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Geomyces destructans, phenotypic features of some Czech isolates

ALENA KUBÁTOVÁ[1*], ONDŘEJ KOUKOL[1], ALENA NOVÁKOVÁ[2]

[1]Department of Botany, Faculty of Science, Charles University in Prague, Benátská 2, CZ–128 01 Praha 2, Czech Republic
[2]Institute of Soil Biology, Biology Centre Czech Academy of Sciences, Na Sádkách 7, CZ–370 05 České Budějovice, Czech Republic
[*]corresponding author; kubatova@natur.cuni.cz

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The microscopic fungus *Geomyces destructans* is a psychrophilic dermatophyte causing since 2006 a serious bat disease in North America called white-nose syndrome (WNS). In Europe, *G. destructans* has also been recorded, however less commonly and without striking lethal effects. Currently, seven isolates of *G. destructans* isolated from greater mouse-eared bat (*Myotis myotis*) from four localities are maintained in the Culture Collection of Fungi (CCF) in Prague. Growth tests at 12 and 15 °C on eight agar media demonstrated that the fungus grows somewhat faster at 12 °C than at 15 °C. Good growth was observed on nutrient rich media. No further isolates of *G. destructans* were recovered during screening of bat-associated environments.

Key words: white-nose syndrome, bat disease, *Geomyces*, *Myxotrichaceae*, Ascomycota.

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Mikroskopická houba *Geomyces destructans* je psychrofilní dermatomycet způsobující od roku 2006 v Severní Americe vážné onemocnění netopýrů zvané syndrom bílého nosu („white-nose syndrome“). V Evropě byla houba *Geomyces destructans* v posledních letech rovněž zaznamenána, avšak v mnohem menší míře a bez rozsáhlých letálních následků. Ve Sbírce kultur hub (CCF) v Praze je uchováváno 7 kultur *G. destructans* izolovaných z netopýra velkého (*Myotis myotis*) ze 4 lokalit v Čechách. Růstové testy při 12 a 15 °C na 8 agarových médiích ukázaly, že houba roste poněkud rychleji při 12 °C než při 15 °C. Dobrý růst byl pozorován na živinami bohatých médiích. Z prostředí obývaného netopýry se nepodařilo získat další izoláty *G. destructans*.

INTRODUCTION

Geomyces is an anamorphic genus (Ascomycota, Leotiomycetes, *Myxotrichaceae*) described by Traaen in 1914. The genus was taxonomically revised by Carmichael (1962) and van Oorschot (1980). Currently, 11 species are recognised (Rice & Currah 2006, Mycobank 2010): *G. asperulatus* Sigler & J.W. Carmich., *G. destructans* Blehert & Gargas, *G. laevis* Zhong Q. Li & C.Q. Cui, *G. luteus*

Kwasna H. & Bateman G.L., *G. pannorum* (Link) Sigler & J.W. Carmich., *G. pulvereus* A.D. Hocking & Pitt, *G. sulphureus* Traaen, *G. vinaceus* Dal Vesco (teleomorph *Pseudogymnoascus roseus* Raillo), and three other *Geomyces* spp. (associated with teleomorphs *P. appendiculatus* Rice & Currah, *P. dendroideus* Locq.-Lin., and *P. verrucosus* Rice & Currah). Rice & Currah (2006) analysed sequences of ITS1, 5,8S, and ITS2 regions of rDNA and found that *G. pannorum* forms a clade with other *Geomyces* species associated with the genus *Pseudogymnoascus* Raillo, whilst the species *Geomyces sulphureus* forms a clade with *Gymnostellatospora* Udagawa, Uchiy. & Kamiya.

So far, three species of *Geomyces* have been recorded in the Czech Republic: *G. pannorum*, *G. vinaceus* and *G. destructans*. *Geomyces pannorum* and *G. vinaceus* are cosmopolitan soil microfungi occurring predominantly in soils of cold regions (Ito & Yokoyama 1985, Mercantini et al. 1993, etc.). The most frequent species in the Czech Republic, *G. pannorum*, was isolated from soil, caves and underground tunnels (see Řepová 1989, Bosák et al. 2001, Kubátová et al. 2005, Kubátová & Váňová 2007, etc.). *Geomyces vinaceus* was found in the rhizosphere of *Picea abies* and in soil (Černý et al. 1987, Kubátová & Kolařík 2007, in both papers as *Pseudogymnoascus roseus*). The last and most recently described species, *G. destructans*, was found on hibernating bats in central Bohemia, central Moravia and Slovakia (Martínková et al. 2010; Šimonovičová et al. 2011).

Species of the genus *Geomyces* were rarely isolated as pathogens; *G. pannorum* was reported to occasionally cause onychomycosis (e.g. Schönborn & Schmoranzer 1970) and was rarely isolated from clinical material in the Czech Republic (Kubátová et al. 2007). *Geomyces destructans* was recently described in the USA as the cause of a serious disease of hibernating bats with fatal progression (Gargas et al. 2009). The disease was called white-nose syndrome of bats (WNS) due the distinct white overgrowth of the fungus on muzzles and first detected in North America in 2006. The fungus invades skin of muzzles, ears or wing membranes, and fills hair follicles, sebaceous glands and sweat glands (Blehert et al. 2009). The pathogenesis mechanism is not yet known. *G. destructans* has also been recorded in Europe during the past years, however, less commonly and without striking lethal effects (Puechmaille et al. 2010, Wibbelt et al. 2010, Martínková et al. 2010).

The aim of this contribution is to report on phenotypic characteristics of six isolates of *G. destructans* isolated in the Czech Republic, to present the results of environmental screening, and draw wider attention to this species.

MATERIALS AND METHODS

Localities and samples. During February and March 2010, WNS-suspected bats were sampled on their muzzles and wings using sterile plastic swabs or transparent adhesive tape (Figs. 2 and 4). We processed samples from twelve bats (ten of *Myotis myotis*, one of *Rhinolophus hipposideros*, and one of a dead *R. hipposideros*) originating from six localities: Malá Amerika mine in the Bohemian Karst (Fig. 1), old mines near Solenice, Nový Knín and Líšnice in central Bohemia, Jan na poušti gallery near Stříbro in NW Bohemia, and Javoříčko Caves in central Moravia (for details, see Martínková et al. 2010).

Direct microscopic observation and isolation of cultures. Both types of samples (swabs and tape) were directly observed for presence of *Geomyces*-like spores, then inoculated on Sabouraud dextrose agar (SDA) and soil extract agar with glucose and rose bengal (SEGA), and incubated for 14 days at c. 7, 13, and 25 °C.

Identification and maintenance. Isolates were identified according to Gargas et al. (2009) based on phenotypic characteristics. Their species identity was confirmed by molecular analyses (Martínková et al. 2010). Isolates were lyophilised and deposited at the Culture Collection of Fungi (CCF), Charles University in Prague (Tab. 1).

Growth tests. Six isolates (Tab. 1) were cultivated for four weeks at 12 and 15 °C on eight mycological agar media (Tab. 2) (three-point inoculation, in duplicates). Measurements of colonies were performed after one, two, three, and four weeks. The main aim of these tests was to find out which agar media are the most suitable for cultivation of this fungus.

Tab. 1. Isolates of *Geomyces destructans* from the Czech Republic deposited at CCF.

Accession no.	Bat species	Locality (old mines)	Date of isolation	Isolated by
CCF 3937	*Myotis myotis*, muzzle	Malá Amerika	II. 2010	O. Koukol
CCF 3938	*M. myotis*, muzzle	Solenice	III. 2010	A. Kubátová
CCF 3939	*M. myotis*, muzzle	Solenice	III. 2010	A. Kubátová
CCF 3941	*M. myotis*, muzzle	Malá Amerika	III. 2010	A. Kubátová
CCF 3942	*M. myotis*, pinna	Malá Amerika	III. 2010	A. Kubátová
CCF 3943	*M. myotis*	Stříbro	III. 2010	A. Kubátová
CCF 3944*	*M. myotis*	Nový Knín	III. 2010	A. Kubátová

* This isolate was not used in growth tests.

Tab. 2. Eight mycological agar media used for growth of *Geomyces destructans* isolates.

Agar medium	Reference	Source of saccharides or proteins (g per 1 litre of medium)	Antibacterial substances (mg per 1litre of medium)
Soil extract agar (SEA)	Gams 1970	–	–
Soil extract agar with glucose and rose bengal (SEGA)	Fassatiová 1986	glucose 10 g	rose bengal 70 mg
Potato carrot agar (PCA)	Fassatiová 1986	boiled potatoes 20 g boiled carrot 20 g	–
Sabouraud dextrose agar (SDA)	Samson et al. 2004	dextrose 40 g peptone 10 g	–
Sabouraud dextrose agar with cycloheximide (SDAC)	Samson et al. 2004, Harrington 1981	dextrose 40 g peptone 10 g	cycloheximide 100 mg
Czapek agar (CZ)	Samson et al. 2004	saccharose 30 g	–
Glucose chloramphenicol agar (GKCH)	Malíř et al. 2003	glucose 20 g yeast extract 5 g	chloramphenicol 100 mg
Synthetic nutrient agar (SNA)	Nirenberg 1976	glucose 0.2 g saccharose 0.2 g	–

Environmental screening. During March and April 2010, isolation of microscopic fungi was carried out from cave sediments of all show caves in the Czech Republic (Chýnov Cave, Koněprusy Caves, Bozkov Dolomite Caves, Na Pomezí Caves, Na Špičáku Cave, Mladeč Caves, Javoříčko Caves, Zbrašov Aragonite Caves, Výpustek Cave, Kateřina's Cave, Punkva Caves, Sloup-Šošůvka Caves, Balcarka Cave) with the aim to detect *Geomyces destructans* in the bat environment. Sixty-seven cave sediment samples were processed. The method used was dilution plating on dichloran rose bengal chloramphenicol agar (DRBC) (Samson et al. 2004) and beer-wort agar with rose bengal (Fassatiová 1986). Petri dishes were incubated at 5 °C in the dark for three months.

Fig. 1. *Myotis myotis* bats in the abandoned Malá Amerika mine in the Bohemian Karst, locality with confirmed *Geomyces destructans* occurrence (February 2010).

Fig. 2. Eppendorf tube with plastic swab used for sampling of *Geomyces* from bat muzzles or wings.

Fig. 3. Primary isolation plate of *Geomyces destructans* CCF 3944 (SDA, 1 month, 15 °C).

Fig. 4. Microphoto of transparent adhesive tape with *Geomyces destructans*-like spores and hair of bat. Prepared in lactic acid with cotton blue. Bar = 20 µm.

Figs. 5, 6. *Geomyces destructans* CCF 3937 (CZ, 21 days, 12 °C). Conidiophores with arthroconidia; prepared in Melzer's reagent. Phase contrast. Bar = 10 µm.

Fig. 7. *Geomyces destructans* CCF 3943 (GKCH, 2 months, 15 °C). Arthroconidia; prepared in lactic acid with cotton blue. Phase contrast. Bar = 5 µm.

Fig. 8. *Geomyces destructans* CCF 3944 (SDA, 2 month, 15 °C). Arthroconidia; SEM (fixed with osmium tetroxide, coated in gold).


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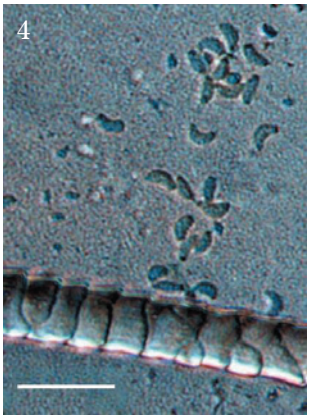
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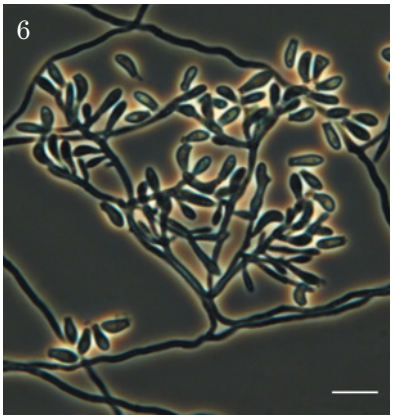
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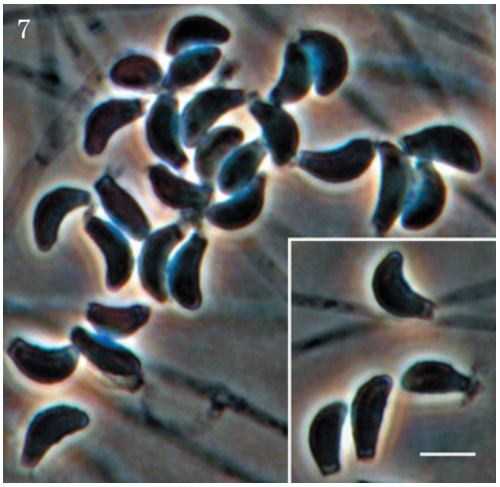
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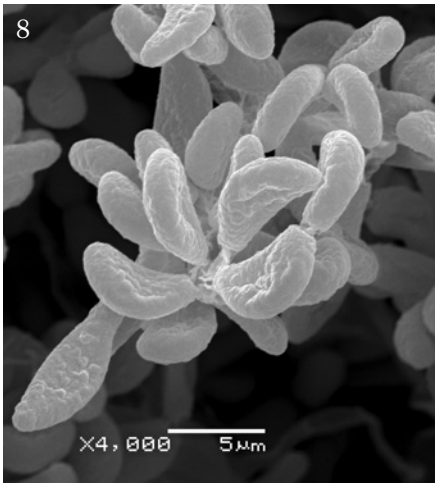
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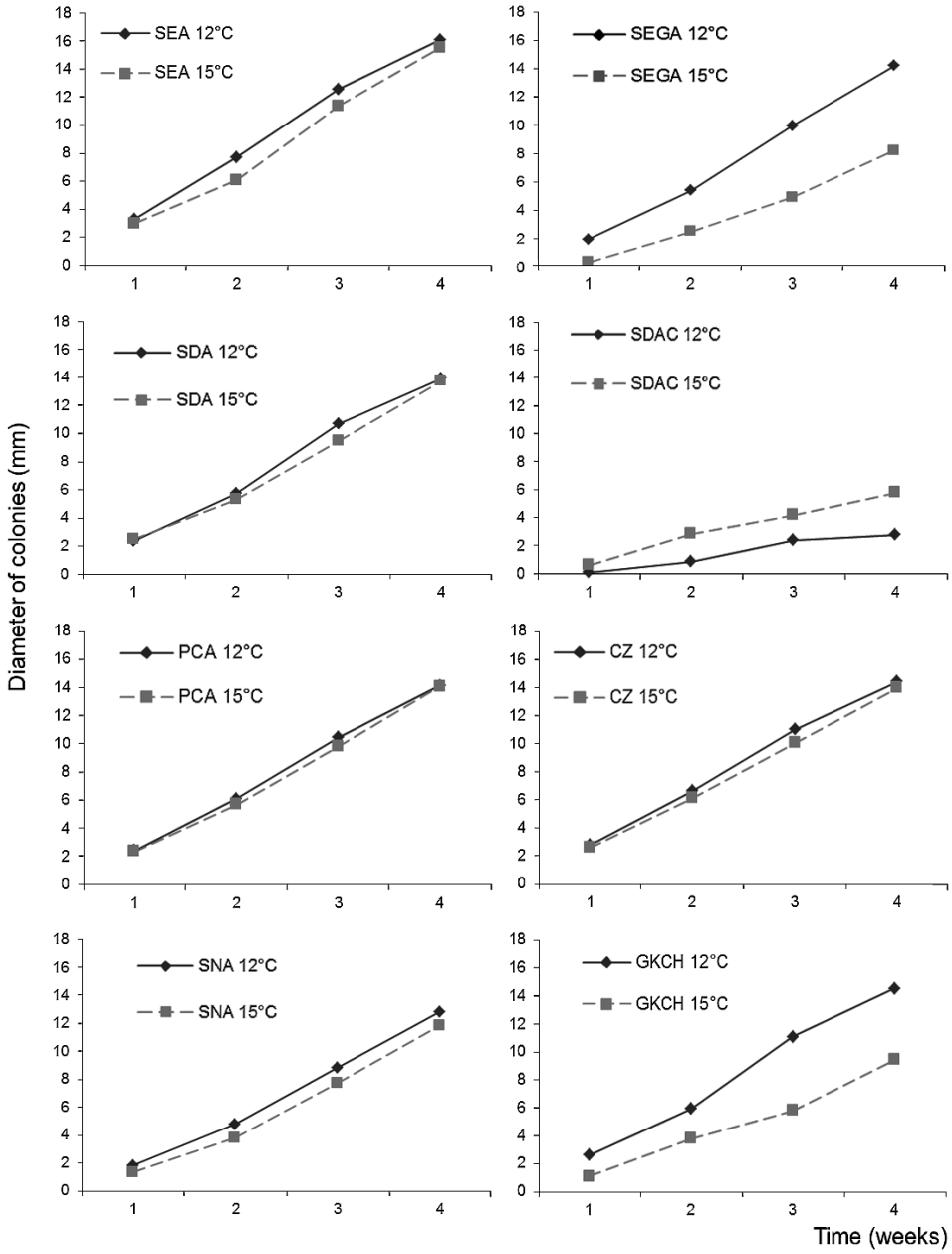

Fig. 9. Growth of *Geomyces destructans* on eight different media at 12 and 15 °C: SEA, SEGA, SDA, SDAC, PCA, CZ, SNA, GKCH (mean values from six isolates, each on two Petri dishes with three-point inoculation).

RESULTS AND DISCUSSION

Isolations

A direct microscopic observation of 12 samples from 12 WNS-suspected bats revealed 11 samples positive for the presence of *Geomyces destructans*-like conidia, ten originating from living *Myotis myotis* and one from a dead *Rhinolophus hipposideros*. Isolation of these 11 samples yielded seven *Geomyces destructans*-like isolates (Fig. 3). In remaining four samples the isolation of *G. destructans* failed due to intensive growth of zygomycetes and other fast growing fungi or bacteria. Chaturvedi et al. (2010) also reported on a high incidence of faster growing microfungi yielded during isolation of *G. destructans* on SDA, which disabled the isolation of *G. destructans*.

From the two media (SDA and SEGA) used for isolation of *G. destructans* from bat samples, only isolation on SDA (both at c. 7 and 15 °C) was successful; no colonies of *G. destructans* were observed on SEGA. No growth was either recorded at 25 °C. This is in accordance with data from the literature confirming the psychrophilic character of this fungus (Gargas et al. 2009, Chaturvedi et al. 2010). Chaturvedi et al. (2010) succeeded in isolation after fortifying the medium with a mixture of antibiotics and cultivation at 4°C.

Environmental screening

From 67 cave sediment samples from 14 localities (show caves) in the Czech Republic no isolates of *G. destructans* were obtained, although a low incubation temperature was used (5 °C). Earlier, long-term mycological examination of caves (e.g. Bosák et al. 2001, Nováková 2009) and case studies of underground tunnels (Kubátová et al. 2005) were made in the Czech Republic and Slovakia. During these studies only *G. pannorum* was isolated from the underground environment, most probably due to the higher incubation temperature used (25 °C). Presence of *G. destructans* in soil samples from bat hibernacula was demonstrated in the USA by Lindner et al. (2010), who detected *G. destructans* by means of a molecular survey in three of 19 soil samples from hibernacula.

Micromorphological features of *Geomyces destructans*

All isolates possessed the characteristic features of *G. destructans* (according to Gargas et al. 2009). The isolates formed hyaline, irregularly branched conidiophores bearing intercalary, lateral and terminal arthroconidia which were smooth under a light microscope (verruculose under a scanning electron microscope, Fig. 8) and slightly pigmented. Intercalary conidia formed short chains, were truncate at both ends and distinctly curved at maturity. Between conidia, less conspicuous

separating cells were formed that collapsed when conidia reached maturity (rhexolytic dehiscence). Lateral and terminal conidia grew singly or in small verticils, were truncate at base, and apically rounded; curvature was often not clearly distinct (Figs. 5–8). Conidia of our isolates measured 5.6–7.7 × 2.7–3.4 µm. These dimensions are within the conidial size range of 5–12 × 2–3.5 µm given by Gargas et al. (2009). The phylogenetically closely related and more frequent species *G. pannorum* differs both in size and shape of conidia. Its conidia are smaller, c. 2–5 × 2–4 µm, verruculose under a light microscope, and not curved (Domsch et al. 2007).

Colony features of *Geomyces destructans*

Morphology of colonies and growth rate differed depending on agar medium tested and on incubation temperature. Colonies on SEA, CZ, PCA, and SNA after 21 days at 15 °C were flat with thin mycelium, occasionally umbonate at centre, mostly whitish, with reverse uncoloured. Colonies on SDA, SDAC, SEGA, and GKCH were felted with dense mycelium, raised at centre, white in non-sporulating parts of colonies, greyish beige in sporulating parts, with coloured reverse (see Tab. 3). The studied isolates sporulated on all tested media.

Tab. 3. Colony pigmentation of *Geomyces destructans* on eight tested mycological media after three weeks at 15 °C.

Agar medium	Colony obverse	Colony reverse
Soil extract agar (SEA)	whitish	pale
Soil extract agar with glucose and rose bengal (SEGA)	white* to greyish beige	pink
Potato carrot agar (PCA)	whitish, at centre greyish	pale to grey at centre
Sabouraud dextrose agar (SDA)	white* to greyish beige	brown at centre
Sabouraud dextrose agar with cycloheximide (SDAC)	white* to greyish beige	dark brown
Czapek agar (CZ)	whitish to grey-green	pale to grey
Glucose chloramphenicol agar (GKCH)	white* to greyish beige	brown at centre
Synthetic nutrient agar (SNA)	whitish	pale to greyish

to* white parts of colonies were formed by non-sporulating mycelium

Growth rates were tested in six isolates at 12 and 15 °C on the eight media (Fig. 9). The growth of *G. destructans* isolates was somewhat faster at 12 °C than at 15 °C on all media with one exception (SDAC). Chaturvedi et al. (2010) reported the highest growth rate on SDA at 15°C, but only compared it to 4°C. It is therefore probable that the optimal temperature for growth of *G. destructans* also depends on the agar medium.

The largest colonies at 12 °C were observed on SEA (c. 16 mm diam. after 28 days), although they were very thin suggesting that the medium was not optimal

because the highest growth rate was reached at the expense of mycelial biomass. A comparable size (14–15 mm diam. after 28 days) was also observed on other media except SDAC (Fig. 9). Chaturvedi et al. (2010) gave a size of 20–27 mm for colonies on SDA after 28 days at 15 °C; we observed colonies of only 14 mm diam. under the same conditions. This discrepancy could be caused by different composition of media used (different producer), or may be attributed to the strain genotype. On the other hand, Gargas et al. (2009) cite 8 mm for colonies on SDA after 16 days at 14 °C; we observed a size of 6–7 mm for colonies on SDA after 16 days at 15 °C.

Somewhat smaller colonies were formed on SNA (c. 13 mm after 28 days), a medium low in nutrients. A very low growth was observed on SDAC (c. 3 mm at 12 °C), which was probably caused by cycloheximide. Chloramphenicol present in GKCH negatively affected fungal growth at 15 °C; differences between growth rates at 12 and 15 °C were greater than on other media. The last antibacterial substance used in the present study, rose bengal in SEGA, had a negative effect on growth compared with SEA. This negative effect was more pronounced at 15 °C again. From this point of view, we suppose that the presence of chloramphenicol or rose bengal in isolation media causes difficulties in isolation of *G. destructans* from cave sediments and could be the reason for our negative results in environmental screening.

Previously, the first author of this study observed the best growth of *G. destructans* on malt extract agar (MEA) and yeast and malt extract agar (YMA), with colonies reaching c. 18 mm diam. after one month at 15 °C (Martínková et al. 2010). Together with the results presented here, this points out the overall preference of nutrient rich media by *G. destructans*, a feature typical of pathogens.

Among the individual isolates of *G. destructans* some small differences in growth rates were also found. Isolate CCF 3942 formed the smallest colonies, isolates CCF 3943 and CCF 3941 the largest colonies. This range seems to be natural in various strains of *G. destructans* and is in agreement with results of Chaturvedi et al. (2010), who also observed slight differences in growth rates among five strains tested on SDA.

CONCLUSIONS

We obtained seven isolates of *G. destructans* from four localities in the Czech Republic which can be used for other studies. The isolates are maintained in CCF, Prague. Phenotypic features of *G. destructans* isolates were studied. The isolates possess the typical characters of the species, i.e. conidium shape and slow growth on agar media. Growth testing at 12 and 15 °C revealed somewhat faster growth at

12 °C confirming the psychrophilic character of the fungus.Testing on eight agar media showed new growth data for the media SEA, SEGA, CZ, PCA, GKCH, and SNA and different sensitivity to three antibiotics. Screening of bat environments for the presence of *Geomyces destructans* was negative probably due to unsuitable isolation media used.

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