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# Entomopathogenic fungi of the genus *Beauveria* and their pathogenicity to *Ips typographus* (Coleoptera: Curculionidae) in the Vitosha National Park, Bulgaria

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**Abstract:** *Ips typographus* is a serious pest for forestry in Eurasia. Effective control is difficult due to its cryptic habits and insect pathogenic microorganisms, including entomopathogenic fungi that are believed to be a promising alternative to the traditional control measures of this pest. In 2018, diversity of entomopathogenic fungi of the genus *Beauveria* was studied in populations of *I. typographus* in the Vitosha National Park, Bulgaria. Two species, *B. bassiana* and *B. caledonica*, were identified and 33 *in vitro* strains were obtained. Phylogenetic positions of the strains were evaluated according to phylogenetic inferences based on ITS and TEF-1α. Pathogenicity of the strains against bark beetles was tested in laboratory. All strains were pathogenic, although there was some variability in the efficacy of *B. bassiana* strains. Virulence of the five most pathogenic strains (four *B. bassiana* strains and one *B. caledonica* strain) was compared with the commercial mycoinsecticide Boverol® and highly-virulent *B. bassiana* strain ARSEF 12957 isolated from *I. typographus* in Slovakia. The strain from Boverol® was least virulent and the Slovak strain ARSEF 12957 was more efficient than the Bulgarian strains, but the difference was not significant. The laboratory experiments suggest that the Bulgarian strains have a potential for the control of bark beetle adults.

**Keywords:** *Beauveria*; bark beetles; natural fungal infection; spruce forests; virulence

The European spruce bark beetle, *Ips typographus* L. (Coleoptera: Curculionidae), is one of the most destructive biotic agents in mature spruce forests of the Palaearctic region (Grégoire, Evans 2004; Wermelinger 2004). In Europe, it is widely spread in the distribution range of its main host, Norway spruce, *Picea abies* (L.) H. Karst. From the ecological viewpoint the spruce bark beetle (SBB) is an integral component of Norway spruce ecosystems. It colonizes wind-felled, stressed and dying spruce trees and thus initiates a natural process of wood decomposition in forests (Wermelinger 2004). However, it is also capable of attacking healthy and vigorous trees in large numbers as soon as its population exceeds critical levels (Wermelinger 2004; Kausrud et al. 2012). Disturbances of spruce stands by abi-

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otic agents are typically the main causes of subsequent SBB outbreaks. Windstorm and snow damage events are regarded the key triggers for outbreaks of SBB populations (Stadelmann et al. 2014; Modlinger, Novotný 2015; Vakula et al. 2015). Extended periods of severe drought exert additional stresses on spruce trees, which reduces forest resistance to SBB attacks (Vakula et al. 2014; Netherer et al. 2015). At population outbreaks the destructive potential of SBB is huge. It was even demonstrated that the volume of trees destroyed by SBB was substantially higher than the volume of windthrows that had triggered the outbreak (Nikolov et al. 2014).

Norway spruce forests covering about 158 000 ha in Bulgaria are economically and ecologically the most important forests in the mountainous regions of the western part of the country (Panayotov et al. 2015). SBB is widely distributed in Bulgaria, but is rarely dangerous unless forest stands are affected by abiotic disturbances like windthrows (Panayotov et al. 2015). For example, a 62-ha windthrow in Bistrishko Branishte Biosphere Reserve in 2001 was followed by a SBB outbreak on a 200-ha area and 50 000 $m^3$ of trees were damaged during the next five years (Rossnev et al. 2005; Georgiev et al. 2006; Panayotov, Georgiev 2012; Panayotov et al. 2015). Initially, the outbreak affected mostly older forests at >100 years of age, but later also younger trees were attacked (Panayotov, Georgiev 2012). By the end of the outbreak, 54% of all Norway spruce forests older than 120 years in the reserve were affected (Panayotov et al. 2015).

Generally, the control of SBB is difficult because of its cryptic habits. In a forestry practice, effective control is usually based on a complex of phytosanitary measures, pheromone trappings and insecticide treatments (Wermelinger 2004). While the pheromone trappings are principally applied for the population density monitoring (Galko et al. 2014), the phytosanitary measures are effective only until the populations reach critical levels (Økland et al. 2016). Insecticides are typically used to protect stored timber, but chemical treatments of log traps are also possible. However, a perfect timing is required and treatments cannot be used in all circumstances (Wermelinger 2004). A specific issue of the SBB control arises in nature forest reserves where standard control measures are limited and insecticide treatments are prohibited. In such areas, effective and ecologically friendly methods of SBB population management are required.

As alternatives to chemical control, insect pathogenic microorganisms can serve as a promising approach to insect pest control (Vega, Kaya 2012; Lacey et al. 2015). Among these microbial agents, entomopathogenic fungi of the genus *Beauveria* (Ascomycota: Hypocreales) are well-known by their potency to kill and regulate insect populations (Zimmerman 2007). *Beauveria* spp. are common insect-pathogenic fungi contributing to natural regulation of insect populations. Generally, *Beauveria* infections are associated with SBB populations at relatively low, but constant, prevalence levels (Barta et al. 2018a). In Europe, several reports have focused on the presence of entomopathogenic fungi in SBB outbreaks and *Beauveria* infections have been reported from larvae and adults (e.g. Landa et al. 2001; Wegensteiner 2007; Draganova et al. 2010, 2017; Takov et al. 2012; Mudrončeková et al. 2013; Wegensteiner et al. 2015a, 2015b; Barta et al. 2018a). In Bulgaria, information on the natural occurrence of these fungi in SBB populations is limited to several notes (Takov et al. 2006, 2007, 2011, 2012; Draganova et al. 2010, 2017) and three species *B. bassiana*, *B. caledonica* Bissett et Widden, and *B. brongniartii* (Sacc.) Petch have been reported. Recently, the genus *Beauveria* was revised and new species were classified using multigene sequence analyses. *B. bassiana s. l.* and *B. brongniartii s. l.* were divided into several cryptic morphologically indistinguishable species (Rehner, Buckley 2005; Rehner et al. 2011). Following the novel molecular approach in *Beauveria* taxonomy several new species were added to the genus during the past decade (e.g. Rehner et al. 2011; Chen et al. 2013; 2017, 2018; Robène-Soustrade et al. 2015; Bustamante et al. 2019; Khonsanit et al. 2020).

There have been developed over 170 microbial preparations by over 80 companies for the pest control during the last 60 years and a great part of the formulations (> 35%) have been based on *Beauveria* spp. (de Faria, Wraight 2007). Although the use of *Beauveria* strains is environmentally safe (Zimmerman 2007) and has already been tested for the biological control of SBB in several studies (e.g. Vaupel, Zimmermann 1996; Kreutz et al. 2004a, 2004b; Landa et al. 2001; Draganova et al. 2010, 2017; Mudrončeková et al. 2013; Barta et al. 2018b), these fungi have not yet been commercially used in forestry. Recent research on testing highly virulent *Beauveria bassiana* (Bals.-Criv.) Vuill. strains, originally isolated from naturally infected SBB in

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Slovakia (Barta et al. 2018a), produced optimistic results in laboratory (Barta et al. 2018b) and a vertical transmission of infection in the population of bark beetles was observed after an augmentative release of the inoculum (Vakula et al. 2019). Based on the latest research, these fungi are believed to have a potential to be successfully implemented into an integrated system managing the problem of bark beetle outbreaks.

In the present study, we aimed to identify the diversity of *Beauveria* species in natural populations of SBB that were active in Norway spruce stands in the Vitosha National Park. Identification was made by a sequencing study of the internal transcribed spacer region of ribosomal DNA and a partial sequence of the translation elongation factor gene of obtained samples. The antagonistic potential of obtained local *Beauveria* strains against adult SBB was also evaluated in laboratory.

## MATERIAL AND METHODS

**Study area.** The collecting of samples was carried out in the Vitosha National Park (42°34'N; 23°17'E), in a mountainous region of 27 079 ha in size in west-central Bulgaria (Pernik province). Vitosha is one of the first nature parks in Europe. Two biosphere reserves are located within its boundaries – Bistrishko branishte and Torfeno branishte. The Bistrishko Branishte Biosphere Reserve was designated in 1934 in order to protect natural Norway spruce stands in the county. The Vitosha region is characterized by cold continental mountain climate. Average annual temperatures range from 4 °C at a middle mountain zone (1 100–1 200 m a.s.l.) to 18 °C at a low mountain zone (700–1 100 m a.s.l.). The annual course of air temperature is minimum in January and maximum in July. The average length of winter, a period with stable average daily temperatures below 0 °C, lasts 35–50 days at altitudes of about 600–700 m and 70–120 days at altitudes of 1 000–1 600 m. The vegetation period, a period with average daily temperatures above 5 °C, is 240 days at 600 m a.s.l. and 210 days at 1 200 to 1 400 m a.s.l. Annual precipitation ranges from 600–700 mm (at a low mountain zone) to 1 000 mm (at a middle mountain zone).

**Specimen collection and fungus isolation.** Fungus-infected bark beetles were collected in the study area in 2018. Cadavers displaying macroscopic symptoms of mycosis were collected from naturally infested spruce trees after removing the bark and placed individually in sterile 1.5-ml plastic microtubes. The specimens were examined under a dissecting microscope (50×) for confirming mycosis and excluding individuals that died from other factors than entomopathogens. Bark beetle species were identified on the basis of external morphology of adults and architecture of gallery systems carved under the bark (Cognato 2015). Samples with confirmed mycosis were stored at 4 °C until being further processed for the isolation of *in vitro* cultures. Conidia developing externally on cadavers were transplanted onto plates of Sabouraud dextrose agar (SDA) (Sigma-Aldrich®, Saint Louis, USA) supplemented with fungicides (250 mg·L$^{-1}$ cycloheximide and 500 mg·L$^{-1}$ dodine) and antibiotics (600 mg·L$^{-1}$ streptomycin sulphate, 50 mg·L$^{-1}$ tetracycline hydrochloride). Colonies of isolated fungi developing on the agar plates were incubated at 25 ± 1 °C for 14 days and transferred onto fresh SDA media without fungicides and antibiotics. The purified fungal strains were maintained at 25 ± 1 °C or transferred to SDA slants and stored at 4 °C. All obtained strains were deposited in the fungal collection of the Institute of Forest Ecology of the Slovak Academy of Sciences (Nitra, Slovakia).

**Identification of entomopa'nic fungi.** *In vitro* cultures were identified microscopically (500×) according to the morphology of microstructures (Rehner, Buckley 2005; Rehner et al. 2011; Humber 2012; Khonsanit et al. 2020). The morphological identification was complemented by a sequencing study of the internal transcribed spacer (ITS) region of ribosomal DNA (ITS1-5.8S-ITS2) and a partial sequence of the translation elongation factor (TEF1-α) gene. DNA was extracted from fungal biomass (mycelium with conidia) scraped off axenic cultures (cultivated at 25 ± 1 °C for 10 days) using EZ-10 Spin Column Fungal Genomic DNA Kit (Bio Basic Canada Inc., Ontario, Canada) according to the manufacturer's protocol. DNA was suspended in 50 µL of elution buffer and stored at –20 °C. The ITS region of ribosomal DNA was amplified with a universal primer pair ITS1-F/ITS4 (Gardens and Bruns 1993). The TEF-1α gene was amplified using primers EF-983F and EF-2218R (Rehner, Buckley 2005). The reaction PCR mixtures (20 µL) contained 5× Hot FirePol® Blend Master Mix (Solis BioDyne OÜ, Tartu, Estonia), 0.2 µM of each primer, deionized water of molecular grade (Aqua pro injectione Braun, Germany) and 50 ng of tem-

https://doi.org/10.17221/123/2020-JFS

plate DNA. The PCRs were carried out in a Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories Inc., CA, USA). PCR amplifications of the ITS region were performed under the following conditions: an initial denaturation step at 95 °C for 14 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s, elongation at 72 °C for 80 s, and a final elongation step at 72 °C for 10 min. For TEF-1α, PCR conditions were as follows: an initial step of 20 s at 95 °C; 10 cycles of 30 s at 94 °C, 55 s at 66–57 °C (decreasing 1°C per cycle), 90 s at 72 °C; plus 35 cycles of 30 s at 94 °C, 30 s at 56 °C, 60 s at 72 °C; followed by 10 min at 72 °C. The resulting PCR products were separated by electrophoresis at 80 V on a 1% agarose gel using SimplySafe™ stain (EURx Ltd., Gdańsk, Poland) and visualized under UV light. To detect the length of the amplified fragments, a 100-bp DNA ladder (Solis, BioDyne, Tartu, Estonia) was used. The target PCR fragments were purified by QIAquick PCR Purification Kit (Qiagen n.v., Venlo, The Netherlands) and sequenced by the Sanger method using Macrogen sequencing service (Macrogen Europe B.V., Amsterdam, The Netherlands) and acquired sequences were processed using SnapGene® Viewer 5.0.7 (GSL Biotech LLC, CA, USA). The sequences were compared by BLAST (Altschul et al. 1997) against DNA sequences deposited in the NCBI GenBank Sequence Database.

**Phylogenetic analysis.** All sequences from *in vitro* cultures (Table 1) were used in the phylogenetic analysis. The sequences were trimmed and edited manually as necessary using Molecular Evolutionary Genetics Analysis software MEGA X, version 10.0.5 (Kumar et al. 2018). Representative sequences of 24 recognized *Beauveria* species (ex-type strains) (Rehner, Buckley 2005; Rehner et al. 2006, 2011; Sung et al. 2007; Zhang et al. 2012; Chen et al. 2013, 2018, 2019; Agrawal et al. 2014; Sanjuan et al. 2014; Ariyawansa et al. 2015; Robène-Soustrade et al. 2015) and sequences of *Metarhizium anisopliae* strain ARSEF 7487 (as an outgroup) were retrieved from GenBank (Table 2) and included in the phylogenetic analysis. Sequences from this study as well as the reference sequences were aligned using MUSCLE (Edgar 2004) in the default settings by MEGA X. Phylogenetic analysis was done on concatenated ITS (58 sequences of 523 bp) and TEF-1α (58 sequences of 874 bp) loci datasets by the Maximum Likelihood (ML) method with the Tamura-Nei substitution model (Tamura, Nei 1993), a N/BioNJ starting tree, and followed by 1 000 bootstrap replications. Alignment gaps were treated as missing data.

**Virulence bioassay.** All *Beauveria in vitro* strains were screened for pathogenicity against SBB adults under standardized laboratory conditions. A stock suspension of conidia was prepared by suspending the fungal biomass of axenic cultures (ca. 2 g) in 250 ml of 0.01% (w/v) Tween®80 inside a 500-ml reagent bottle. The suspension was thoroughly mixed by hand for ca. 60 s and mycelial debris was removed by filtration through a sterile 10-μm nylon membrane (Spectra Mesh®, Spectrum Laboratories, Inc., USA). Conidial concentration in the stock suspension was determined using Neubauer haemocytometer and adjusted to the required concentration by diluting in 0.01% Tween®80. Viability of conidia in the suspensions was determined prior to each bioassay by germination tests on agar plates at 25 °C. Conidial viability of all axenic cultures was > 90% ( = 94.11 ± 2.28%) measured after 12-h incubation. SBB adults for pathogenicity tests were obtained from a laboratory colony reared in Norway spruce logs at 25 ± 2 °C, 60 ± 10% relative humidity, and 12/12 h (L/D) photoperiod. Only fully mature adults (having the dark brown exoskeleton coloration) were used in the assays. SBB adults were inoculated by their submersion in a conidia suspension at a concentration of $1 \times 10^6$ conidia·ml$^{-1}$. For each strain, 50 individuals were immersed together in the suspension for 5 s. Additional 50 individuals were treated with sterile 0.01% Tween®80 as controls. The groups of treated and control beetles were incubated in Petri dishes (200 × 35 mm) lined with wet filter paper for a period of 14 days at 25 ± 2 °C, saturated humidity and 12/12 h (L/D) photoperiod. A piece of fresh spruce bark (100 × 100 mm) was provided as food to each Petri dish. The pieces of bark were disinfected by ultraviolet germicidal (UV-C) irradiation for 30 min under aseptic conditions of a laminar flow cabinet before their use as food. Mortality of beetles in Petri dishes was monitored at 24-h intervals and cadavers were removed from the dish to prevent a horizontal transmission of conidia in the test groups. The cadavers were incubated separately in sterile Petri dishes (60 × 15 mm) on a piece of wet filter paper for 5 days and mortality caused by the fungi was confirmed by microscopic examination. Only cadavers with confirmed *Beauveria* mycosis were used to estimate pathogenicity. The bioassay was repeated four times under the same conditions.

https://doi.org/10.17221/123/2020-JFS

Table 1. List of *Beauveria* spp. strains isolated from naturally infected *Ips typographus* adults in Vitosha Mountain (Bulgaria) in 2018

| No. | Fungal species | Strain code | GenBank accession no.[1] | | Pathogenicity[2] mean mortality ± SE (%) |
|---|---|---|---|---|---|
| | | | ITS | TEF-1α | |
| 1 | *B. bassiana* | BG19 | MT180394 | MT215036 | 35.17 ± 6.45$^{abc}$ |
| 2 | *B. bassiana* | BG20 | MT180395 | MT215037 | 30.58 ± 5.19$^{abc}$ |
| 3 | *B. bassiana* | BG21 | MT180396 | MT215038 | 25.03 ± 6.50$^{abc}$ |
| 4 | *B. bassiana* | BG22 | MT180397 | MT215039 | 24.61 ± 7.43$^{abc}$ |
| 5 | *B. bassiana* | BG23 | MT180398 | MT215040 | 44.27 ± 6.10$^{abc}$ |
| 6 | *B. bassiana* | BG24 | MT180399 | MT215041 | 25.94 ± 6.51$^{abc}$ |
| 7 | *B. bassiana* | BG25 | MT180400 | MT215042 | 44.41 ± 5.14$^{abc}$ |
| 8 | *B. bassiana* | BG27 | MT180401 | MT215043 | 43.97 ± 8.26$^{abc}$ |
| 9 | *B. bassiana* | BG28 | MT180402 | MT215044 | 44.82 ± 9.09$^{abc}$ |
| 10 | *B. bassiana* | BG29 | MT180403 | MT215045 | 30.16 ± 6.40$^{abc}$ |
| 11 | *B. bassiana* | BG30 | MT180404 | MT215046 | 20.98 ± 6.09$^{abc}$ |
| 12 | *B. bassiana* | BG31 | MT180405 | MT215047 | 12.18 ± 3.80$^{c}$ |
| 13 | *B. bassiana* | BG32 | MT180406 | MT215048 | 43.75 ± 9.11$^{abc}$ |
| 14 | *B. bassiana* | BG33 | MT180407 | MT215049 | 57.05 ± 10.46$^{a}$ |
| 15 | *B. bassiana* | BG34 | MT180408 | MT215050 | 20.31 ± 5.96$^{abc}$ |
| 16 | *B. bassiana* | BG35 | MT180409 | MT215051 | 55.63 ± 9.33$^{a}$ |
| 17 | *B. bassiana* | BG36 | MT180410 | MT215052 | 55.18 ± 9.22$^{a}$ |
| 18 | *B. bassiana* | BG37 | MT180411 | MT215053 | 33.73 ± 7.88$^{abc}$ |
| 19 | *B. bassiana* | BG38 | MT180412 | MT215054 | 13.30 ± 3.40$^{bc}$ |
| 20 | *B. bassiana* | BG39 | MT180413 | MT215055 | 19.47 ± 5.69$^{abc}$ |
| 21 | *B. bassiana* | BG40 | MT180414 | MT215056 | 11.24 ± 4.61$^{c}$ |
| 22 | *B. bassiana* | BG41 | MT180415 | MT215057 | 43.88 ± 9.44$^{abc}$ |
| 23 | *B. bassiana* | BG42 | MT180416 | MT215058 | 35.09 ± 10.34$^{abc}$ |
| 24 | *B. bassiana* | BG43 | MT180417 | MT215059 | 34.27 ± 8.22$^{abc}$ |
| 25 | *B. bassiana* | BG44 | MT180418 | MT215060 | 56.27 ± 6.05$^{a}$ |
| 26 | *B. bassiana* | BG45 | MT180419 | MT215061 | 41.89 ± 9.71$^{abc}$ |
| 27 | *B. bassiana* | BG46 | MT180420 | MT215062 | 42.15 ± 10.94$^{abc}$ |
| 28 | *B. bassiana* | BG48 | MT180421 | MT215063 | 42.76 ± 8.08$^{abc}$ |
| 29 | *B. bassiana* | BG49 | MT180422 | MT215064 | 19.97 ± 7.08$^{abc}$ |
| 30 | *B. bassiana* | BG50 | MT180423 | MT215065 | 15.67 ± 6.87$^{bc}$ |
| 31 | *B. bassiana* | BG51 | MT180424 | MT215066 | 7.70 ± 2.16$^{c}$ |
| 32 | *B. caledonica* | BG26 | MT180426 | MT215067 | 55.18 ± 10.16$^{a}$ |
| 33 | *B. caledonica* | BG47 | MT180427 | MT215068 | 41.91 ± 8.61$^{abc}$ |

ITS – internal transcribed spacer, TEF1-α – translation elongation factor, [1]GenBank accession numbers of DNA sequences for ITS and TEF-1α regions submitted to GenBank database, [2]mean mortality data (%) of adult *Ips typographus* from the pathogenicity tests, SE – standard error of the mean, means followed by the same letter are not significantly different (Tukey's HSD test, $P = 0.05$), mean mortality in the controls reached 2.0 ± 0.82%

Five fungal strains showing the highest pathogenicity to SBB were selected for a virulence assay. Two additional strains of *B. bassiana*, one isolated from the commercial mycoinsecticide Boverol® (Fytovita s.r.o., Ostrožská Lhota, Czech Republic) and the ARSEF 12957 strain obtained from previous studies (Barta et al. 2008a, b), were also included in the bioassay as reference strains. The strain ARSEF 12957 was isolated from a naturally infected SBB in Slovakia and has recently been selected as a promising bioagent against SBB (SK Patent No. 5046-2015, Barta et al. 2018c). Boverol® was selected

https://doi.org/10.17221/123/2020-JFS

Table 2. List of *Beauveria* spp. and *Metarhizium anisopliae* sequences from GenBank used in the phylogenetic analysis

| Species | Strain code | Locality | Host/Substrate | GenBank Accession no. | | References |
|---|---|---|---|---|---|---|
| | | | | ITS | TEF-1α | |
| *B. amorpha* | ARSEF 7542 | USA, Colorado | Hymenoptera: Formicidae | HQ880805 | HQ881007 | Rehner et al. (2011) |
| *B. asiatica* | ARSEF 4850 | South Korea, Chiag Mt. | Coleoptera: Cerambycidae | HQ880787 | AY531937 | Rehner et al. (2011) |
| *B. australis* | ARSEF 4598 | Australia, Tasmania | Soil | HQ880789 | HQ880995 | Rehner et al. (2011) |
| *B. baoshanensis* | BUB283 | China, Gaoligong Mt. | Lepidoptera: Lymantridae | MG642828 | MG642898 | Chen et al. (2019) |
| *B. bassiana* | ARSEF 1564 | Italy, Villa Cade | Lepidoptera: Hyphantria cunea | HQ880761 | HQ880974 | Rehner et al. (2011) |
| *B. brongniartii* | Je276 | Switzerland | Coleoptera: Scarabaeidae | HQ880784 | HQ880993 | Rehner et al. (2011) |
| *B. caledonica* | ARSEF 2567 | Scotland | Soil | HQ880817 | EF469057 | Rehner et al. (2011), Sung et al. (2007) |
| *B. hoplocheli* | Bt96 | Madagascar | Coleoptera: Melolonthidae | KC339697 | KC339709 | Robène-Soustrade et al. (2015) |
| *B. kipukae* | ARSEF 7032 | USA, Hawaii | Homoptera: Delphacidae | HQ880803 | HQ881005 | Rehner et al. (2011) |
| *B. lii* | RCEF5500 | China, Xianyang | Coleoptera: Coccinellidae | JN689372 | JN689371 | Zhang et al. (2012) |
| *B. majiangensis* | GZU12141 | China, Guizhou: Majiang | Coleoptera: Scarabaeoidea | MG052643 | MG052640 | Chen et al. (2018) |
| *B. malawiensis* | IMI 228343 | Malawi, Zomba | Coleoptera: Cerambycidae | DQ376247 | DQ376246 | Rehner et al. (2006) |
| *B. medogensis* | BUB426 | China, Gaoligong Mt. | Hymenoptera: Formicidae | MG642832 | MG642904 | Chen et al. (2019) |
| *B. pseudobassiana* | ARSEF 3405 | USA, Virginia | Lepidoptera: Erebidae | HQ880792 | AY531931 | Rehner et al. (2011) |
| *B. rudraprayagi* | MTCC 8017 | India, Rudraprayag | Lepidoptera: Bombycidae | JQ266173 | JQ990914 | Agrawal et al. (2014) |
| *B. scarabaeidicola* | ARSEF 7281 | South Korea: Guryungryung | Coleoptera: Scarabaeidae | HQ880815 | HQ881011 | Rehner et al. (2011) |
| *B. sinensis* | RCEF3903 | China, Anhui | Lepidoptera: Geometridae | HQ270152 | HQ270151 | Chen et al. (2013) |
| *B. varroae* | ARSEF 8257 | France, Montdardier | Acari: Varroidae | HQ880800 | HQ881002 | Rehner et al. (2011) |
| *B. vermiconia* | ARSEF 2922 | Chile, Valdivia | Soil | HQ880822 | AY531920 | Rehner et al. (2011) |
| *B. acridophila* | AV1875 | Colombia, Zaphire Reserve | Orthoptera: Proscopiidae | JQ958602 | JQ958616 | Sanjuan et al. (2014) |
| *B. diapheromeriphila* | MV2492 | Ecuador, Jatun-sacha Reserve | Phasmatodea: Diapheromeridae | JQ958603 | JQ958611 | Sanjuan et al. (2014) |
| *B. gryllotalpidicola* | BCC26300 | Thailand, Nakhon Ratchasima | Orthoptera | FJ459787 | FJ459795 | Ariyawansa et al. (2015) |
| *B. locustiphila* | TS881 | Colombia, Tolima | Orthoptera: Romaleidae | JQ958606 | JQ958619 | Sanjuan et al. (2014) |
| *B. loeiensis* | BCC23104 | Thailand, Loei | Orthoptera | FJ459784 | FJ459792 | Ariyawansa et al. (2015) |
| *M. anisopliae* | ARSEF 7487 | Eritrea | Orthoptera: Schistocerca gregaria | HQ331446 | DQ463996 | Schneider et al. (2011) |

ITS – internal transcribed spacer, TEF1-α – translation elongation factor

https://doi.org/10.17221/123/2020-JFS

as a reference strain in this study because it was tested against SBB in several previous studies (e.g. Wegensteiner 1996; Kreutz et al. 2004a, 2004b; Jakuš, Blaženec 2011; Grodzki, Kosibowicz 2015; Barta et al. 2018a, 2018b) and also because it was originally isolated from a coleopteran host (the family Chrysomelidae). Virulence of the tested strains was estimated from cumulative mortality data of SBB treated with four different conidial concentrations ranging from $1 \times 10^5$ to $1 \times 10^8$ conidia·ml$^{-1}$. Groups of 50 adults were treated by their submersion in the suspensions for 5 s and 50 adults were treated with sterile 0.01% Tween®80 as controls. The treated and control beetles were incubated, monitored, and handled as mentioned above. The bioassay was repeated three times under the same conditions.

**Data analysis.** Mortality data from pathogenicity tests were corrected for natural (control) mortality using Abbott's formula and subjected to ANOVA. Prior to ANOVA, the mortality data were tested for normality (Shapiro-Wilk test) and then arcsine transformed ($n'$ = arcsin $\sqrt{n}$) to obtain a normally distributed data set. Post-hoc Tukey's HSD test was performed to separate and compare means if significant differences ($P = 0.05$) were detected. Cumulative percentage mortality data of SBB from the virulence tests of the five most pathogenic strains and the two reference strains were subjected to probit analysis (Finney 1971) and median lethal concentrations ($LC_{50}$) with associated 95% confidence intervals were calculated. Values of median lethal concentrations were subjected to ANOVA and post-hoc Tukey's HSD test. All the analyses were conducted using Minitab 17® (© 2013 Minitab Inc.).

## RESULTS

### Entomopathogenic fungi from collected samples

During the survey of entomopathogens of SBB in Vitosha Mountain 89 dead individuals displaying symptoms of fungal infection were collected. Based on a microscopic observation, 60 cadavers were suspected to be killed by *Beauveria* mycosis and were used for the isolation of *in vitro* cultures. Altogether, 33 *in vitro* strains were obtained (Table 1) and two *Beauveria* species were identified by sequencing of ITS and TEF-1α regions. The sequences of obtained DNA fragments were compared with data from the GenBank and the Bulgarian strains were characterized as *B. bassiana* (31 strains) or *B. caledonica* (two strains) supported by a high degree (approx. 99%) of identity with sequences of the neotype *B. bassiana* strain (ARSEF 1564) or the type strain of *B. caledonica* (ARSEF 2567), respectively. All obtained ITS and TEF-1α sequences were deposited in the NCBI GenBank and accession numbers are given in Table 1. The ITS sequences of all Bulgarian *B. bassiana* strains differed from the neotype *B. bassiana* strain at 5 positions. The differences were detected at positions 54 (C instead of T), 148 (T instead of C), 183 (gap instead of A), 253 (C instead of T) and 369 (C instead of T). An additional difference at position 179 (G instead A) was identified for the strain BG23. The *B. bassiana* TEF-1α sequences of all Bulgarian strains differed from the neotype strain at positions 121 (T vs. C), 151, 349, 400, 664 and 772 (C vs. T at all positions) and at position 358 (A vs. C, only for BG19 strain). The ITS sequences of *B. caledonica* strains (BG26 and BG47) were identical with the sequence of the type *B. caledonica* strain. On the other hand, differences at positions 918 and 959 (C vs. T at both positions) for both *B. caledonica* strains and one more difference for the strain BG46 at position 766 (gap vs. G) were observed when compared with TEF-1α sequence of the type strain.

### Phylogenetic analysis

The amplicons of ITS and TEF-1α regions ranged from 464 to 546 bp and from 891 to 952 bp, respectively. The concatenated two-locus dataset included 58 sequences: 33 Bulgarian strains (Table 1), sequences of 24 recognized *Beauveria* species (Table 2) and *Metarhizium anisopliae* strain ARSEF 7487 as an outgroup. The final dataset consisted of 77 453 bp of sequence data and ML analysis produced a consensus tree (Figure 1) with the log-likelihood of –3 356.39. The Bulgarian strains are clustered in two independent groups. All strains identified as *B. bassiana* are clustered together (82% bootstrap support) with the neotype ARSEF 1564 strain of *B. bassiana*. However, it should be noted that the *B. bassiana* clade comprises two well-supported (ML bootstrap = 98%) subclades and all Bulgarian strains were placed in an independent sub-group. Two strains identified as *B. caledonica* are grouped together in one strongly supported (ML bootstrap = 99%) clade with the type strain of *B. caledonica* (ARSEF 2567).

### Virulence bioassay

Under laboratory conditions, all *Beauveria* strains were pathogenic to SBB adults and were able

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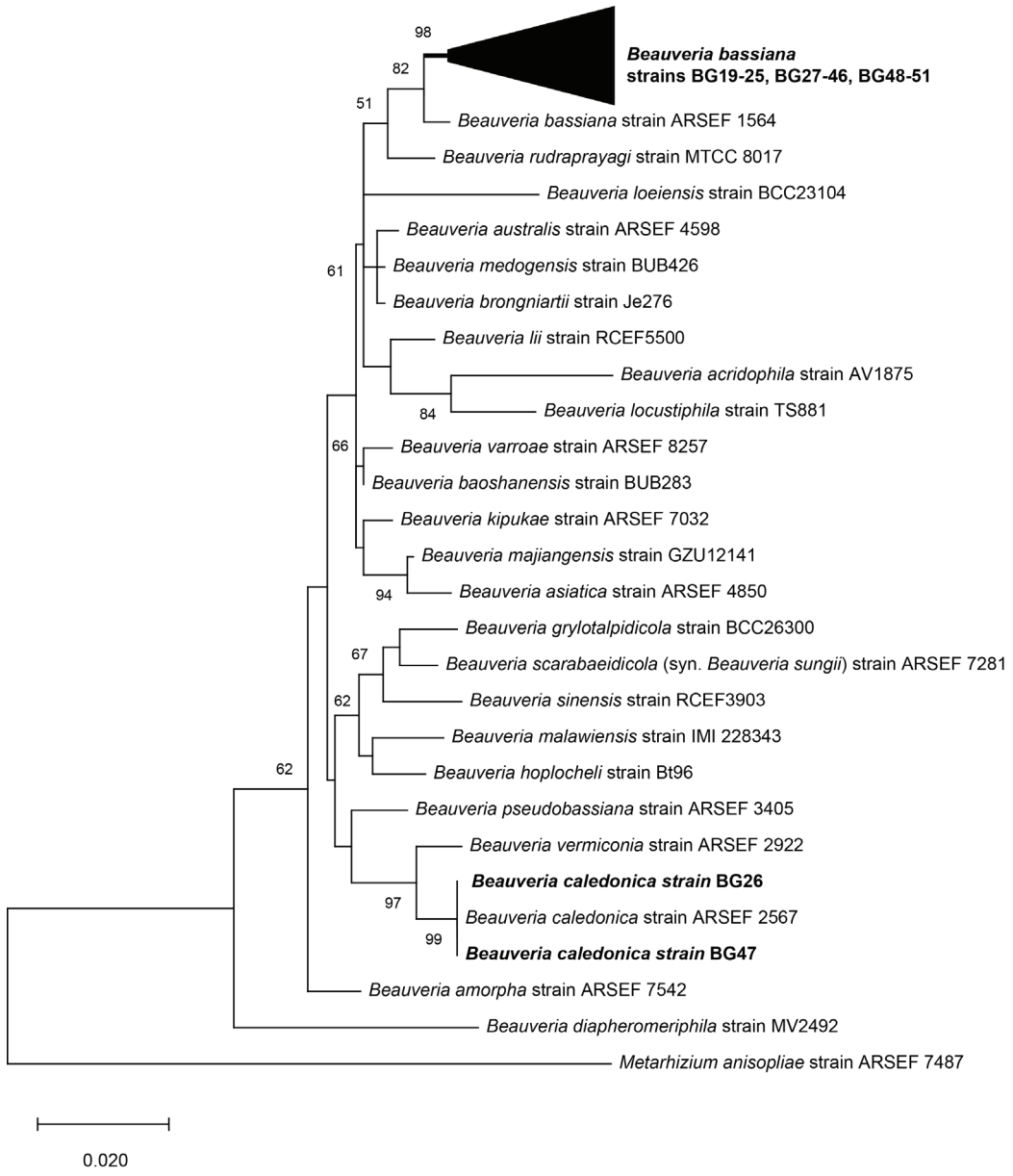


Figure 1. Phylogenetic tree showing *Beauveria* species relationships inferred by using the maximum likelihood method and Tamura-Nei model (Tamura, Nei 1993), tree with the highest log-likelihood (–3 356.39) is shown, percentage of trees (> 50%) in which the associated taxa clustered together is shown next to the branches, *Metarhizium anisopliae* was used as outgroup, strains collected and sequenced in this study are shown in bold type

to reduce the viability of bark beetles compared to the control. Pathogenicity of all *Beauveria* strains represented by mean mortality data is shown in Table 1. The mortality rate demonstrates significant ($F_{(32,99)}$ = 3.67, $P$ < 0.01) variability among the strains and three groupings of strains could be distinguished when assessing the mean cumulative mortality: a group of least virulent strains (< 20% cumulative mortality, seven strains), a group of intermediate virulence (21–49% cumulative mortality, 21 strains), and highly-virulent strains (> 50% cumulative mortality, five strains). Tukey's HSD test ($P$ = 0.05) identified three homogeneous groups and the significantly highest mean cumulative mortality of SBB was detected for four *B. bassiana* strains BG33, BG35, BG36 and BG44 (57.05, 55.63, 55.18 and 56.27%, respectively) and one *B. caledonica* strain BG26 (55.18%). A general pattern of my-

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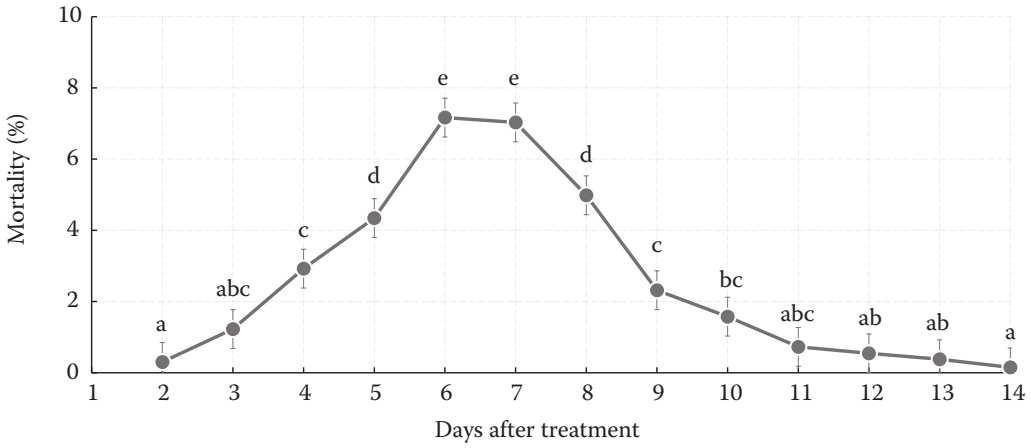


Figure 2. Mean daily mortality of *Ips typographus* adults calculated from pooled data of all tested *Beauveria* strains in laboratory pathogenicity tests

bars around points represent 95% intervals based on Tukey's honestly significant difference (HSD) procedure, points labelled with the same letter are not significantly different ($P = 0.05$)

cosis progress by the *Beauveria* strains in treated SBB groups is depicted by the mean daily mortality rate in Figure 2. The first death due to mycosis occurred 48 h after treatment in case of 11 strains. On day four, fungal infection was already observed in all tested strains. The most rapid increase in mortality rate was recorded between days five and six after treatment. The mean daily mortality for all strains rose significantly ($F_{(1,262)} = 30.94$, $P < 0.01$) from day five (4.34%) to day six (7.17%), followed by a moderate decrease on day seven (7.03%) and a distinct decrease over the next two days. The mean mortality dropped below 3% on day nine and only 0.15% mortality was recorded on day 14, when the bioassay was ceased. The mean daily mortality recorded during days 5–8 (5.88%) was significantly higher than that for days 1–4 (1.48%) or days 9–14 (0.95%) in the bioassay ($F_{(2,1713)} = 499.28$, $P < 0.01$). In order to assess the dynamics of pathogenesis in the test populations, we compared cumulative mortality among strains recorded on days five and ten after treatment (Figure 3). A great variability in these parameters was detected among strains ($F_{(32,99)} = 45.39$, $P < 0.01$ for day five and $F_{(32,99)} = 69.17$, $P < 0.01$ for day ten) and strains BG26, BG33, BG36, and BG44 demonstrated the highest efficacy. These strains brought about over 20% mortality yet five days post-inoculation and more than 50% mortality ten days after treatment. Out of all strains included in the pathogenicity tests, four *B. bassiana* strains (BG33, BG35, BG36, and BG44) and one *B. caledonica* strain (BG26) demonstrated the greatest biological activity against SBB. Mycosis development induced by these strains in tested bark beetle groups had a trend similar to the remaining strains, but the highest daily mortality occurred one day earlier, between day four (mean mortality was 7.20%) and day six, when mean daily mortality reached the maximum of 11.46%. Based on the above results, the five strains were selected for virulence bioassays.

In a series of virulence bioassays, the percentage mortality of SBB increased with the conidia concentration of selected *Beauveria* strains that allowed estimating a dose-response relationship by probit analysis. The mean values of $LC_{50}$ are shown in Table 3 and varied from 2.00 to 2.60 × $10^6$ conidia·$ml^{-1}$ with probit regression slopes of 0.33–0.37. The *B. bassiana* strain BG35 showed the greatest virulence, however mean values of median lethal concentrations among the strains were not significantly different ($P > 0.05$). The *B. bassiana* strain from the commercial mycoinsecticide Boverol® was least virulent against bark beetles and the Slovak strain ARSEF 12957 was more efficient than the Bulgarian strains, but the differences were not statistically significant ($P > 0.05$). However, the strain ARSEF 12957 was significantly ($P < 0.05$) more virulent than the Boverol® strain.

## DISCUSSION

Entomopathogenic fungi belong to important natural control agents of SBB in Europe (Wegen-

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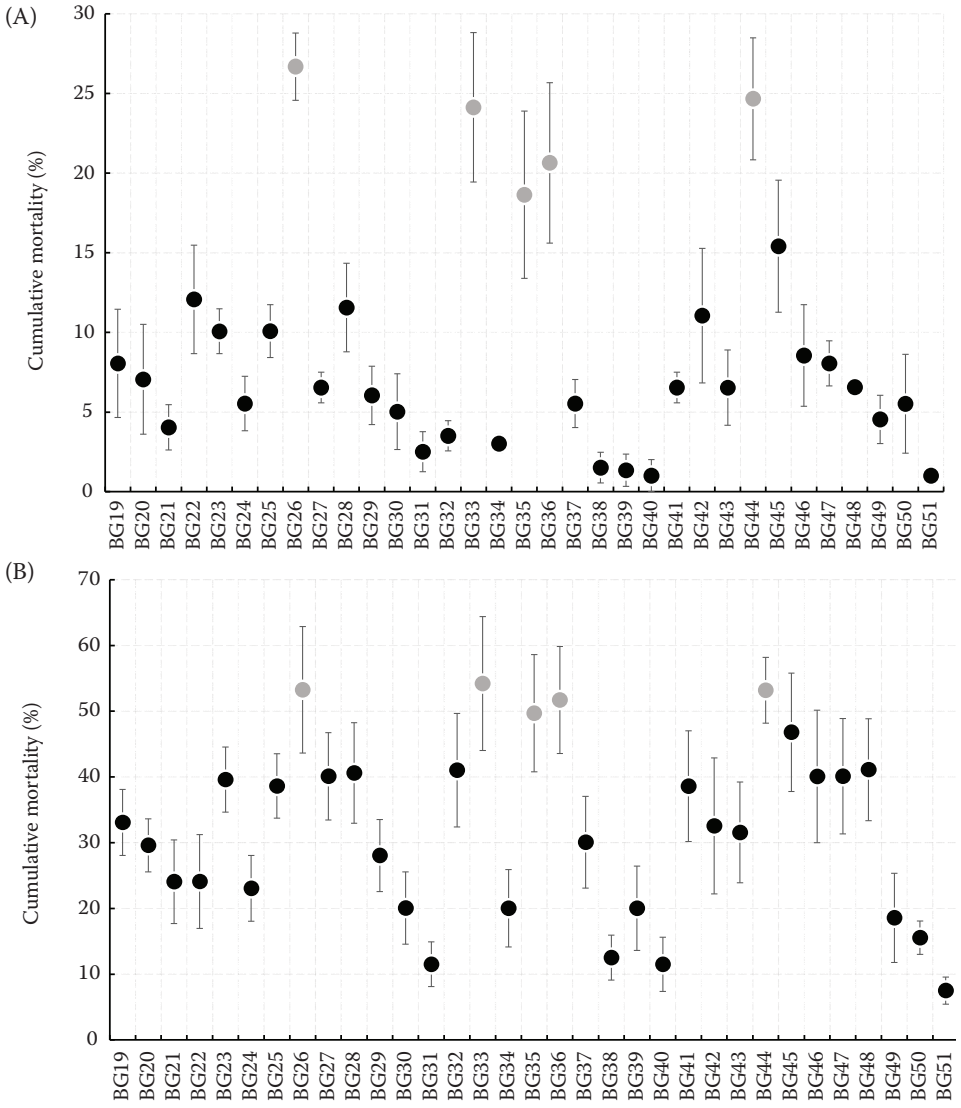


Figure 3. Mean cumulative mortality of *Ips typographus* adults recorded five (A) and ten (B) days after treatment with conidia suspensions ($1 \times 10^6$ conidia·ml$^{-1}$) of *Beauveria* strains during pathogenicity tests (error bars represent standard errors of the means, grey points show mortalities of the five most pathogenic strains selected for virulence assay)

Table 3. Results of probit analyses testing the virulence of selected *Beauveria* strains against adults of *Ips typographus* after their treatment with conidia suspensions in laboratory bioassays

| Fungal strains | Probit analysis parameters | | | | |
|---|---|---|---|---|---|
| | $LC_{50}$ ($\times 10^6$)[1] | 95% fiducial CI ($\times 10^6$) | Slope[1] | $P$[2] | ($\chi^2$)[3] |
| BG26 | 2.45 ± 0.50$^{ab}$ | 1.59–3.57 | 0.35 ± 0.03 | < 0.01 | 2.29 |
| BG33 | 2.60 ± 0.55$^{ab}$ | 1.66–3.85 | 0.33 ± 0.03 | < 0.01 | 2.18 |
| BG35 | 2.00 ± 0.42$^{ab}$ | 1.27–2.93 | 0.34 ± 0.03 | < 0.01 | 2.54 |
| BG36 | 2.11 ± 0.43$^{ab}$ | 1.37–3.06 | 0.35 ± 0.03 | < 0.01 | 1.99 |
| BG44 | 2.22 ± 0.43$^{ab}$ | 1.47–3.17 | 0.37 ± 0.03 | < 0.01 | 3.17 |
| ARSEF 12957 | 0.33 ± 0.09$^{a}$ | 0.17–0.55 | 0.28 ± 0.02 | < 0.01 | 2.53 |
| Boverol® | 3.33 ± 0.72$^{b}$ | 2.33–5.17 | 0.34 ± 0.03 | < 0.01 | 2.99 |

[1]Mean values ± standard errors of the means; $LC_{50}$ – values of lethal concentrations; CI – 95%-fiducial confidence intervals are in conidia per millilitre of suspension; [2]$P$-value of a slope from regression analysis; [3]Pearson $\chi^2$ goodness-of-fit test on the probit model ($P$ = 0.05, $df$ = 2); ean mortality in the controls reached 2.67 ± 0.67%

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steiner 2007; Wegensteiner et al. 2015b). From the viewpoint of mass production and use in biocontrol, the most promising entomopathogens are those from the order Hypocreales (Ascomycota) (Lacey et al. 2015). The activity of hypocrealean entomopathogenic fungi was well documented in populations of bark beetles across Europe and species of the genus *Beauveria* were often listed among the common pathogens (e.g. Landa et al. 2001; Kreutz et al. 2004a; Wegensteiner 2007; Draganova et al. 2010; Takov et al. 2012; Mudrončeková et al. 2013; Wegensteiner et al. 2015a; Barta et al. 2018a). As demonstrated in this study, *Beauveria* infection is a natural mortality factor of SBB populations in Bulgaria. Although only a limited number of bark beetle samples in a relatively small geographic area was evaluated during the present survey, infected individuals could be observed at any sampling occasion. *Beauveria* infections of bark beetles were also reported at low prevalence levels in Bulgaria in previous studies (Georgiev et al. 2006; Takov et al. 2006, 2007, 2012; Draganova et al. 2010). The genus *Beauveria* is considered a cosmopolitan group of soil-borne arthropod pathogenic fungi with a broad host range including the insect orders 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). Up to now, 24 *Beauveria* species have been described and based on the published data there are 14 *Beauveria* species that parasitize coleopteran hosts (Rehner et al. 2011; Kepler et al. 2017; Chen et al. 2018; Bustamante et al. 2019; Khonsanit et al. 2020). Only five *Beauveria* species (*B. bassiana*, *B. brongniartii*, *B. caledonica*, *B. pseudobassiana* S.A. Rehner et R.A. Humber, and *B. varroae* S.A. Rehner et R.A. Humber) have been documented from Europe. While *B. bassiana*, *B. brongniartii* and *B. pseudobassiana* are globally distributed fungi with a broad host range occurring in a variety of habitats, *B. varroae* is originally known from ectoparasitic mites of honeybee in France, but can also infect coleopterans (Rehner et al. 2011) and lepidopteran pupae (Barta et al. 2020). *B. caledonica* is a species originally described from soil in Scotland (Bissett, Widden 1988), but it has also been reported outside of Europe (Glare et al. 2008). Out of the five *Beauveria* species present in Europe, four species, *B. bassiana*, *B. caledonica, B. pseudobassiana* and *B. brongniartii*, have already been recorded in natural populations of bark beetles. While the former two species were also identified from SBB during the present survey, we did not record *B. brongniartii* and *B. pseudobassiana* from any of collected cadavers. *B. brongniartii* is a well-known and globally distributed pathogen of soil-inhabiting coleopteran larvae (Zimmermann 2007). In two previous studies on SBB pathogens carried out in Bulgaria, *B. brongniartii* was reported from five individuals of bark beetles. However, the identification was based on the traditional morphotaxonomic study of collected material and morphological details of fungal structures were either not provided (Takov et al. 2012) or limited to only a conidial shape and size (Draganova et al. 2010). Since *B. bassiana* and *B. brongniartii* are morphologically very similar, all records made by a microscopic determination must be judged with caution. Generally, species identification in the genus *Beauveria*, especially in the anamorphic state, is difficult due to structural simplicity and extensive overlap in morphological characters, which entails a lack of distinctive diagnostic features. Therefore, the application of molecular methods in the fungal taxonomy is becoming a standard for the species determination. For example, the comprehensive taxonomic revision of the genus *Beauveria* performed by a molecular multigene phylogenetic analysis (Rehner, Buckley 2005; Rehner et al. 2011) revealed that the most commonly reported species, namely *B. bassiana* and *B. brongniartii,* encompass cryptic lineages and new *Beauveria* species were described. *B. pseudobassiana* was recorded in natural populations of SBB in Slovakia (Barta et al. 2018a), where it was considered a common entomopathogenic fungus in bark beetle populations. In a laboratory bioassay, this fungus even demonstrated high virulence against bark beetles and was reported as a promising biocontrol agent of these pests (Kocaçevik et al. 2016). *B. pseudobassiana* is known to prefer forests over agricultural or meadow habitats (Medo et al. 2016). Although this fungus was not observed during the present survey in the Vitosha National Park, its activity in SBB populations can be expected there as it has already been reported from south-east and central Bulgaria in 2018 and 2019. It was identified from mycosed pupae of *Thaumetopoea pityocampa* (Den. et Schiff.) (Lepidoptera: Notodontidae) (Barta et al. 2020) and caterpillars

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of *Euproctis chrysorrhoea* L. (Lepidoptera: Erebidae) (Barta, unpublished data). *B. bassiana* is the most commonly reported *Beauveria* from natural populations of SBB in Europe (e.g. Landa et al. 2001; Takov et al. 2007, 2012; Wegensteiner 2007; Wegensteiner et al. 2015a; Barta et al. 2018a). In Bulgaria, this fungus is associated with SBB populations at a relatively low but constant prevalence level (Takov et al. 2007, 2012). Although natural epizootics by this fungus do not occur in SBB populations, numerous laboratory experiments showed its high efficacy against SBB adults (e.g. Kreutz et al. 2004a, 2004b; Herrmann, Wegensteiner 2010; Mudrončeková et al. 2013; Kocaçevik et al. 2016; Barta et al. 2018a, 2018b). For instance, two superior *B. bassiana* strains were selected and patented as promising candidates for the biocontrol of SBB in Slovakia (Barta et al. 2018a, 2018b). *B. caledonica* was originally known only from the soil, but it was later observed from insect hosts, including bark beetles, in Europe (Kirschner 2001; Wegensteiner et al. 2015a; Draganova et al. 2017; Barta et al. 2018a) and New Zealand (Glare et al. 2008). While isolates of this species from New Zealand were highly pathogenic to adults of scolytid beetles (Glare et al. 2008), Slovak strains induced just low or medium mortality of SBB (Barta et al. 2018a). In this paper we report *B. caledonica* for the first time from SBB in Bulgaria. The first record of this fungus in Bulgaria was reported in 2013. It was identified by a microscopic examination of two individuals of *Hylastes cunicularius* Erichson (Coleoptera: Scolytinae) in the Vitosha National Park (Draganova et al. 2017).

The laboratory experiments in this study suggest that the Bulgarian *Beauveria* strains have a potential for the effective control of SBB adults, however the efficacy was strain-dependent. Significant variability in the efficacy of *Beauveria* strains against SBB was also demonstrated in other similar studies (e.g. Barta et al. 2018a, b). Based on the interspecific variability we can conclude that both species *B. bassiana* and *B. caledonica* can be pathogenic to SBB, however, mortality induced by *B. bassiana* strains was more variable. Although Barta et al. (2018a) demonstrated that *B. bassiana* induced higher mortality of SBB than did *B. caledonica,* the current results do not show that *B. caledonica* would be less pathogenic to SBB than *B. bassiana*. It seems that efficacy was rather strain-specific than species-dependent. The strain variability in the virulence of entomopathogenic fungi has been reported in many studies and it is generally admitted that strains tend to have higher virulence to their original host or species closely related to the original host (Wang et al. 2020). For example, *B. bassiana* strains isolated from SBB or related to coleopteran hosts were more effective against SBB than strains from lepidopteran hosts (Kreutz et al. 2004b). However, this was not the case in other studies (e.g. Draganova et al. 2007; Barta et al. 2018a). In pathogenicity tests we preselected a group of five highly pathogenic strains and compared their virulence with the commercial mycoinsecticide Boverol® and the recently patented highly virulent *B. bassiana* strain ARSEF 12957 (Barta et al. 2018c). The virulence tests confirmed the high capacity of selected strains as appropriate candidates for the biocontrol of SBB. The *B. bassiana* strain from the commercial mycoinsecticide Boverol® was least virulent against bark beetles and the Slovak strain ARSEF 12957 was more efficient than the Bulgarian strains, but the difference was not statistically significant.

Based on the results of this study, we conclude that *Beauveria* spp. are promising biocontrol agents for use in the management of SBB. Laboratory bioassays are carried out under optimal conditions for pathogenesis (humidity, temperatures etc.), which can be very different from environmental conditions in the fields. Therefore, the efficacy of strains demonstrated in laboratory might be different after their application in forests. For example, the promising efficacy of Boverol® against SBB in standardized laboratory conditions (Wegensteiner 1996; Kreutz et al. 2004a, b) was not demonstrated in outdoor conditions when applied by spraying to trap logs or combined with modified pheromone traps (Jakuš, Blaženec 2011; Grodzki, Kosibowicz 2015). Additional research under simulated and/or actual field conditions is necessary to confirm whether the laboratory efficacy reflects the performance of the strains in the field. Further research is currently conducted to search for appropriate inoculum formulation and method of inoculum introduction into SBB populations in forests.

## CONCLUSION

We confirmed that entomopathogenic fungi of the genus *Beauveria* are a natural component of insect pathogenic mycoflora in populations of spruce bark beetles in the Vitosha National Park. Two species,

https://doi.org/10.17221/123/2020-JFS

*B. bassiana* and *B. caledonica*, were identified from collected cadavers of bark beetles. Pathogenicity of obtained *Beauveria* strains was demonstrated against bark beetle adults in laboratory bioassays. Virulence varied among the strains and the five most virulent strains showed efficacy comparable with the commercial mycoinsecticide Boverol® and the recently selected *B. bassiana* strain patented for use against spruce bark beetle in Slovakia. The results suggest that the Bulgarian strains have a potential for the control of bark beetle adults and deserve further investigation under semi-field and field conditions.

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