, M.J., 1970. *Ballocephala verrucospora* sp. nov., parasitizing tardigrades. Trans. British Mycol. Soc. 55 (2), 307–IN3.

Saikawa, M., Oyama, M., Yamaguchi, K., 1991. Harposporium anguillulae and Haptoglossa intermedia parasitizing tardigrades. Trans. Mycol. Soc. Japan (Japan).

Sharon, E., Bar-Eyal, M., Chet, I., Herrera-Estrella, A., Kleifeld, O., Spiegel, Y., 2001. Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Phytopathology 91 (7), 687–693.

Tripp, E.A., Lendemer, J.C., 2020. Field Guide to the Lichens of Great Smoky Mountains National Park, first ed. The University of Tennessee Press. Knoxville, Tennessee, pp. 466–467.

Vitt, D.H., Buck, W.R., 2019. Key to the moss genera of North America and Mexico. In: Flora of North America Editorial Committee (eds) Flora of North America North of Mexico [online], vol. 28. Available from: http://beta.floranorthamerica.org/Key_to_the_Moss_Genera_of_North_America_North_of_Mexico [accessed 3 Oct. 2020].

White, T.J., Bruns, T., Lee, S.J.W.T., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., PCR protocols: a guide to methods and applications. Academic Press, Inc. New York, N.Y., pp. 315–322.