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Entomopathogenic Fungi (Ascomycota: Hypocreales) as Natural Antagonists of the Pine Processionary Moth *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae) in Bulgaria

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Abstract: *Thaumetopoea pityocampa*, a moth naturally distributed in pine ecosystems of Mediterranean region, is considered the most dangerous defoliator in Bulgarian pine forests. Caterpillars of this species also represent a health hazard because their hairs, which contain an urticating protein – thaumetopoein, are responsible for painful skin irritations, rashes and allergic reactions in some people. Therefore, *T. pityocampa* is not only a serious forest pest, but also a public health problem. Populations of the moth are regulated by a complex of natural enemies. There is only limited information about a parasitic activity of entomopathogenic fungi. These fungi are natural antagonists of insects helping to control of host population and prevention of outbreaks formation. A goal of the present study was to identify species of entomopathogenic fungi in natural populations of *T. pityocampa* in Bulgaria. During the study, 908 larvae and pupae were collected from two sites (Fotinovo and Kandilka villages) in the south-eastern Rhodopes. In laboratory conditions, 147 larvae or pupae showing characteristic symptoms of mycoses were observed and on 27 of them *in vitro* isolates were obtained. The cultures were microscopically identified and subsequently analysed by a sequencing study of the internal transcribed region of rDNA and a partial sequence of TEF1-α gene. Altogether, two *Beauveria* species (*B. pseudobassiana* – 7 isolates and *B. varroae* – 6 isolates) and *Purpureocillium lilacinum* (14 isolates) were identified. The three entomopathogens are reported from *T. pityocampa* for the first time.

Key words: forest pests, biological control, *Beauveria*, *Purpureocillium*, pine forest

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Introduction

The pine processionary moth (PPM) *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae), is a forest pest that is currently extending its geographical distribution in Europe in response to the climate warming (Roques 2015). The problem with PPM's economic, environmental and social impact within its existing distribution is common to the countries from the Mediterranean region. The pest is a serious defoliator of *Pinus* spp. in countries where it occurs in high densities. For this reason, some European Mediterranean countries, with the leading role of France and Italy, performed a large-scale monitoring programme for expanding the range of *T. pityocampa* (Battisti et al. 2005, 2006, Robinet et al. 2007). It was reported that, due to defoliations by this pest, losses in total biomass were up to 50% in moderately defoliated pine trees and up to 64% in completely defoliated trees (Markalas 1998). PPM may also cause a tree death indirectly by weakening trees and facilitating attacks by other pests, mostly bark beetles. Aesthetic problems may also occur in public greenery due to defoliation and larval nests (Rossi et al. 2016). Additionally, social impacts are caused by the urticating hairs of the older larvae which become detached from the larvae and contaminate the environment. If they come into contact with skin, these setae cause severe rashes in both humans and other mammals due to a toxic protein they contain. If airborne setae are inhaled, they may cause breathing difficulties, e.g. asthma. If the setae enter the eye, severe corneal inflammation can occur. The larval nest also contains shed hairs that continue to pose a risk to those that handle it for months or even years afterwards (Portero et al. 2012).

In Bulgaria, PPM's biological and ecological characteristics are relatively well studied and two forms were determined, which can be distinguished by their different phenology (Tsankov et al. 1996a, 1996b, Mirchev et al. 2017, 2019). A typical Mediterranean winter form reproduces in late summer having a winter larval development and an early developing summer form reproduces in spring with larval development during summer. Both the summer and winter forms may coexist in the same pine stands (Mirchev et al. 2019). While effects of environmental conditions on PPM's population density have already been investigated (Hódar et al. 2003, Netherer & Schopf 2010, Berardi et al. 2015), there is only limited actual information about the impact of biological factors on PPM's populations in Europe. Generally, populations of the moth are regulated by a complex of natural enemies. The effects of parasitoids (e.g. Tsankov et al. 1996a, 1996b, Mirchev & Tsankov 2001, Mirchev et al. 2015a, 2015b) and bacteria (Ince et al. 2008, Cebeci et al. 2010) on PPM's population have been well documented but there is scarce information about a parasitic activity of entomopathogenic fungi in Bulgaria (Draganova et al. 2013). Entomopathogenic fungi are important natural control agents of insects and have been a subject of intense study for more than 120 years. There is estimated > 700 species, but a majority of perspective species for a mass production and their use in biocontrol are those from the order Hypocreales (Ascomycota) (Lacey et al. 2015). Investigating the effectiveness of hypocrealean entomopathogenic fungi has drawn the attention of researchers in different countries and several fungal species have already been reported from PPM including, *Metarhizium anisopliae* (Metschn.) Sorokin, *Lecanicillium lecanii* (Zimm.) Zare et W. Gams, *Beauveria bassiana* (Bals.-Criv.) Vuill., *Isaria farinosa* (Holmsk.) Fr. or *Isaria fumosorosea* Wize (Paparatti & Fabozzi 1988, Vargas-Osuna et al. 1994, Er et al. 2007, Sevim et al. 2010, Tarasco et al. 2015, Akinci et al. 2017). Entomopathogenic fungi are cosmopolitan insect parasites helping in a natural regulation of host populations (Vega et al. 2012). There has been an extensive research on fungal entomopathogens for their use as biocontrol agents in agriculture or forestry (Lacey et al. 2015). Plenty of strains have been isolated and tested against insect pests and some of them have been successfully licensed for a commercial use (Ravensberg 2011). Until now, no entomopathogenic fungi have been incorporated into the integrated pest management of PPM.

A goal of this study was to identify the species spectrum of biological control agents of *T. pityocampa* with a focus to entomopathogenic fungi in pine forests of the south-east Rhodope Mountains in Bulgaria.

Materials and Methods

Experimental material was collected at two localities in the south-east Rhodopes: near Fotinovo and Kandilka villages (Fig. 1). The localities fall into the Continental-Mediterranean climatic region, characterised by mild and very humid winter and hot and dry summer. Unlike the habitats of PPM in southwest Bulgaria, the south-east Rhodopes are considerably less protected from cold air. Considering a physical-geographical aspect, the characteristics of the two sites are similar (Table 1). They are located

Table 1. Main characteristics of the localities where collecting of samples was carried out.

Locality name	Date of collection	Altitude, m a.s.l.	Latitude	Longitude	Slope aspect	Host tree
Fotinovo	Feb. 6, 2019	459	41°37'82"	25°32'16"	western	*Pinus nigra*
Kandilka	March 26, 2019	465	41°40'94"	25°58'18"	northern	*Pinus nigra*

Fig. 1. Map of Bulgaria showing sites where collecting of samples was carried out

in the Bulgarian part of the south-east Rhodopes, which have a low mountainous and hilly relief. The host plant in both localities is *Pinus nigra* (Arn.).

Samples of PPM's larvae and pupae with typical macroscopic symptoms of infection by entomopathogenic fungi were placed individually in 1.5-ml microtubes and stored at cool conditions. Dead individuals displaying no external symptoms were incubated in 1.5-ml microtubes with a piece of wet cotton at 25 ± 1°C for 48 h to stimulate fungal growth. All individuals with typical symptoms of mycosis (Humber 2012) were examined under a dissecting microscope (40×) and those with confirmed fungal infection were used for obtaining fungal cultures. *In vitro* cultures of entomopathogens were obtained from infected samples using a selective culture medium based on Sabouraud-dextrose agar (Barta et al. 2018). Cadavers overgrown by mycelium were placed on the surface of the selective culture medium in Petri dishes (60 × 15 mm) and incubated at 25 ± 1°C in the dark for 10-14 days. Fungal colonies on the medium growing out of cadavers were sub-cultured on Sabouraud-dextrose agar in culture glass tubes (120 × 17 mm) at 25 ± 1°C until sporulation (ca. 10 days). Sporulating cultures in the tubes were stored at 5°C. All isolates were deposited in the fungal collection of Institute of Forest Ecology of Slovak Academy of Sciences.

Identification of fungi isolated from infected PPM was based on the morphology of microstructures (Humber 2012, Rehner & Buckley 2005, Rehner et al. 2011) and was supported by sequencing of internal transcribed region (ITS) of ribosomal DNA and a partial sequence of TEF1-α gene. Fungal biomass was harvested from *in vitro* cultures, ground with liquid nitrogen using pestle in 1.5-ml microtubes. Genomic DNA was extracted from the biomass using EZ-10 Spin Column Fungal Genomic DNA Kit (Bio Basic Canada Inc., Ontario, Canada) according to the manufacturer's instructions. PCR

amplifications of the ITS region with universal primer pair ITS1F/ITS4 (Gardens & Bruns 1993) and the TEF-1α with primers 983F and 2218R (Rehner & Buckley 2005) were performed in 20 µl reaction volumes consisting of 5 × Hot FirePolymerase Blend Master Mix, 0.2 µm of each primer, deionised water of molecular grade (Pro injectione –B. Braun) and 1 µl of template DNA. The PCRs were carried out on a BIO-RAD T100TM Thermal Cycler. PCR amplifications of ITS region were performed under following conditions: initial denaturation step for 14 min at 95°C, followed by 35 cycles for 15 s at 95°C denaturation, annealing for 30 s at 50°C, elongation for 80 s at 72°C and a final elongation step for 10 min at 72°C. For TEF-1α, PCR conditions were an initial step of 20 s at 95°C; 10 cycles of 30 s at 94°C, 55 s 66°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 on a 1% agarose gel using Simply Safe stain (EURx, Gdansk, Poland) and visualised under UV light. To detect the length of the amplified fragments, a 100-bp marker (Solis BioDyne, Tartu, Estonia) was used. The target fragments were purified using a QIAquick PCR purification Kit (Qiagen, Venlo, Netherlands) and sequenced using an ABI3130 × l sequencer (Applied Biosystems). The retrieved sequences were assembled and processed using SnapGene® Viewer 4.3.11 (GSL Biotech; available at snapgene.com) and compared by BLAST (Altschul et al. 1997) against DNA sequences deposited in GenBank. Alignment was made using MUSCLE in MEGA 7.0.26 (Kumar et al. 2016; available at megasoftware.net) and phylogenetic tree was constructed using Maximum likelihood method with Hasegawa-Kishino-Yano substitution model, a N/BioNJ starting tree and followed by 500 bootstrap replications.

Results

During this study, a total number of collected *T. pityocampa* samples reached 642 pupae and 88 caterpillars in Fotinovo, and 178 pupae in Kandilka. Out of the collected samples, 147 ones (16.2% of all samples) were showing characteristic symptoms of mycosis. *In vitro* isolates were obtained from selected samples (Table 2) and as many as 23 isolates were obtained from Kandilka and four isolates are from samples collected in Fotinovo. The isolates were identified as either *Beauveria* sp. or *Purpureocillium* sp. by microscopic examination. DNA fragments of 494-540 pb of the partial ITS1-5.8S-ITS2 gene region and fragments of the partial TEF-1α (874-959 bp) were sequenced. Based on the genomic analysis two *Beauveria* species (*B. pseudobassiana* S.A. Rehner & R.A. Humber – seven isolates and *B. varroae* S.A. Rehner & R.A. Humber – six isolates) and *Purpureocillium lilacinum* (Thom) Luangsa-ard (14 isolates) were identified. While *B. pseudobassiana* and *P. lilacinum* were detected in both localities, *B. varroae* was only observed in Kandilka. All the three entomopathogens are reported from *T. pityocampa* for the first time.

Discussion

Activity of hypocrealean entomopathogens in populations of PPM has been documented in several European countries and species of *Beauveria* genus have always been listed among the pathogens. Interestingly, this genus was exclusively represented by *B. bassiana*, the species that was not detected in the current study. Vargas-Osuna et al. (1994), Sevim et al. (2010) and Draganova et al. (2013) isolated *B. bassiana* from PPM larvae and Tarasco et al. (2015) isolated it from pupae. It is important to mention that the traditional morpho-taxonomic determination of *Beauveria* species is insufficient. Before the comprehensive taxonomic revision of *Beauveria* genus was performed by a multigene phylogenetic analysis (Rehner & Buckley 2005, Rehner et al. 2011), *B. bassiana*, in a broad sense, was a complex of species consisting of morphologically similar but cryptic lineages. Soon after the novel molecular approach in *Beauveria* taxonomy was designed, several new *Beauveria* species have been described (Rehner et al. 2011, Zhang et al. 2012, Chen et al. 2013, Agrawal et al. 2014, Robène-Soustrade et al. 2015, Imoulan et al. 2016). Because the majority of records on *B. bassiana* from PPM were made on the basis of morpho-taxonomic principles (Vargas-Osuna et al. 1994, Draganova et al. 2013, Tarasco et al. 2015), the results must be judged with caution. And some species, morphologically very similar, might remain undetermined. In the study by Sevim et al. (2010), the molecular identification was applied, but again only *B. bassiana* (from two different clades by Rehner & Buckley 2005) was identified; together only five isolates were analysed.

The three fungal species identified in this study are known from a wide range of substrates, including insects, mites and nematodes. *P. lilacinum* is a ubiquitous saprobic fungus commonly isolated from soil, decaying vegetation, nematode eggs, but insects can also be infected (Luangsa-ard et al. 2011). *B. pseudobassiana* and *B. varroae*

Table 2. List of *in vitro* isolates of entomopathogenic fungi obtained from *T. pityocampa* collected in Bulgaria during 2019

Isolate name	Entomopathogenic fungus	Source	Name of locality
BG-ep_52	*Beauveria pseudobassiana*	pupa	Fotinovo
BG-ep_53	*Beauveria pseudobassiana*	larva	Fotinovo
BG-ep_54	*Purpureocillium lilacinum*	pupa	Fotinovo
BG-ep_55	*Purpureocillium lilacinum*	pupa	Fotinovo
BG-ep_57	*Purpureocillium lilacinum*	pupa	Kandilka
BG-ep_58	*Purpureocillium lilacinum*	pupa	Kandilka
BG-ep_59	*Purpureocillium lilacinum*	pupa	Kandilka
BG-ep_60	*Purpureocillium lilacinum*	pupa	Kandilka
BG-ep_61	*Purpureocillium lilacinum*	pupa	Kandilka
BG-ep_62	*Purpureocillium lilacinum*	pupa	Kandilka
BG-ep_63	*Purpureocillium lilacinum*	pupa	Kandilka
BG-ep_64	*Beauveria varroae*	pupa	Kandilka
BG-ep_65	*Beauveria varroae*	pupa	Kandilka
BG-ep_66	*Beauveria varroae*	pupa	Kandilka
BG-ep_67	*Beauveria pseudobassiana*	pupa	Kandilka
BG-ep_68	*Beauveria pseudobassiana*	pupa	Kandilka
BG-ep_69	*Beauveria varroae*	pupa	Kandilka
BG-ep_70	*Beauveria pseudobassiana*	pupa	Kandilka
BG-ep_71	*Purpureocillium lilacinum*	pupa	Kandilka
BG-ep_72	*Purpureocillium lilacinum*	pupa	Kandilka
BG-ep_73	*Purpureocillium lilacinum*	pupa	Kandilka
BG-ep_74	*Purpureocillium lilacinum*	pupa	Kandilka
BG-ep_75	*Purpureocillium lilacinum*	pupa	Kandilka
BG-ep_76	*Beauveria varroae*	pupa	Kandilka
BG-ep_77	*Beauveria varroae*	pupa	Kandilka
BG-ep_78	*Beauveria pseudobassiana*	pupa	Kandilka
BG-ep_79	*Beauveria pseudobassiana*	pupa	Kandilka

are morphologically indistinguishable. While *B. varroae* is primarily known as pathogen of mites, *B. pseudobassiana* is a globally distributed soilborne and broad host-range generalist entomopathogen that occurs in a wide range of habitats (Rehner et al. 2011). Recent soil analyses in Slovakia indicated that *B. pseudobassiana* preferred forest habitats over arable soils or meadows (Medo et al. 2016). *B. varroae* is known from ectoparasitic mites of honeybee in France, but it can infect coleopteran hosts (*Larinus* sp.) as well (Rehner et al. 2011). We report this fungus from the lepidopteran host for the first time. *B. pseudobassiana* and *B. varroae* were identified from 13 PPM cadavers (48.15% of all mycosed samples) and can be thus considered common elements of entomopathogenic communities in the pest populations. It is not clear why *B. bassiana*, the species observed in PPM in other studies, was not detected during this survey.

Pathogenicity of the obtained isolates against PPM larvae was not determined in this study, however, several other studies tested *B. bassiana*, *M. anisopliae*, *I. farinosa* or *L. lecanii* strains against PPM larvae and reported optimistic results (e.g. Er et al. 2007, Akinici et al. 2017, Sönmez et al. 2017, Ozdemir et al. 2019). Because the fungi could be considered as perspective candidate for microbial control agents for suppressing *T. pityocampa* populations, the Bulgarian isolates obtained in the current survey deserve further attention to determine their effectiveness in laboratory and field conditions.

Conclusions

Three species of entomopathogenic fungi were identified from naturally infected caterpillars or pupae of *T. pityocampa* in the south-eastern Rho-

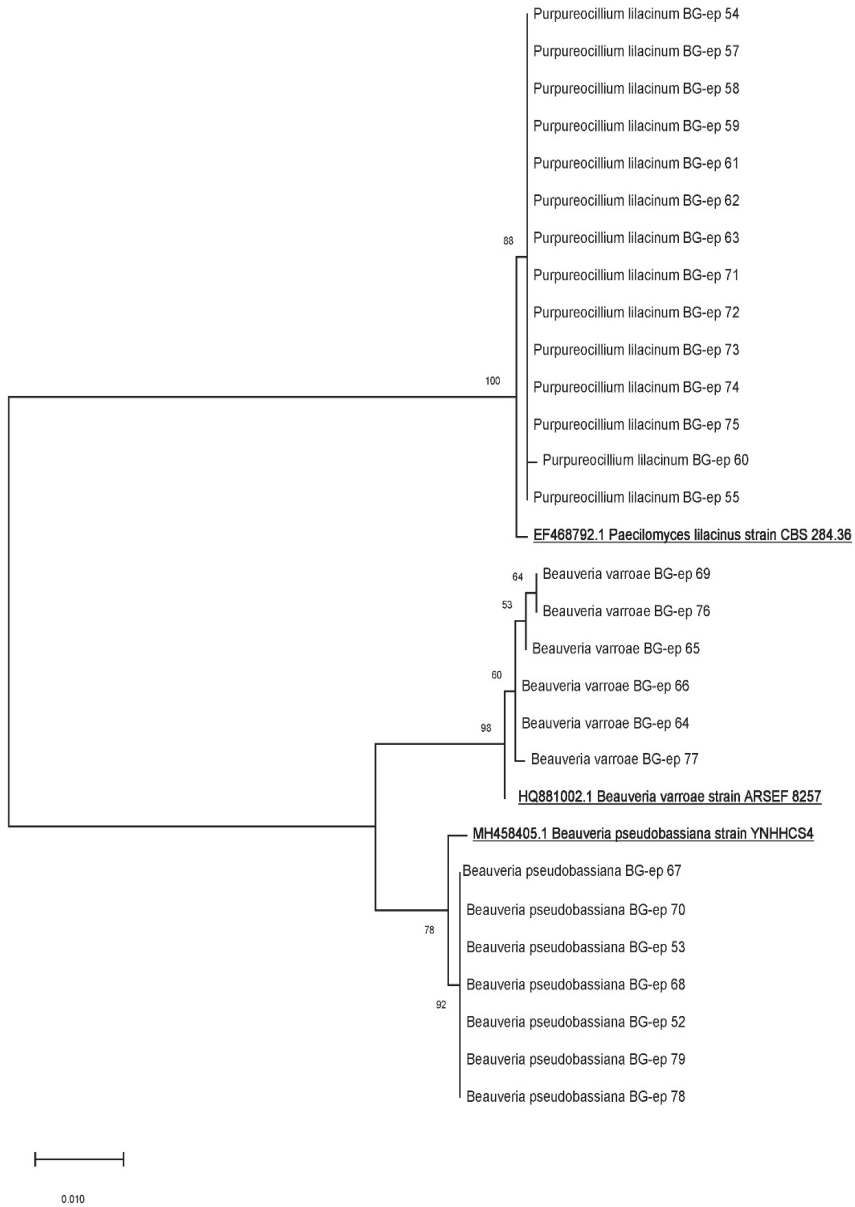

Fig. 2. Phylogeny of fungal isolates from Bulgaria inferred by maximum likelihood analysis of partial elongation factor TEF-1α data. Isolates from Bulgaria are in a normal text and sequences of reference isolates (bold and underlined text) were obtained from GenBank. The reference sequences are labelled by GenBank accession numbers.

dopes in Bulgaria. Prevalence of infection in the pest population reached to 16.2%. Two *Beauveria* species (*B. pseudobassiana* and *B. varroae*) and *Purpureocillium lilacinum* were identified. All the three entomopathogenic species are reported from *T. pityocampa* for the first time. This is also the first record of *B. varroae* from a lepidopteran host.

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