CZECH MYCOLOGY 70(2): 101–121, AUGUST 19, 2018 (ONLINE VERSION, ISSN 1805-1421)

Microscopic fungi on cadavers and skeletons from cave and mine environments

ALENA NOVÁKOVÁ[1]*, ALENA KUBÁTOVÁ[2], FRANTIŠEK SKLENÁŘ[1,2], VÍT HUBKA[1,2]

[1] Laboratory of Fungal Genetics and Metabolism, Institute of Microbiology of the CAS, v.v.i., Vídeňská 1083, CZ-142 20 Praha 4, Czech Republic
[2] Department of Botany, Faculty of Science, Charles University, Benátská 2, CZ-128 01 Praha 2, Czech Republic
*corresponding author: ANmicrofungi@seznam.cz

Nováková A., Kubátová A., Sklenář F., Hubka V. (2018): Microscopic fungi on cadavers and skeletons from cave and mine environments. – Czech Mycol. 70(2): 101–121.

During long-term studies of microscopic fungi in 80 European caves and mine environments many cadavers and skeletons of animals inhabiting these environments and various animal visitors were found, some of them with visible microfungal growth. Direct isolation, the dilution plate method and various types of isolation media were used. The resulting spectrum of isolated fungi is presented and compared with records about their previous isolation.

Compared to former studies focused mainly on bat mycobiota, this paper contributes to a wider knowledge of fungal assemblages colonising various animal bodies in underground environments. The most interesting findings include ascocarps of *Acaulium caviariforme* found abundant on mammals cadavers, while *Botryosporium longibrachiatum* isolated from frogs, *Chaetocladium jonesiae* from bats and *Penicillium vulpinum* from spiders represent the first records of these species from cadavers or skeletons.

Key words: European caves, abandoned mines, dead bodies, bones, mammals, frogs, spiders, isopods, micromycetes.

Article history: received 11 May 2018, revised 17 June 2018, accepted 20 June 2018, published online 19 August 2018.

Nováková A., Kubátová A., Sklenář F., Hubka V. (2018): Mikroskopické houby na mrtvých tělech a kostrách živočichů v prostředí jeskyní a dolů. – Czech Mycol. 70(2): 101–121.

V průběhu dlouhodobého studia mikroskopických hub v osmdesáti evropských podzemních prostorách (jeskyně, opuštěné doly) byly nalezeny také mrtvá těla a kostry živočichů žijících v tomto prostředí či nahodilých návštěvníků, některé s viditelnými nárosty mikroskopických hub. Přímá izolace, zřeďovací metoda a různá média byly použity pro izolaci konkrétních druhů. Získané spektrum hub je prezentováno a srovnáno s předchozími nálezy.

Oproti dosavadním studiím, zaměřeným hlavně na houby napadající netopýry, je tato práce příspěvkem k širšímu poznání společenstev hub, kolonizujících těla různých živočichů v podzemním prostředí. Mezi nejzajímavější nálezy lze zařadit *Acaulium caviariforme*, jehož plodnice byly hojně nacházeny na mrtvých tělech savců, zatímco druhy *Botryosporium longibrachiatum* (izolované z žab), *Chaetocladium jonesiae* (z netopýrů) a také *Penicillium vulpinum* (z pavouků) představují první nálezy těchto druhů na mrvých tělech nebo kostrách živočichů.

INTRODUCTION

Hitherto, the majority of studies into microfungal assemblages in underground spaces have been predominantly focused on cave air and sediments or on rock and speleothem surfaces. This seemingly nutrient-poor ecosystem can however also include many spots with organic matter, such as plant material (e.g. seeds, twigs, detritus) washed from the environment above as well as various deposits of animal inhabitants, excretions of cave-inhabiting invertebrates and vertebrates, bat droppings and guano.

Underground environments including caves are often visited by various animals which seek refuge from unfavourable weather conditions or search for food, and some of them also fall into the underground (frogs, lizards). All of these animals may die after some time as a consequence of age, food absence or collapse from exhaustion. Cadavers of cave-inhabiting animals, mainly bats, are utilised as food for cave-inhabiting terrestrial invertebrates such as earthworms, isopods, diplopods, springtails, mites etc. which, together with cave microbiota, gradually decompose dead organic matter. Cadavers in various stage of decomposition are found on cave sediment or speleothems, from fresh dead bodies to skeletons or particular bones. Given the high air humidity and constant air temperature in the cave environment, successional decomposition is not limited to dry periods, as in above-ground conditions, but goes on continually. Some cadavers are covered by visible mycelia of microscopic fungi, other ones are without visible microfungal infection.

During long-term studies of cave mycobiota in various underground environments, from 2002 up to the present, mainly focused on microscopic fungi in cave air, cave sediments and bat guano, we found a number of recently deceased animals as well as skeletons and separate bones. We used various isolation techniques to elucidate which microfungal species colonise these substrates and are responsible for their decomposition. This report provides an overview of microfungal species which have been found on skeletons/bones and dead animals (with the exception of insects) inhabiting underground environments.

MATERIAL AND METHODS

Sampling sites. Studies of microfungal assemblages have been carried out since 2002, in a total of 80 underground environments of several European countries, mainly in the Czech Republic, Slovakia, Spain and Romania. Their original and English names and other characteristics are given in Tab. 1. Studied caves are karstic, oligotrophic or eutrophic, one is chemoautotrophic (Movile Cave), one is of marine origin (Treasure Cave), and two sites belong to non-karstic environments (Na Rozhraní Cave and Simon and Jude Mines).

Tab. 1. Overview of the studied caves. Caves in which cadavers or skeletons were found are in bold.

Country	Underground site	Original name	Underground type	Air temperature (°C)*
Czech Republic	Chýnov Cave	Chýnovská jeskyně	Karstic cave, show cave	5.0–9.0
	Koněprusy Caves	Koněpruské jeskyně	Karstic cave, show cave	10.6
	Bozkov Dolomite Caves	Bozkovské dolomitové jeskyně	Karstic cave, show cave	7.5–9.0
	Na Pomezí Caves	Jeskyně Na Pomezí	Karstic cave, show cave	7.0–8.0
	Na Špičáku Cave	Jeskyně Na Špičáku	Karstic cave, show cave	7.0–9.0
	Mladeč Caves	Mladečské jeskyně	Karstic cave, show cave	8.0
	Javoříčko Caves	Javoříčské jeskyně	Karstic cave, show cave	7.0–8.0
	Zbrašov Aragonite Caves	Zbrašovské aragonitové jeskyně	Hydrothermal cave, show cave	14.0–16.0
	Hranická Abyss – Rotunda	Hranická propast – Rotunda	Hydrothermal abyss	14.3–18.0
	Na Turoldu Cave	Jeskyně Na Turoldu	Karstic cave, show cave	7.1–9.1
	Punkva Caves	Punkevní jeskyně	Karstic cave, show cave	7.0–8.0
	(Catherine's) Kateřina's Cave	Kateřinská jeskyně	Karstic cave, show cave	7.0–8.0
	Balcarka Cave	Jeskyně Balcarka	Karstic cave, show cave	7.0–8.0
	Sloup-Šošůvka Caves	Sloupsko-šošůvské jeskyně	Karstic cave, show cave	7.0–8.0
	Výpustek Cave	Jeskyně Výpustek	Karstic cave, show cave	7.0–9.0
	Pustožlebská zazděná Cave	Pustožlebská zazděná jeskyně	Karstic cave, closed to the public	7.0–9.0
	New Amateur Cave	Nová Amatérská jeskyně	Karstic cave, closed to the public	7.0–9.0
	Býčí skála Cave	Jeskyně Býčí skála	Karstic cave, closed to the public	7.0–9.0
	Harbešská Cave	Harbešská jeskyně	Karstic cave, closed to the public	7.0–9.0
	Manželský Sinkhole	Manželský závrt	Karstic cave, closed to the public	7.0–9.0
	Králova Cave	Králova jeskyně	Karstic cave, closed to the public	9.0–10.0
	Pod křížem Cave	Jeskyně Pod křížem	Karstic cave, closed to the public	9.0–10.0
	Na Rozhraní Cave	Jeskyně Na Rozhraní	Non-karstic cave, closed to the public	9.0–10.0
	Ochozská Cave	Ochozská jeskyně	Karstic cave, closed to the public	8.0
	Slámova sluj Cave	Jeskyně Slámova sluj	Karstic cave, closed to the public	8.0–10.0
	Simon and Jude Mines	Doly Šimona a Judy	Abandoned iron ore mines	5.0–10.0

Country	Underground site	Original name	Underground type	Air temperature (°C)*
Slovakia	**Domica Cave**	Jaskyňa Domica	Karstic cave, show cave	10.2–11.4
	Čertova diera Cave	Jaskyňa Čertova diera	Karstic cave, closed to the public	10.2–11.4
	Ardovská Cave	Ardovská jaskyňa	Karstic cave, closed to the public	7.9–11.5
	Gombasecká Cave	Gombasecká jaskyňa	Karstic cave, show cave	9.0–9.4
	Krásnohorská Cave	Krásnohorská jaskyňa	Karstic cave, show cave	8.7–8.9
	Hrušovská Cave	Hrušovská jaskyňa	Karstic cave, closed to the public	9.0
	Jasovská Cave	Jasovská jaskyňa	Karstic cave, show cave	8.8–9.4
	Šingliarova Abyss	Šingliarova priepasť	Karstic cave, closed to the public	9.0–10.0
	Stará Brzotínska Cave	Stará Brzotínska jaskyňa	Karstic cave, closed to the public	9.5–10.4
	Drienovská Cave	Drienovská jaskyňa	Karstic cave, closed to the public	9.3–11.3
	Silická Ice Cave	Silická ľadová jaskyňa	Karstic cave, closed to the public	4.0–10.0 (–1.0 to –11.0)**
	Ochtinská Aragonite Cave	Ochtinská aragonitová jaskyňa	Karstic cave, show cave	7.6–8.4
	Dobšinská Ice Cave	Dobšinská ľadová jaskyňa	Karstic cave, show cave	–0.4 to –1.0 (0.8–3.5)**
	Demänovská Ice Cave	Demänovská ľadová jaskyňa	Karstic cave, show cave	0 (1.3–5.7)**
	Demänovská Peace Cave	Demänovská jaskyňa Mieru	Karstic cave, closed to the public	6.5–7.0
	Demänovská Cave of Liberty	Demänovská jaskyňa Slobody	Karstic cave, show cave	6.1–7.0
	Dead Bats Cave	Jaskyňa mŕtvych netopierov	Karstic cave, show cave	3.0–3.5
	Bobačka Cave	Jaskyňa Bobačka	Karstic cave, closed to the public	7.3–9.1
	Harmanecká Cave	Harmanecká jaskyňa	Karstic cave, show cave	5.8–6.4
	Modrovská Cave	Modrovská jaskyňa	Karstic cave, closed to the public	8.9–9.1
	Suchá Cave	Suchá jaskyňa	Karstic cave, closed to the public	3.4–9.5
	Pružinská Dúpna Cave	Pružinská Dúpna jaskyňa	Karstic cave, show cave	7.4–8.9
	Belianska Cave	Belianska jaskyňa	Karstic cave, show cave	5.0–5.8
	Milada Cave	Jaskyňa Milada	Karstic cave, closed to the public	9.0–10.0
	Michňová Abyss	Michňová priepasť	Karstic cave, closed to the public	8.0–10.0
	Brestovská Cave	Brestovská jaskyňa	Karstic cave, show cave	4.9–5.4
	Perlová Cave	Perlová jaskyňa	Karstic cave, closed to the public	9.0–10.0
Spain	Altamira Cave	Cueva de Altamira	Karstic cave, closed to the public	18.0
	Ardales Cave	Cueva de Doña Trinidad	Karstic cave, show cave	17.6

Country	Underground site	Original name	Underground type	Air temperature (°C)*
	Castañar de Ibor Cave	Cueva de Castañar	Karstic cave, show cave	16.9
	Treasure Cave	Cueva del Tesoro	Cave of marine origin, show cave	17.6–19.2
	Grotto of the Marvels	Gruta de las Maravillas	Karstic cave, show cave	18.1–19.0
	Nerja Cave	Cueva de Nerja	Karstic cave, show cave	19.5–20.5
Romania	Scărişoara Ice Cave	Peştera ghetarul Scărişoara	Karstic cave, show cave	0–1.0 (–7.0 in winter)
	Vârtop Ice Cave	Peştera ghetarul Vârtop	Karstic cave, show cave	0–3.0
	Coiba Mare Cave	Peştera Coiba Mare	Karstic cave, closed to the public	9.0–10.0
	Poarta lui Ionele Cave	Peştera Poarta lui Ionele	Karstic cave, show cave	9.0–10.0
	Fânaţe Cave	Peştera de la Fânaţe	Karstic cave, closed to the public	9.0–10.0
	Ferice Cave	Peştera de la Ferice	Karstic cave, closed to the public	9.0–10.0
	Măgura Cave	Peştera Măgura	Karstic cave, closed to the public	11.0–12.0
	Urşilor Cave (Bears Cave)	Peştera Urşilor	Karstic cave, show cave	9.8
	Meziad Cave	Peştera Meziad	Karstic cave, show cave	9.0–10.0
	Limanu Cave	Peştera Limanu	Karstic cave, closed to the public	**15.0–18.0**
	Liliecilor de la Gura Dobrogei Cave	Peştera Liliecilor de la Gura Dobrogei	Karstic cave, closed to the public	**18.0–19.0**
	Movile Cave	Peştera Movile	Chemoautotrophic cave, closed to the public	**21.5**
	Zidita Cave	Peştera Zidita	Karstic cave, closed to the public	9.0–10.0
	Dracoaia Cave	Peştera Dracoaia	Karstic cave, closed to the public	9.0–10.0
	Ungurului Cave	Peştera Ungurului	Karstic cave, closed to the public	9.0–10.0
	Coliboaia Cave	Peştera Coliboaia	Karstic cave, closed to the public	9.0–10.0
	Ungureasca Cave	Peştera Ungureasca	Karstic cave, show cave	9.0–10.0
Hungary	**Baradla Cave**	Baradla barlang	Karstic cave, show cave	**10.2**
Croatia	Hrustovača Cave	Hrustovača pečina	Karstic cave, closed to the public	10.0–12.0
Slovenia	Škocjan Caves	Škocjanske jame	Karstic cave, show cave	12.0
France	Lascaux Cave	Grotte de Lascaux	Karstic cave, closed to the public	12.7

* Temperature values were taken from official websites of the caves or the Czech and Slovak Caves Administration. Temperatures measured by the authors (digital Multi-thermometer, Guangdong, China) are in bold.

** Different values indicate temperatures in different parts of the cave (with or without ice deposition).

All studied sites are characterised by high air humidity (c. 98–99%) but air temperature differed according to their geographical location as well as to cave type. While Czech, Slovak, and some Romanian caves in the Apuseni Mts. are characterised by an air temperature of 7 to 11 °C, most of the studied Spanish caves and Romanian ones in the Dobrogea region are warmer cave systems with an air temperature of 16–21 °C. Some of the studied caves are abundantly inhabited by bats (e.g. the Czech Javoříčko Caves or Na Turoldu Cave, the Slovak Domica Cave, Drienovská Cave, Dead Bats Cave, Jasovská Cave, and Ardovská Cave, the Romanian Liliecilor de la Gura Dobrogei Cave, Meziad Cave, Ferice Cave and Fânaţe Cave), in others bats are less common (e.g. the Slovak Demänovská Peace Cave, Demänovská Cave of Liberty, Gombasecká Cave and Krásnohorská Cave, the Romanian Limanu Cave, the Spanish Nerja Cave, the Czech Kateřina's Cave and Sloup-Šošůvka Caves). In contrast, bats are absent from the Romanian Movile Cave, the Spanish Grotto of the Marvels, Ardales Cave, Altamira Cave, and the Slovak Ochtinská Aragonite Cave. Several caves, such as Altamira Cave, Grotto of the Marvels, and Ardales Cave (Spain), are regularly visited by various species of rodents.

Isolation and identification. Samples of cadavers – dead bodies – with visible microfungal growth and degrading organic matter were collected using sterile forceps into sterile microtubes or directly on agar isolation media in Petri dishes. In the laboratory, isolations were carried out by transferring the collected material on agar discs in Petri dishes or preparing a dilution in sterile water. Dichloran rose Bengal chloramphenicol agar (DRBC), Sabouraud's glucose agar and beer wort agar, both with rose Bengal (0.1 g/l) and chroramphenicol (0.1 g/l) (Atlas 2010) were used as isolation media. Cultivation lasted for 7 days at 25 °C in the dark.

Microfungal identification was performed according to the macro- and micromorphological characters using relevant taxonomic literature focused on particular genera (e.g. de Hoog 2000, Samson & Varga 2004, 2007, Samson & Houbraken 2011, Seifert et al. 2011, Samson et al. 2011, 2013, Bensch et al. 2012, Guarro et el. 2012, Dijksterhuis et al. 2013, Hubka et al. 2016, Sandoval-Denis et al. 2016, Chen et al. 2016, 2017, etc.). Malt extract agar (MEA), Czapek yeast autolysate agar (CYA) and Czapek-Dox agar (CZA) were used for microfungal identification (Atlas 2010).

Molecular analysis. Some isolates were also identified with molecular methods. DNA was extracted from 7-day-old colonies with the ArchivePure DNA yeast and Gram2 +kit (5PRIME Inc., Gaithersburg, Maryland, USA) with modified incubation times: lytic enzyme solution (2 h, 37 °C) and cell lysis solution (4 h, 64 °C). Partial *caM* gene encoding calmodulin was amplified using forward primers CF1M or CF1L and reverse primer CF4 (Peterson 2008). The PCR mixture (total volume 25 µl) contained 0.1 µl MyTaq DNA polymerase (Bioline GmbH, Luckenwalde, Germany), 5 µl MyTaq Reaction Buffer, 1 µl of each primer (10 µM stock concentration), and 1 µl (50 ng) genomic DNA. The standard thermal cycle profile was 93 °C for 2 min; 38 cycles of 93 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and final extension 72 °C for 10 min. Automated sequencing was performed at Macrogen Sequencing Service (Amsterdam, The Netherlands) using the forward and reverse primers. The obtained sequences were inspected and assembled with BioEdit version 7.1.8 (www.mbio. ncsu.edu/BioEdit/bioedit.html) and then compared with those derived from ex-type strains deposited in the GenBank database in order to identify the isolates at the species level.

Representative strains of the sequenced species are deposited in the Culture Collection of Fungi at the Department of Botany, Charles University in Prague (CCF). Obtained DNA sequences were deposited in the European Nucleotide Archive (ENA) database (Tab. 2).

Tab. 2. GenBank database accession numbers of the sequences obtained in this study.

Species	Locality	Strain number / culture collection code*	ITS rDNA	*caM*
Aspergillus aureolatus	Fânaţe Cave	S193		LS974072
Aspergillus baeticus	Movile Cave	S349 = CCF 5046		LT558749
Aspergillus baeticus	Demänovská Peace Cave	CMF ISB 2181 = CCF 4231		HE615119
Aspergillus creber	Demänovská Peace Cave	S321		LS974073
Aspergillus movilensis	Movile Cave	Mo10 = CCF 4410 = CMF ISB 2614 = NRRL 62819 = CBS 134395		HG916740
Aspergillus parasiticus	Harmanecká Cave	S146		LS974074
Aspergillus tennesseensis	Movile Cave	S135		LS974075
Aspergillus thesauricus	Movile Cave	S552 = CCF 4968		LT558753
Aspergillus thesauricus	Movile Cave	S741		LS974076
Botryosporium longibrachiatum	Domica-Baradla cave system	CCF 5732	LS974068	

* CCF – Culture Collection of Fungi, Prague; CMF – Collection of Microscopic Fungi ISB, České Budějovice; S and Mo – strains from A. Nováková's working collection.

RESULTS AND DISCUSSION

Fungi on bat cadavers

Bats belong to the most commonly known inhabitants in caves and other underground environments. Unfortunately, until recently studies were focused on the occurrence of microfungi on bat fur (Larcher et al. 2003, Beguin et al. 2005) and later mainly on *Pseudogymnoascus* (*Geomyces*) *destructans* as an infection agent of WNS (white nose syndrome) (Blehert et al. 2008, Gargas et al. 2009,

Tab. 3. Overview of microfungal taxa isolated from dead animal bodies or bones.
Sites of origin: 1 – Javoříčko Caves, 2 – Sloup-Šošůvka Caves, 3 – Simon and Jude Mines, 4 – Domica Cave, 5 – Gombasecká Cave, 6 – Demänovská Peace Cave, 7 – Dead Bats Cave, 8 – Harmanecká Cave, 9 –Krásnohorská Cave, 10 – Nerja Cave, 11 – Fânaţe Cave, 12 – Liliecilor de la Gura Dobrogei Cave, 13 – Movile Cave, 14 – Baradla Cave.

Dead animals	**marten**	**dormouse**	**bat**										**spider**			**isopod**	**frog**	
Caves	**6**	**5**	**1**	**2**	**3**	**4**	**6**	**7**	**8**	**10**	**11**	**12**	**5**	**9**	**13**	**13**	**4**	**14**
Mucoromycota																		
Chaetocladium jonesiae (Berk. & Broome) Fresen.			+		+													
Mortierella horticola Linnem.								+										
Mortierella humilis Linnem. ex W. Gams				+		+		+										
Mortierella sp.										+		+						
Mucor hiemalis Wehmer f. *hiemalis*								+										
Mucor hiemalis f. *luteus* (Linnem.) Schipper				+														
Mucor hiemalis f. *silvaticus* (Hagem) Schipper								+										
Mucor mucedo P. Micheli ex St.-Amans								+										
Mucor racemosus Fresen. var. *racemosus*				+			+	+										
Mucor wosnessenskii Schostak.							+	+										
Rhizomucor pusillus (Lindt) Schipper								+										
Thamnidium elegans Link				+														
Ascomycota																		
Acaulium caviariforme (Malloch & Hubart) Sandoval-Denis, Guarro & Gené*		+					+		+									
Aspergillus aureolatus Munt.-Cvetk. & Bata											+							
Aspergillus baeticus A. Nováková & Hubka							+								+			
Aspergillus creber Jurjević, S.W. Peterson & B.W. Horn	+																	
Aspergillus movilensis Nováková, Hubka, M. Kolařík & S.W. Peterson																+		
Aspergillus parasiticus Speare									+									
Aspergillus tennesseensis Jurjević, S.W. Peterson & B.W. Horn															+			
Aspergillus thesauricus Hubka & A. Nováková																+		
Botryosporium longibrachiatum (Oudem.) Maire																	+	+
Cephalotrichum stemonitis (Pers.) Nees							+											
Chrysosporium sp.								+										
Cladosporium cladosporioides (Fresen.) G.A. de Vries							+											
Cladosporium herbarum (Pers.) Link								+										
Cladosporium sphaerospermum Penz.								+										
Cylindrocarpon obtusiusculum (Sacc.) U. Braun							+											
Fusarium merismoides Corda							+											
Gliomastix chartarum (Corda) S. Hughes								+										
Oidiodendron griseum Robak							+	+										
Penicillium aurantiogriseum Dierckx							+											

Dead animals	**marten**	**dormouse**	**bat**										**spider**			**isopod**	**frog**	
Caves	**6**	**5**	**1**	**2**	**3**	**4**	**6**	**7**	**8**	**10**	**11**	**12**	**5**	**9**	**13**	**13**	**4**	**14**
Penicillium chrysogenum Thom							+											
Penicillium corylophilum Dierckx							+											
Penicillium olsonii Bainier & Sartory								+										
Penicillium vulpinum (Cooke & Massee) Seifert & Samson													+	+				
Pseudogymnoascus pannorum complex							+											
Undetermined strains																		
Sterile dark pigmented mycelium								+										
Undetermined yeast							+											
Undetermined basidiomycetes								+										

* The identification was carried out only on microscopic features of collected material, not isolated.

Martínková et al. 2010, Wibbelt et al. 2010, Puechmaille et al. 2011, Johnson et al. 2013, Zukal et al. 2014, Vanderwolf et al. 2016 etc.) and microfungal colonisation of bat cadavers was neglected.

At the place of their roosting or hibernation as well as in places where they move through, their cadavers can be found in various stages of decomposition and with progressive microfungal colonisation. Bat cadavers with white cottony overgrowth (mostly of *Myotis myotis*, but in some case unidentifiable due to disintegration) were found in several caves during 2005–2011, e.g. Domica Cave, Dobšinská Ice Cave and Dead Bats Cave (Slovakia), Nerja Cave (Spain), and Liliecilor de la Gura Dobrogei Cave (Romania) (Fig. 1a–c). From all these cadavers, non-sporulating strains of the genus *Mortierella* were isolated, which were identified on the genus level according to their characteristic colony growth (typical lobate colonies) and garlic odour. A hanging dead bat (*Myotis myotis*) with white mycelial overgrowth (Fig. 1d) identified according to its macro- and micromorphological properties as *Mortierella humilis* (Fig. 1f–g) was found in Sloup-Šošůvka Caves (Moravian Karst, Czech Republic). This species was also isolated from fine mycelium growing on bat bones (Fig. 1e) on display in the Dead Bats Cave (Slovakia). *Aspergillus aureolatus* was isolated from bat cadavers found in the entrance corridor of the Liliecilor de la Gura Dobrogei Cave in Romania. During our sampling in galleries of the Simon and Jude Mines near the village of Malá Morávka (northern Moravia, Czech Republic) in spring 2015 we found several fresh bat cadavers. These bats had been killed by marten (*Martes foina*), after which their cadavers in an early stage of decomposition were colonised by predominantly zygomycetous fungi (Fig. 2), especially by *Mucor* spp. and *Chaetocladium jonesiae*. *Chaetocladium* sp. was also found on a bat cadaver collected in Javoříčko Caves (Czech Republic). Species of the genus

Fig. 1. *Mortierella* spp. on dead bat bodies. **a** – Domica Cave (Slovakia); **b** – Nerja Cave (Spain); **c** – Liliecilor de la Gura Dobrogei Cave (Romania); **d** – growth of *Mortierella humilis* on dead *Myotis myotis* (Sloup-Šošůvka Caves, Czech Republic); **e** – exhibition of bones in Dead Bats Cave (Slovakia); **f** – 14-day old colony of *M. humilis* on malt extract agar; **g** – *M. humilis*, sporangiophore with young sporangium. Scale bar = 20 µm. Photo A. Nováková (a–c, e–g), P. Zajíček (d).

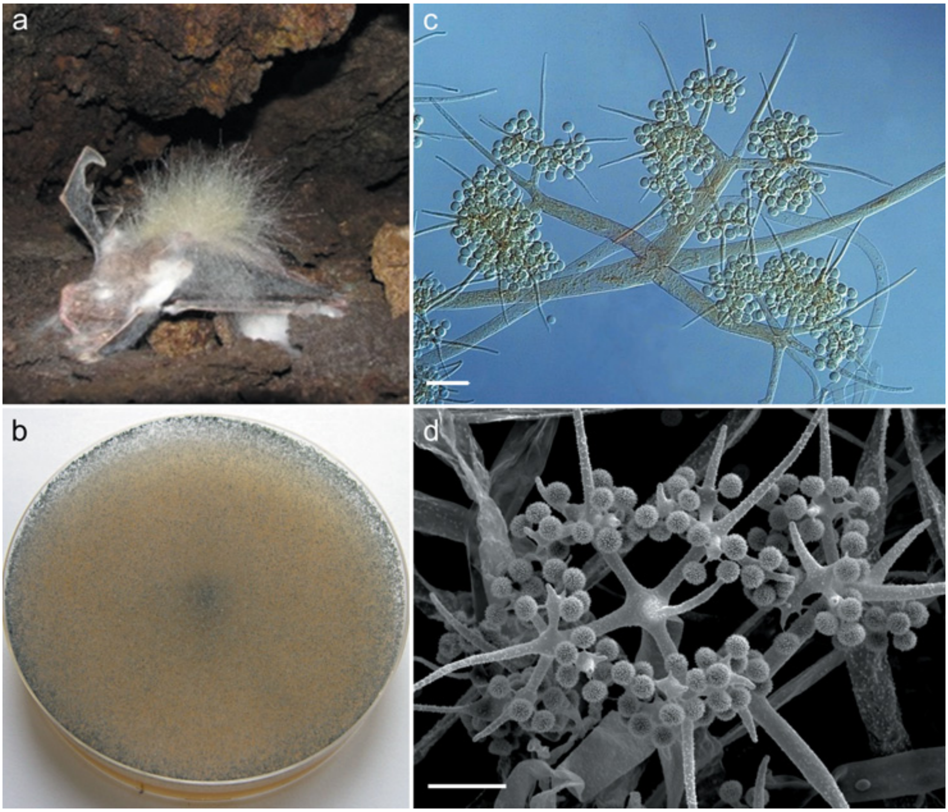


Fig. 2. *Chaetocladium jonesiae*. **a** – colonies on dead bats in Simon and Jude Mines, Malá Morávka (Czech Republic); **b** – 10-day old colony on MEA; **c, d** – branched fertile hyphae with unispored sporangia and sterile spines (c – Nomarski interference contrast, d – SEM). Scale bars = 20 μm. Photo A. Kubátová.

Chaetocladium mainly belong to facultative, gall-forming parasites on other *Mucorales* (Benny 2005) and strains of this genus were earlier isolated from marten dung in Ardovská Cave as well as from cave sediment of the Domica Cave (Nováková, unpublished).

On the contrary, older bat cadavers found in Demänovská Peace Cave and Harmanecká Cave (Fig. 3) as well as dormouse cadavers found in Gombasecká Cave (Slovak Karst, Slovakia) were found to be covered by a mass of black ascocarps of *Acaulium caviariforme.* The isolation of this species was unsuccessful but its typical morphology enabled reliable identification. In the case of two dormouse (*Dryomys nitedula*) cadavers found in Gombasecká Cave after summer floods, we could observe a fungal succession on dead bodies: the occurrence of zygomycetous fungi (*Mucor* spp. and *Mortierella* spp. in an early stage) was fol-

Fig. 3. *Acaulium caviariforme*. **a** – black ascocarps on dead dormouse body; **b** – detail of dead dormouse body with black ascocarps of *A. caviariforme* and white *Mortierella* sp. growth; **c–f** – a mass of black ascocarps on bat skeletons from Slovak caves: Guličková Passage in Demänovská Peace Cave (c, d), Ruins Passage in Demänovská Peace Cave (e), Stray Dome in Harmanecká Cave (f). Photo A. Nováková.

lowed by large numbers of ascocarps of *Acaulium caviariforme* after several months. This fungus was first found in the Cave of Ramioul (Belgium) and described as *Microascus caviariformis* (Malloch & Hubart 1987) and recently transferred to the genus *Acaulium* (Sandoval-Denis et al. 2016). Vanderwolf et al.

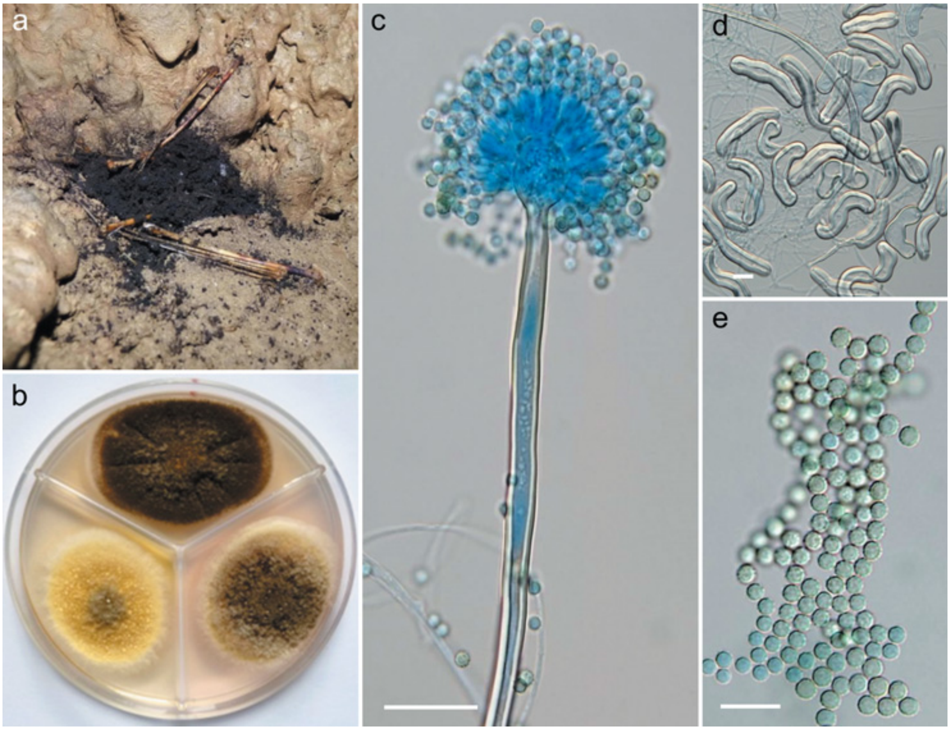


Fig. 4. *Aspergillus baeticus*. **a** – bat skeleton with a mass of black material from which this species was isolated (Guličková Passage in Demänovská Peace Cave, Slovakia); **b** – 7-day old colonies on CYA (top), MEA (bottom left) and CZA (bottom right); **c** – conidiophore; **d** – Hülle cells; **e** – conidia. Scale bars = 20 µm (c, d), 10 µm (e). Photo A. Nováková (a, b), A. Kubátová (c–e).

(2013a) isolated this species from fur of hibernating bats in three New Brunswick caves, Canada. The occurrence of *A. caviariforme* ascomata on bat carcasses in an advanced stage of decomposition, just consisting of remnant fur and bone, was observed in the New Brunswick hibernacula (Vanderwolf et al. 2016a). In spite of the fact that this species has been rarely reported from underground environments, it is probably a common microfungal species participating in the decomposition of dead animals in caves and mines.

Using the dilution isolation method (Garrett 1981), several filamentous fungi were isolated during our surveys (Tab. 3). *Aspergillus baeticus*, *Cephalotrichum stemonitis*, *Cylindrocarpon obtusiusculum*, *Fusarium merismoides*, *Mucor racemosus* var. *racemosus*, *Mucor wosnessenskii*, *Oidiodendron griseum* and *Pseudogymnoascus pannorum* s.l. were isolated from dead bats collected in the Demänovská Peace Cave. *Aspergillus baeticus* (Fig. 4) had already been isolated from Spanish Treasure Cave, Grotto of the Marvels (Nováková et al. 2012) and

Tab. 4. An overview of microfungal taxa isolated from dead bats, reported in previous studies.

References	Country	Species
Zeller (1966)	Hungary	*Chrysosporium merdarium*
Wibbelt et al. (2010)	United Kingdom	*Penicillium* sp.
Voyron et al. (2011)	Italy	*Alternaria* sp., *Aspergillus* sp., *Candida palmioleophila, Chrysosporium/Gymnoascus*, *Chrysosporium merdarium*, *Cladosporium cladosporioides*, *Fusarium dimerum*, *Fusarium equiseti*, *Gymnoascaceae*, *Lecanicillium lecanii*, *Mortierella gamsii*, *Mortierella polycephala*, *Mucor hiemalis* f. *hiemalis*, *Mucor plumbeus*, *Mucor racemosus*, *Ophiostomataceae*, *Penicillium griseofulvum*, *Penicillium* sp., *Thielavia* sp., *Trichosporon chiropterorum*
Nováková (2012)	Slovakia	*Mortierella humilis*
Vanderwolf et al. (2013b)	world	*Acremonium implicatum*, *Alternaria* sp., *Arthroderma silverae*, *Aspergillus baeticus*, *Aspergillus* sp., *Candida palmioleophila*, *Chrysosporium merdarium*, *Chrysosporium* sp., *Cladosporium cladosporioides*, *Clonostachys rosea* f. *catenulata*, *Fusarium dimerum*, *Fusarium equiseti*, *Gliocladium atrum*, *Gymnoascus* sp., *Gymnoascaceae* unidentified, *Lecanicillium lecanii*, *Mortierella gamsii*, *Mortierella polycephala*, *Mucor hiemalis* f. *hiemalis*, *Mucor piriformis*, *Mucor plumbeus*, *Mucor racemosus*, *Ochroconis constricta*, *Ophiostomataceae* unidentified, *Penicillium glandicola*, *Penicillium restrictum*, *Penicillium* sp., *Scolecobasidium tenerum*, *Sporobolomyces pruinosum*, *Thielavia terricola*, *Thielavia* sp., *Trichosporon chiropterorum*
Vanderwolf et al. (2016)	Canada	*Acaulium caviariforme*, *Acremonium* spp., *Arachniotus* sp., *Arthroderma silverae*, *Cephalotrichum stemonitis*, *Chrysosporium merdarium*, *Chrysosporium* sp., *Fusarium* sp., *Leuconeurospora capsici*, *Mortierella* sp., *Mucor* sp., *Oidiodendron truncatum*, *Pseudogymnoascus destructans*, *Pseudogymnoascus pannorum* s.l., *Trichoderma* sp., *Trichosporon dulcitum*

Nerja Cave (Nováková, unpublished) and was later isolated from the Romanian Movile Cave (Nováková et al. 2018). *Aspergillus parasiticus*, *Gliomastix chartarum*, *Mortierella humilis*, *M. horticola*, *Mucor hiemalis* var. *hiemalis*, *M. hiemalis* var. *silvaticus*, *M. mucedo*, *M. wosnessenskii*, *Cladosporium herbarum* s.l., *Rhizomucor pusillus* and a sterile dark pigmented fungus were isolated from a dead bat collected in Harmanecká Cave and *Clonostachys rosea* f. *rosea*, *Mortierella* sp. and *Mucor mucedo* were isolated from dead dormouse bodies found in the Gombasecká Cave.

Previously published records of micromycetes including yeasts isolated from dead bats in underground environments are given in Tab. 4. A total of 48 fungal species were isolated from bat cadavers. Twenty microfungal taxa are reported in the study on dead bats in the Grotta del Palummaro located in southern Italy and in the Grotta delle Vene, Piedmont, Italy (Voyron et al. 2011), while 16 microfungal species were isolated from dead bats in New Brunswick hibernacula (Vanderwolf et al. 2016). A world review of Canadian researchers (Vanderwolf et al. 2013b) reported a survey of published records of fungi, yeasts and slime

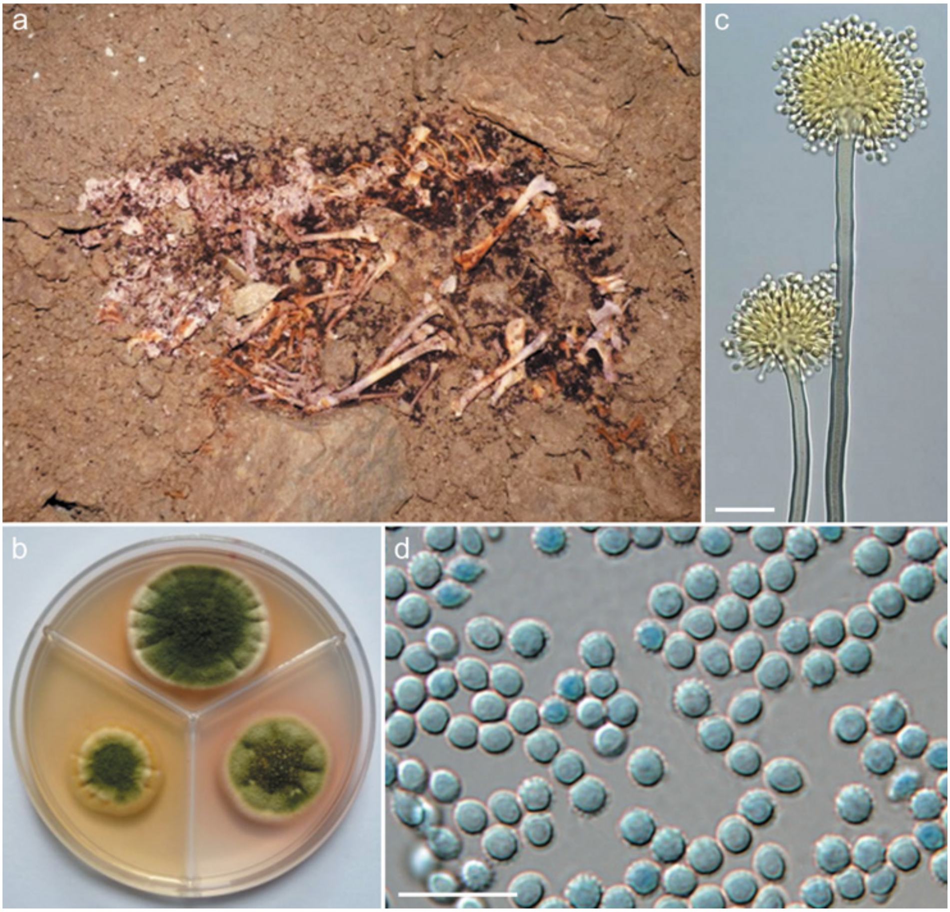
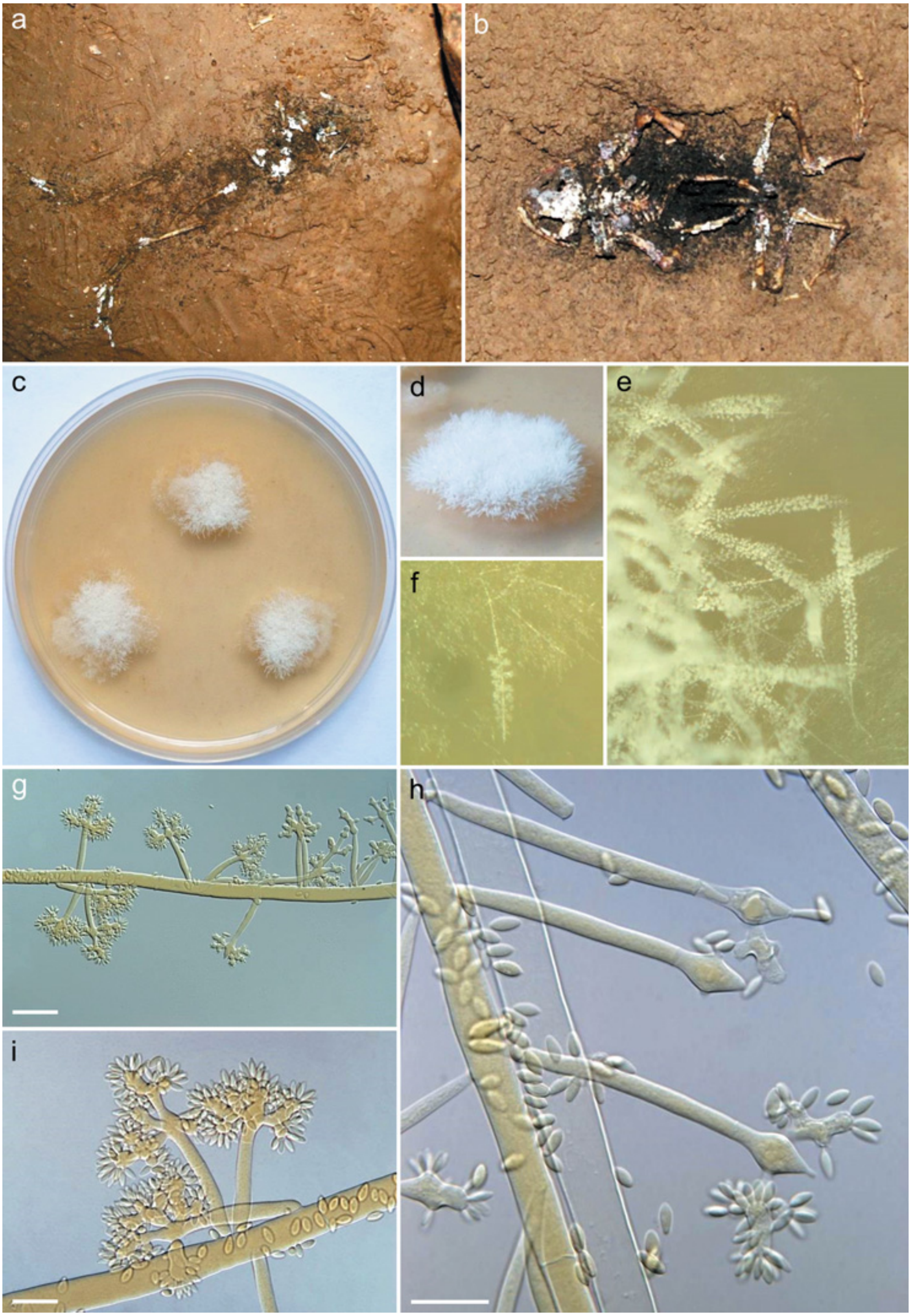


Fig. 5. *Aspergillus creber.* **a** – marten skeleton in Guličková Passage, Demänovská Peace Cave (Slovakia) from which the species was isolated; **b** – 7-day old colonies on CYA (top), MEA (bottom left) and CZA (bottom right); **c** – conidiophore; **d** – conidia. Scale bars = 20 µm (c), 10 µm (d). Photo A. Nováková (a, b), A. Kubátová (c, d).

moulds in caves including 32 microfungal species isolated from bat cadavers. On the contrary, Zeller (1966) and Wibbelt et al. (2010) presented only one isolated species. The most frequently reported species from dead bats is *Chrysosporium merdarium*. Nováková (2009) isolated a species identified as *Mortierella humilis* only once (Nováková 2012).

Our records are rather poor in comparison with the results of studies by Voyron et al. (2011) and Vanderwolf et al. (2016) only targeted at mycobiota of dead bats, because they originate from random finds during studies focused on


a
b
c
d
e
f
g
h
i

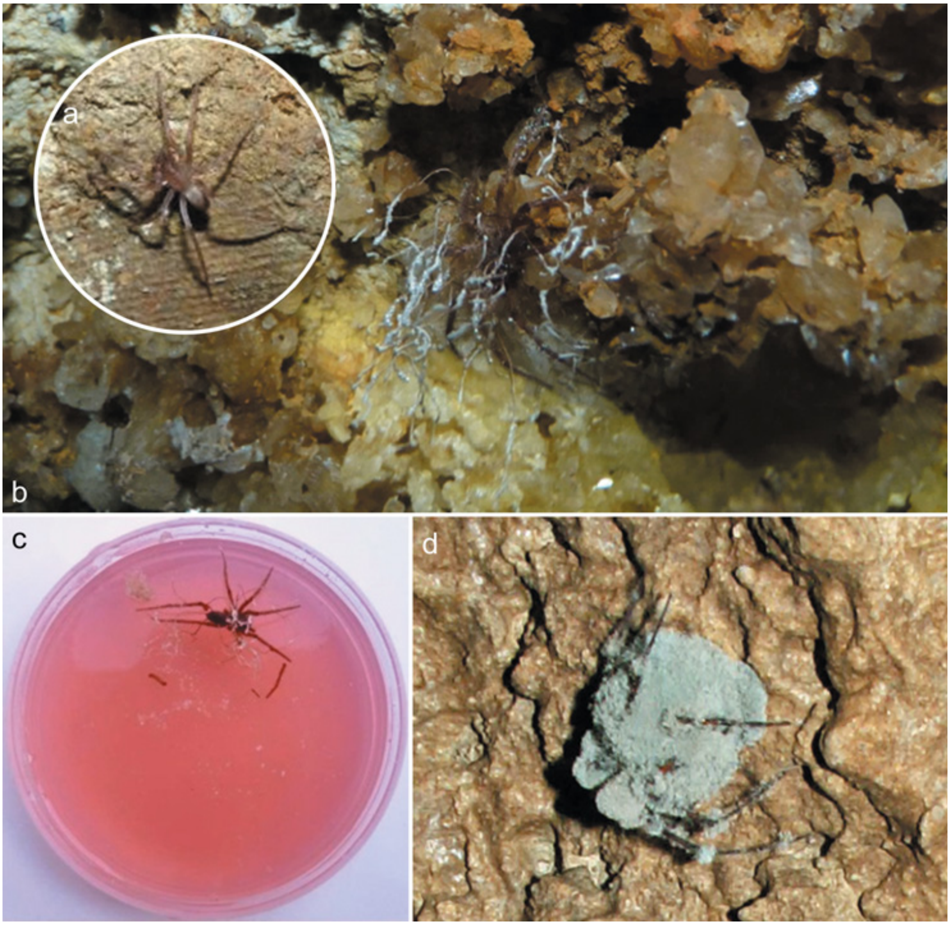


Fig. 7. Spiders. **a** – *Agraecina cristianii*; **b** – dead *A. cristianii* with synnematal microfungal growth, cave wall (Lake Room, Movile Cave, Romania); **c** – collected sample on Petri dish with DRBC; **d** – colonies of *Penicillium vulpinum* on dead spider body (*Meta menardi*) in entrance corridor of Krásnohorská Cave (Slovakia). Photo A. Nováková.

◀ **Fig. 6.** *Botryosporium longibrachiatum*. **a, b** – frog skeletons with visible white growth (Domica-Baradla cave system, Slovakia); **c, d** – 14-day old colonies of *B. longibrachiatum* on MEA; **e, f** – detail of colony margin with visible conidiophores; **g–i** – conidiophore details: branches with conidiogenous cells and conidia. Scale bars = 50 µm (g), 20 µm (h, i). Photo A. Nováková (a–f), A. Kubátová (g–i).

cave mycobiota including fungal occurrence in cave air, sediments, bat guano, and animal excretions. Nevertheless, some of our records are very interesting and include rarely isolated species and first reports from this substrate.

Fungi on other animals

Underground environments are also visited by other animals, e.g. martens (*Martes foina*), dormice (*Dryomys nitedula*), frogs (*Pelophylax* sp.), and spiders (*Meta menardi* in entrance sections but also some true cave-inhabitants such as the endemic *Agraecina cristianii* in Movile Cave). Besides the abovementioned dormice, martens are very frequent visitors to caves and abandoned mines. Some of them enter underground sites to protect themselves from bad weather conditions or in order to search for food. Therefore marten cadavers or skeletons were also occasionally found in caves – e.g. a well-preserved marten skeleton was found in the Guličková Passage of the Demänovská Peace Cave (Fig. 5). Bones lacked visible microfungal growth, but cultivation yielded *Aspergillus creber* (identified using the *caM* gene, see Tab. 2).

Other animals such as frogs and lizards visit cave environments occasionally after summer floods or storms, because of downfall or during searching for a colder site during a hot summer. Their long-term stay in the underground mostly ends in death because of starvation. Frog skeletons with visible white microfungal growth identified as *Botryosporium longibrachiatum* were discovered in the Domica-Baradla cave system (Fig. 6). Our find is the first record of this fungus from an animal substrate as well as from a cave environment. Thus far, this species had been recorded predominantly from plant material including *Curcuma rubricaulis* leaves (Tribe & Weber 2001) and later from a wide variety of dead and decaying plant material (Tribe & Weber 2001, Park & Park 2013) or as an airborne species (Anonymus 2016).

During our study of the mycobiota of the Movile Cave (Romania), we isolated microscopic fungi from dead invertebrate bodies. Several species of the genus *Aspergillus* were isolated from a dead endemic spider, *Agraecina cristianii*, i.e. *Aspergillus baeticus* and *A. tennesseensis*, whilst *A. movilensis* and *A. thesauricus* were isolated from a dead diplopod, *Trachelipus troglobius* (Nováková et al. 2018).

Synnematal microfungal colonies (Fig. 7b) were found on some cadavers of the spider *Agraecina cristianii* in the Lake Room of Movile Cave in 2013. It was impossible to identify this fungus based on morphological characters and isolation was unsuccessful. Unfortunately, similar dead spider bodies with synnematal colonies were not found during sampling in the following years. Dead spider bodies (*Meta menardi*) were also found in Gombasecká Cave and Krásnohorská Cave (Slovak Karst) (Fig. 7d) and in both cases, *Penicillium vulpinum* was isolated from them. This species is a coprophilous fungus frequently found in caves on various sorts of dung.

CONCLUSIONS

During our studies, a total of 39 fungal taxa (classified as species, forms or identified at a supraspecific level) on various cadavers and skeletons found in underground environment were identified in several European caves and mines. Thirty-eight species were isolated in pure cultures – 12 of them belonging to Mucoromycota, 23 species to Ascomycota, while 3 strains were not identified to the species level. Species of the genera *Mucor* and *Aspergillus* were isolated most abundantly. The richest microfungal spectrum was found in the Demänovská Peace Cave and Dead Bats Cave, from which 14 and 16 microfungal species were isolated, respectively. The ascomycete *Acaulium caviariforme* was reported repeatedly from several Slovak caves, but unfortunately the isolation of these strains failed. However, this fungus is probably a common fungal species participating in the decomposition process of dead animals in underground environments.

ACKNOWLEDGEMENTS

We thank Milada Chudíčková and Alena Gabrielová (Institute of Microbiology of the CAS, Prague) and Ivana Borovičková (Department of Botany, Charles University, Prague) for their invaluable assistance in the laboratory.

REFERENCES

ANONYMUS (2016): *Botryosporium longibrachiatum*. – http://www.mycobank.org/BioloMICS.aspx?Table=Mycobank&Rec=3319&Fields=All. [accessed May 2018]

ATLAS R.M. (2010): Handbook of microbiological media. – CRC Press, Washington, D.C.

BEGUIN H., LARCHER G., NOLARD N., CHABASSE D. (2005): *Chrysosporium chiropterorum* sp. nov., isolated in France, resembling *Chrysosporium* state of *Ajellomyces capsulatus* (*Histoplasma capsulatum*). – Medical Mycology 43(2): 161–169.

BENNY G.L. (2005): *Chaetocladium*. – http://zygomycetes.org/index.php?id=102. [accessed May 2018]

BENSCH K., BRAUN U., GROENEWALD J.Z., CROUS P.W. (2012): The genus *Cladosporium*. – Studies in Mycology 72: 1–401.

BLEHERT D.S., HICKS A.C., BEHR M., METEYER C.U., BERLOWSKI-ZIER B.M., BUCKLES E.L., COLEMAN J.T., DARLING S.R., GARGAS A., NIVER R., OKONIEWSKI J.C., RUDD R.J., STONE W.B. (2008): Bat white-nose syndrome: an emerging fungal pathogen? – Science 323(5911): 227.

CHEN A.J., FRISVAD J.C., SUN B.D., VARGA J., KOCSUBÉ S., DIJKSTERHUIS J., KIM D.H., HONG S.-B., HOUBRAKEN J., SAMSON R.A. (2016): *Aspergillus* section *Nidulantes* (formerly *Emericella*): Polyphasic taxonomy, chemistry and biology. – Studies in Mycology 84: 1–118.

CHEN A.J., HUBKA V., FRISVAD J.C., HOUBRAKEN J., MEIJER M., VARGA J., DEMIREL R., JURJEVIĆ Ž., KUBÁTOVÁ A., SKLENÁŘ F., ZHOU Y.G., SAMSON R.A. (2017): Polyphasic taxonomy of *Aspergillus* section *Aspergillus* (formerly *Eurotium*), and its occurrence in indoor environments and food. – Studies in Mycology 88: 37–135.

DIJKSTERHUIS J., WÖSTEN H., eds. (2013): Development of *Aspergillus niger*. – Studies in Mycology 74: 1–85.

GARGAS A., TREST M.T., CHRISTENSEN M., VOLK T.J., BLEHERT D.S. (2009): *Geomyces destructans* sp. nov. associated with bat white-nose syndrome. – Mycotaxon 108: 147–154.

GARRETT S.D. (1981): Soil fungi and soil fertility: an introduction to soil mycology, 2nd ed. – Pergamon Press, Oxford.

GUARRO J., GENÉ J., STCHIGEL A.M., FIGUERAS M.J. (2012): Atlas of soil Ascomycetes. – CBS Biodiversity Series 10, 486 p., Centraalbureau voor Schimmelcultures, Utrecht.

DE HOOG G.S. (2000): Atlas of clinical fungi, 2nd ed. – 1126 p., Centraalbureau voor Schimmelcultures, Utrecht / Universitat Rovira i Virgili, Reus.

HUBKA V., NOVÁKOVÁ A., PETERSON S.W., FRISVAD J., SKLENÁŘ F., MATSUZAWA T., KUBÁTOVÁ A., KOLAŘÍK M. (2016): A reappraisal of *Aspergillus* section *Nidulantes* with descriptions of two new sterigmatocystin-producing species. – Plant Systematics and Evolution 302: 1267–1299.

JOHNSON L.J.A.N., MILLER A.N., MCCLEERY R.A., MCCLANAHAN R., KATH J.A., LUESCHOW S., PORRAS-ALFARO A. (2013): Psychrophilic and psychrotolerant fungi on bats and the presence of *Geomyces* spp. on bat wings prior to the arrival of white nose syndrome. – Applied and Environmental Microbiology 79(18): 54–65.

LARCHER G., BOUCHARA J.P., PAILLEY P., MONTFORT D., BÉGUIN H., DE BIÈVRE C., CHABASSE D. (2003): Fungal biota associated with bats in western France. – Journal de Mycologie Médical 13: 29–34.

LORCH J.M., MULLER L.K., RUSSELL R.F., O'CONNOR M., LINDNER D.L., BLEHERT D.S. (2013): Distribution and environmental persistence of the causative agent of white-nose syndrome, *Geomyces destructans*, in bat hibernacula of the eastern United States. – Applied and Environmental Microbiology 79(4): 1293–1301.

MALLOCH D., HUBART J.-M. (1987): An undescribed species of *Microascus* from the Cave of Ramioul. – Canadian Journal of Botany 65(11): 2384–2388.

MARTÍNKOVÁ N. et al. (2010): Increasing incidence of *Geomyces destructans* fungus in bats from Czech Republic and Slovakia. – PLOS ONE 5(11): e13853.

NOVÁKOVÁ A. (2009): Microscopic fungi from the Domica Cave system (Slovak Karst National Park, Slovakia). A review. – International Journal of Speleology 38: 71–82.

NOVÁKOVÁ A. (2012): Microscopic fungi associated with bats. – In: Kováč Ľ., Uhrin M., Ľuptáčik P., eds., 21st International Conference on Subterranean Biology, 2nd–7th September 2012, Košice, Slovakia. Abstract book, p. 78.

NOVÁKOVÁ A., HUBKA V., SAIZ-JIMENEZ C., KOLAŘÍK M. (2012): *Aspergillus baeticus* sp. nov. and *Aspergillus thesauricus* sp. nov., two species in section *Usti* from Spanish caves. – International Journal of Systematic and Evolutionary Microbiology 62: 2778–2785.

NOVÁKOVÁ A., HUBKA V., VALINOVÁ Š., KOLAŘÍK M., HILLEBRAND-VOICULESCU A.M. (2018): Cultivable microscopic fungi from an underground chemosynthesis-based ecosystem: a preliminary study. – Folia Microbiologica 6: 1–13.

PARK J.H., PARK M.J. (2013): First report of black stem caused by *Botryosporium longibrachiatum* on sweet basil in Korea. – Plant Disease 97(3): 425.

PETERSON S.W. (2008): Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. – Mycologia 100: 205–226.

PUECHMAILLE S.J. et al. (2011): Pan-European distribution of white-nose syndrome fungus (*Geomyces destructans*) not associated with mass mortality. – PLOS ONE 6(4): e19167.

SAMSON R.A., COBUS M., VISAGIE C.M., HOUBRAKEN J., eds. (2014): Species diversity in *Aspergillus*, *Penicillium* and *Talaromyces*. – Studies in Mycology 78: 1–451.

SAMSON R.A., FRISVAD J.C. (2004): *Penicillium* subgenus *Penicillium*: new taxonomic schemes and mycotoxins and other extrolites. – Studies in Mycology 49: 1–174.

SAMSON R.A., HOUBRAKEN J. (2011): Phylogenetic and taxonomic studies on the genera *Penicillium* and *Talaromyces*. – Studies in Mycology 70: 1–183.

SAMSON R.A., VARGA J. (2007): *Aspergillus* systematics in the genomic era. – Studies in Mycology 59: 1–206.

SAMSON R.A., VARGA J., FRISVAD J.C. (2011): Taxonomic studies on the genus *Aspergillus*. – Studies in Mycology 69: 1–97.

SANDOVAL-DENIS M., GUARRO J., CANO-LIRA J.F., SUTTON D.A., WIEDERHOLD N.P., DE HOOG G.S., ABBOTT S.P., DECOCK C., SIGLER L., GENÉ J. (2016): Phylogeny and taxonomic revision of *Microascaceae* with emphasis on synnematous fungi. – Studies in Mycology 83: 193–233.

SEIFERT K., MORGAN-JONES G., GAMS W., KENDRICK B. (2011): The genera of Hyphomycetes. – CBS Biodiversity Series 9, 997 p., Centraalbureau voor Schimmelcultures, Utrecht.

TRIBE H.T., WEBER R.W.S. (2001): Dead basil stems – a possible ecological niche for the hoar-frost fungus *Botryosporium longibrachiatum*. – Mycologist 15(4): 158–161.

VANDERWOLF K.J., MCALPINE D.F., MALLOCH D., FORBES G.J. (2013a): Ectomycota associated with hibernating cave bats in eastern Canada prior to the emergence of white-nose syndrome. – Northeastern Naturalist 20(1): 115–130.

VANDERWOLF K.J., MALLOCH D., MCALPINE D.F., FORBES G.J. (2013b): A world review of fungi, yeasts and slime molds in caves. – International Journal of Speleology 42(1): 77–96.

VANDERWOLF K.J., MALLOCH D., MCALPINE D.F. (2016): Fungi on white-nose infected bats (*Myotis* spp.) in Eastern Canada show no decline in diversity associated with *Pseudogymnoascus destructans* (Ascomycota: *Pseudeurotiaceae*). – International Journal of Speleology 45(1): 43–50.

VOYRON S., LAZZARI A., RICCUCCI M., CALVINI M., VARESE G.C. (2011): First mycological investigation on Italian bats. – Hystrix 22: 189–197.

WIBBELT G., KURTH A., HELLMANN D., WEISHAAR M., BARLOW A., VEITH M., PRUGER J., GORFOL T., GROSCHE L., BONTADINA F., ZOPHEL U., SEIDL H.P., CRYAN P.M., BLEHERT D.S. (2010): White nose syndrome fungus (*Geomyces destructans*) in bats, Europe. – Emerging Infectious Diseases 16: 1237–1243.

ZELLER L. (1966): Keratinophilic fungi from the Baradla cave in Aggtelek (Biospeleologica Hungarica, XXII). – Annals of the University of Sciences Budapest, Section Biology, 8: 375–388.

ZUKAL J., BANĎOUCHOVÁ H., BARTONIČKA T., BERKOVÁ H., BRACK V., BRICHTA J., DOLINAY M., JAROŇ K.S., KOVÁČOVÁ V., KOVAŘÍK M., MARTÍNKOVÁ N., ONDRÁČEK K., ŘEHÁK Z., TURNER G.G., PIKULA J. (2014): White-nose syndrome fungus: a generalist pathogen of hibernating bats. – PLOS ONE 9: e97224.