Emerging Animal Species 3 (2022) 100001

Contents lists available at ScienceDirect

Emerging Animal Species

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

Fungal infection in free-ranging snakes caused by opportunistic species

Sylvain Dubey [a,b,c,*], Sébastien Pellaud [a], Katia Gindro [d], Johan Schuerch [c], Joaquim Golay [c], Robin Gloor [c], Karim Ghali [c], Olga Dubey [a]

[a] AgroSustain SA, Rue de Lausanne 64, 1020 Renens, Switzerland
[b] Department of Ecology and Evolution, Biophore Building, University of Lausanne, 1015 Lausanne, Switzerland
[c] HW Romandie SA, Avenue des Alpes 25, 1820 Montreux, Switzerland
[d] Agroscope, Swiss Federal Agricultural Research Station in Changins, CH-1260 Nyon, Switzerland

ARTICLE INFO

Keywords:
Reptiles
Snakes
Fungal pathogens
Genetic analyses

ABSTRACT

Emerging infectious fungal diseases such as chytridiomycosis, caused by *Batrachochytrium dendrobatidis* and *B. salamandrivorans* in amphibians, or ophidiomycosis in reptiles (*Ophidiomyces ophiodiicola*), are major threats that can drive species or entire populations to extinction over a short period of time. Besides these well-documented pathogens, such diseases can be caused by numerous opportunistic fungal species that can target e.g. immunocompromised individuals from various species of vertebrates. In reptiles, opportunistic species are mainly documented in captive animals kept in inadequate conditions, but data remains scarce for wild individuals. In the present study, we isolated and genetically identified fungal species responsible of skin lesions in wild-caught smooth snakes (*Coronella austriaca*) during a field survey targeting endangered reptile species in Switzerland. A total of 18 fungal species were isolated and genetically identified from the lesions of the two wild-caught snakes and included several species known for being opportunistic pathogens in vertebrates and infecting mainly immunocompromised individuals, such as *Alternaria infectoria* and *Rhodotorula* spp. It is not possible to establish whether the snakes had such an issue. However, the exceptional wet and cold conditions experienced in spring 2021 might have trigger the infections. Indeed, high humidity has been recorded as a predisposing factor for mycoses in captive reptiles.

Introduction

Emerging infectious fungal diseases such as chytridiomycosis, caused by *Batrachochytrium dendrobatidis* and *B. salamandrivorans* in amphibians, or ophidiomycosis in reptiles (*Ophidiomyces ophiodiicola*), are major threats that can drive species or entire populations to extinction over a short period of time. They have attracted much attention as chytridiomycosis has impacted more 500 species and caused the extinction of 90 species worldwide in few decades (Scheele et al., 2019). For this reason, it is a key factor in what is considered as the ongoing sixth mass extinction (Ceballos et al., 2020). Involuntary human-mediated dispersal is one of the core reasons of such a dramatic propagation and impact of fungal diseases (Lorch et al., 2016). Concerning ophidiomycosis, first described in 2006, it has not yet led species to extinction, but remains a major conservation issue in North America, where it has dramatic impact on rare snake species and can be considered as an endemic disease (Davy et al., 2021). In contrary, it is still an emerging disease in Europe. Indeed, its presence in Europe has been highlighted only recently, in e.g. wild snakes from Great Britain and Czech Republik, and its impact on snake populations has not yet been fully evaluated (Franklinos et al., 2017).

Besides these well-documented pathogens, such diseases can be caused by numerous opportunistic fungal species that can target e.g. immunocompromised individuals from various species of vertebrates (Yockey et al., 2019; Huggins et al., 2020). In reptiles, opportunistic species are mainly documented in captive animals kept in inadequate conditions, but data remains scarce for wild individuals (Jacobson and Cheatwood, 2000; Lukac et al., 2017).

In the present study, we isolated and genetically identified fungal species involved in multiple skin lesions in wild-caught smooth snakes (*Coronella austriaca*) during a field survey targeting endangered reptile species in Switzerland.

* Corresponding author at: AgroSustain SA, Rue de Lausanne 64, 1020 Renens, Switzerland.
E-mail address: sylvain.dubey@unil.ch (S. Dubey).

https://doi.org/10.1016/j.eas.2022.100001
Received 15 December 2021; Revised 4 March 2022; Accepted 11 March 2022

Materials and methods

Samples, fungal culture and DNA extraction

A field survey targeting endangered reptile species (*Coronella austriaca* and *Vipera aspis*) in western Switzerland was performed in spring 2021, in an area exhibiting important snake populations that might be impacted negatively by future road works (Canton of Vaud; latitude: 46.475, longitude: 6.804). During this survey, two smooth snakes (*Coronella austriaca*) out of four captured (Authorization number: 2021–340; Direction générale de l'environnement, Canton of Vaud, Switzerland; Fig. 1) were exhibiting important skin lesions increasing in size and spreading quickly throughout their body over a two-days period (Fig. 1).

We isolated and genetically identified fungal species from the observed lesions. Infected tissues, throughout the body of the two individuals, were collected with swabs and fragments were placed on sterile PDA plates (Condalab, Madrid, Spain; 39 g/L). The snakes were then kept in dry conditions (by replacing the natural substrate by household paper) at a room temperature oscillating between 16 °C at night to 22 °C in the afternoon. A 50 W day light basking spot from Exo Terra was available for basking 8 h per day. The snakes were successfully treated with a Povidone-Iodine solution (Betadine), a broad-spectrum topical microbicide, over a two-weeks period (Fig. 1). Once fungal growth was observed on the plates, and before fungi overlapped, each of the 21 observed fungi were isolated, each on a new sterile PDA plate to get a clean culture. Fungal cultures were left growing for 1 week at room temperature before being harvested for DNA extraction and identification. Similarly, fungal species from the substrate (topsoil with a high content of organic matter) that was directly in contact with snakes before the treatment were isolated and genetically identified, as well as the fungal species present after treatment on the skin and from the substrate (subsoil with a relatively low content of organic matter), in order to better understand, which species were responsible of the observed lesions.

Small pieces of each pathogen candidate (3 mm × 3 mm) were cut from cultures, collected in 1.5 mL microfuge tubes together with 2 glass beads (3 mm diameter) and frozen in liquid nitrogen. DNA was prepared using a modified Shorty Method (Visscher et al., 2010). Samples were ground in mixer mill (Retsch, Haan, Germany) and 450 μL of shorty buffer (200 mM Tris pH9, 400 mM LiCl, 25 mM EDTA, 1% SDS) were added to each sample. Samples were mixed for 5 min and centrifuged at 13,000 rpm for 5 min. 350 μL of supernatant were transferred into new tubes and 350 μL of isopropanol were added to each tube. Samples were mixed by inversion 30 times and centrifuged 10 min at 13′000 rpm. Liquid was poured off and tubes were dried upside down for at least 30 min. Once dry, DNA was resuspended in 200 μL of deionized water prior to PCR amplification.

Fungal identification

Fungal strains were identified by sequencing of PCR products. A PCR master mix was prepared using 2.5 μL PCR buffer (Qiagen, Hilden, Germany), 2.25 μL $MgCl_2$ 25 mM (Qiagen, Hilden, Germany), 1.25 μL of primer 1 (10 mM), 1.25 μL of primer 2 (10 mM), 10.625 μL of deionized water, 1 μL dNTPs (10 mM each), 1 μL betain and 0.125 μL Taq polymerase (Qiagen, Hilden, Germany), for a total of 24 μL per sample. 1 μL of DNA template was added to each reaction. A fragment of the 5.8S rRNA (420–825 bp, depending on species) was amplified by PCR using the primers ITS1_F (5′-CCT GGT CAT TTA GAG GAA GTA A-3′; Gardes and Bruns, 1993) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′; White et al., 1990). For *Penicillium* spp., a fragment of the 28S large subunit rRNA (1132–1145 bp, depending on species) was amplified by PCR using the primers LR7 (5′-TAC TAC CAC CAA GAT CT-3′) and LROR (5′-GTA CCC GCT GAA CTT AAG C-3′; Rehner and Samuels, 1994). Samples were amplified as follows: 2 min at 94 °C, 30× (30 sec at 94 °C, 30 sec at 55 °C, 30 sec at 72 °C) and 10 min at 72 °C. PCR products were sent for purification and Sanger sequencing to Fasteris SA (Plan-les-Ouates, Switzerland). Sequences were aligned with the Multiple Sequence Alignment Tool

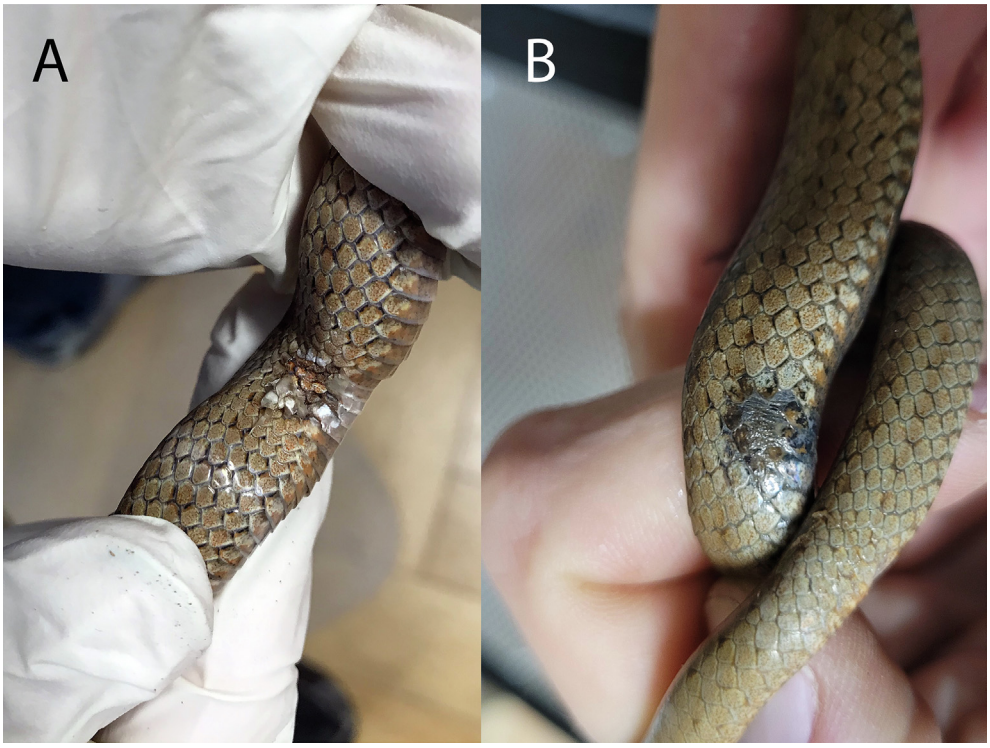

Fig. 1. Example of a skin lesion of a smooth snake (*Coronella austriaca*) analysed in this study before (A) and after treatment with a betadine solution (B).

(https://www.evi.ac.uk/Tools/msa/clustalo/) and both ends of each sequence were manually cut using the shortest sequence as a reference. Sequences were finally compared with the NCBI nucleotide database for species identification using the nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/).

Results

A total of 18 fungal species (from 21 different fungal colonies) were isolated and genetically identified from the two wild-caught snakes exhibiting skin lesions and included species of the following genera: *Alternaria*, *Arthrinium*, *Aureobasidium*, *Cephalotrichum*, *Cladosporium*, *Diatrype*, *Fusarium*, *Mucor*, *Penicillium*, *Rhodorotula*, and *Xylaria*. *Ophidiomyces ophiodiicola* was not detected. Concerning the fungal species present in the substrate before the treatment of snakes, only two *Mucor* species and one *Penicillium* were detected (from six different fungal colonies). After treatment, species of the genera *Cladosporium*, *Fusarium, Mucor*, *Penicillium*, *Pichia*, *Sporidiobolus* and *Trichoderma* were isolated from the skin of snakes (from 13 different fungal colonies) and the genera *Cladosporium*, *Fusarium*, *Geotrichum, Mucor*, *Penicillium*, *Rhodosporidiobolus* and *Trichoderma* from the substrate (from 14 different colonies; Genbank accession numbers OL840781–OL840801, OM541309–OM541327 and OM535924–OM535926). For more details, see Table 1.

Discussion

In the present study, we highlighted that several genetically identified fungal species from the skin lesions of two smooth snakes are already known for being opportunistic pathogens in vertebrates, such as *Alternaria infectoria, Cladosporium* sp., *Rhodotorula* spp. or *Mucor* spp.

Cladosporium spp. have been identified in granulomatous lesions from the mandibule of an anaconda (*Eunectes murinus*), as well as from a skin lesion of Boa constrictor (*Boa constrictor*) and can infect immunocompromised humans and cause subcutaneous abscesses and central nervous system infections (such as cerebral abscesses and meningitis; Henao-Martínez and Vela-Duarte 2018). Similarly, Mucor spp. is responsible of mycosis in immunocompromised and diabetic humans and can infect their brain, respiratory and digestive tracts,

Table 1
Identified fungal species from skin lesions of two smooth snakes (*Coronella austriaca*) based on genetic analyses (5.8S rRNA and 28S sequences). In addition, fungal species from the substrate that was directly in contact with snakes were isolated and genetically identified, as well as the fungal species present after treatment on the skin and from the substrate. Sequences were compared with the NCBI nucleotide database for species identification. When more than one species is mentioned for one line, NCBI nucleotide database returned several species with a 100% sequence identity.

Division	Order	Family	Species	Genbank
Skin lesions before treatment				
Ascomycota	Capnodiales	Mycosphaerellaceae	*Cladosporium* sp.	OL840795/OL840797/OL840799
Ascomycota	Chaetothyriales	Trichomeriaceae	Not defined	OL840784
Ascomycota	Dothideales	Dothioraceae	*Aureobasidium pullulans*	OL840787/OL840789
Ascomycota	Eurotiales	Trichocomaceae	*Penicillium citrinum*	OL840792
Ascomycota	Eurotiales	Trichocomaceae	*Penicillium simplicissimum/P. mariae-crucis*	OL840793
Ascomycota	Hypocreales	Nectriaceae	*Fusarium culmorum*	OL840794
Ascomycota	Hypocreales	Nectriaceae	*Fusarium* sp1.	OL840801
Ascomycota	Incertae sedis	Apiosporaceae	*Arthrinium arundinis/A. sacchari*	OL840786
Ascomycota	Microascales	Microascaceae	*Cephalotrichum stemonitis*	OL840790
Ascomycota	Pleosporales	Pleosporaceae	*Alternaria infectoria*	OL840800
Ascomycota	Pleosporales	Pleosporaceae	*Alternaria* sp.	OL840798
Ascomycota	Xylariales	Diatrypaceae	*Diatrype stigma*	OL840791
Ascomycota	Xylariales	Xylariaceae/Diatrypaceae	*Xylaria* sp./*Diatrype* sp.	OL840785
Basidiomycota	Sporidiobolales	Sporidiobolaceae	*Rhodotorula fujisanensis*	OL840788
Basidiomycota	Sporidiobolales	Sporidiobolaceae	*Rhodotorula mucilaginosa*	OL840781
Zygomycota	Mucorales	Mucoraceae	*Mucor circinelloides*	OL840782
Zygomycota	Mucorales	Mucoraceae	*Mucor flavus*	OL840796
Zygomycota	Mucorales	Mucoraceae	*Mucor* sp.	OL840783
Substrate before treatment				
Zygomycota	Mucorales	Mucoraceae	*Mucor circinelloides*	OM541309
Ascomycota	Eurotiales	Trichocomaceae	*Penicillium* sp. *1*	OM541310
Zygomycota	Mucorales	Mucoraceae	*Mucor* sp.	OM541311
Skin after treatment				
Zygomycota	Mucorales	Mucoraceae	*Mucor circinelloides*	OM541312
Zygomycota	Mucorales	Mucoraceae	*Mucor hiemalis*	OM541313
Ascomycota	Capnodiales	Mycosphaerellaceae	*Cladosporium* sp.	OM541314
Basidiomycota	Sporidiobolales	Sporidiobolaceae	*Sporidiobolus pararoseus*	OM541315
Ascomycota	Eurotiales	Trichocomaceae	*Penicillium* sp. 2	OM535924
Zygomycota	Mucorales	Mucoraceae	*Mucor* sp.	OM541316
Ascomycota	Saccharomycetales	Saccharomycetaceae	*Pichia terricola*	OM541317
Ascomycota	Hypocreales	Nectriaceae	*Fusarium oxysporum/F. proliferatum*	OM541318
Ascomycota	Hypocreales	Hypocreaceae	*Trichoderma hamatum/T. asperellum*	OM541319
Substrate after treatment				
Ascomycota	Capnodiales	Mycosphaerellaceae	*Cladosporium* sp.	OM541320
Zygomycota	Mucorales	Mucoraceae	*Mucor laxorrhizus*	OM541321
Ascomycota	Hypocreales	Hypocreaceae	*Trichoderma hamatum/T. asperellum*	OM541322
Ascomycota	Eurotiales	Trichocomaceae	*Penicillium crustosum*	OM535925
Ascomycota	Eurotiales	Trichocomaceae	*Penicillium* sp. 2	OM535926
Ascomycota	Hypocreales	Nectriaceae	*Fusarium oxysporum*	OM541323
Zygomycota	Mucorales	Mucoraceae	*Mucor hiemalis*	OM541324
Basidiomycota	Sporidiobolales	Sporidiobolaceae	*Rhodosporidiobolus colostri*	OM541325
Ascomycota	Hypocreales	Nectriaceae	*Fusarium oxysporum/F. proliferatum*	OM541326
Ascomycota	Saccharomycetales	Dipodascaceae	*Geotrichum* sp.	OM541327

as well as skin (Herbrecht et al. 2013). In snakes, it is also recognized as responsible of mycoses (Jacobson and Cheatwood, 2000; Barbosa et al., 2018). *Alternaria* spp., such as *A. infectoria*, can be responsible of subcutaneous and cutaneous infections and rhinosinusitis in human and various infections in e.g. cat and horse (Roosje et al., 1993; Pastor and Guarro, 2008; Dworecka-Kaszak et al., 2020). *Rhodotorula* spp. were considered as nonpathogenic species, but recent studies revealed that they can infect susceptible individuals from birds to mammals, including humans where it can cause e.g. meningeal, skin, ocular, peritoneal infections (Wirth and Goldani 2012; Tligui et al. 2018). *Aureobasidium pullulans* has been documented in a nosocomial fungal infection and can be systemic (Bolignano and Criseo, 2003; Joshi et al., 2010).

It is important to note that several species identified on snakes before the treatment of skin lesions were also detected on their skin after the treatment as well as in the substrate, such as *Cladosporium* sp. and *Mucor* sp. Hence, this result suggests that these taxa were likely not involved in the observed lesions (Table 1).

Concerning the other detected fungi such as *Penicillium* spp., they are commonly isolated from lesions in reptiles (Jacobson and Cheatwood, 2000), but in human data are scarce and mainly involved superficial infections such as keratitis and otomycosis (Lyratzopoulos et al., 2002). Similarly, *Fusarium* spp. can infect reptiles (Jacobson and Cheatwood, 2000), but are known to cause mainly superficial and localized infections in healthy individuals, whereas it can disseminate and be invasive in immunocompromised ones (Nucci and Anaissie, 2007). Finally, other detected fungal species i.e. *Cephalotrichum stemonitis* and Xylariales are not known for being pathogenic in animals and hence were very likely not involved in the observed skin lesions.

As a conclusion, the observed skin lesions were likely caused by a cocktail of opportunistic species, known for infecting mainly immunocompromised individuals. At this stage, it is not possible to establish whether the snakes had such an issue. However, the exceptional wet and cold conditions experienced in spring 2021 might have trigger the infections. Indeed, high humidity has been recorded as a predisposing factor for mycoses in captive reptiles (Jacobson and Cheatwood, 2000; Paré and Jacobson, 2007).

Ethical statement

The project was performed under the Authorization number: 2021-340 (Direction générale de l'environnement, Canton of Vaud, Switzerland).

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

References

Barbosa, L.N., Ferreira Jr., R.S., Mello, P.L., Mello, P.L., Garces, H.G., Chechi, J.L., Frachin, T., De Barros, L.C., De Moraes Guimenes Bosco, S., Bagagli, E., Fernandes Júnior, A., Barraviera, B., Delazari Dos Santos, L., 2018. Molecular identification and phylogenetic analysis of *Bothrops insularis* bacterial and fungal microbiota. J. Toxicol. Environ. Health, Part A 81, 142–153.

Bolignano, G., Criseo, G., 2003. Disseminated nosocomial fungal infection by *Aureobasidium pullulans* var. *melanigenum*: a case report. J. Clin. Microbiol. 41, 4483–4485.

Ceballos, G., Ehrlich, P.R., Raven, P.H., 2020. Vertebrates on the brink as indicators of biological annihilation and the sixth mass extinction. Proc. Natl. Acad. Sci. 117, 13596–13602.

Davy, C.M., Shirose, L., Campbell, D., Dillon, R., McKenzie, C., Nemeth, N., Braithwaite, T., Cai, H., Degazio, T., Dobbie, T., Egan, S., Fotherby, H., Litzgus, J.D., Manorome, P., Marks, S., Paterson, J.E., Sigler, L., Slavic, D., Slavik, E., Urquhart, J., Jardine, C., 2021. Revisiting ophidiomycosis (snake fungal disease) after a decade of targeted research. Front. Vet. Sci. 8, 448.

Dworecka-Kaszak, B., Biegańska, M.J., Dąbrowska, I., 2020. Occurrence of various pathogenic and opportunistic fungi in skin diseases of domestic animals: a retrospective study. BMC Vet. 16, 248.

Franklinos, L.H.V., Lorch, J.M., Bohuski, E., Rodriguez-Ramos Fernandez, J., Wright, O. N., Fitzpatrick, L., Petrovan, S., Durrant, C., Linton, C., Baláž, V., Cunningham, A.A., Lawson, B., 2017. Emerging fungal pathogen *Ophidiomyces ophiodiicola* in wild European snakes. Sci. Rep. 7, 3844.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes — application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113–118.

Henao-Martínez, A.F., Vela-Duarte, D., 2018. Cryptococcal meningitis and other opportunistic fungal infections of the central nervous system: epidemiology, pathogenesis, diagnosis, and treatment. Microbiol. Central Nerv. Syst. Infect., 261–278.

Herbrecht, R., Sabou, M., Ledoux, M.-P., 2013. Clinical and radiological aspects of mucormycosis. Med. Sci. (Paris) 29, 19–24.

Huggins, J.L., Garner, M.M., Raverty, S.A., Lambourn, D.M., Norman, S.A., Rhodes, L.D., Gaydos, J.K., Olson, J.K., Haulena, M., Hanson, M.B., 2020. The emergence of Mucormycosis in free-ranging marine mammals of the pacific Northwest. Front. Mar. Sci. 7, 555.

Jacobson, E.R., Cheatwood, J.L., 2000. Mycotic diseases of reptiles. Semin. Avian Exotic Pet Med. 9, 94–101.

Joshi, A., Singh, R., Shah, M.S., Umesh, S., Khattry, N., 2010. Subcutaneous mycosis and fungemia by *Aureobasidium pullulans*: a rare pathogenic fungus in a post allogeneic BM transplant patient. Bone Marrow Transplant. 45, 203–204.

Lorch, J.M., Knowles, S., Lankton, J.S., et al, 2016. Snake fungal disease: an emerging threat to wild snakes. Philos. Trans. R. Soc. B: Biol. Sci. 371, 20150457.

Lukac, M., Horvatek, D., Mihokovic, S., Mandac, Z., 2017. Oral and cloacal aerobic bacterial and fungal flora of free-living four-lined snakes (*Elaphe quatuorlineata*) from Croatia. Vet. Arch. 87, 351–361.

Lyratzopoulos, G., Ellis, M., Nerringer, R., Denning, D.W., 2002. Invasive infection due to penicillium species other than *P. marneffei*. J. Infect. 45 (3), 184–195.

Nucci, M., Anaissie, E., 2007. Fusarium infections in immunocompromised patients. Clin. Microbiol. Rev. 20 (4), 695–704.

Paré, J.A., Jacobson, E.R., 2007. Mycotic diseases of reptiles. In: Infectious Diseases and Pathology of Reptiles: Color Atlas and Text. p. 1030.

Pastor, F.J., Guarro, J., 2008. Alternaria infections: laboratory diagnosis and relevant clinical features. Clin. Microbiol. Infect. 14 (8), 734–746.

Rehner, S., Samuels, G.J., 1994. Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. Mycol. Res. 98, 625–634.

Roosje, P.J., de Hoog, G.S., Koeman, J.P., Willemse, T., 1993. Phaeohyphomycosis in a cat caused by Alternaria infectoria E. G. Simmons. Mycoses 36, 451–454.

Scheele, B.C., Pasmans, F., Skeerat, L.F., Berger, L., et al, 2019. Amphibian fungal panzootic causes catastrophic and ongoing loss of biodiversity. Science 363, 1459–1463.

Tligui, H., Oudaina, W., El Ftouh, S., Madda, F., Hesseissen, L., 2018. A skin ulcer infection due to *Rhodotorula mucilaginosa* in an immunocompromised child. J. Med. Mycol. 28, 215–217.

Visscher, A.M., Paul, A.L., Kirst, M., Guy, C.L., Schuerger, C., Ferl, R.J., 2010. Growth performance and root transcriptome remodeling of Arabidopsis in response to Mars-like levels of magnesium sulfate. PLoS ONE 5.

White, T.J., Bruns, T.D., Lee, S.B., Taylor, J.W., 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. (Eds.), PCR Protocols — A Guide to Methods and Applications. Academic Press, San Diego, CA, pp. 315–322.

Wirth, F., Goldani, L.Z., 2012. Epidemiology of *Rhodotorula*: An emerging pathogen. Interdisciplinary perspectives on infectious diseases 2012, 465717.

Yockey, J., Andres, L., Carson, M., Ory, J.J., Reese, A.J., Mitchell, A.P., 2019. Cell envelope integrity and capsule characterization of *Rhodotorula mucilaginosa* strains from clinical and environmental sources. Host-Microbe Biol. 4 (3).