Bulletin of Insectology ***69*** *(2): 311-317, 2016*
ISSN 1721-8861

First record of *Beauveria bassiana* on *Tomicus minor* in Sicily

Martin SCHEBECK[1], Marta WRZOSEK[2], Mirella CLAUSI[3], Eustachio TARASCO[4], Cezary TKACZUK[5], Rudolf WEGENSTEINER[1]

[1]*Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences Vienna (BOKU), Austria*
[2]*Department of Molecular Phylogenetics and Evolution, University of Warsaw, Poland*
[3]*Department of Biological, Geological and Environmental Sciences, Animal Biology Section, University of Catania, Italy*
[4]*Department of Plant, Soil & Food Sciences, University of Bari "Aldo Moro", Italy*
[5]*Department of Plant Protection and Breeding, Siedlce University, Poland*

Abstract

Tomicus minor (Hartig) (Coleoptera Curculionidae Scolytinae) was found breeding in *Pinus nigra calabrica* (for the first time in this subspecies) in the Etna national park (Sicily) in two sites in 2013 and in another site in 2015. We used morphological features of the beetle, a molecular marker (barcoding region of the COI gene) and characteristics of the breeding system to verify the species affiliation. The results of these three traits were in accordance. Moreover, we screened living and dead beetles for pathogens. Dissecting living beetles did not exhibit any pathogens except unidentified hyphae of entomopathogenic fungi. *Beauveria bassiana* was found on dead *T. minor* collected from galleries. Species affiliation was studied by applying cultural, morphological, and molecular (ITS marker) methods. This is the first evidence of the fungus on this pest confirmed by molecular tools and the first record of *B. bassiana* on *T. minor* in Sicily. These data contribute to the knowledge of the distribution of this beetle in Italy. Moreover, the findings of *B. bassiana* bring more light in the pathogen complex of *T. minor* and help to understand the biology of this important forest insect.

Key words: lesser pine shoot beetle, entomopathogenic fungus, Etna national park, ITS, COI, barcoding.

Introduction

The Curculionid subfamily Scolytinae comprises about 6,000 described species and includes bark and wood boring beetles (Knižek and Beaver, 2004; Hulcr *et al*., 2015). These insects spend most of their life cycle inside their host plants. Bark beetles can be serious pests mainly in conifer forests in the Holarctic. Especially after abiotic disturbances like storm, wet snow, icing or drought, population densities can increase rapidly leading to heavy ecological impacts and economical losses (Schroeder and Lindelöw, 2002).

The lesser pine shoot beetle, *Tomicus minor* (Hartig) (Coleoptera Curculionidae Scolytinae), infests standing pine trees (*Pinus* spp.), especially all thin barked parts of the crown. At high population densities even healthy trees can be attacked (Fernández Fernández *et al*., 1999). Mainly pine species (e.g. *Pinus sylvestris* L., *Pinus nigra* Arnold) are known to be infested (Pfeffer *et al*., 1995; Lundgren, 2004), only few records report about occurrence on *Larix* spp. and *Picea* spp. (Postner, 1974). Maturation feeding of immature offspring and regeneration feeding of parental beetles is conducted in fresh pine shoots which can be broken by wind afterwards (Pfeffer *et al*., 1995; Lundgren, 2004). Young shoots are also affected by overwintering beetles (Fernández Fernández *et al*., 1999). Pine trees damaged by shoot feeding of *T. minor* might show a substantial decline of increment (Borkowski, 2001). This beetle mainly establishes one generation per year, under favourable climatic conditions two generations per year can develop (Pfeffer *et al*., 1995; Lundgren, 2004).

Until now, *T. minor* was reported to occur in northern, central and southern Italy, but is not mentioned for Sicily (Stoch, 2003; Alonzo-Zarazaga, 2004). However, there is already a record of *T. minor* in the Etna region collected from *P. nigra* (Longo *et al*., 2001).

Up to now, the entire pathogen spectrum of *T. minor* was never investigated in detail (Wegensteiner, 2004). There is one note on the occurrence of a *Gregarina* sp. in the midgut lumen of *T. minor* (Kohlmayr, 2001). Some infection experiments focused on entomopathogenic fungi with *T. minor* (Ruiz-Portero *et al*., 2002), but *Beauveria bassiana* [(Balsamo-Crivelli) Vuillemin] was described only a few times to occur naturally in *T. minor* (Jankevica, 2004; Humber and Hansen, 2005), though that fungal pathogen is known from several other bark beetle species (Wegensteiner *et al.*, 2015a; 2015b). Among entomopathogenic fungi *B. bassiana* is known to occur in different habitats and in many insect species and was found to cause high mortality in its hosts (Zimmermann, 2007; Vega *et al.*, 2012). The soil is an important reservoir for entomopathogenic fungi. These fungi are an important and widespread component of most terrestrial ecosystems and are commonly found in the soil and leaf litter of forests worldwide. However, compared to tropical habitats their diversity in temperate forests is relatively low (Evans, 1982; Aung *et al.*, 2008). Many fungal species belonging to the order Hypocreales (Ascomycota), for example the genera *Beauveria*, *Isaria* and *Metarhizium*, inhabit the soil for a significant part of their life cycles (Keller and Zimmermann, 1989; Jackson *et al.*, 2000). Bidochka *et al.* (1998) found *B. bassiana* to be affiliated with shaded and uncultivated habitats (i.e. forests) and it has been isolated from the surface and the interior of plants. Using selective media this fungus was isolated from the bark of elm trees (Doberski and Tribe, 1980) and from

the bark of the hardwood *Carpinus caroliniana* Walter (Bills and Polishook, 1991). Reay *et al.* (2010) found widespread occurrence of *B. bassiana* as endophyte of pine (*Pinus radiata* Don) in New Zealand forests.

As experts for certain taxa (or certain developmental stages) are getting rare and knowledge on classic species determination by morphological traits might get lost, the importance of molecular markers for species determination has increased over the last years (Hebert *et al.*, 2003). This has implications for contributing to the knowledge of species' distribution but also for pest management, even for non-experts.

The main aims of our study were 1) to identify exactly the presence of *T. minor* in Sicily by using morphological and molecular traits and characters of the galleries for species determination and 2) to investigate the presence of pathogens in this bark beetle species using morphological, cultural and molecular features.

Materials and methods

Insect collection

T. minor was collected from Calabrian pine, *Pinus nigra calabrica* (Loudon) Murray, in Etna national park in several sampling sites situated in the protection zone "B" of the park. In the year 2013 *T. minor* was collected from pines in two sites and in 2015 from one site (table 1).

In both years, beetles were collected in spring (April) from logs of fresh cut tree-tops in three stands in total. One tree-top per location and year was sampled. In all cases the stands were dominated by *P. nigra calabrica.* The number of galleries was not counted because of numerous branches and the impracticality of turning the tree-top. However, tree-tops were fresh but sparsely colonized by *T. minor*. Beside that species no other infestation could be observed in all three years. In old and dry tree-tops former infestations of *T. minor* were recognized because of old galleries.

In total 130 individuals were collected, 117 living beetles and 13 dead beetles (table 1). The majority of samples was obtained in 2013, most cadavers in breeding systems were found in 2015. Samples for observations on fungi associated with these beetles were transferred to humid conditions using plastic Petri dishes with moistened filter paper and stored after transport at room temperature (about 22 °C). Living individuals for molecular studies were immediately transferred to 98% DNA free ethanol and stored at −20 °C afterwards.

After morphological identification, in total 85 living and 26 dead (died during transport) beetles from all three locations were dissected on microscopical slides and checked for the presence of pathogens in a bright field microscope (at magnification 40× to 1000×; table 2). Beside living beetles, in 2013 one dead individual from 'Monte Crisimo 1' and in 2015 twelve cadavers from 'Rifugio Ragabo' were collected from galleries of the same trees. The twelve dead individuals collected in 2015 were incubated in the laboratory on moistened filter paper in a Petri dish (9 cm in diameter) to provide humid conditions for promotion of fungal growth. Small parts of the mycelium with conidia were transferred to microscopical slides, fixed with methanol and stained with Giemsa dye. After a first preliminary diagnosis in a bright field microscope (at magnification 40× to 1000×) six fungus overgrown beetles were transferred to the laboratory in Siedlce in 2015 for further cultural studies and after that to Warsaw for molecular studies.

Preparing fungal cultures

After a first observation of fungus overgrown beetles under a dissecting microscope and tentative confirmation as entomopathogens, pure cultures were prepared from each of the six cadavers by transferring conidia with a dissecting needle onto Sabouraud-Dextrose-Agar (SDA) and on Potato-Dextrose-Agar (PDA) supplemented with antibiotics (streptomycin sulphate 250 mg/L, chlorotetracycline hydrochloride 50 mg/L; Sigma, St Louis, USA). Colony descriptions and measurements were done from cultures grown on SDA at 22 °C in darkness. Radial growth of the colony was measured by drawing two orthogonal diameters after ten days after incubation. Conidia and mycelia were mounted from plates in lactophenol-Cotton blue (Merck) and examined by light microscopy (Nikon Eclipse E 600) with "phase contrast" and magnification 400×, following Inglis *et al.* (2012). Measurements of length and width of fungal structures (conidia, conidiogenous cells and mycelia)

Table 1. Information on sampling sites of *T. minor* in Sicily in spring 2013 and 2015. Numbers of living and dead beetles collected from galleries of one infested *P. nigra calabrica* on each site in each year.

Location	Year	Geographical data	Altitude	N living beetles	N dead beetles
Monte Crisimo 1	2013	37.818206N 15.106192E	1000 m	50	1
Monte Crisimo 2	2013	37.809536N 15.084529E	1000 m	34	-
Rifugio Ragabo	2015	37.808370N 15.074498E	1425 m	33	12

Table 2. Living *T. minor* from three locations and two years; N dissected beetles (living males and females) and numbers of dead during transport; beetles having an unidentified fungal infection (n.i. = sex not identified).

Location	Collection date	Dissection date	N dissected	Living	♀	♂	Dead during transport	Fungal infection
Monte Crisimo 1	9 IV 2013	15 IV 2013	50	33	14	19	17	5 n.i.
Monte Crisimo 2	10 IV 2013	15 IV 2013	34	25	11	14	9	3 n.i.
Rifugio Ragabo	16 IV 2015	23 IV 2015	27	27	13	14	-	1 ♂

were done with an eyepiece micrometer to enable specific identification. Fungi were identified after Samson *et al.* (1988) and Humber (1997).

Moreover, molecular studies were conducted with the fungus isolate to confirm the proper identification. The ITS marker was chosen for identification as it has been proposed as universal DNA barcode marker for fungi (Schoch *et al*., 2012).

Fungal DNA extraction, PCR amplification and sequencing

Genomic DNA extraction was done from pure cultures growing on PDA mentioned above. For isolation the II Plant DNA kit (Bioline Ltd., UK) was used following the manufacturer's instructions. Sequencing of the region ITS1-5.8SDNA-ITS2 was performed using the universal primer pair ITS1f and ITS4 (White *et al*., 1990) as reported earlier (Różalska *et al*., 2013). Forward and reverse sequences were assembled and edited with ChromasPro version 1.7.1 (Technelysium, Australia), aligned with BioEdit (Hall, 1999) and the resulting consensus sequences were searched against the GenBank database (National Center for Biotechnology Information, NCBI) using the 'blastn' algorithm (http://blast.ncbi.nlm.nih.gov/). Species assignment was performed by comparing the samples with the top BLAST hits (following parameters were used: E value = 0.0, > 99% sequence identity for species delimitation, > 92% sequence identity for genus delimitation). Sequence data generated for this study is available in NCBI under accession number KU163450.

DNA extraction from *T. minor*, PCR amplification and sequencing

To confirm the species determination of collected bark beetles we used the barcoding region of the mitochondrial COI gene. For molecular analyses, living individuals from 'Rifugio Ragabo' were immediately transferred to 98% DNA free ethanol. DNA was extracted from six whole beetles - determined by morphology before (Pfeffer *et al*., 1995) - using the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich, St. Louis, USA) following the manufacturer's instructions. A part of the mitochondrial COI gene was amplified using the forward primer LCO1490 and the reverse primer HCO2190 (Folmer *et al*., 1994; Hebert *et al*., 2003). PCR reactions were carried out in total reaction volumes of 20 µl, containing 1× reaction buffer, 800 µM dNTP-mix, 0.4 µM forward and reverse primer, respectively, 0.05 U/µl Taq-DNA-polymerase (peqlab/VWR, Erlangen, Germany), 20 mM $MgCl_2$ and ~50 ng template DNA. PCR reactions were performed with an initial denaturation step at 94 °C for 3 min; denaturation at 94 °C for 30 sec, annealing at 49 °C for 1 min and an extension at 72 °C for 1 min for 33 cycles; and a final extensions step at 72 °C for 7 min.

Sequence chromatograms were checked by eye using ChromasLite version 2.0 (Technelysium, Australia) for the presence of ambiguous peaks so that only clear sequences were used for further analyses. Sequences were edited using GeneRunner version 5.0 (www.generunner.net). A BLAST search was performed using the algorithm 'blastn' (GenBank, NCBI, http://blast.ncbi.nlm.nih.gov/). The sequences of these six specimens were aligned to the data of the top BLAST hit in ClustalX version 2.0 (Larkin *et al*., 2007). The alignments were checked for mutations by eye using MEGA version 6 (Tamura *et al*., 2013).

Results and discussion

Beetle discovery and determination using morphological traits

T. minor was found breeding in *P. nigra calabrica* for the first time in three locations in the Etna national park in Sicily, in two sites in 2013 (Monte Crisimo 1 and 2) and in another location in 2015 (Rifugio Ragabo). In total 130 beetles, 117 living and 13 dead, were collected from fresh tree-tops (table 1).

For a first identification the elytral declivity was checked, it exhibited bristles and no grooves. Furthermore, female galleries consisted of two horizontal mother galleries crossing the phloem fibres (figure 1). Both features are characteristic for *T. minor* (Postner, 1974; Pfeffer *et al*., 1995). To confirm the species affiliation with molecular tools a sub-sample of individuals was studied by using the COI barcoding region.

Beetle determination using molecular traits

The DNA barcode region - fragment of ~ 640 bp of the mitochondrial COI gene - was used for identifying the *Tomicus* spp. specimens collected in Sicily. The six specimens yielded in one consensus sequence in all individuals analyzed. The BLAST search conducted with this sequence showed highest identity (99%) with accession number KM439268, a sequence from *T. minor* (Hendrich *et al*., 2015). Aligning the sequences yielded in five transitions. All mutations were confirmed as detected in all six individuals. Thus, all specimens studied were identified as *T. minor*. Hence, the results of DNA barcoding confirmed the species determination using

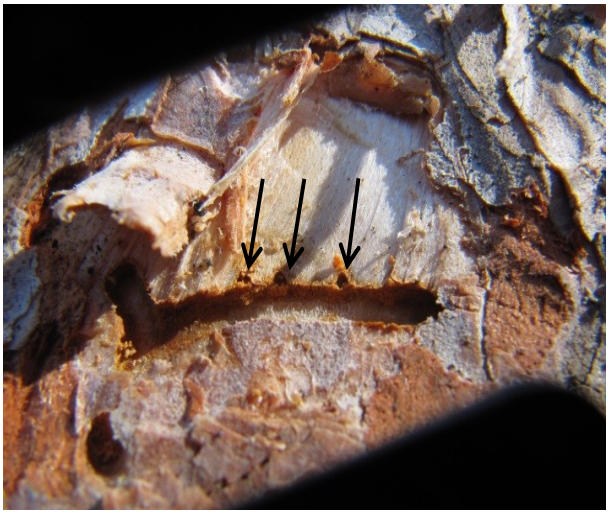

Figure 1. Mother gallery of *T. minor* with egg niches (arrows).
(In colour at www.bulletinofinsectology.org)

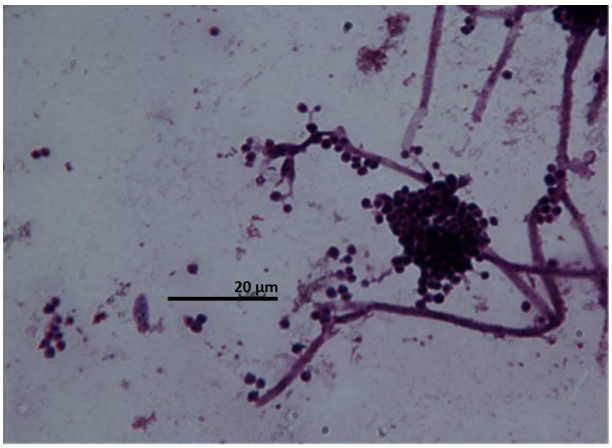

Figure 2. *B. bassiana* conidia isolated from *T. minor* (Giemsa stained smear) (bar = 20 µm).
(In colour at www.bulletinofinsectology.org)

morphological traits and characteristics of the breeding system.

Surprisingly the occurrence of *T. minor* was never reported to the Checklist of the species of the Italian Fauna (Stoch, 2003) and to the Catalogus Fauna Europaea (Alonzo-Zarazaga, 2004) even the species was already found in Sicily during faunistic surveys in the Etna national park (Longo *et al*., 2001). However, various bark beetle species of different genera have been reported from Sicily so far. The only one from the genus *Tomicus* is *T. destruens* (Wollaston) (Stoch, 2003).

Pathogen identification

When dissecting living *T. minor* surprisingly no pathogens were found except hyphae of unidentified entomopathogenic fungi growing in the body cavity of some beetles. In 2013 out of 50 dissected beetles from 'Monte Crisimo 1' five showed these symptoms and out of 34 dissected beetles from 'Monte Crisimo 2' three individuals also had hyphae in the body cavity. In 2015 27 beetles from 'Rifugio Ragabo' were dissected but only one had a fungus growing in the body (table 2). The absence of pathogens except entomopathogenic fungi is similar to the results of Kohlmayr (2001) who found a *Gregarina* sp. in one single *T. minor* beside a not exactly identified fungal species.

Cadavers were examined in 2015 too. Twelve *T. minor* were found dead in their galleries. When incubating them in the laboratory a white mycelium started growing out of the cadavers quickly followed by superficial growth of hyphae on the surface. Conidia developing on the mycelium were identified as *B. bassiana* in a bright field light microscope (figure 2). The isolate was characterized microscopically by conidiophores consisting of whorls and dense clusters of sympodial, short and globose conidiogenous cells with apical zig-zag appearance of the rachis and one-celled spherical conidia. Conidial length and width were 2.12 ± 0.35 µm and 1.78 ± 0.23 µm, respectively, with a length/width ratio of 1.20. Colonies on SDA were normally white to pale yellow and the average diameter of the colonies after ten days of growth at 22 °C was 23.1 ± 3.2 mm.

The dry culture of *B. bassiana* is stored at the University of Warsaw Herbarium under the number WA51509. Sub-culturing the conidia and applying molecular tools confirmed the identity of the fungus as *B. bassiana*. The obtained sequence KU163450 showed 100% identity with 26 sequences available in NCBI. Among them 21 were assigned to *B. bassiana*, two to *B. pseudobassiana* from Mexico (KC355186, KC355187, unpublished), two to unidentified species belonging to *Beauveria* and one to an uncultured fungus isolated from a soil sample from Alaska (Taylor *et al*., 2007).

This is the first information about the infection of *T. minor* beetles by *B. bassiana* in natural forest conditions in Sicily. *B. bassiana* infected *T. minor* have been recorded from Latvia (Jankevica, 2004) and France (Humber and Hansen, 2005), but it is the first one confirming the identity of the infection of *T. minor* by *B. bassiana* by molecular tools.

B. bassiana in bark beetle management

The influence of bark beetle pathogens on the host density is still not sufficiently investigated. Several studies found that pathogen prevalence depends on various factors, like pathogen characteristics, its life cycle, population density and immunity of the host, or host development (Wegensteiner, 2004; Bałazy, 2012; Grodzki and Kosibowicz, 2015).

The effects of temperature on germination of conidia and on fungal growth were demonstrated in several entomopathogenic fungi (Mwamburi *et al.*, 2015) and differences in fungal isolates from different regions were found as well (Fargues *et al.*, 1997; Bidochka *et al*., 2002). Considering these facts, the origin of an isolate seems to be of great importance in relation to its ecological valence and with regard to the possible use of entomopathogenic fungi as biological control agent.

Already in 1935, Karpiński reported *B. bassiana* as the reason of *Ips typographus* (L.) mortality. Moreover, *B. bassiana* was found to have a wide range of forest insects as hosts (Fuxa and Tanada, 1987; Zimmermann, 2007; Augustyniuk-Kram and Kram, 2012). However, little effects of entomopathogenic fungi on their hosts under natural conditions were found. Bałazy (2012) reported mortality rates of 0.7-3% in one *I. typographus* generation.

Laboratory bioassay with *B. bassiana* showed high virulence of some isolates against bark beetles, especially of those obtained from pure cultures from infected natural populations (Draganova *et al*., 2010; Steinwender *et al*., 2010). Control measures with *B. bassiana* under natural conditions did not show clear results. In a study conducted over three years in a managed forest in Poland, no satisfactory effects in terms of controlling *I. typographus* populations infesting Norway spruce were found (Grodzki and Kosibowicz, 2015). Positive results were obtained when *B. bassiana* was sprayed on Norway spruce logs in the Czech Republic (Landa *et al*., 2007; 2008) and in Slovakia (Vakula *et al.*, 2010; Jakuš and Blaženec, 2011).

Media of *B. bassiana* against bark beetles usually contain spores being applied by direct spraying, dusting or in pheromone traps with dry fungal inoculums. In the latter

case, bark beetles are trapped and fungus-infested individuals are released afterwards (Kreutz *et al*., 2004; Grodzki and Kosibowicz, 2015). Kreutz *et al.* (2004) studied the effects of artificially initiated *B. bassiana* infections of *I. typographus* adults with the use of modified pheromone traps. The fungus was transmitted into bark beetle galleries and led to high infection rates. Moreover, a decreased intensity of bark beetle infestations as well as a reduced length of galleries was observed.

T. minor adults usually overwinter in the forest litter or in broken pine shoots. Therefore, adults could easily be infected with various fungi (Jankowiak, 2008). According to Hajek (1997), entomopathogenic fungi are dispersed by migrating insects which die somewhere else. As mentioned before, forest soil and litter are the main reservoirs for entomopathogenic fungi where species like *B. bassiana* develop on insect hosts (e.g. overwintering bark beetles) and as saprophyte on organic matter (Zimmermann, 2007; Augustyniuk-Kram and Kram, 2012). However, the long-term survival of *B. bassiana* seems to depend mainly on its spread to secondary hosts (Gottwald and Tedders, 1982). Moreover, *Beauveria* spp. are poor competitors for organic resources compared to other opportunistic saprophytic fungi that are ubiquitous in the forest litter and the soil (Keller and Zimmermann, 1989; Hajek, 1997). It seems more likely that conidia of the fungus remain in the soil or litter to infect new hosts. Nevertheless, the organic matter as potential source for building up fungus populations should be considered.

Reay *et al*. (2010) describe the ability of *B. bassiana* to colonize pine tissues as an endophyte. This may be a possible unique way of regulating cryptic species such as bark beetles difficult to control with common biological or chemical application techniques. Endophytic isolates of *B. bassiana* have been shown to reduce insect damage, probably as a result of *in planta* production of insecticidal metabolites (Vega *et al*., 2008).

Summing up, studies on other bark beetle species showed the potential of controlling populations with entomopathogenic fungi. More research has to be done to clarify the effects of the *B. bassiana* isolate found in this study on *T. minor* for developing control measures against this forest pest.

Conclusions

This is the first time that *T. minor* was found breeding on *Pinus nigra calabrica* in Sicily. We confirmed the occurrence of this beetle using morphological and molecular traits and characteristics of the breeding system. The occurrence of this species in Sicily was reported by Longo *et al.* (2001) but not yet by databases. These findings are important contributions to the knowledge of the distribution of this important forest insect.

Moreover, we diagnosed the entomopathogenic fungus *B. bassiana* in beetles from Sicily by morphological and cultural characteristics and by molecular traits for the first time. The origin of the fungal isolate can be especially interesting with regard to possible control measures against this bark beetle species.

Acknowledgements

We thank the Erasmus staff mobility program of the European Commission who financed some travels. We thank Diego Leone (University of Catania) for his help in locating infested trees, Andrea Stradner (BOKU University) who helped with dissection of beetles and Susanne Krumböck (BOKU University) for help with genetic lab work.

References

ALONZO-ZARAZAGA M. A., 2004.- Fauna Europaea: *Tomicus minor*.- *Fauna Europaea version 2.6.2*, [online] URL: http://www.faunaeur.org (accessed July 13, 2015).

AUGUSTYNIUK-KRAM A., KRAM K. J., 2012.- Entomopathogenic fungi as an important natural regulator of insect outbreaks in forests (review), pp. 265-295. In: *Forest ecosystems - more than just trees* (BLANCO J. A., LO Y-H., Eds).- InTech, Rijeka, Croatia.

AUNG O. M., SOYTONG K., HYDE K. D., 2008.- Diversity of entomopathogenic fungi in rainforests of Chiang Mai Province, Thailand.- *Fungal Diversity*, 30: 15-22.

BAŁAZY S., 2012.- Antagonistyczne oddziaływania pomiędzy bezkręgowcami a ich patogenami grzybowymi w żerowiskach kambio i ksylofagów, pp. 51-63. In: *Ochrana lasu - wybrane problemy historyczne i współczesne* (MAZUR A., Ed.).- Uniwersytetu Przyrodniczego w Poznaniu, Poznań, Poland.

BIDOCHKA M. J., KASPERSKI J. E., WILD G. A., 1998.- Occurrence of the insect-pathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near-northern habitats.- *Canadian Journal of Botany*, 76: 1198-1204.

BIDOCHKA M. J., MENZIES F. V., KAMP A. M., 2002.- Genetic groups of the insect-pathogenic fungus *Beauveria bassiana* are associated with habitat and thermal growth preferences.- *Archives of Microbiology*, 178 (6): 531-537.

BILLS G. F., POLISHOOK J. D., 1991.- Microfungi from *Carpinus caroliniana*.- *Canadian Journal of Botany*, 69: 1477-1482.

BORKOWSKI A., 2001.- Threats to pine stands by the pine shoot beetles *Tomicus piniperda* (L.) and *Tomicus minor* (Hart.) (Col., Scolytidae) around a sawmill in southern Poland.- *Journal of Applied Entomology*, 125: 489-492.

DOBERSKI J. W., TRIBE H. T., 1980.- Isolation of entomogenous fungi from elm bark and soil with reference to ecology of *Beauveria bassiana* and *Metarhizium anisopliae*.- *Transactions of the British Mycological Society*, 74: 95-100.

DRAGANOVA S., TAKOV D., DOYCHEV D., 2010.- Naturally occurring entomopathogenic fungi on three bark beetle species (Coleoptera: Curculionidae) in Bulgaria.- *Pesticides and Phytomedicine*, 25: 59-63.

EVANS H. C., 1982.- Entomogenous fungi in tropical forest ecosystems: an appraisal.- *Ecological Entomology*, 11 (1): 47-60.

FARGUES J., GOETTEL M. S., SMITS N., OUEDRAOGO A., ROUGIER M., 1997.- Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins.- *Mycologia*, 89: 383-392.

FERNÁNDEZ FERNÁNDEZ M. M., PAJARES ALONSO J. A., SALGADO COSTAS J. M., 1999.- Shoot feeding and overwintering in the lesser pine shoot beetle *Tomicus minor* (Col., Scolytidae) in north-west Spain.- *Journal of Applied Entomology*, 123: 321-327.

FOLMER O., BLACK M., HOEH W., LUTZ R., VRIJENHOEK R., 1994.- DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates.- *Molecular Marine Biology and Biotechnology*, 3 (5): 294-299.

FUXA J. R., TANADA Y., 1987.- *Epizootiology of insect diseases*.- Wiley-Interscience, New York, NY, USA.

GOTTWALD T. R., TEDDERS W. L., 1982.- Studies on conidia release by the entomogenous fungi *Beauveria bassiana* and *Metarhizium anisopliae* (Deuteromycotina, Hyphomycetes) from adult pecan weevil (Coleoptera, Curculionidae) cadavers.- *Environmental Entomology*, 11: 1274-1279.

GRODZKI W., KOSIBOWICZ M., 2015.- An attempt to use the fungus *Beauveria bassiana* (Bals.) Vuill. in forest protection against the bark beetle *Ips typographus* (L.) in the field.- *Leśne Prace Badawcze/Forest Research Papers*, 76 (10): 5-17.

HAJEK A. E., 1997.- Ecology of terrestrial fungal entomopathogens.- *Advances in Microbial Ecology*, 15: 193-249.

HALL T., 1999.- BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.- *Nucleic Acids Symposium Series*, 41: 95-98.

HEBERT P. D. N., CYWINSKA A., BALL S. L., DE WAARD J. R., 2003.- Biological identifications through DNA barcodes.- *Proceedings of the Royal Society of London B*, 270: 313-321.

HENDRICH L., MORINIERE J., HASZPRUNAR G., HEBERT P. D. N., HAUSMANN A., KOHLER F., BALKE M., 2015.- A comprehensive DNA barcode database for Central European beetles with a focus on Germany: adding more than 3500 identified species to BOLD.- *Molecular Ecology Resources*, 15 (4): 795-818.

HULCR J., ATKINSON T. H., COGNATO A. I., JORDAL B. H., MCKENNA D. D., 2015.- Morphology, taxonomy, and phylogenetics of bark beetles, pp. 41-84. In: *Bark beetles: biology and ecology of native and invasive species* (VEGA F. E., HOFSTETTER R. W., Eds).- Elsevier, London, UK.

HUMBER R., 1997.- Fungi: identification, pp. 153-163. In: *Manual of techniques in insect pathology* (LACEY L. A., Ed.).- Academic Press, San Diego, CA, USA.

HUMBER R. A., HANSEN K. S., 2005.- Beauveria *and allied fungi*.- USDA-ARS Plant Protection Research Unit, US Plant, Soil & Nutrition Laboratory, Ithaca, NY, USA.

INGLIS G. D., ENKERLI J., GOETTEL M. S., 2012.- Laboratory techniques used for entomopathogenic fungi: Hypocreales, pp. 189-253. In: *Manual of techniques in invertebrate pathology* (LACEY L. A., Ed.).- Academic Press, Amsterdam, The Netherlands.

JACKSON T. A., ALVES S. B., PEREIRA R. M., 2000.- Success in biological control of soil-dwelling insects by pathogens and nematodes, pp. 271-296. In: *Biological control: measures of success* (GURR G., WRATTEN S., Eds).- Kluwer Academic Publisher, Dordrecht, The Netherlands.

JAKUŠ R., BLAŽENEC M., 2011.- Treatment of bark beetle attacked trees with entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin.- *Folia Forestalia Polonica. Series A-Forestry*, 53 (2): 150-155.

JANKEVICA L., 2004.- Ecological associations between entomopathogenic fungi and pest insects recorded in Latvia.- *Latvijas Entomologs*, 41: 60-65.

JANKOWIAK R., 2008.- Fungi associated with *Tomicus minor* on *Pinus sylvestris* in Poland and their succession into the sapwood of beetle-infested windblown trees.- *Canadian Journal of Forest Research*, 38: 2579-2588.

KARPIŃSKI J. J., 1935.- Przyczny ograniczające rozmnażanie się korników drukarzy (*Ips typographus* L. i *Ips duplicatus* Sahlb.) w lesie pierwotnym.- *Instytut Badawczy Lasów Państwowych, Warszawa, Pologne, Rozprawy I sprawozdania, Ser. A*, 15: 1-86.

KELLER S., ZIMMERMANN G., 1989.- Mycopathogens of soil insects, pp. 240-270. In: *Insect-fungus interactions* (WILDING N., COLLINS N. M., HAMMOND P. M., WEBBER J. F., Eds).- Academic Press, London, UK.

KNÍŽEK M., BEAVER R., 2004.- Taxonomy and systematic of bark and ambrosia beetles, pp. 41-54. In: *Bark and wood boring insects in living trees in Europe, a synthesis* (LIEUTIER F., DAY K. R., BATTISTI A., GRÉGOIRE J.-C., EVANS H. F., Eds).- Kluwer Academic Publishers, Dordrecht, The Netherlands.

KOHLMAYR B., 2001.- Zum Auftreten von Krankheitserregern in den Europäischen Waldgärtnerarten *Tomicus piniperda*, *Tomicus minor* und *Tomicus destruens* (Coleoptera; Scolytidae). *PhD thesis*, University of Natural Resources and Life Sciences, Vienna, Vienna, Austria.

KREUTZ J., ZIMMERMANN G., VAUPEL O., 2004.- Horizontal transmission of the entomopathogenic fungus *Beauveria bassiana* among the spruce bark beetle, *Ips typographus* (Col., Scolytidae) in the laboratory and under field conditions.- *Biocontrol Science and Technology*, 14 (8): 837-848.

LANDA Z., KŘENOVÁ Z., VOJTĚCH O., 2007.- Využiti houby *Beauveria bassiana* w ochraně proti lýkožroutu smrkovému.- *Lesnická práce*, 10: 14-15.

LANDA Z., KALISTA M., KŘENOVÁ Z., VOJTĚCH O., 2008.- Praktické využiti entomopatogenni houby *Beauveria bassiana* proti lýkožroutu smrkovému *Ips typographus*, pp. 62-71. In: *Ekologické metody ochrany lesa před podkornim hmyzem* (VOJTĚCH O., ŠUSTR P., Eds).- Sborniky z výzkumu na Šumavé-sešit 1, Správa NP a CHKO, Šumava, Vimperk, Czech Republic.

LARKIN M. A., BLACKSHIELDS G., BROWN N. P., CHENNA R., MCGETTIGAN P. A., MCWILLIAM H., VALENTIN F., WALLACE I. M., WILM A., LOPEZ R., THOMPSON J. D., GIBSON T. J., HIGGINS D. G., 2007.- Clustal W and Clustal X version 2.0.- *Bioinformatics*, 23: 2947-2948.

LONGO S., BARBAGALLO S., RAPISARDA C., TROPEA GARZIA G., MAZZEO G., SISCARO G., BELLA S., 2001.- Note sull'artropodofauna degli ambienti forestali e agrari del parco dell'Etna.- *Tecnica Agricola*, 53 (3-4): 3-61.

LUNDGREN J. G., 2004.- Exotic forest pest information system for North America: *Tomicus minor*.- North American Forest Commission, EXFOR Database, [online] URL: http//:download.ceris.purdue.edu, (accessed 2015/01/14).

MWAMBURI L. A., LAING M. D., MILLER R. M., 2015.- Effect of surfactants and temperature on germination and vegetative growth of *Beauveria bassiana*.- *Brazilian Journal of Microbiology*, 46: 67-74.

PFEFFER A., KNÍŽEK M., ZUMR V., ZUBER M., 1995.- *Zentral- und westpaläarktische Borken- und Kernkäfer*.- Pro Entomologica, Naturhistorisches Museum Basel, Basel, Switzerland.

POSTNER M., 1974.- Scolytidae (=Ipidae), Borkenkäfer, pp. 334-487. In: *Die Forstschädlinge Europas. Bd. 2 Käfer*. (SCHWENKE W., Ed.).- P. Parey, Hamburg, Berlin, Germany.

REAY S. D., BROWNBRIGDE M., GICQUEL B., CUMMINGS N. J., NELSON T. L., 2010.- Isolation and characterization of endophytic *Beauveria* spp. (Ascomycota: Hypocreales) from *Pinus radiata* in New Zealand forest.- *Biological Control*, 54: 52-60.

RÓŻALSKA S., PAWŁOWSKA J., WRZOSEK M., TKACZUK C., DŁUGOŃSKI J., 2013.- Utilization of 4-n-nonylphenol by *Metarhizium* sp. isolates.- *Acta Biochimica Polonica*, 60: 677-682.

RUIZ-PORTERO C., BARRANCO P., DE LA PEÑA J., CABELLO T., 2002.- Bioensayo con entomopatógenos para el control de escolídos plagas forestales (Col.: Scolytidae).- *Boletin de Sanidad Vegetal*, 28: 367-373.

SAMSON R. A., EVANS H. C., LAGÉ J.-P., 1988.- *Atlas of entomopathogenic fungi*.- Springer, Berlin, Germany.

SCHOCH C. L., SEIFERT K. A., HUHNDORF S., ROBERT V., SPOUGE J. L., LEVESQUE C. A., CHEN W., 2012.- Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi.- *Proceedings of the National Academy of Sciences of the United States of America*, 109: 6241-6246.

SCHROEDER L. M., LINDELÖW Å., 2002.- Attacks on living spruce trees by the bark beetle *Ips typographus* (Col.: Scolytidae) following a storm felling: a comparison between stands with and without removal of wind-felled trees.- *Agricultural and Forest Entomology*, 4: 47-56.

STEINWENDER B. M., KRENN H. W., WEGENSTEINER R., 2010.- Different effects of the insectpathogenic fungus *Beauveria bassiana* (Deuteromycota) on the bark beetle *Ips sexdentatus* (Coleoptera: Curculionidae) and on its predator *Thanasimus formicarius* (Coleoptera: Cleridae).- *Journal of Plant Diseases and Protection*, 117 (1): 33-38.

STOCH F., 2003.- *Checklist of the species of the Italian Fauna*.- [online] URL: http://www.faunaitalia.it/checklist/ (accessed July 13, 2015).

TAMURA K., STECHER G., PETERSON D., FILIPSKI A., KUMAR S., 2013.- MEGA6: Molecular Evolutionary Genetics Analysis version 6.0.- *Molecular Biology and Evolution*, 30: 2725-2729.

TAYLOR D. L., HERRIOTT I. C., LONG J., O'NEILL K., 2007.- TOPO TA is A-OK: a test of phylogenetic bias in fungal environmental clone library construction.- *Journal of Environmental Microbiology*, 9 (5): 1329-1334.

VAKULA J., VARKONDA Š., GALKO J., GUBKA A., KUNCA A., ZÚBRIK M., 2010.- Rozvoj sučasných technických možnosti pri štúdiu niektorých spôsobov aplikácie entomopatogénnej huby *Beauveria bassiana* v rámci biologických metód ochrany lesa, pp. 63-67. In: *Aktuálne problemy v ochrane lesa* (KUNCA A., Ed.).- Národné lesnicke centrum Zvolen, Slovakia.

VEGA F. E., POSADA E., AIME M. C., PAVA-RIPOLL M., INFANTE F., REHNER S. A., 2008.- Entomopathogenic fungal endophytes.- *Biological Control*, 46: 72-82.

VEGA F. E., MEYLING N. V., LUANGSAARD J. J., BLACKWELL M., 2012.- Fungal entomopathogens, pp. 171-220. In: *Insect pathology, second edition* (VEGA F. E., KAYA H .K., Eds).- Academic Press, San Diego, CA, USA.

WEGENSTEINER R., 2004.- Pathogens in bark beetles, pp. 291-313. In: *European bark and wood boring insects in living trees, a synthesis* (LIEUTIER F., DAY K. R., BATTISTI A., GRÉGOIRE J.-C., EVANS H. F., Eds).- Kluwer Academic Publishers, Dordrecht, The Netherlands.

WEGENSTEINER R., TKACZUK C., BAŁAZY S., GRIESSER S., ROUFFAUD M.-A., STRADNER A., STEINWENDER B., HAGER H., PAPIEROK B., 2015a.- Occurrence of pathogens in populations of *Ips typographus*, *Ips sexdentatus* (Coleoptera, Curculionidae, Scolytinae) and *Hylobius* spp. (Coleoptera, Curculionidae, Curculioninae) from Austria, Poland and France.- *Acta Protozoologica*, 54: 219-232.

WEGENSTEINER R., WERMELINGER B., HERRMANN M., 2015b.- Natural enemies of bark beetles: predators, parasitoids, pathogens and nematodes, pp. 247-304. In: *Bark beetles: biology and ecology of native and invasive species* (VEGA F. E., HOFSTETTER R. W., Eds).- Elsevier, London, UK.

WHITE T. J., BRUNS T. D., LEE S., TAYLOR J. W., 1990.- Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315-322. In: *PCR protocols: a guide to methods and applications* (INNIS M. A., GELFAND D. H., SNINSKY J. J., WHITE T. J., Eds).- Academic Press, New York, NY, USA.

ZIMMERMANN G., 2007.- Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*.- *Biocontrol Science and Technology*, 17 (5/6): 553-596.

Authors' addresses: Eustachio TARASCO (corresponding author: eustachio.tarasco@uniba.it), Department of Plant, Soil & Food Sciences, University of Bari "Aldo Moro", Bari, Italy; Martin SCHEBECK, Rudolf WEGENSTEINER, Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences Vienna (BOKU), Vienna, Austria; Marta WRZOSEK, Department of Molecular Phylogenetics and Evolution, University of Warsaw, Warsaw, Poland; Mirella CLAUSI, Department of Biological, Geological and Environmental Sciences, Animal Biology Section, University of Catania, Catania, Italy; Cezary TKACZUK, Department of Plant Protection and Breeding, Siedlce University, Siedlce, Poland.

Received January 22, 2016. Accepted September 2, 2016.