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Dimargaris bacillispora – novel records from cave environment and its isolation in culture

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The coprophilous *Dimargaris bacillispora* is a rarely found and more rarely isolated microfungal species. In this study, we report the repeated observation and isolation of this fungus from a cave sediment used to rear cave-inhabiting isopods and springtails in the laboratory, originating from the Domica Cave, Slovakia. *Dimargaris bacillispora* was also observed and isolated from the faeces and cadavers of these laboratory-reared invertebrates. Subsequently, pure culture isolates of *D. bacillispora* were obtained from these substrates, characterised and identified. Finally, this species was then isolated directly from the cave sediment of the Domica Cave after several days of exposure of agar slants to the cave environment. This is the first record of this species from Europe and from a subterranean environment.

Additionally, this study reports a new growth substrate for a fungus which was considered to be strictly coprophilous and mycoparasitic. This study suggests that this species is probably more widely distributed in nature than is reported in the literature, due to the difficulties of viewing its delicate sporophores and growing it in culture.

Key words: *Dimargitales*, cave sediment, cadaver, cave-inhabiting invertebrates, faecal pellets.

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Koprofilní houba *Dimargaris bacillispora* je vzácně nacházeným a ještě vzácněji izolovaným druhem mikroskopických hub. V této studii uvádíme opakované pozorování a izolaci této houby z jeskynního sedimentu používaného pro chovy jeskynních stejnonožců a chvostoskoků v laboratorních podmínkách a pocházejícího z jeskyně Domica na Slovensku. *Dimargaris bacillispora* byla dále pozorována a izolována z fekálních pelet a mrtvých těl těchto živočichů z laboratorních chovů. Následně byly čisté kultury *D. bacillispora* identifikovány a charakterizovány. Tento druh byl dále izolován přímo z jeskynního sedimentu v jeskyni Domica po několikadenní expozici agarových ploten na povrchu sedimentu. Jedná se o první údaj o výskytu tohoto druhu v Evropě a současně i z podzemního prostředí.

Studie současně uvádí i nový substrát pro tento druh, který byl dosud považován za striktně koprofilní a mykoparizitickou houbu. Dále studie ukazuje, že tento druh má pravděpodobně mnohem větší rozšíření v přírodě, než bylo dosud uváděno v literatuře, vzhledem k obtížnému nalezení jemných sporoforů a problematické kultivaci.

INTRODUCTION

Caves offer a highly specialised environment characterised by stable temperatures and humidity, deficiency of organic matter, and complete absence of light. Most organisms inhabiting caves are adapted to these conditions, although some may be considered visitors which can survive in these conditions only temporarily. Those which are cave-adapted, such as troglodytic springtails, often require growth conditions mimicking the cave environment in factors such as temperature, humidity, and source of nutrients.

While many of the uniquely troglodytic organisms described are animals, both invertebrate and vertebrate, a number of microorganisms are also known from subterranean environments only. New bacterial species were described from the Altamira Cave by Schabereiter-Gurtner et al. (2002). Novel micro-fungal species have also been described, such as the white-nose fungus *Pseudogymnoascus destructans* (Gargas et al. 2009, Minnis & Lindner 2013), *Chrysosporium chiropterorum* and *C. speluncarum*, isolated from bat fur and guano, respectively (Beguin et al. 2005, Nováková & Kolařík 2010), *Mucor troglophilus*, isolated from cave crickets (Gunde-Cimerman et al. 1998), *Microascus caviariformis*, isolated from cooked meat baits (Malloch & Hubart 1987), *Ochroconis anomala* and *O. lascauxensis*, isolated from black stains on Paleolithic paintings in the Lascaux Cave (Martin-Sanchez et al. 2012), *Trichosporon akiyoshidainum*, *T. cavernicola*, and *T. chiropterorum*, isolated from cave air (Sugita et al. 2005), and several *Aspergillus* species, e.g. *A. baeticus*, *A. thesauricus*, *A. movilensis*, *A. spelunceus*, and *A. spelaeus* from cave air, sediments and arthropod frass (Nováková et al. 2012a, Raper & Fennell 1965, Hubka et al. 2015). Others, although not unique to caves, are rarely reported in the literature and include *Pidoplitchkoviella terricola* from earthworm casts and *Dimargaris bacillispora*, both of which were reported from the Domica Cave, Slovakia, but not isolated (Nováková 2009a, 2009b, 2010).

The family *Dimargitaceae* (Kickxellomycotina, *Dimargitales*) (Benjamin 1965, Tretter et al. 2014) contains several genera forming two-spored merosporangia and hyaline sporangiospores. The type genus *Dimargaris* Tiegh. was described in 1875 with type species *D. cristalligena* Tiegh. (isolated from rat dung in Paris, France), and is characterised by erect, septate sporangiophores, cymosely or verticillately branched, producing sporiferous branchlets arising by apical or lateral budding in which each cell creates distal whorls of two-spored merosporangia producing smooth spores (Van Tieghem 1875, Benny 2007). After its original description, the type species *D. cristalligena* was isolated from mouse dung in California, USA in 1956 (Benjamin 1959) and again from *Helicostylum nigricans* growth in Java (Boedijn 1958). Originally, three species

(*D. cristalligena*, *D. verticillata*, and *D. bacillispora*) were presented by Benjamin (1959), all from mouse dung in California, USA. By 1965, several other species had also been reported for *Dimargaris* (Benjamin 1965). These include *D. arida* isolated from soil in Texas, USA, *D. xerosporica* (originally described as *D. verticillata* var. *xerosporica*), *D. simplex*, and *D. oblongispora*, which were described by Mehrotra and Baijal (1963, 1964) from India. *Dimargaris oblongispora* was isolated from cow dung manure (Mehrotra & Baijal 1963), *D. verticillata* var. *xerosporica* from snail excreta and *D. simplex* from soil near tiger dung piles (Mehrotra & Baijal 1964). According to Benjamin (1965), *D. oblongispora* and *D. simplex* resemble *D. bacillispora*. Unfortunately however, living cultures of these species have not been preserved and are not available for study by other authors. Kirk & Kirk (1984), who presented the first record of *D. verticillata* from the British Isles, reported that the published description and illustration of *D. oblongispora* did not differ significantly from *D. bacillispora*, so that this species is probably a synonym of *D. bacillispora*. Nevertheless, *D. bacillispora* and *D. oblongispora* were listed by Benny (2007) as being distinct. According to Benny (2007), seven species are currently recognised in this genus: *D. cristalligena*, *D. verticillata*, *D. bacillispora*, *D. oblongispora*, *D. arida*, *D. simplex*, and *D. xerosporica*.

The species of the genus *Dimargaris* are considered coprophilous, historically found mainly on rodent dung. The apparent coprophilous nature of these species has been supported by collection records. Three species of *Dimargaris*, *D. verticillata*, *D. cristalligena*, and *D. arida*, were isolated from mouse and rat dung in Japan by Mikava (1976). Mirza et al. (1979) reported isolation of *D. arida* and *D. bacillispora* from bat dung in Faisalabad, Punjab, Pakistan. *Dimargaris cristalligena* was also recorded on the dung of several rodents in Wielkopolski National Park, Poland (Wrzosek & Gajowniczek 1998) and from house mice dung in Taiwan (Chien 2002). An additional report of *D. bacillispora* on tapir dung in Brazil was published by Cabral et al. (2009). Five additional records of *D. bacillispora* from the Rancho Santa Ana Botanic Garden collection (California, USA) are known (Benny, pers. comm.), although some of them were not published, including three strains originating from San Bernardino County, California, USA. These were isolated in 1957, 1969 and 1970 from dung; one by Benjamin from lizard dung in Mexico, one by Benjamin from rodent dung in the Chiraqua Mts., Arizona, USA, and one strain of *D. bacillispora* by Benny from dung of *Podomys floridanus* in Ordway Nature Preserve, Florida, USA (Benny, pers. comm.).

While commonly found on dung, these species are also known to be haustorial parasites of *Mortierellales* and *Mucorales* (Benny 2007). In fact, successful cultivation of these species has been conducted with co-cultivation on *Cokeromyces recurvatus* on Emerson's YpSs (yeast extract peptone soluble starch medium) agar (Benjamin 1959, 1965) and V8® juice agar (Benny 2007).

The presence of possible prey species on bat dung indicates that this may be an ideal substrate for their isolation, although their presence in subterranean environments has never been reported. Although infrequently encountered, the members of this genus appear to be widely distributed.

In this study we detail the discovery and isolation of the rarely encountered *Dimargaris bacillispora* from cave sediments used in the rearing of cave invertebrates. This study provides evidence of novel growth substrates utilised by this fungus: invertebrate cadavers, invertebrate frass, and cave sediments. Finally, it also characterises a variety of agar media in the isolation and cultivation of this *D. bacillispora* to aid in future studies aimed at further investigating its physiology and trophic interactions. This report provides the first published record of *D. bacillispora* from the European continent.

MATERIAL AND METHODS

Study site. Samples of cave sediments (ca 500 g) were collected from the Domica Cave (Slovakia) for use in routine long-term rearing of cave invertebrates in the laboratory and for subsequent use in laboratory food preference tests. The Domica Cave is located on the south-western edge of the Silická Plateau in Slovak Karst National Park, close to the state border with Hungary. The cave entrance is situated on the southern foothill of Domica Hill at an elevation of 339 m. Domica Cave is connected with the Čertova diera Cave, which together reach a length of 5,368 m. The cave was formed in the Middle Triassic, in pale Wetterstein limestones of the Silica Nappe along tectonic faults by corrosive and erosive activities of the Styx stream, Domický Stream and smaller underground tributaries mainly draining water from the non-karstic part of the basin. The lowest passages are filled up with gravels and loam. Air temperature ranges from 10.2 to 11.4 °C and relative humidity from 95 to 98% (Bella 2000).

Substrate collection and invertebrate microcosm maintenance. The use of microcosms to mimic the cave environment for the rearing of cave invertebrates has been described by Lynch (2001–2005). In this study, two setups were used. Initially, microcosms were created using a damp layer of plaster of Paris. Another system, utilising cave sediment instead of plaster of Paris, was used to mimic the natural cave environment more closely. All cave sediments were subjected to a disinfestation procedure to reduce microbial contamination. This procedure included rapid heating of sediments in a microwave oven for 10 min. followed by rapid cooling and freezing at –18 °C in a freezer overnight. This procedure was favoured to minimise substrate transformation which might occur during autoclave sterilisation. After disinfestation, arthropods were intro-

duced to several microcosms for each collembolan and isopod species and maintained in the dark at 10–15 °C for three to six months. Special edible yeasts (Country Life, Ltd., Nenačovice, Czech Republic) were supplied as nutrition for these animals and all dishes were regularly moistened with tap water. The microcosms were observed for invertebrate population viability and microbial growth throughout their maintenance. Invertebrate specimens referenced in this report (representing springtails *Folsomia candida*, *Heteromurus nitidus*, *Hypogastrura* sp. (cf. *aquaepilosa*), *Ceratophysella denticulata*, and the isopod *Mesoniscus graniger*) were also collected from the Domica Cave system in Slovakia, and were maintained in plastic microcosms as described above.

Dimargaris spp. isolation and identification. Attempts to isolate *Dimargaris* spp. were made for both microcosms, as described above, and from agar slants exposed directly to the cave environment. In-cave isolation directly from cave sediments was attempted by exposing agar slants resting on the cave sediment surface in several places in the Domica Cave. In the initial attempt, beer-wort agar slants with various microfungal growths were exposed (16 microfungal species were offered as nutrition during a selection preference trial for invertebrates carried out in the cave in 2009) to the cave environment for one month (Nováková 2010). A second attempt was made by placing dichloran rose Bengal chloramphenicol (DRBC) and beer-wort agar slants on the cave sediments for four days in 2010. Agar slants were collected in Petri dishes and were maintained in damp chambers at 5 °C in the dark.

Sporangiophores of *D. bacillispora* from microcosm sediment, from *Mesoniscus graniger* or *Hypogastrura* sp. cadavers, and from agar plates placed on cave sediments in the Domica cave were transferred to one of several isolation media in Petri dishes using a sterile injection needle. Several isolation media were used (Tab. 1), including malt extract agar, potato dextrose agar, cornmeal agar, cornmeal agar with yeast extract, Emerson's YpSs agar, V8 juice agar, and carrot agar (Benjamin 1959, Kreisel & Schauer 1987, Atlas 2010). Plates were cultivated at 10 and 25 °C in the dark for 14–21 days. At lower temperature the cultivation time was even longer, up to several weeks. Additionally, in order to encourage growth in culture, combinations of various isolation media were utilised with living cultures of *Cokeromyces recurvatus*, sterilised cadavers of *M. graniger*, and sterilised collembolan faecal pellets. Cultivation on sterile cadavers of *M. graniger* alone in a damp chamber was also used for the isolation of *Dimargaris* spp. Pure cultures of *D. bacillispora* were maintained on carrot agar. Isolates were preserved in the Collection of Microscopic Fungi ISB (CMF 2332) (Nováková 2010, Nováková et al. 2012b).

The specimens were studied both directly on cave sediment and dead animal bodies using a stereomicroscope and microscopic mount slides prepared using

lactofuchsin or lactophenol solutions. Preparations for scanning electron microscopy were also prepared from substrates with *Dimargaris* sporophores. Briefly, samples were fixed in a 2.5% glutaraldehyde solution in an 0.2 M phosphate buffer followed by fixation in a 2% osmium tetroxide solution. Fixed samples were then dehydrated in a graded series of acetone solutions using the critical point drying method. A second procedure involved deep-freezing samples with crystalline osmium tetroxide for 3 weeks followed by sputter coating with gold after two days in a digester. Observations were made with a JEOL JSM-7401F scanning electron microscope (JEOL, Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Microcosm maintenance and observation of *Dimargaris bacillispora*

The laboratory rearing of invertebrate animals (mites, springtails, terrestrial isopods, etc.) is a commonly used technique for long-term maintenance and study in laboratory conditions. Oftentimes, they must be raised in an environment simulating their natural one, including environmental factors like temperature, humidity, source of nutrients, etc. For collembolans in this study, both plaster and cave sediment microcosms were used. In the case of microcosms lined with cave sediment, filamentous fungi, not supplied as feedstock, were found to colonise the microcosm substrate. These fungi were found growing on the cave sediment surface in spite of attempts to disinfest the sediment using high (microwave oven) and low (freezer) temperature treatments, suggesting the contaminating fungi were either not killed during disinfestation or were associated with the arthropods themselves. As these fungi were not observed in the plaster-lined microcosms, it is assumed that the filamentous fungi originated from the cave sediment.

Fungal hyphae were observed on sediment surfaces 1 to 3 days after microcosm establishment. Initial visible microfungal colonies frequently grew in close proximity to the deposition sites of the edible yeasts which were regularly supplied as nutrition for invertebrate animals. These were later identified to be various species of the genus *Mortierella* or *Verticillium* s.l. After several months, distinct microfungal colonies were found in some of the microcosms. Delicate, verticillately branching sporophores were found on the sediment, particularly close to white microfungal colonies (Fig. 1A–D) on the offered nutrition, but they were also found on sediment surfaces without visible microfungal growth nearby. The microscopic characters – verticillately branched sporophores with merosporangia producing white, bacilliform, curved spores which then turned grey (Fig. 3) – led to the identification of this fungus as *Dimargaris bacillispora*. *Dimargaris* sporophores were also observed on some collembolan faecal pellets, where they grew

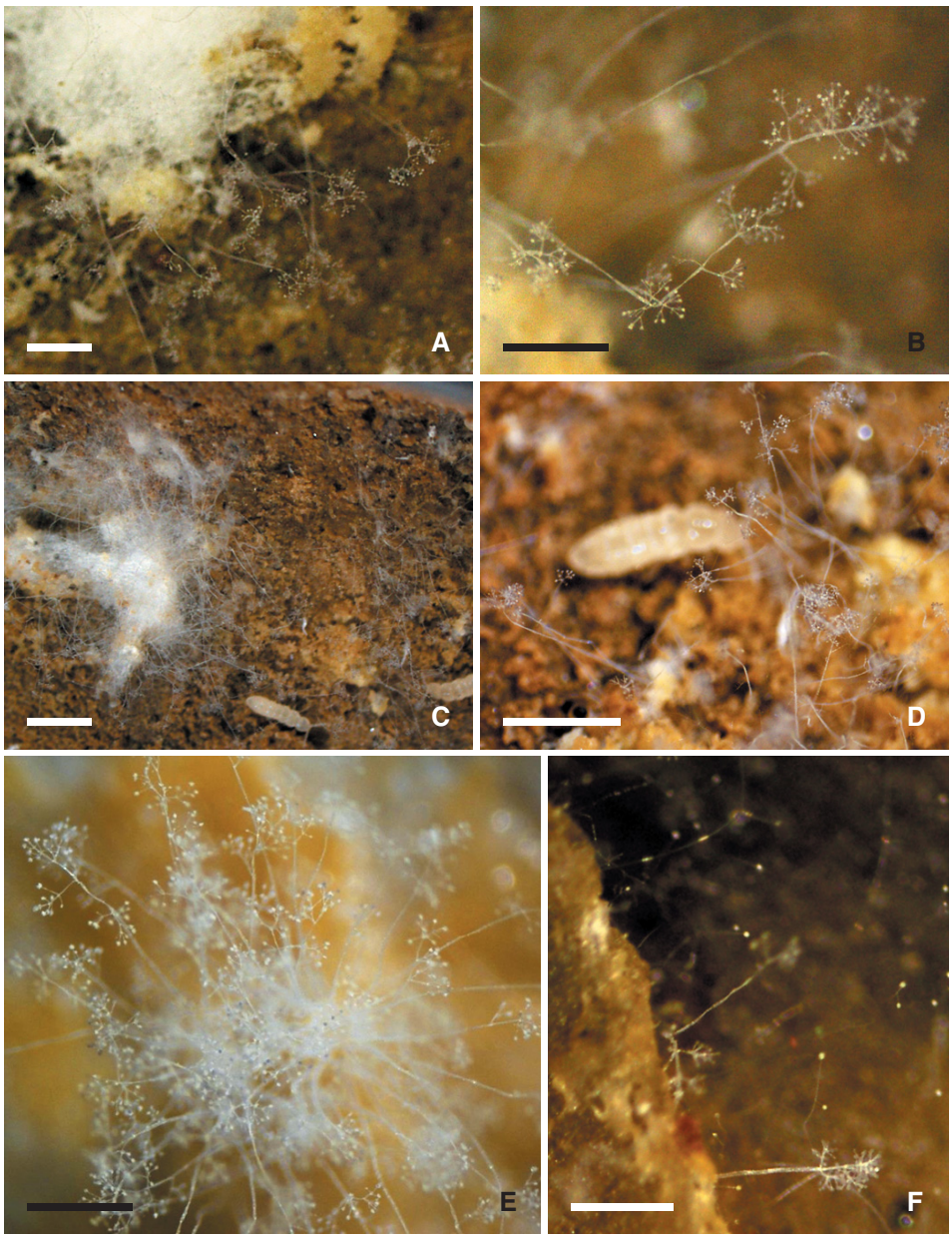

Fig. 1. *Dimargaris bacillispora* (CMF 2332). **A–D** – ramified sporophores growing on cave sediment in collembolan rearing microcosms in the laboratory; **E** – detail of colony on cave sediment; **F** – sporophores growing on agar slant after three-month cultivation of exposed slant at 10 °C in the dark. Scale bars 1 mm (A, C, D, and F), 0.25 mm (B and E). Photo A. Nováková.

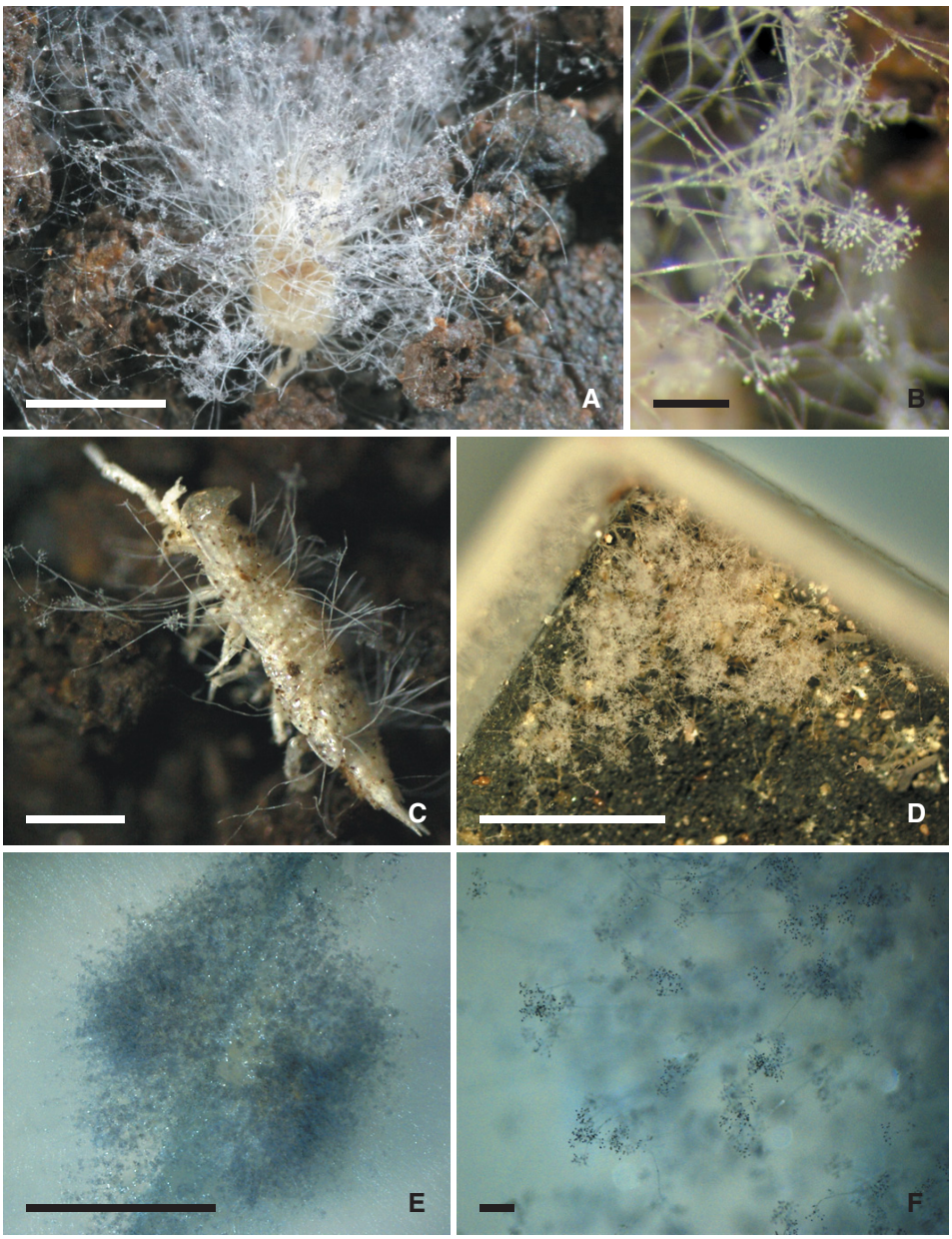

Fig. 2. *Dimargaris bacillispora* (CMF 2332). **A–C** – colony on dead body of *Mesoniscus graniger*; **D** – colony on dead *Hypogastrura* springtails; **E, F** – colony on carrot agar. Scale bars 1 mm (A, B, and C), 1 cm (D and E), 0.25 mm (F). Photo A. Nováková.

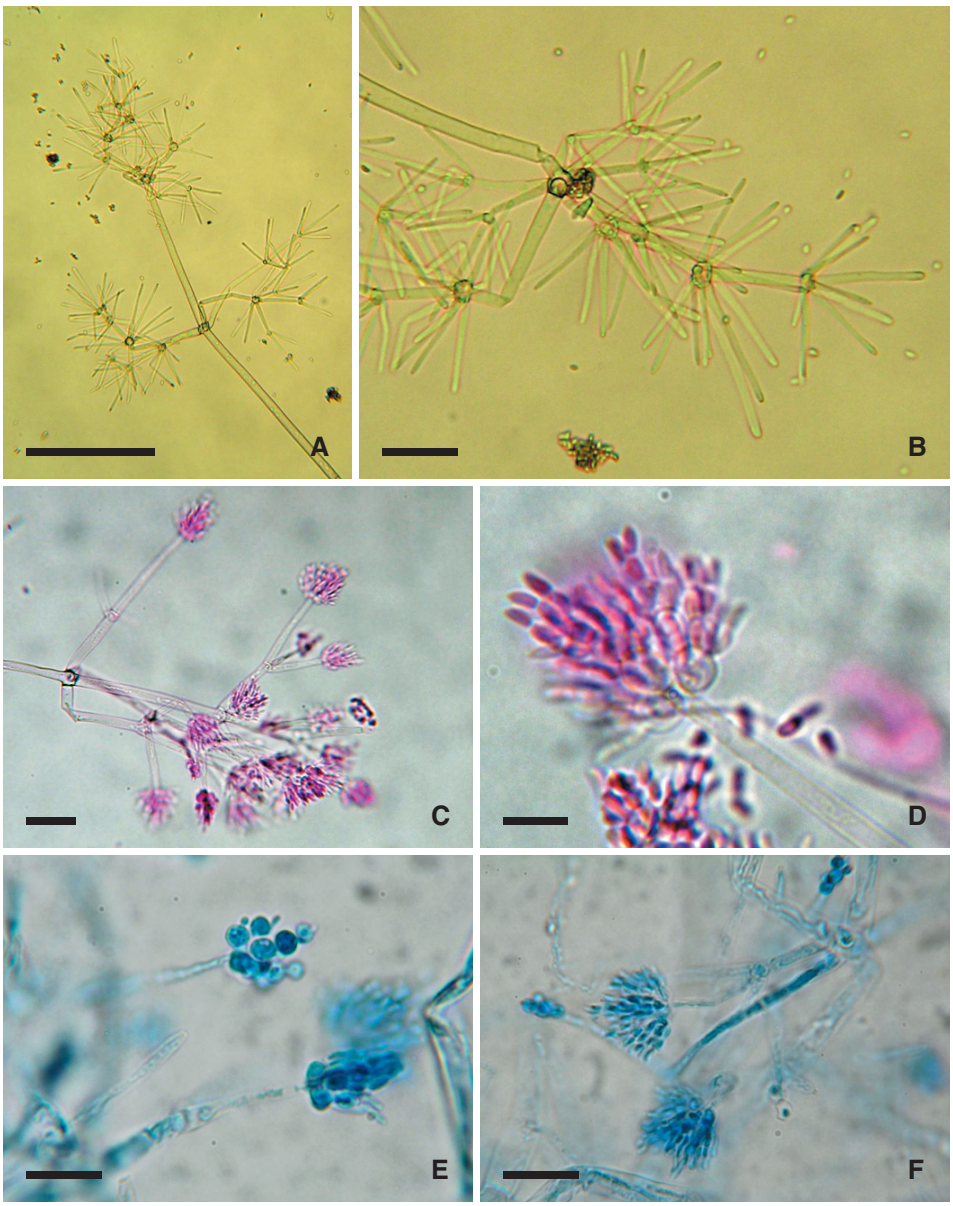

Fig. 3. *Dimargaris bacillispora* (CMF 2332). **A–C** – verticillate sporophore; **D–F** – merosporangia. Scale bars 50 µm (A), 10 µm (B and D), 25 µm (C, E, and F). Photo A. Nováková.

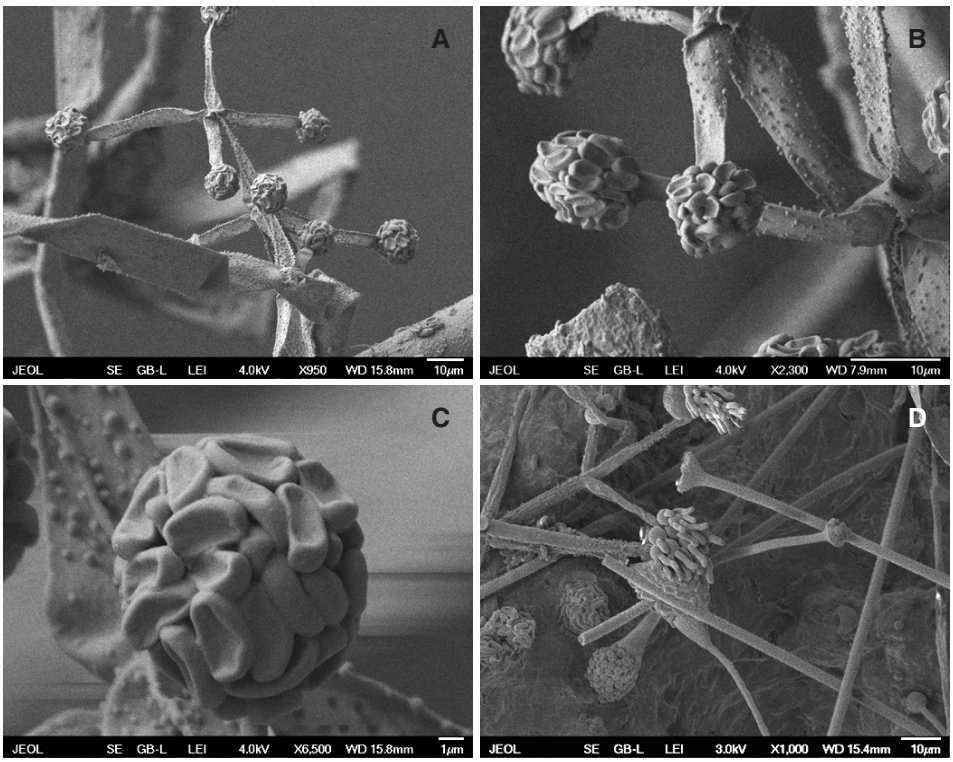

Fig. 4. *Dimargaris bacillispora* (CMF 2332). **A** – verticillately branched sporophore; **B, C** – fertile head and tuberculate surface of sporangiophores; **D** – merosporangium, SEM. Scale bars 1 µm (C) and 10 µm (A, B, and D). Photo M. Tesařová.

very frequently. Unfortunately, they were often destroyed by subsequent deposition of faecal pellets and other springtail activity. Although consumption was not directly measured in this study, observations of springtail behaviour indicated that *D. bacillispora* may be consumed by cave-inhabiting springtails. Inclusion of *D. bacillispora* in future food preference experiments will be needed to confirm this.

In addition to their appearance in microcosm substrates, massive *D. bacillispora* colonies were observed on dead bodies of *Mesoniscus graniger* (Fig. 2A–C) and *Hypogastrura* sp. (Fig. 2D), covered by well-developed sporophores (details of a verticillately branching sporophore and young colonies on a dead *M. graniger* body are provided in Fig. 2B and Fig. 1F, respectively). Zygospores were not observed. Sporophores of *D. bacillispora* were also found on agar slants with *Coemansia aciculifera* colonies after one month exposure on cave sediment in the Domica Cave, Slovakia followed by cultivation in a damp chamber in a laboratory (Fig. 1F).

Isolation of *Dimargaris bacillispora*

Initial attempts to isolate the colonies or sporophores of *D. bacillispora* found in these microcosms on a wide range of isolation media (Tab. 1), using several isolation methods, were unsuccessful. Surprisingly, the methods reported in Benjamin (1959, 1965) and Benny (2007), including co-cultivation with *Cokeromyces recurvatus*, were unsuccessful. This could have been due to weaker mycoparasitism by these isolates, although this was not tested directly and was not within the scope of this study. Using media-based sterilised collembolan faecal pellets and direct cultivation on sterile cadavers of *Mesoniscus graniger* were initially successful, showing strong *D. bacillispora* growth. However, after transferring its spores to an isolation medium, the isolates were quickly overgrown by an unidentified yeast species, possibly outcompeting *D. bacillispora.* The fact that no growth was observed on the non-inoculated faecal pellets and cadavers indicates that the contaminating yeast was transferred in close association with the *D. bacillispora* spores, but was unsuited to grow on the faecal pellet and cadaver substrates. Even if these initial isolation attempts were unsuccessful, likely due to the unsuitability of the media for *D. bacillispora* growth vs. other fungi, *D. bacillispora* could be observed in mixed cultures using optical and scanning electron microscopy (Figs. 3 and 4). These observations revealed the tuberculate surfaces of sporophores and branches, the merosporangia, budding cells, and smooth merospores (Fig. 3C–F), confirming the identity of the *D. bacillispora* colonies.

In the second round of isolation attempts in April 2010, a new medium, Dichloran rose Bengal chloramphenicol (DRBC) agar, was utilised to slow down aggressively growing fungi. These slants were placed on the cave sediment in several places in the Domica Cave, and after several months of cultivation in a damp chamber at 5 °C, several sporangiospores of *D. bacillispora* were found on agar surface in one Petri dish. These sporangiospores were transferred to the DRBC with a sterile needle and further cultivated at 5 °C in the dark. By December 2010, growth of *D. bacillispora* was observed, but growing with other microfungal species such as *Oidiodendron* spp. and *Mortierella* spp. The inclusion of rose Bengal in the isolation medium improves the selectivity of the medium for fungi, and can aid in the enumeration and isolation of particular strains by slowing down the growth of aggressive fungal cultures. However, since rose Bengal only inhibits growth, the presence of multiple fungal species from these environmental samples is not unexpected. Those wishing to use the DRBC to isolate *D. bacillispora* will find it helpful in preventing overgrowth of non-target fungi, although further isolation will be required.

Further attempts in testing a broad spectrum of isolation media (Tab. 1) resulted in achieving a pure culture of *D. bacillispora*, which was isolated and put

to pure culture on carrot agar, which appears to be the best medium for isolating and maintaining it. This isolate, originally from sporophores found in laboratory microcosms rearing *Hypogastrura*, was preserved in the Collection of Microscopic Fungi ISB (CMF 2332) (Nováková 2010, 2012, Nováková et al. 2012b).

Tab. 1. Media used in attempts to bait and isolate *Dimargaris bacillispora* from the cave environment, microcosms, and agar plates. Growth of the target organisms varied and was often outcompeted by more aggressive fungi.
Symbols: – no growth, ± limited growth (colony diameter of 3–5 mm), +++ very good growth (more than 1 cm colony diameter).

Nutrient medium	***Dimargaris* growth after 21 days**
Beer-wort agar	±
Carrot agar	+++
Cornmeal agar	–
Cornmeal agar with yeast extract	–
Emerson's YpSs agar	–
Malt extract agar	±
Malt extract agar with *Cokeromyces recurvatus*	–
Malt extract with *Mesoniscus graniger* cadavers	–
Potato dextrose agar	±
V8 juice agar	±

While many members of *Dimargaris* are known to be mycoparasites, they have also been known to grow axenically on media with distinct nutritional profiles (Tab. 1). These media often have a high protein content and possess growth factors such as glycerol, organic nitrogen and vitamins (Gams et al. 2004). The growth of *D. bacillispora* on carrot agar indicates that this medium must contain the factors required for its growth, although additional experimentation will be needed to determine what these factors are. The delicate nature of the sporophores was partly responsible for the small success rate during the isolation process, as it necessitated the transfer of sporophores together with the agar medium. This technique often resulted in contamination of growing colonies by other microfungal species. The delicate sporulating structures also complicated microscopy, as transferring the undamaged sporophores into a mounting medium was very difficult.

Novel substrate for the coprophilous *Dimargaris bacillispora*

To date, *D. bacillispora* has been reported predominantly from various sorts of vertebrate animal dung. These reports included mouse dung in California, Florida, and Arizona, USA (Benjamin 1959, 1965, Benny 2007, Cabral et al. 2009), lizard dung in Mexico (Benny, pers. comm,), bat dung in Pakistan (Mirza et al. 1979), and tapir dung in Brazil (Cabral et al. 2009). Understandably, all of these reports, with the notable exception of Benjamin and Benny (Benny, pers. comm.), were not accompanied by isolation of *D. bacillispora*.

Our results are the first records of *D. bacillispora* for Europe. Additionally, this is the first report of *D. bacillispora* from substrates other than animal dung or soil. We also demonstrated for the first time that *D. bacillispora* can grow on cadavers of the cave-inhabiting isopod *Mesoniscus graniger* and the springtail *Hypogastrura* sp., on collembolan faecal pellets, and in laboratory collembolan microcosms containing cave sediments (originally from the Domica Cave in Slovakia). Other studies have suggested that members of *Dimargaris* may be entomopathogenic, but whether or not *D. bacillispora* can utilise this life strategy needs to be confirmed in future experimentation (Hussain et al. 2014).

The occurrence of *D. bacillispora* in cave sediments in the Domica Cave was also confirmed in two separate surveys by sporophore finds on agar disc after exposure to the cave environment. Additional sporophores have also been found on faecal pellets of the cave oribatid *Pantelozetes cavaticus* in laboratory rearings in microcosms with plaster of Paris. Further, the mycoparasitic properties of *D. bacillispora* (Tretter et al. 2014) do not appear to be obligate as it has the ability to grow either on collembolan and oribatid faecal pellets, on dead isopod bodies, and on cave sediment without the presence of other microfungal growths, as confirmed by inspection with a stereomicroscope. Coupled with the fact that pure isolates of *D. bacillispora* can be maintained on carrot agar suggest that while mycoparasitism could be a preferred life strategy, it can to some extent grow also saprotrophically.

CONCLUSIONS

Dimaragris bacillispora was recorded from a cave sediment used in laboratory rearings of cave invertebrates and, later, isolated from a cave sediment of the Domica Cave, Slovakia. This is the first record of isolation of this rarely recorded, and even more rarely isolated, species from an underground environment. Additionally, this is also the first record of this fungus from several novel substrates, including invertebrate faecal pellets, cadavers, and cave sediment. These observations indicate a life strategy unknown for this species, long consid-

ered strictly coprophilous and mycoparasitic. It seems likely that this species occurs in nature more often than reported in the literature due to difficulties in viewing the delicate sporophores and its recalcitrance to culturing. In cave environments, this species probably facultatively participates in the processes of decomposition of invertebrate dead bodies and faecal pellets.

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