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Pathogens and nematodes associated to three bark beetle species of the genus *Orthotomicus* (Coleoptera Curculionidae) in central-south Europe

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Abstract

The aim of the research was to determine the occurrence of insect's pathogens and nematodes in *Orthotomicus laricis* (F.), *Orthotomicus erosus* (Wollaston), and *Orthotomicus nobilis* (Wollaston) in central-south Europe. We found three insect's pathogens *Chytridiopsis* sp., *Gregarina* sp., *Mattesia* sp. and nonspecific gut nematodes and nematodes in the haemolymph, where adult nematodes were identified as *Contortylenchus laricis* (Fuchs). A total of 1,183 beetles from galleries in pine bark were dissected and examined; these included 912 *O. laricis* beetles from seven localities, 164 *O. erosus* beetles from three localities, and 107 *O. nobilis* beetles from one locality. At one locality, microsporidium *Chytridiopsis* sp. was detected in almost 15% of the *O. laricis* beetles. Protozoan pathogen *Gregarina* sp. was found in *O. laricis* beetles at three localities, but its incidence did not exceed 5%. *Chytridiopsis* sp., *Gregarina* sp., and neogregarine *Mattesia* sp. were detected in *O. erosus* beetles at one of three localities. No pathogen was found in *O. nobilis*. The genus *Mattesia* was reported in an *Orthotomicus* species for the first time. The incidence of pathogens and nematodes in the three *Orthotomicus* species seemed unrelated to the beetle species: the deposition of eggs singly or in clusters, the ability to outbreak, or the beetle distribution range. A literature review and the current results indicated that the number of pathogens detected in a particular species of pine bark beetle is related mainly to the number of specimens examined.

Key words: bark beetle, Scolytinae, pine, gregarine, microsporidium, *Mattesia*, *Orthotomicus*, pathogens, nematodes.

Introduction

Orthotomicus bark beetles are frequently detected in international ports and in plant quarantine inspections (Rassati *et al*., 2015). They are often found in timber, wood products, and woody packaging materials (Knížek, 2006). Several species have been introduced to new areas, such as *Orthotomicus caelatus* (Eichhoff) and *Orthotomicus erosus* (Wollaston) to South Africa, *Orthotomicus proximus* (Eichhoff) to Madagascar, *Orthotomicus angulatus* (Eichhoff) to many Pacific areas, and *Orthotomicus laricis* (F.) to South Africa and Chile (Wood and Bright, 1992; Brockerhoff *et al*., 2006; Knížek, 2006). In the USA, the introduction of *O. erosus* has raised concerns about its further spread (Colunga-Garcia *et al*., 2010). The potential for damage caused by introduced *Orthotomicus* spp. has led to intensive efforts to develop pheromone lures for *O. erosus* (Giesen *et al*., 1984; Klimetzek and Vite, 1986; Mendel, 1988; Paiva *et al*., 1988; Seybold *et al*., 2006; Fan *et al*., 2010) and for *Orthotomicus latidens* (LeConte) (Miller *et al*., 2005; Steed and Wagner, 2008).

Only a few papers have reported on the pathogens (Wegensteiner, 2004; Takov *et al*., 2011; 2012) and nematodes (Rühm, 1956; Korentchenko, 1987; 1992) associated to *Orthotomicus* bark beetles, and the number of dissected beetles has been small. There is no known pathogen of *O. laricis*, but only nine individuals have been studied (Takov *et al*., 2011). Protozoan pathogen *Gregarina* sp. and microsporidia *Chytridiopsis typographi* Weiser were found in *O. erosus* (Purrini and Weiser, 1985; Takov *et al*., 2011). Species spectrum of pathogens was analysed for the first time in our study. On the other hand, pathogen infection rates were high in the small number of *Orthotomicus longicollis* (Gyllenhal) that were studied (Takov *et al*., 2012).

The first aim of the current research was to determine pathogen infection levels and nematode parasitism rates in three species of *Orthotomicus* bark beetles in their native distribution range: *O. laricis*, *O. erosus*, and *Orthotomicus nobilis* (Wollaston). These species differ in geographical range, life history, and damage potential. *O. laricis* has an extensive Palearctic range, does not cause damage, and lays dusters of eggs in the mother gallery (Schwenke, 1972; Knížek, 2006). *O. erosus* is considered a polygamous pest across the Mediterranean and southern Europe, Asia and North Africa (Gregoire and Evans, 2004). *O. nobilis* is an endemic species with an area from the Canary Islands. The last two species lay eggs individually in the mother gallery; the larvae dig galleries, which are generally perpendicular to the axis of the mother galleries (Schwenke, 1972; Knížek, 2006). The second aim of this study was to determine whether the infection levels of pathogens in *Orthotomicus* species and in other bark beetles living on pines are associated with beetle bionomic characteristics and outbreak performance. We assume that the more abundant and widespread species of bark beetles would have relatively high infection rates because the contact between individuals in intersecting galleries is likely to increase as the population density increases.

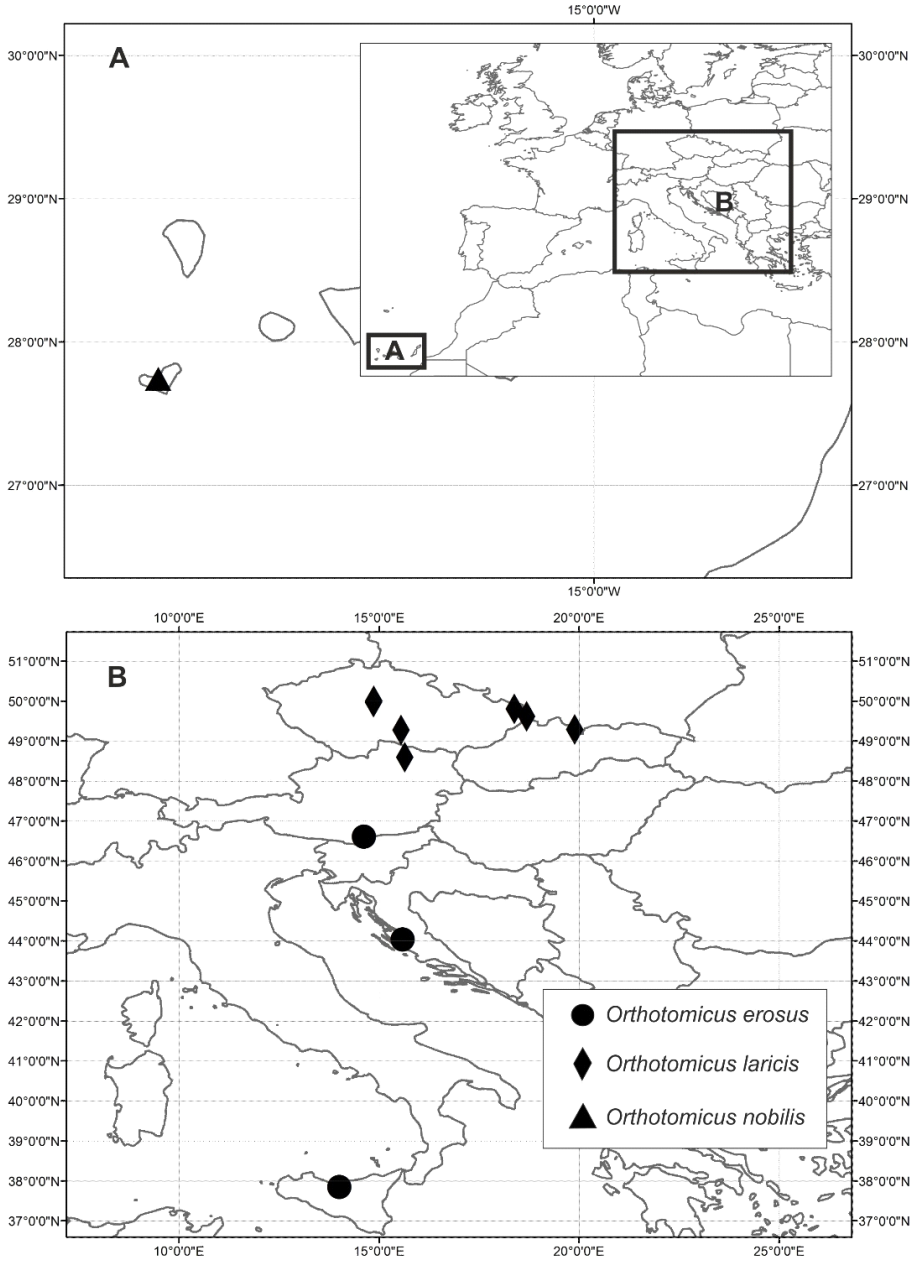

Figure 1. Localities in Europe where beetles of *Orthotomicus* species were collected.

Materials and methods

Beetles were collected from *Picea* or *Pinus* trees at 10 localities in two ways (figure 1, table 1). We (i) directly collected mature beetles in mature galleries under bark from one to three different trees per site from April to July, or (ii) we sampled pine bark from stumps after logging that contained overwintering beetles from five to ten trees per site in February or March (table 1). For the second method, pine bark was placed in emergence traps in the laboratory at 20 °C. All collected beetles were placed in 2 ml Eppendorf microtubes (ca 50 beetles per microtube), which contained a piece of damp gauze to maintain 100% relative humidity. The beetles were immediately frozen and stored at −4 °C. All internal organs, including the fat body, were later dissected in a water drop using surgical tweezers. Each dissected beetle was examined with a Nikon Eclipse Ni microscope at 40-400× magnification to determine its sex and to determine whether it contained one or more pathogens. The presence and identify of viruses, microsporidia, protozoa, and nematodes were determined under the light microscope according to reports summarised by Wegensteiner (2004). For each *Orthotomicus* sp. at each locality, the number of males vs. females with pathogens or nematodes was compared with a χ^2 test.

Data from the current study and from previously published studies concerning bark beetles of pines were reviewed to determine which of the following factors were most closely associated with the number of pathogen species detected per bark beetle species: host tree (according to Pfeffer 1989; a continuous independent variable), sexual behaviour (monogamy vs. polygamy; a categorical independent variable), outbreak ability

Table 1. Details of studied localities. Mature beetles (MB) were obtained directly from infested trees, and overwintering beetles (OB) were obtained from infested pine bark that was collected in the field and incubated in emergence traps in the laboratory. O.e. = *Orthotomicus erosus*; O.l. = *Orthotomicus laricis*; O.n. = *Orthotomicus nobilis.*

Locality	Species	Latitude	Longitude	Altitude (m a.s.l.)	Year of sampling	MB or OB	Tree species
Benkovac (HR)	O.e.	44.0417142N	15.5896736E	153	2010	MB	pine
Polizzi Generosa (I)	O.e.	37.8487153N	14.0069958E	1,180	2009	MB	pine
Wasserhofen (A)	O.e.	46.6147222N	14.6202778E	440	2000	MB	pine
Bystřice (CZ)	O.l.	49.6176681N	18.6914086E	440	2012	MB	spruce
Dolina Koscieliska (PL)	O.l.	49.2907178N	19.8885728E	1,026	2016	OB	spruce
Goldberg (A)	O.l.	48.5938889N	15.6425000E	375	2000	MB	pine
Kostelec N.Č.L. (CZ)	O.l.	50.0040633N	14.8666322E	345	2013	OB	pine
Kostelec N.Č.L. (CZ)	O.l.	50.0040633N	14.8666322E	345	2014	OB	pine
Kostelec N.Č.L. (CZ)	O.l.	49.9752925N	14.8613858E	415	2016	OB	pine
Lužná (CZ)	O.l.	50.1237703N	13.7700422E	348	2016	OB	pine
Pežgov (CZ)	O.l.	49.8068131N	18.3853578E	290	2012	MB	spruce
Stonařov (CZ)	O.l.	49.2762394N	15.5517864E	640	2014	MB	spruce
El Pinar (E)	O.n.	27.7435097N	18.0381783W	437	2016	MB	pine

(according to Postner 1974; a categorical independent variable), and number of specimens dissected (a continuous independent variable). The number of known pathogen (from the current study and from previously published studies) was used as the dependent variable in the generalized linear model (GLZ) using a Poisson distribution. All statistical analyses were performed with Statistica 12.0 software (Dell Inc., Round Rock, TX, USA).

Results

In total, we dissected 1,183 beetles: 912 *O. laricis* beetles from seven localities (471 females and 441 males), 164 *O. erosus* beetles (103 females and 61 males) from three localities, and 107 *O. nobilis* beetles (57 females and 50 males) from one locality. At most localities, gut nematodes but no pathogens were detected, i.e., pathogens were detected in beetles in only a small number of localities. The parasitism rate for gut nematodes ranged from 3 to 20% in *O. laricis* and *O. erosus* but sometimes exceeded 70% in *O. nobilis* (table 2).

The percentage of beetles with nematodes in the haemolymph ranged from 3 to 25% in the three *Orthotomicus* species. The adult nematodes in the haemolymph were identified as *Contortylenchus laricis* (Fuchs).

Three pathogens were found (*Chytridiopsis* sp., *Gregarina* sp., and *Mattesia* sp.). *Chytridiopsis* sp. was detected in *O. laricis* and infected almost 15% of the beetles at one locality. *Gregarina* sp. was found in *O. laricis* at three localities but the infection level did not exceed 5% (table 2). In *O. laricis* were infected 3.2% females and 0.5% males of the beetles by *Gregarina* sp.. *Chytridiopsis* sp. infected 0.2% males and no females.

Chytridiopsis sp., *Gregarina* sp., and *Mattesia* sp. were found in *O. erosus* at one of the three localities with this beetle (table 2). In *O. erosus* were infected 1.9% females and 3.3% males of the beetles by *Chytridiopsis* sp.; 8.7% females and 11.5% males of the beetles were infected by *Gregarina* sp.. *Mattesia* sp. were infected only 1% males. No pathogen was found in *O. nobilis* (table 2). Proportions of males and females with infections were equal in all cases (table 2).

Information regarding pathogens of the main bark beetle species on pines in Europe, based on the current data and on previously published data, is summarized in table 3. Ten pathogens have been reported from bark beetles on pine in Europe. More pathogen species have been reported from *Pityogenes chalcographus* (L.) and *Ips amitinus* (Eichhoff) than from the other bark beetles (table 3) but this was significantly associated with the number of dissected beetles ($\chi^2 = 9.25$; $p < 0.01$), which was much greater for *P. chalcographus* and *I. amitinus* than for the other species, but in most cases the samples were analysed from spruce. The number of pathogens reported for a species was not significantly associated with outbreak ability ($\chi^2 = 0.17$), monogamy/polygamy ($\chi^2 = 1.94$), or number of host species ($\chi^2 = 0.31$).

Discussion and conclusions

In the current study, the pathogens *Chytridiopsis* sp., *Gregarina* sp., and *Mattesia* sp. were detected in *O. laricis* and *O. erosus* in Europe. No pathogen was found in *O. nobilis*.

Because only a small number of *O. laricis* beetles have been studied for the presence of pathogens (Takov *et al.*, 2011), it is impossible to compare the number of pathogens reported from this species with those reported from other species. One report from Germany mentions that *C. typographi* was found in *O. laricis* but no quantitative data were provided (Purrini and Weiser, 1985). The protozoan pathogen *Gregarina* sp. and the microsporidium *Chytridiopsis* sp. have been reported in *O. longicollis* but at higher infection rates (Takov *et al.*, 2012). *Gregarina typographi* Fuchs and *C. typographi* are both the quite common and nonspecific pathogens of bark beetles. It has been found in the midgut lumen in a number of members of the Scolytinae subfamily (Wegensteiner,

Table 2. The percentages of beetles of the three *Orthotomicus* species containing the indicated nematodes and pathogens, and comparisons of percentages of males vs. females with a particular pathogen or nematode based on χ^2 tests. O.e. = *Orthotomicus erosus*; O.l. = *Orthotomicus laricis*; O.n. = *Orthotomicus nobilis*; N = number of beetles; *Chytridiopsis* sp., *Gregarina* sp., *Mattesia* sp. = pathogens; Ni = gut nematodes; Nh = haemolyph nematodes; *C. laricis* = adult of haemolyph nematodes; ns = not studied; n.s. = nonsignificant.

Locality	Species	N	*Chytridiopsis* sp. (%)	χ^2	*Gregarina* sp. (%)	χ^2	*Mattesia* sp. (%)	χ^2	*C. laricis* (%)	χ^2	Ni (%)	χ^2	Nh (%)	χ^2
Benkovac (HR)	O.e.	72	4.2	1.4 n.s.	-		-		-		19.4	2.3 n.s.	2.8	1.1 n.s.
Polizzi Generosa (I)	O.e.	53	-		-		-				ns			
Wasserhofen (A)	O.e.	39	2.7	0.6 n.s.	37.2	0 n.s.	2.6	0.2 n.s.			ns			
Bystřice (CZ)	O.l.	21	-		-		-		20.0	1.9 n.s.	-	0.7 n.s.	20.0	0.6 n.s.
Dolina Koscieliska (PL)	O.l.	163	-		-		-		2.5	0.9 n.s.	-	1.7 n.s.	7.4	0.3 n.s.
Goldberg (A)	O.l.	7	14.3	1.0 n.s.	-		-				ns			
Kostelec N.Č.L. (CZ)	O.l.	315	-		2.9	1.6 n.s.	-		1.6	0.7 n.s.	3.8	2.4 n.s.	14.0	0.1 n.s.
Kostelec N.Č.L. (CZ)	O.l.	172	-		-		-		4.1	0.1 n.s.	8.1	0.2 n.s.	17.4	1.3 n.s.
Kostelec N.Č.L. (CZ)	O.l.	47	-		-		-		-	0.7 n.s.	-		25.5	1.2 n.s.
Lužná (CZ)	O.l.	29	-		3.5		-		-		3.4	0.3 n.s.	6.9	0.6 n.s.
Pežgov (CZ)	O.l.	5	-		4.8	0.7 n.s.	-		4.8	1.0 n.s.	-	1.5 n.s.	4.8	1.5 n.s.
Stonařov (CZ)	O.l.	153	-		3.5	1.8 n.s.	-		4.6	1.3 n.s.	7.0	0.6 n.s.	20.4	0.2 n.s.
El Pinar (E)	O.n.	107	-		-		-		-	2.1 n.s.	66.3	0.4 n.s.	8.4	0.0 n.s.

2004; Takov *et al*., 2010). These pathogens are currently not considered a lethal pathogens and are characterized as having very low virulence (Yaman, 2007; Wegensteiner *et al*., 2010; Lukášová and Holuša, 2012).

The current study provides the first report of *Mattesia* (a neogregarine genus) in an *Orthotomicus* species. As pathogens, *Mattesia* spp. have been detected in the adipose tissue of bark beetles (Wegensteiner, 2004). *Mattesia schwenkei* (Purrini) is a common species in *Ips typographus* (L.) and other species (Purrini, 1978; Händel *et al*., 2003). This disease causes high mortality of beetles during the overwintering in the bark (Weiser et al., 2000). *M. schwenkei* was first described from *Dryocoetes autographus* (Ratzeburg) (Purrini, 1977). We did not determine the species of *Mattesia* detected in the current study (doing so would require DNA analysis).

We also didn't analyse in detail the species spectrum of fungi of *Orthotomicus* species. E.g. many papers refer to bark beetle pathogenic fungi, mostly *Beauveria* spp. and several assays have even been performed to test their possible use in bark beetle control and has been observed naturally killing the adults *O. erosus* and *O. laricis* (Wegensteiner, 2004; Wegensteiner *et al*., 2015a; Lieutier *et al*., 2016).

Most gut nematodes in bark beetles are parasitic species that are difficult to identify because they are usually parasitic juveniles that lack distinguishing morphological characteristics, and because identification in certain groups is impossible without preservation (Rühm, 1956). Our results were in line with a previously reported 17.6% parasitism rate of intestinal nematodes in *O. longicollis* (Takov *et al*., 2012). The nematode *C. laricis*, which was found in the haemolymph of beetles in the current study, has been reported from *Orthotomicus suturalis* (Gyllenhal) and *O. laricis* (Rühm, 1956).

According to our results and literature data, the levels of pathogens detected in *Orthotomicus* spp. seemed un-

Table 3. Summary of information concerning pathogens of bark beetles that attack pine trees in Europe. The data include those of the current and previous studies. P = polygamous; M = monogamous. References: [1]Takov *et al*., 2007; [2]Takov *et al*., 2010; [3]Takov *et al*., 2011; [4]Takov and Pilarska, 2008; [5]Yaman, 2016; [6]Zitterer, 2002; [7]Wegensteiner *et al*., 2014; [8]Haidler, 1998; [9]Händel *et al*., 2003; [10]Lukášová *et al*., 2013; [11]Holuša *et al*., 2016; [12]Unal, 2009; [13]Wegensteiner *et al*., 2015b; [14]Yaman, 2007; [15]Weiser, 1955; [16]Purrini, 1978; [17]Kohlmayr *et al*., 2003.

Species	Monogamous/polygamous	Host trees	Outbreak ability	*Cannigia tomici*	*G. typographi* and *Gregarina* sp.	*Chytridiopsis typographi*	ItEPV	*Malamoeba scolyti*	*Mattesia schwenkei*	*Menzbieria chalcographi*	*Metschnikowia typographi*	*Microsporidium* sp.	*Unikaryon montanum*	Numbers of dissected beetles	References
Ips acuminatus (Gyllenhal 1827)	P	*Pinus* sp.	Yes		x	x		x	x					1,470	1, 2, 3, 4, 5, 6
Ips amitinus (Eichhoff 1871)	P	*Pinus* sp., *Picea* sp.	Yes		x	x	x	x	x		x	x	x	4,816	7, 8, 9, 10, 11
Ips sexdentatus (Boerner 1767)	P	*Pinus* sp., *Picea* sp.	Yes		x	x					x			1,874	1, 2, 3, 4, 12, 13, 14
Orthotomicus erosus (Wollaston 1857)	P	*Pinus* sp.	Yes		x	x			x					264	3, 4, this study
Orthotomicus laricis (F. 1792)	M	*Pinus* sp., *Picea* sp., *Larix* sp., *Abies* sp.	No		x	x								771	3, 4, this study
Orthotomicus longicollis (Gyllenhal 1827)	M	*Pinus* sp.	No		x	x								20	3
Orthotomicus nobilis (Wollaston 1862)	P	*Pinus* sp.	No			x								107	this study
Orthotomicus proximus (Eichhoff 1867)	M	*Pinus* sp.	No		x	x								25	1, 3
Pityogenes chalcographus (L. 1761)	P	*Pinus* sp., *Picea* sp., *Larix* sp., *Pseudotsuga* sp.	Yes		x	x		x	x	x			x	6,174	1, 2, 3, 7, 8, 9, 15, 16
Tomicus minor (Hartig 1834)	M	*Pinus* sp.	Yes		x									13	3
Tomicus piniperda (L. 1758)	M	*Pinus* sp.	Yes	x										2,472	3, 4, 17

related to beetle oviposition behaviour (deposition of single eggs or clusters of eggs), outbreak vs. non-outbreak species, or the distribution area. The levels of nematodes were similar in *O. laricis* and *O. erosus* but were much higher in *O. nobilis* than in the other two species. Pathogens were found in *O. laricis* at three of the seven localities and were found in *O. erosus* in two of the three localities. No pathogens were detected in *O. nobilis*, which was studied at only one isolated locality. Pathogen persistence in a population of beetles probably requires some stable and minimum host density, and pathogens are likely to increase more rapidly in populations of polygamous than monogamous species.

Regardless of the pathogen, the risk of infection appeared similar for both sexes of all three *Orthotomicus* spp. regardless life cycle or bionomy of species, which is consistent with Wegensteiner *et al*. (1996) and Lukašová and Holuša (2011). Both sexes come in contact with pathogen-contaminated faeces at the same time during breeding, egg-laying, and removal of frass from galleries. These findings show that the risk of infection is similar for both sexes and that the infection spreads more or less evenly among males and females.

The largest numbers of pathogen species of pine bark beetles have been reported from *P. chalcographus* and *I. amitinus* (table 3), but only from spruce. These polygamous bark beetles are highly studied and have outbreak ability. Our review of the literature and the current data suggests that the most important factor explaining the number of pathogens detected in a particu-

lar species is the number of specimens examined, which probably reflects the real abundance of the species in nature. For species of bark beetles with low density, pathogen levels may be low because the beetles do not encounter individuals from other galleries, pathogen spores are only transferred among beetles in one gallery system, and infection of other beetles by faeces and the remains of dead bodies is infrequent (Wegensteiner and Weiser, 1996). In addition to being in close contact with conspecifics, species with high abundance are also in close contact with other species sharing the same host. The possibility of natural transfer between species has been confirmed in the laboratory, for instance *Malamoeba scolyti* (Purrini) was transferred from *D. autographus* to many other bark beetle species (Kirchhoff, 1982; Kirchhoff and Führer, 1990).

Overall, we infer that although *Orthotomicus* species are common, their numbers are usually too low to support substantial levels of pathogens.

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