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Diversity and prevalence of entomopathogenic fungi (Ascomycota, Hypocreales) in epidemic populations of bark beetles (Coleoptera, Scolytinae) in spruce forests of the Tatra National Park in Slovakia

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Abstract Bark beetles are serious forest pests in Slovakia. Their outbreaks may have significant ecological and economic impacts on spruce forests. There is a variety of natural enemies that activate themselves during population outbreaks of insects and entomopathogenic fungi belong to important antagonists with a potential to regulate populations of their hosts. In 2014–2016, species richness and prevalence of entomopathogenic fungi were evaluated during the bark beetle outbreaks in spruce forests affected by windstorms in the Tatra National Park in Slovakia. Three *Beauveria* species, *B. bassiana*, *B. caledonica* and *B. pseudobassiana*, with *Metapochonia bulbillosa* were identified from 271 specimens of three bark beetle species, *Ips typographus*, *Ips amitinus* and *Pityogenes chalcographus*. *Beauveria bassiana* was the dominant pathogen and infected all three bark beetle species. Phylogenetic analysis identified three phylogenetic groups of *B. bassiana* in the evaluated host populations. *M. bulbillosa* was reported for the first time from bark beetle hosts and Slovakia. The prevalence of fungal infection in natural populations of *I. typographus* was low, varied between 0.07 and 0.72%, and have little influence on the bark beetle abundance.

Keywords: bark beetle outbreaks, *Beauveria*, *Metapochonia*, *Picea abies*, Slovakia.

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Introduction

Norway spruce, *Picea abies* L. H. Karst., is one of the most common and economically most important tree species in Central Europe. In Slovakia, spruce forests cover, mostly in monocultures, 22% (approximately 430 000 ha) of the total forest area. Recently, these forest ecosystems have been confronted with severe destabilisation in Central Europe due to various disturbance agents (Nopp & Führer 2000) and outbreaks of insect pests belong among the most important biotic disturbance factors. Bark beetles (Coleoptera, Scolytinae) are a specific group of insect pests having significant ecological and economic impacts on forest ecosystems (Grégoire & Evans 2007). This relatively large group of insects is represented approximately by 220 genera with 6 000 species worldwide (Knížek & Beaver 2007), but only few of them pose a serious threat to Norway spruce in Europe. The European spruce bark beetle, *Ips typographus* L., is the most important species of this group (Grodzki et al. 2004, Wermelinger 2004, Økland et al. 2016). It is mainly damaging to mature spruce stands. *Pityogenes chalcographus* L., *Ips amitinus* Eichhoff, *Ips duplicatus* Sahlberg and *Polygraphus poligraphus* L. are also common bark beetle species attacking spruce stands, but their eruptive potential is less prominent. *Pityogenes chalcographus* prefers to colonise young (10-20 years old) or middle aged (20-40 years old) spruce forests. *Ips amitinus* often occurs together with *I. typographus* at higher elevations and spruce stands in lower elevations are frequently infested by *I. duplicatus. Polygraphus poligraphus* attacks older spruce forests stressed by air pollution and weakened by root fungal infections (Turčáni & Hlásny 2007, Grodzki et al. 2010). *Ips typographus*, the most destructive pest of spruce forests in Eurasia, attacks damaged, physiologically stressed, and dying trees. However, it can also colonise healthy trees and may act as primary mortality agent of spruce trees if the population density exceeds critical levels (Wermelinger 2004). In Europe, outbreaks of *I. typographus* have intensified significantly during the past two decades and they were usually triggered by wind disturbances and climatic drivers (e.g. Økland & Bjørnstad 2006, Kautz et al. 2011, Vakula et al. 2013, Stadelmann et al. 2014, Seidl et al. 2015, Økland et al. 2015, 2016, Jakoby et al. 2019, Hlásny et al. 2021). The current massive outbreaks of bark beetles in Slovakia were activated by 'Alžbeta' windstorm that struck the northern areas of Slovakia in November 2004 (Kunca & Zúbrik 2006, Vakula et al. 2013, Ferenčík 2016). The spruce stands in the Tatra National Park were the most damaged and about 30% (12 500 ha) of the total forest cover was affected in the park (Falťan et al. 2011, Ferenčík 2016). Due to pest management restrictions in this reserve area, the damaged wood could not be processed and became attractive to bark beetles. Subsequent outbreaks destroyed nearly 20 000 ha of spruce forests that was a half of the park area (Fleischer et al. 2016). The ongoing bark beetle outbreaks have developed into the largest and most severe calamity in Slovakia and the total damage to timber due to the bark beetle outbreaks has already exceeded the damage caused by the windstorm (Vakula et al. 2013).

A combination of phytosanitary measures, insecticide treatments and pheromone trappings are usually applied to control bark beetle populations in spruce forests (Wermelinger 2004). However, specific problems arise in nature reserves where chemical control is not allowed and other measures are also limited. In such areas, biocontrol is under consideration as an alternative pest management strategy with the exploitation of different natural enemies, including entomopathogenic fungi (e.g. Landa et al. 2001, Hilszczański et al. 2007, Wegensteiner 2007, Wegensteiner et al. 2010, 2015a, 2015b, Fora et al. 2014, Barta et al. 2018a, 2020). Limitations in successful use of entomopathogenic fungi against bark beetles arising from environmental conditions unique

to bark beetle habitats have been recently reviewed and a framework for standardising and improving laboratory assays to enhance field applications has been provided (Mann & Davis 2021). Entomopathogenic fungi (EPF) are insect parasites that participate in a natural regulation of host populations (Augustyniuk-Kram & Kram 2012, Vega et al. 2012). Hypocrealean entomopathogenic fungi (Ascomycota, Hypocreales), acting as natural regulators of insect populations, are constantly present in bark beetle populations (e.g. Wegensteiner et al. 2015b, Barta et al. 2018a) and their activity usually increases with a culmination of host population density (Augustyniuk-Kram & Kram 2012). A relatively broad range of EPF is known from bark beetles in Europe (e.g. Wegensteiner et al. 2015b) and *Beauveria* species were often recognised as the principal pathogens (e.g. Landa et al. 2001, Draganova et al. 2010, Wegensteiner et al. 2015a, Barta et al. 2018a, 2020). Many studies on entomopathogens of bark beetles have been targeted to *I. typographus* and there is only limited information on pathogens of other bark beetle species like *I. duplicatus*, *Ips sexdentatus* Börner or *Dryocoetes autographus* Ratzeburg (e.g. Draganova et al. 2010, Dinu et al. 2012, Wegensteiner et al. 2015a). In Slovakia, a species diversity of EPF in *I. typographus* has been recently studied, but special attention was put to the *Beauveria* genus and to a selection of highly virulent strains suitable for the bark beetle biocontrol (Barta et al. 2018a). In this study, we were interested in the activation of natural antagonists - entomopathogenic fungi - during the ongoing population outbreaks of bark beetles in Norway spruce forests of the Tatra National Park (Slovakia) affected by windstorms. We determined a species richness of hypocrealean EPF in communities of different bark beetle species and evaluated a prevalence of the fungi in *I. typographus* populations during its massive gradation.

Materials and Methods

Study area

The study was performed in the Tatra National Park (49.180278 N, 19.919444 E), the high-altitude region (600-2 600 m a.s.l.) of the Tatra Mountains in north central Slovakia. The Tatra Mountains are the highest mountain range in the Carpathian Arch forming a natural border between Slovakia and Poland. The park was established in 1949 and currently extends over 738 km2, with 307 km^2 of a protection zone. Forests cover an area of 50 000 ha and create almost a continuous vegetation belt spreading from 700 m a.s.l. up to the timberline at 1 550 m a.s.l. (Fleischer et al. 2005). The forests are dominated by *P. abies* with *Larix decidua* Mill., *Pinus cembra* L., and few broadleaved trees (beech, sycamore, birch, alder etc.) as admixture species. The climate in the area is cold and humid with the average annual air temperature of 5.8 °C and the average annual precipitation of 1 100-1 900 mm. The mean duration of snow cover varies from 124 days at the base to 228-236 days at 2 000 m a.s.l. The maximum thickness of snow cover occurs in February in the lower parts and in the second half of March at higher altitudes (Niedzwiedz 1992). In November 2004, heavy windstorm 'Alžbeta' struck the park and damaged more than five million cubic metres of Norway spruce. In the following years, bark beetle populations rapidly increased because practically no sanitation felling or salvage logging of fallen trees could be conducted due to the protection status of the affected areas. Ten years after the windstorm, 15 sites of uniform Norway spruce stands of age >80 years were selected in the damaged areas for evaluation of species diversity of entomopathogenic fungi in bark beetle populations (Figure 1). The population dynamics of the dominating bark beetle species *I. typographus* reached the outbreak phase (defined by Biedermann et al. 2019) during the sampling. The sites

were selected with the purpose to be evenly distributed throughout the park and to reflect the existing foci of bark beetle outbreaks.

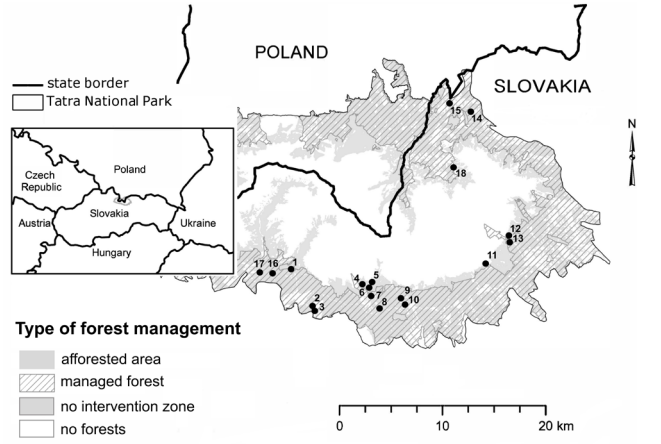

Figure 1 A map of the Tatra National Park showing sites (●) where species diversity and prevalence of entomopathogenic fungi in bark beetle populations were studied during 2014–2016.

Bark beetle sampling and fungus isolation

To determine a species richness of entomopathogenic fungi, fungus-infected bark beetles were collected in the park from July to September in 2014 and 2015. The specimens were collected from windthrown spruce trees that were naturally infested in the spring of the same year. Adult bark beetles with typical macroscopic symptoms of fungal infection were placed individually in sterile 2.0-ml cryogenic vials. The specimens were screened for the presence of entomopathogenic fungi by a dissecting microscope (50×) and individuals that had died from other factors were excluded from further analyses. Bark beetle species were identified according to the external morphology of adults and the architecture of gallery systems under the bark (Cognato 2015). Samples with confirmed fungal infection were used for isolation of axenic cultures. Axenic cultures were obtained by incubating individuals with fungal infection onto Sabouraud dextrose agar (SDA) plates supplemented with fungicides (250 mg/L cycloheximide and 500 mg/L dodine) and antibiotics (600 mg/L streptomycin sulphate, 50 mg/L tetracycline hydrochloride). The plates were incubated at 25 ± 1 °C for 10 days and fungal colonies were transferred onto fresh SDA plates without fungicides and antibiotics. The obtained cultures were stored at 4 °C.

Identification of entomopathogenic fungi

Axenic cultures were identified to a genus level by a microscopic (500×) investigation of fungal microstructures (Rehner & Buckley 2005, Rehner et al. 2011, Humber 2012). The morphological identification was coupled to the rDNA-ITS sequencing study. The DNA was extracted from the biomass of 10-day-old fungal cultures. Approximately 50 mg of mycelium was put into a 2-ml microtube with 200 µl of PrepManTM Ultra Sample Preparation Reagent (Life Technologies, USA) and homogenised with glass beads (diam. 2 mm) by BeadBug Microtube Homogenizer (Benchmark scientific, USA) for 2 min. After a 15-min treatment at 110 °C in thermoblock BioTDB 120 (Biosan, Latvia), the samples were centrifuged for 5 min at 24 000 × g. As many as 1 µl of the resulting supernatant was used in the PCR reactions. The internal transcribed spacer (ITS) region of the nuclear DNA was amplified with the primer pairs ITS5/ITS4 (White et al. 1990). A PCR mixture (30 µl) contained 3 µl of 10X Dream Taq DNA buffer, 3 µl of 2 mM dNTP mix, 1 µl of 25mM MgCl2, 1.2 µl of primers (10 mM), 1 µl of bovine serum albumin (25 mg/ml), 0.5 U of Dream Taq DNA polymerase and 1 µl of genomic DNA. The PCR amplification was

performed in a thermocycler MJ Mini (Biorad, USA) under the following cycling conditions: 95 °C for 60 s followed by 45 cycles of 95 °C for 30 s, annealing at temperature 56 °C for 30 s, 72 °C for 30 s and a final elongation at 72 °C for 5 min. Amplification products were purified using exonuclease I and FastAP (Life Technologies, USA). Sequencing was performed by Macrogen Europe B.V. (Amsterdam, the Netherlands), sequences were deposited in the NCBI GenBank database and compared with data from the GenBank using the BLASTn algorithm for identification of fungi. The sequences from this study and the reference sequences of all currently available ex-type strains of *Beauveria* and *Metapochonia* in the GenBank (Table 1) were aligned using MUSCLE (Edgar 2004) and used for the phylogenetic analysis in MEGA X software (Kumar et al. 2018). A phylogenetic tree was constructed using the Maximum likelihood method (ML) with TN93 substitution model (Tamura & Nei 1993), BioNJ starting tree with the best of NNI tree searching, and followed by 1 000 bootstrap replications. Alignment gaps were treated as missing data.

Table 1 List of species of entomopathogenic fungi with ITS sequences retrieved from the NCBI GenBank database used in the phylogenetic analysis.

Fungal species	Strain code	Locality	Host/Substrate	GenBank	References
Beauveria amorpha	ARSEF 7542	USA, Colorado	Hymenoptera: Formicidae	HQ880805	Rehner et al. (2011)
B. asiatica	ARSEF 4850	South Korea, Chiag Mt.	Coleoptera: Cerambycidae	HQ880787	Rehner et al. (2011)
B. australis	ARSEF 4598	Australia, Tasmania	Soil	HQ880789	Rehner et al. (2011)
B. baoshanensis	BUB283	China, Gaoligong Mt.	Lepidoptera: Lymantridae	MG642828	Chen et al. (2019)
B. bassiana	ARSEF 1564	Italy, Villa Cade	Lepidoptera: *Hyphantria cunea*	HQ880761	Rehner et al. (2011)
B. bassiana	ARSEF 1848	Belgium	Coleoptera: Rhizophagidae	AY531995	Rehner and Buckley (2005)
B. bassiana	B78-5	Slovakia, Tatranská Lomnica	*Ips typographus*	MW051928	This study
B. bassiana	B2110-1	Slovakia, Podbanské	*Pityogenes chalcographus*	MW051979	This study
B. bassiana	A14-5	Slovakia, Tatranská Lomnica	*Ips typographus*	MW051976	This study
B. bassiana	NREP100	Slovakia	*Hylobius abietis*	MK490878	Barta et al. (2019)
B. bassiana	IK10	Slovakia, Michalovo	*Ips typographus*	KY352660	Barta et al. (2018)
B. bassiana	MK167	Slovakia, Lysá Poľana	*Ips typographus*	KY352659	Barta et al. (2018)
B. bassiana	BG21	Bulgaria: Vitosha	*Ips typographus*	MT180396	Barta et al. (2020)
B. bassiana	SUAh03	Slovakia	Soil	KJ489072	Medo et al. (2016)
B. bassiana	SUAo38	Slovakia	Soil	KJ489065	Medo et al. (2016)
B. bassiana	SUAa46	Slovakia	Soil	MT239412	Medo et al. (2021)
B. bassiana	SUAe81	Slovakia	Soil	KJ489075	Medo et al. (2016)
B. brongniartii	Je276	Switzerland	Coleoptera: Scarabaeidae	HQ880784	Rehner et al. (2011)
B. caledonica	ARSEF 2567	Scotland	Soil	HQ880817	Rehner et al. (2011)
B. caledonica	Ab16-1	Slovakia, Krivánska cesta	*Ips typographus*	MW052024	This study
B. caledonica	MK206	Slovakia, Hnilčík	*Ips typographus*	KY352729	Barta et al. (2018)
B. hoplocheli	Bt96	Madagascar	Coleoptera: Melolonthidae	KC339697	Robène-Soustrade et al. (2015)
B. kipukae	ARSEF 7032	USA, Hawaii	Homoptera: Delphacidae	HQ880803	Rehner et al. (2011)
B. lii	RCEF5500	China, Xianyang	Coleoptera: Coccinellidae	JN689372	Zhang et al. (2012)

Table 1 List of species of entomopathogenic fungi with ITS sequences retrieved from the NCBI GenBank database used in the phylogenetic analysis.

Fungal species	Strain code	Locality	Host/Substrate	GenBank	References
B. majiangensis	GZU12141	China, Guizhou: Majiang	Coleoptera: Scarabaeoidea	MG052643	Chen et al. (2018)
B. malawiensis	IMI 228343	Malawi, Zomba	Coleoptera: Cerambycidae	DQ376247	Rehner et al. (2006)
B. medogensis	BUB426	China, Gaoligong Mt.	Hymenoptera: Formicidae	MG642832	Chen et al. (2019)
B. pseudobassiana	ARSEF 3405	USA, Virginia	Lepidoptera: Erebidae	HQ880792	Rehner et al. (2011)
B. pseudobassiana	B2710-13	Slovakia, Tatranská Lomnica	*Ips typographus*	MW052021	This study
B. pseudobassiana	MK394	Slovakia, Čierny Váh	*Ips typographus*	KY352725	Barta et al. (2018)
B. rudraprayagi	MTCC 8017	India, Rudraprayag	Lepidoptera: Bombycidae	JQ266173	Agrawal et al. (2014)
B. scarabaeidicola	ARSEF 7281	South Korea: Guryungryung	Coleoptera: Scarabaeidae	HQ880815	Rehner et al. (2011)
B. sinensis	RCEF3903	China, Anhui	Lepidoptera: Geometridae	HQ270152	Chen et al. (2013)
B. varroae	ARSEF 8257	France, Montdardier	Acari: Varroidae	HQ880800	Rehner et al. (2011)
B. vermiconia	ARSEF 2922	Chile, Valdivia	Soil	HQ880822	Rehner et al. (2011)
B. acridophila	AV1875	Colombia, Zaphire Reserve	Orthoptera: Proscopiidae	JQ958602	Sanjuan et al. (2014)
B. diapheromeriphila	MV2492	Ecuador, Jatunsacha Reserve	Phasmatodea: Diapheromeridae	JQ958603	Sanjuan et al. (2014)
B. gryllotalpidicola	BCC26300	Thailand, Nakhon Ratchasima	Orthoptera	FJ459787	Ariyawansa et al. (2015)
B. locustiphila	TS881	Colombia, Tolima	Orthoptera: Romaleidae	JQ958606	Sanjuan et al. (2014)
B. loeiensis	BCC23104	Thailand, Loei	Orthoptera	FJ459784	Ariyawansa et al. (2015)
Metapochonia bulbillosa	CBS 145.70	Denmark	Roots of *Picea abies*	NR154142	Zare et al. (2001)
M. bulbillosa	B18-13	Slovakia, Vyšné Hágy	*Ips typographus*	MW052028	This study
Metarhizium anisopliae	ARSEF 7487	Eritrea	Orthoptera: Schistocerca gregaria	HQ331446	Schneider et al. (2011)

Prevalence of fungal infection in Ips typographus populations

The prevalence of EPF was evaluated in populations of *I. typographus* that was the dominating and most destructive species in the Tatra National Park. For this purpose, spruce tree logs were collected at nine most affected sites (Table 2) during May-October 2016. At each sampling site, 3-15 logs (approximately 2 m long) of naturally infested trees were transferred to the laboratory. The circumference of the logs was measured at both ends and the average circumference was calculated for each log. The bark was stripped of the logs so that the size of debarked area had the length of 0.5 m and the width reached a half the circumference of the particular log. The total debarked area was calculated for each log. The effort was put to get the bark in a single piece from each log because it was used for the evaluation of infestation by bark beetles and prevalence of fungal infection. On each piece of bark, the total number of completely developed larval galleries and fungus-killed individuals was recorded. The dead insects were handled as mentioned above and used for fungal species identification. The infestation of logs by bark beetles was

expressed as the number of larval galleries counted per area of one square metre.

Prevalence of infection in host populations was calculated using the formula:

$$infection\ prevalence = \frac{NGI}{TNG} \times 100,$$

where *NGI* is number of galleries with infected individuals and *TNG* is total number of galleries.

Table 2 List of sites in the Tatra National Park where prevalence of fungal infection was studied in bark beetles populations during May-October 2016.

Name of collection site*	Altitude(m)	Coordinates of site	No. of logs**
Podbanské I (1)	1131	49.144722° N; 19.958611° E	15
Podbanské II (16)	1168	49.141667° N; 19.938889° E	4
Podbanské III (17)	1140	49.142163° N; 19.926575° E	6
Vyšné Hágy I (9)	1133	49.125181° N; 20.108297° E	8
Vyšné Hágy II (10)	1128	49.118803° N; 20.112651° E	4
Javorová dolina (18)	1293	49.216925° N; 20.159143° E	10
Podspády - Krupovky (14)	908	49.285008° N; 20.175114° E	3
Tatranská Lomnica I (12)	857	49.073611° N; 20.145000° E	9
Tatranská Lomnica II (13)	1400	49.183861° N; 20.245046° E	11

*Numbers in the parenthesis refer to the particular collection sites as displayed in Figure 1.

**Number of spruce tree logs collected for evaluation of fungal prevalence at a particular site.

Data analysis

The mean values of infection prevalence and infestation of logs by bark beetles per collection sites were tested for normality by the Kolmogorov-Smirnov test. Data for infestation were square-root transformed and data for infection prevalence were arscine transformed to get normally distributed data. The transformed data were subjected to ANOVA with the aim to compare infestation and infection prevalence means among the sites ($p = 0.05$). The post-hoc Tukey's HSD test was performed to separate and compare the means if significant differences ($p = 0.05$) were detected. Pearson correlation was performed to test the relationship between the infestation levels of spruce logs with bark beetles and the prevalence of fungal infection. All the analyses were conducted using Minitab 17® (© 2013 Minitab Inc.).

Results

Species spectrum of entomopathogenic fungi in bark beetle populations

During the survey, 271 samples of dead bark beetles (205 samples of *I. typographus*, 18 samples of *I. amitinus*, 24 individuals of *P. chalcographus*) showing external symptoms of fungal infection were collected. As many as 24 dead beetles could not be identified to the species level and were excluded from the further analyses. Dead bark beetles with confirmed mycosis by microscopic observations were used for isolation of in vitro cultures on the agar plates. Axenic cultures were obtained from 171 dead beetles. The remaining samples produced no fungal colonies. From a single dead individual one axenic culture was always isolated. Altogether, 120 isolates were identified as entomopathogenic species by molecular analyses (Table 3). The amplicons of ITS region were deposited in the NCBI GenBank database under the accession numbers given in Table 4. The obtained sequences were compared with the GenBank database and three *Beauveria* species were identified, *Beauveria bassiana* (Bals.-Criv.) Vuill. (111 isolates), *B. caledonica* Bissett & Widden (five isolates) and *B. pseudobassiana* S.A. Rehner & R.A. Humber (two isolates). All sequences were supported by a high degree (> 99%) of identity with sequences of the neotype *B. bassiana* strain ARSEF 1564 and the type strains of *B. caledonica* (ARSEF 2567) or *B.*

pseudobassiana (ARSEF 3405). Two fungal isolates were identified as *Metapochonia bulbillosa* (W. Gams & Malla) Kepler, S.A. Rehner & Humber. *Beauveria bassiana* as the dominant pathogen in the bark beetle populations was detected in 13 sites. The less frequent species *B. caledonica* and *B. pseudobassiana* were isolated from samples collected in just three or two sites, respectively. *Metapochonia bulbillosa* was observed in a single site. The EPF were isolated from three bark beetle species, *I. typographus*, *I. amitinus* and *P. chalcographus*, but not all pathogens were observed in each bark beetle species. While *B. bassiana* was isolated from all species, *B. pseudobassiana* and *B. caledonica* were not detected in *P. chalcographus* or *I. amitinus*, respectively. *Metapochonia bulbillosa* was detected in *I. typographus* and *P. chalcographus*. This fungus is reported from the bark beetle hosts for the first time.

Table 3 List of species of entomopathogenic fungi identified from bark beetles in the Tatra National Park during 2014-2015.

Bark beetle species	Fungal species	Name of collection site*	No. of fungal isolates
Ips typographus	*Beauveria bassiana*	Podbanské I (3)	16
		Štrbské Pleso I (8)	10
		Podbanské II (2)	2
		Štrbské Pleso II (7)	4
		Vyšné Hágy II (10)	24
		Vyšné Hágy I (9)	3
		Štrbské Pleso III (6)	2
		Popradské Pleso (5)	1
		Vodopád Skok (4)	3
		Tatranská Lomnica II (13)	19
		Tatranská Lomnica I (12)	6
	Beauveria caledonica	Krivánska cesta (1)	1
		Tatranská Lomnica II (13)	3
	Beauveria pseudobassiana	Tatranská Lomnica II (13)	1
	Metapochonia bulbillosa	Vyšné Hágy (10)	1
Ips amitinus	*Beauveria bassiana*	Štrbské Pleso I (8)	2
		Vyšné Hágy II (10)	7
		Tatranská Lomnica II (13)	1
	Beauveria pseudobassiana	Vyšné Hágy II (10)	1
Pityogenes chalcographus	*Beauveria bassiana*	Podbanské I (3)	1
		Štrbské Pleso I (8)	2
		Štrbské Pleso II (7)	1
		Vyšné Hágy II (10)	2
		Popradské Pleso (5)	1
		Tatranská Lomnica II (13)	2
		Podspády - Krupovky (14)	1
		Podspády - Vojtašová (15)	1
	Beauveria caledonica	Podspády - Vojtašová (15)	1
	Metapochonia bulbillosa	Vyšné Hágy II (10)	1

*Numbers in the parenthesis represents the particular collection sites as displayed in Figure 1.

Dataset for phylogenetic analysis included 43 sequences (Table 1): six sequences of representative isolates from this survey, 36 sequences of *Beauveria* species or *M. bulbillosa* from other studies and a sequence of *Metarhizium anisopliae* (strain ARSEF 7487) designated as the outgroup. The final dataset of sequences consisted of 22 852 bp and ML analysis produced a consensus tree (Figure 2) with a log-likelihood of -1720.18. In the phylogenetic tree, the isolates of *B. pseudobassiana*, *B. caledonica* and *M. bulbillosa* each represented a single phylogenetic clade with the sequences of particular species. The *B. bassiana* isolates were clustered together with the neotype strain of *B. bassiana* (ARSEF 1564), however the clade comprises three distinct sub-clades. Almost 95% (n = 105) of *B. bassiana* isolates obtained in this study are organised in the phylogenetic group A1 (Table 4).

Table 4 Phylogenetic classification of fungal isolates obtained from bark beetles in spruce forests of the Tatra National Park during 2014-2015.

Fungal species	Phylogenetic group	Number of isolates	GenBank Accession numbers of sequences from this study*
Beauveria bassiana	A1	105	MW051915-MW051928, MW051929-MW051951, MW051953-MW051975, MW051977-MW051978, MW051980, MW051982-MW052010, MW052012-MW052015, MW052017-MW052019
	A2	4	MW051952, MW051979, MW052011, MW052016
	A3	2	MW051976, MW051981
Beauveria pseudobassiana	B	2	MW052020, MW052021
Beauveria caledonica	C	5	MW052022-MW052024, MW052025, MW052026
Metapochonia bulbillosa	D	2	MW052027, MW052028

*the GenBank Accession numbers highlighted in the bold type were selected for the phylogenetic analysis (Figure 2).

Prevalence of fungal infection in populations of *Ips typographus*

The prevalence of fungal infection in natural populations of *I. typographus* was low in the study area (Table 5). The mean percentage of galleries with infected individuals varied between 0.07 and 0.72% depending on the collection sites, but there was not significant difference in prevalence of fungal infection among the collection sites ($F_{(8,61)}$ = 2.402; $p > 0.05$). The mean infestation level of spruce logs by bark beetles varied significantly among collection sites ($F_{(8,61)}$ = 12.443; $p < 0.05$). The mean number of infected individuals per 1-m^2 area of debarked spruce logs reached 0.66-3.87 beetles and did not correlate (r = -0.325; p = 0.393) with the total number of galleries per 1-m^2 area. For example, the highest disease prevalence (0.72% resp. 3.87 ind./m^2) was detected in the collection site (Podbanské I) with the lowest infestation of logs (5 164 galleries/m^2). The lowest fungal prevalence (0.07% resp. 0.66 ind./m^2) was observed in the site (Tatranská Lomnica I) with low infestation (6 434 galleries/m^2) but, in contrast, in the site (Podbanské II) with the highest infestation by *I. typographus* (24 624 galleries/m^2) a moderate fungal prevalence (0.10% resp. 1.17 ind./m^2) was detected.

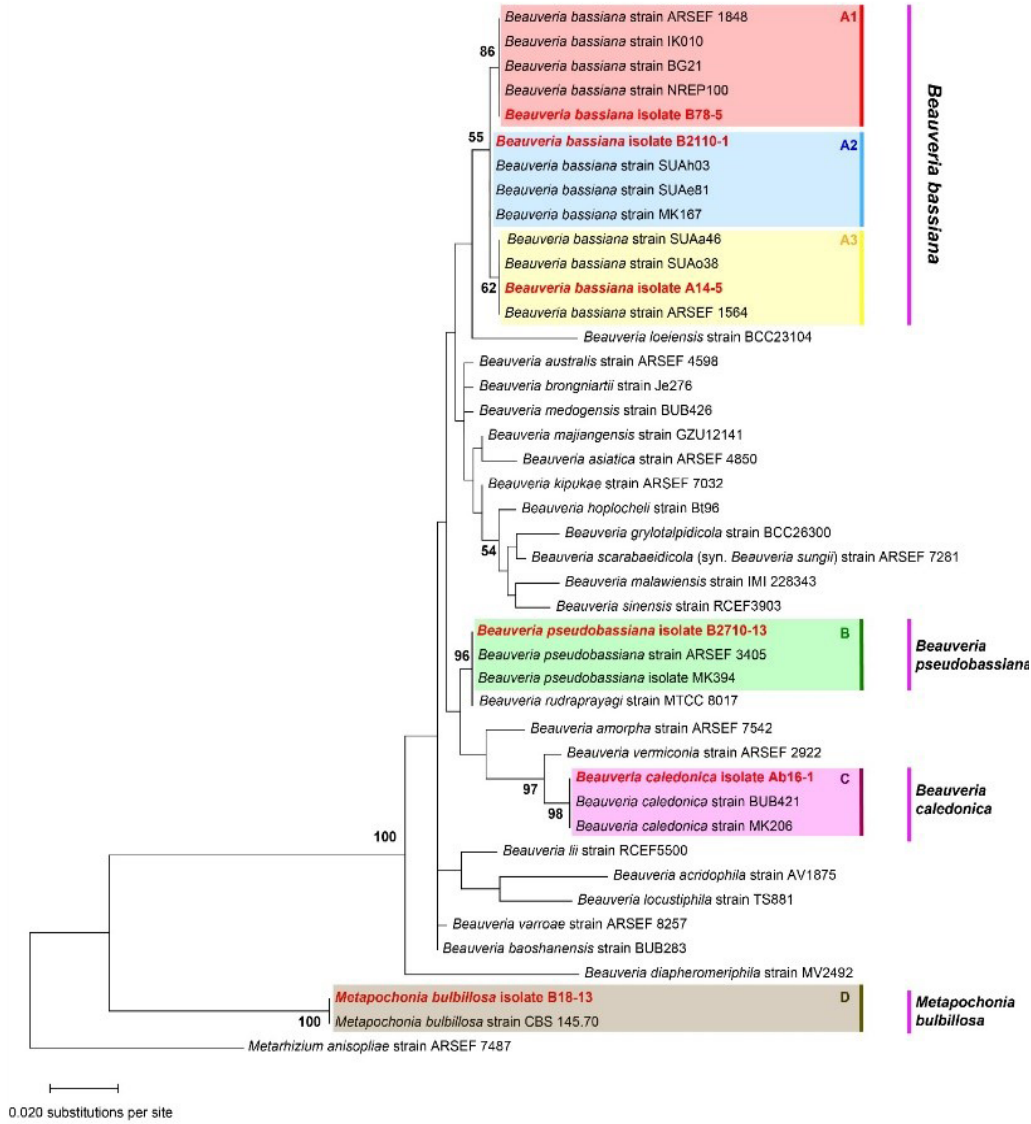

Figure 2 Phylogenetic tree showing relationships of isolates of entomopathogenic fungi inferred by using the maximum likelihood method and Tamura-Nei model (Tamura & Nei 1993), tree with the highest log-likelihood (-1720.18) is shown, percentage of trees (> 50%) in which the associated taxa clustered together is shown next to the branches, *Metarhizium anisopliae* was used as outgroup, isolates collected and sequenced in this study are shown in red colour and bold type.

Table 5 Infestation of spruce tree logs by *Ips typographus* with prevalence of fungal infection in the population in sites of the Tatra National Park during May-October 2016.

Collection site*	TNG per 1 m² mean ± SE**	NGI per 1 m² mean ± SE	IP (%)** mean ± SE
Podbanské I (1)	5164.52 ± 237.22 a	3.87 ± 0.46	0.72 ± 0.15 a
Podbanské II (16)	24624.83 ± 6115.23 b	1.17 ± 0.51	0.10 ± 0.07 a
Podbanské III (17)	7581.67 ± 896.42 a	0.98 ± 0.40	0.08 ± 0.03 a
Vyšné Hágy I (9)	7862.84 ± 714.88 a	1.38 ± 0.37	0.09 ± 0.02 a
Vyšné Hágy II (10)	9490.70 ± 1576.27 a	0.94 ± 0.49	0.09 ± 0.05 a
Javorová dolina (18)	6349.42 ± 354.37 a	1.40 ± 0.22	0.14 ± 0.03 a
Podspády - Krupovky (14)	9485.58 ± 765.15 a	1.60 ± 0.65	0.11 ± 0.04 a
Tatranská Lomnica I (12)	6434.86 ± 828.74 a	0.66 ± 0.34	0.07 ± 0.09 a
Tatranská Lomnica II (13)	5660.32 ± 866.56 a	3.10 ± 1.93	0.36 ± 0.17 a
Mean for all sites	**9183.86 ± 571.17**	**1.68 ± 0.29**	**0.20 ± 0.05**

*Numbers in the parenthesis refer to the particular collection sites as displayed in Figure 1.

**Means in the columns followed by the same letter are not significantly different (Tukey's HSD test, $p = 0.05$)

TNG - total number of larval galleries

NGI - number of larval galleries with infected individuals

IP - infection prevalence in population

Discussion and conclusion

Species spectrum of entomopathogenic fungi in bark beetle populations

During the past two decades the occurrence of EPF in bark beetle populations has been documented in Europe in several studies (e.g. Landa et al. 2001, Kreutz et al. 2004, Wegensteiner 2007, Draganova et al. 2010, Takov et al. 2012, Mudrončeková et al. 2013, Wegensteiner et al. 2015a, Barta et al. 2018a, 2020) and *Beauveria* species have been reported as common pathogens. The current study also demonstrates that infection by *Beauveria* is a natural mortality factor of bark beetles and the species composition of EPF in the Tatra National Park is similar to those reported from other European countries. The exception is the record of *M. bulbillosa* in this study. This species was originally described as *Verticillium cephalosporum* W. Gams and isolated from the tissue of spruce roots in Denmark (Gams 1971). Later, it was renamed to *Verticillium bulbillosum* W. Gams & Malla (Gams 1988) and *Pochonia bulbillosa* (W. Gams & Malla) Zare & W. Gams (Zare et al. 2001). In a recent phylogenetic study of anamorphic insect-pathogenic fungi linked to the sexual state *Metacordyceps*, a new genus *Metapochonia* was described for species of verticillium-like morphology and seven species, with *M. bulbillosa*, were included in the genus (Kepler et al. 2014). Although *M. bulbillosa* has not been reported from bark beetles or other insect hosts before, its former synonym *V. bulbillosum* was rarely recorded as a parasite of cyst nematodes (*Heterodera* sp.) (Dackman & Nordbring-Hertz 1985). In 2014, *M. bulbillosa* was identified from soil samples and unidentified heteropteran hosts in Slovakia (Medo, personal communication, November 2020).

The *Beauveria* genus includes globally distributed species pathogenic to a broad range of insect hosts from many orders including Blattodea, Coleoptera, Diptera, Embioptera, Hemiptera, Hymenoptera, Lepidoptera, Mantodea, Neuroptera, Orthoptera, Phasmatodea, Siphonaptera and Thysanoptera (e.g. Zimmermann 2007, Rehner et al. 2011, Chen et al. 2017, Kepler et al. 2017, Khonsanit et al. 2020). Until now, as many as 24 species of the genus have been recognised and 14 of them

have been reported as parasites of coleopterans (e.g. Rehner et al. 2011, Kepler et al. 2017, Chen et al. 2018, Bustamante et al. 2019, Khonsanit et al. 2020). Five species, including *B. bassiana*, *B. brongniartii* (Sacc.) Petch, *B. caledonica*, *B. pseudobassiana* and *B. varroae* S. A. Rehner & R. A. Humber, have been recorded in Europe. While *B. bassiana*, *B. brongniartii* and *B. pseudobassiana* are cosmopolitan species infecting insect hosts living in various habitats, *B. caledonica* is originally known from the soil in Scotland (Bissett & Widden 1988), but recently it was also reported from weevils (Curculionidae, Scolytinae) in New Zealand and Ireland (Glare et al. 2008) as well as from *I. typographus* in Slovakia (Barta et al. 2018a) and Bulgaria (Barta et al. 2020). *Beauveria varroae* was formerly known from ectoparasitic mites of honeybee in France, but recently it was also identified from coleopterans (Rehner et al. 2011) and lepidopteran pupae (Barta et al. 2020). Of the five *Beauveria* species known in Europe, four have been recorded in bark beetle populations. They are *B. bassiana*, *B. caledonica*, *B. pseudobassiana* and *B. brongniartii* (e.g. Landa et al. 2001, Wegensteiner 2007, Draganova et al. 2010, Takov et al. 2012, Mudrončeková et al. 2013, Wegensteiner et al. 2015a, Barta et al. 2018a, 2020). In the current survey, we recorded all of them except for *B. brongniartii*, which is a well-known parasite of coleopteran larvae living in soil (Zimmermann 2007). Recently, this species was reported from *I. typographus* in Bulgaria, but the identification was based on morphotaxonomic analysis and no detailed morphological characteristics were provided (Takov et al. 2012) or the morphological description was only limited to conidia (Draganova et al. 2010). *Beauveria bassiana* and *B. brongniartii* are morphologically very similar and all identifications supported by just a microscopic investigation must be judged with a caution. Generally, the identification of *Beauveria* species, especially in the anamorphic state, is rather problematic due to the structural simplicity and overlap in morphological characteristics. Thus, DNA barcoding is becoming the standard approach for species determination. For example, recent phylogenetic studies based on multilocus analyses revealed that *B. brongniartii* and *B. bassiana* include cryptic lineages and new species were described (Rehner & Buckley 2005, Rehner et al. 2011). *Beauveria bassiana* belongs among the most frequently reported *Beauveria* species in natural populations of *I. typographus* in Europe (e.g. Landa et al. 2001, Takov et al. 2007, 2012, Wegensteiner 2007, Wegensteiner et al. 2015a, Barta et al. 2018a, 2020) and it also prevailed in the populations studied in the current survey. Although we identified *B. pseudobassiana* only in two individuals of bark beetles, in previous study this fungus was reported a common pathogen of bark beetles in Slovakia (Barta et al. 2018a). In laboratory bioassays, a high virulence against bark beetles was reported by this fungus and it was recommended as a perspective biocontrol agent of these pests (Kocaçevik et al. 2016). It also seems that *B. pseudobassiana* is adapted to a forest ecosystem since it was recognised to prefer forests to agricultural or meadow habitats (Medo et al. 2016).

Prevalence of fungal infection in populations of *Ips typographus*

The prevalence of fungal infection in natural populations of *I. typographus* was low and corresponds to results of similar studies in Europe (Draganova et al. 2010, Takov et al. 2012, Barta et al. 2018a, 2020). The observations of this study indicate that the fungal infection had no capacity to significantly influence populations of *I. typographus* during the massive outbreak. Biotic and abiotic factors that could contribute to the efficacy of entomopathogenic fungi in regulating eruptive forest insects like bark beetles have been recently analysed. Besides general abiotic factors that limit the efficacy of the fungi like temperature and ultraviolet light, biotic factors specific to the bark beetle environment like the existence of associated microorganisms, plant

secondary metabolites and bark beetle behaviour have been discussed (Mann & Thomas 2021). The transmission of fungal infection within and among populations occurs by direct contact between infected dead insects and susceptible hosts or indirectly via airborne spores or spores deposited on the plant surface or soil particles. We found no significant relationship between host density and infection cases in the analysed populations. Due to a cryptic behaviour of bark beetles during a significant part of their life cycle, the factor of host density is probably not crucial for disease spreading. Several other studies confirm that this group of EPF is usually associated with bark beetle populations at low but constant prevalence level (Takov et al. 2006, 2007, 2012, Draganova et al. 2010, Barta et al. 2018a, 2020) and natural epizootics do not occur in host populations. From the viewpoint of bark beetle outbreak management, our observations indicate that a simple reliance on the natural activity of entomopathogenic fungi would not reduce the bark beetle abundance. Therefore, there have been numerous efforts for developing of effective augmentation techniques for introduction of fungal inoculum into bark beetle populations (e.g. Kreutz et al. 2004, Grégoire & Evans 2007, Vakula et al. 2012, Barta et al. 2018b). Especially in protected forests, where the standard management practices are not allowed, an efficient method of *Beauveria* spores augmentation would be valuable (Barta et al. 2018b).

In the phylogenetic tree, all fungal species identified from the bark beetles were clustered with the type strains of corresponding species. The Slovak isolate of *M. bulbillosa* is clustered together with the type strain CBS 145.70 (Zare et al. 2001). *Beauveria caledonica* isolate from this survey is grouped into one strongly supported clade together with the type strain ARSEF 2567 (Rehner et al. 2011) as well as the strain MK206 (Barta et al. 2018a) also isolated from *I. typographus* in Slovakia. All *B. pseudobassiana* sequences are clustered together with the type strain ARSEF 3405 (Rehner et al. 2011). However, the type strain MTCC 8017 of *B. rudraprayagi* (Agrawal et al. 2014) is also placed in this clade. *Beauveria rudraprayagi* was originally described from a silkworm in India through a multi-gene phylogenetic analysis. It could not be distinguished from *B. pseudobassiana* (98% bootstrap support) by ITS-based analysis as mentioned in the original report (Agrawal et al. 2014), what is also documented by the phylogenetic tree of this study. *Beauveria bassiana* sequences of the Slovak isolates are clustered together, however, the clade comprises three distinct sub-clades. All the *B. bassiana* sub-clades were previously reported in isolates from soil or insect hosts in Europe (Meyling et al. 2009, 2012). The majority of *B. bassiana* isolates from this study are organised in the phylogenetic group A1. This group corresponds to the group Eu4 reported for isolates from soil or insect hosts in Denmark (Meyling et al. 2009, 2012) and interestingly, it was not detected from the soil during the extensive survey conducted in Slovakia in 2008 (Medo et al. 2016). Four *B. bassiana* isolates are placed in the group A2 that was previously identified as the group Eu6 by Meyling et al. (2009, 2012). Two *B. bassiana* isolates are placed in the phylogenetic group A3 that corresponds to the group Eu1 (Meyling et al. 2009, 2012). This phylogenetic group of *B. bassiana* was considered the most common in agricultural soil in recent studies (Medo et al. 2016, Meyling et al. 2009). Genetic variability of *B. bassiana* isolates obtained from bark beetles was lower than the variability of isolates obtained from soils in Denmark and Slovakia (Medo et al. 2016, Meyling et al. 2009). The ITS region, which was used in this study, is considered a universal barcode for fungi and is widely used in taxonomic analysis, however the application of multiple gene regions can significantly enhance the taxonomic resolution at inter-species level (Rehner et al. 2011). For example, eight genetic lineages comprising 15 haplotypes were detected in soil samples from Slovakia, when two DNA loci (ITS and Bloc regions) were analysed (Medo et al. 2016, 2021).

Conclusions

The survey on the occurrence of entomopathogenic fungi in populations of bark beetles in the Tatra National Park revealed activity of four fungal species, *B. bassiana*, *B. pseudobassiana*, *B. caledonica* and *M. bulbillosa*. *Beauveria bassiana* was the dominant pathogen infecting three bark beetle species, in particular *I. typographus*, *I. amitinus* and *P. chalcographus*. Fungal infection in populations of *I. amitinus* and *P. chalcographus* is reported for the first time in Slovakia. *Metapochonia bulbillosa*, the fungus originally isolated from the tissue of spruce roots or reported as an occasional parasite of cyst nematodes, was detected from bark beetles for the first time. Phylogenetic analysis identified three genetic lineages in population of *B. bassiana* parasitising bark beetles in the Tatra National Park, but the majority of analysed isolates were placed in a single phylogenetic group. The low natural prevalence of infected individuals during the population outbreaks indicates that *Beauveria* infection does not have sufficient capacity to regulate bark beetle abundances. Therefore, from the practical perspective, a searching for effective augmentation techniques how to release *Beauveria* inoculum into bark beetle populations is necessary.

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