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Metschnikowia cf. *typographi* and other pathogens from the bark beetle *Ips sexdentatus* – Prevalence, histological and ultrastructural evidence, and molecular characterization

Regina G. Kleespies [a,*], Young Woon Lim [b], Cezary Tkaczuk [c], Marta Wrzosek [d], Bernhardt M. Steinwender [e,f], Rudolf Wegensteiner [f]

[a] Julius Kühn-Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Biological Control, Heinrichstrasse 243, 64287 Darmstadt, Germany
[b] School of Biological Sciences, Seoul National University, Seoul 151-747, Republic of Korea
[c] Siedlce University of Natural Sciences and Humanities, Department of Plant Protection and Breeding, Prusa 14, 08-110 Siedlce, Poland
[d] Biological and Chemical Research Center, Faculty of Biology, Al. Żwirki i Wigury 101, Warsaw, Poland
[e] University of Copenhagen, Department of Plant and Environmental Sciences, Denmark
[f] University of Natural Resources and Life Sciences, Vienna, Gregor Mendel Str. 33, A-1180 Vienna, Austria

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ABSTRACT

Ips sexdentatus (six-spined engraver beetle) from Austria and Poland were dissected and examined for the presence of pathogens. Specimens collected in Austria were found to contain the ascomycetous fungus *Metschnikowia* cf. *typographi*. Infection rates ranged from 3.6% to 26.8% at different collection sites. *M.* cf. *typographi* infected midguts were investigated by histological, ultrastructural and molecular techniques. Extraordinary ultrastructural details are shown, such as ascospores with bilateral flattened flanks resembling alar rims at both sides of their attenuating tube-like ends. These have not yet been described in other yeast species. Molecular investigations showed a close phylogenetic relationship to the fungi *Metschnikowia agaves* and *Candida wancherniae*. Presence of the entomopathogenic fungus *Beauveria bassiana* found in Austria was confirmed both morphologically and molecularly. The eugregarine *Gregarina typographi* was diagnosed most frequently. Infection rates of all *I. sexdentatus* specimens ranged from 21.4% to 71.9% in Austria and 54.1% to 68.8% in Poland. Other entomopathogenic protists, bacteria, or viruses were not detected.

1. Introduction

Altogether, 39 *Metschnikowia* species are listed. Some are found in sea-water, freshwater crustaceans, or insects, and are suggested to cause host mortality. Others have been isolated from plants (flowers, fruits, floral nectar, floral honey or honeydew), some of which have antimicrobial activity against yeasts (Magyar et al., 2005; Lachance, 2011; Guzmán et al., 2013; Oro et al., 2014). *Metschnikowia* spp. described from insects have been detected mainly in the gut lumen or on eggs, in most cases in flower-associated insects (Lachance, 2011). However, *M. pulcherrima* has been isolated from *Aesculus*, *Betula* and *Salix* exudates (Lachance, 2011), and from rotten wood in *Quercus* and other tree species (Hui et al., 2013).

Metschnikowia typographi (Ascomycota, Metschnikowiaceae) was originally described from two bark beetle species, *Ips typographus* (L.) and *Ips amitinus* (Eichhoff, 1871). Both species have been collected on Norway spruce (*Picea abies* Karst) in Austria and Finland (Weiser et al., 2003; Wegensteiner and Weiser, 2009). *M. typographi* was described based on morphological description of asci in infected midguts from the limited number of available electron micrographs (Weiser et al., 2003). Unfortunately, this species was not cultivable on any medium. Later, *Metschnikowia* cf. *typographi* was found in *Ips sexdentatus* (Börner) (Coleoptera, Curculionidae) from Austria (Wegensteiner et al., 2007). The same yeast was subsequently reported in *I. sexdentatus* (Unal et al., 2009) and *Dendroctonus micans* (Kugelann) (Yaman and Radek, 2008) in Turkey. Until now, all of these studies were based upon morphological data of the asci and have been mainly conducted by light microscopy, with the exception of one micrograph showing the

* Corresponding author.
E-mail addresses: regina.kleespies@julius-kuehn.de (R.G. Kleespies), ywlim@snu.ac.kr (Y.W. Lim), tkaczuk@uph.edu.pl (C. Tkaczuk), martawrzosek@gmail.com (M. Wrzosek), b.steinwender@t-online.de (B.M. Steinwender), rudolf.wegensteiner@boku.ac (R. Wegensteiner).

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ultrastructure of an early stage of *M. typographi* in *I. sexdentatus* (Unal et al., 2009). Further micrographs of the ultrastructure of a *Metschnikowia* sp. in *D. micans* have been presented by Yaman and Radek (2008). *M. typographi* has been found in *I. typographus* in Georgia (Burjanadze et al., 2011) and in *I. amitinus* in Czech Republic (Lukášová et al., 2013), always in a very low number of beetles.

Detailed ultrastructural descriptions or molecular characterizations of *Metschnikowia* spp. from *I. typographus*, *I. amitinus* or *I. sexdentatus* have not been provided in previous studies. Due to its unclear status, *M. typographi* was not included in the review by Lachance (2011).

Since the partial sequencing (D1/D2 region) of the 26S rRNA genes was first used for *Metschnikowia* phylogeny (Mendoça-Hagler et al., 1993), sequencing data from the D1/D2 region have continued to accumulate and be applied to the identification and phylogenetic study of *Metschnikowia* spp. Two different lineages were delimited in the phylogenetic relationship of *Metschnikowia* based on the D1/D2 rRNA gene (Lachance, 2011), and are well supported by four protein-coding gene phylogenies (Guzmán et al., 2013).

Ips sexdentatus, the six-spined engraver beetle, is found in Europe and Asia (Pfeffer et al., 1995) on different *Pinus* spp. This pest is known to attack fresh logs from recently felled trees, as well as standing trees suffering from drought or forest fire. According to Grégoire and Evans (2004), the economic importance of *I. sexdentatus* in Europe varies from negligible to highly important, but recent reports from Spain and France have categorized it as important (Etxebeste and Pajares, 2011; López and Goldarazena, 2012).

Little is known about pathogen occurrence in *I. sexdentatus*. In addition to *M.* cf. *typographi*, only one other eugregarine, *Gregarina typographi*, has been described. Two records of the fungus *Beauveria bassiana* (Ascomycota, Cordycipitaceae) have been described based on morphological observations (Thèodoridés, 1960; Wegensteiner, 2004; Jankevica, 2004; Wegensteiner et al., 2015a, b). Presently, no detailed ultrastructural descriptions or molecular characterizations of any of these pathogens are available.

The aim of this study was to collect *I. sexdentatus* in Austria and in Poland for dissection, identify the presence of any pathogens, and to apply histological, ultrastructural and molecular techniques to more clearly define the status of *Metschnikowia* cf. *typographi* in *I. sexdentatus*, in order to better understand possible natural microbial control agents that could be used against the six-toothed engraver beetle.

2. Materials and methods

2.1. Collection of bark beetles

Ips sexdentatus was collected in Austria (Weikersdorf, Lanzenkirchen and Baden) from infested *Pinus nigra* (Arnold) logs and in Poland (Białowieża and Dąbrowa) from infested *Pinus sylvestris* (L.) logs (Table 1) in years 2009, 2010, 2012, and 2015.

Beetles were cut out from the bark, excepting one case (in Weikersdorf at 2009) where beetles were allowed to emerge from an infested log section in the laboratory (stored at 23 °C, ±2 °C, at long day conditions L:D = 16:8). Parental (black-brown) and offspring beetles (light brown) were separated by color. Beetles were stored in a refrigerator (at 12 °C, ±1 °C, without light) prior to inspection, for a maximum of one week.

2.2. Light and electron microscopy

Living beetles were dissected under a stereo microscope (WILD M3C) at magnification 10× to 40×. The sex and presence of pathogens was identified during evaluation of fresh smears in a light microscope (Reichert Polyvar; 400× to 1000× magnification). In addition to living beetles, dead beetles were also collected and examined for the presence of pathogens.

For histology, *M.* cf. *typographi* infected guts were fixed with alcoholic Bouin's Dubosq-Brazil (Romeis, 1968), embedded in synthetic resin (Technovit 7100, Heraeus Kulzer GmbH) and cut with a rotary microtome (Reichert Jung, Mod. 1165/Rotocut) in 2 µm thick histological slices. Histological sections were stained with 0.1% Toluidinblue solution and enclosed with Eukitt.

For transmission electron microscopy, infected guts were transferred into phosphate buffered Glutaraldehyde (3.5%) for 48 h, then transferred to buffer solution for transport to the 'Laboratory for diagnosis, histo- and cytopathology of arthropod diseases' of the 'JKI-Institute for Biological Control' in Darmstadt, where they were dissected, fixed with 2% osmium tetroxide in Veronal buffer for 6 or 24 h, and then stained with uranyl acetate wolfram phosphoric acid for 5 h. Gut tissues were stepwise dehydrated in increasing concentrations of ethanol and embedded in a 7:3 mixture of butyl- and *n*-methylmethacrylate. Thin sections were examined in a Zeiss TEM 902 transmission electron microscope (Zeiss, Oberkochen, Germany) after double-staining with uranyl acetate and lead citrate. Micrographs were taken with a CCD camera, TRS sharp:eye (Troendle, Moorenwies, Germany).

2.3. Molecular investigations

For molecular studies of *M.* cf. *typographus*, infected guts of *I. sexdentatus* were transferred to DNA free Ethanol (98%) and shipped to the laboratory at Seoul National University, Republic of Korea. Guts were frozen at −80 °C and DNA was extracted using a modified CTAB method (Rogers and Bendich, 1994) with the addition of proteinase K. In order to detect *Metschnikowia* species, a *Metschnikowia* specific reverse primer (MetschR382, 5′-GTG TCT GCT TGC AAG CC-3′) was designed from a clustal alignment of partial 26S rRNA sequences of nine *Metschnikowia* species: *M. agaves* (U84243), *M. andauensis* (AJ745110), *M. fructicola* (AF360542), *M. gruessii* (U45737), *M. koreensis* (AF296438), *M. kunwiensis* (AF389527), *M. pulcherrima* (U45736), *M. reukaufii* (U44825), and *M. zobellii* (U44823). This primer annealed within the D1/D2 region. The forward primer for this amplification was LROR (5′-ACC CGC TGA ACT TAA GC-3′) which has previously been used to amplify this locus. Polymerase chain reaction (PCR) was performed using the Maxime PCR PreMix-StarTaq (Intron Biotechnology Inc., Seoul, Korea) with 1 µl each of primers and genomic DNA at the following PCR conditions: 95 °C for 3 min, 5 cycles of 95 °C, 45 °C, 72 °C, and 30 cycles of 95 °C, 50 °C, 72 °C for 1 min each, and a final

Table 1
Geographical data of *I. sexdentatus* sampling sites in Austria (A) and Poland (PL).

	Nation	Latitude (North)	Longitude (East)	Altitude
Weikersdorf	A	47.80254 N	16.16482 E	295 m
Lanzenkirchen	A	47.75759 N	16.23504 E	298 m
Baden	A	48.00086 N	16.19508 E	340 m
Białowieża	PL	52.70372 N	23.92173 E	170 m
Dąbrowa (Poznań)	PL	52.34755 N	17.17000 E	78 m

extension at 72 °C for 5 min. PCR product was examined in a 1% agarose gel electrophoresis stained with loading STAR (Dyne Bio, Seoul, Korea), and purified using the Expin™ PCR Purification Kit (GeneAll Biotechnology, Korea) following the manufacturer's instructions. The DNA sequencing was performed by Macrogen (Seoul, Korea) in both forward and reverse directions with PCR primers using an ABI3700 automated DNA sequencer (Applied Biosystems, CA, USA). Sequences were assembled, proofread, and aligned using MEGA v5 (Tamura et al., 2011). According to a previous study by Guzmán et al. (2013), 34 reference sequences were retrieved from the GenBank and aligned using MAFFT v7 with l-INS-i algorithm (Katoh and Standley, 2013). Maximum likelihood (ML) phylogenetic analysis was conducted using RAxML v8.0.2 (Stamatakis, 2014) based on the GTRGAMMA substitution model with 1000 bootstrap replicates. Sequence similarity was calculated between *M.* cf. *typographi* and closely related species using MEGA v5.

2.4. Attempts to cultivate M. cf. typographi on artificial media

Cultivation experiments of *M.* cf. *typographi* were conducted with infected *I. sexdentatus* specimens from Austria. Midguts of *M.* cf. *typographi* infected specimens of *I. sexdentatus* were macerated and dispersed on malt extract agar and Sabouraud dextrose agar in Petri dishes. YM agar was used as a first step selective medium (Nguyen et al., 2006). Samples were incubated at 22 °C (±1 °C) for seven days. All attempts to cultivate *M.* cf. *typographi* on artificial media were unsuccessful.

2.5. Identification of a hyphal ascomycete fungus

Filamentous fungus was isolated from infected beetles and cultured on Sabouraud-Dextrose-Agar (SDA) (in Siedlce University). The primary identification of a fungus was based on phase contrast microscopic analyses at magnification of 400×, following Inglis et al. (2012) and Humber (2012). Colony descriptions and measurements were taken at 10 days post-inoculation from cultures grown on SDA at 22 °C in darkness.

Additional molecular studies were conducted with the same isolate for exact identification (at University of Warszawa). The ITS marker was chosen for identification as it has been proposed as the universal DNA barcode marker for Fungi (Schoch et al., 2012).

2.6. Fungal DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from fresh mycelium growing on PDA medium using an Isolate II Plant DNA kit (Bioline Ltd., UK) following the manufacturer's instructions. The complete ITS region was amplified and sequenced using the primer pair ITS1f and ITS4 (White et al., 1990) as described by Różalska et al. (2013). Forward and reverse sequences were matched using the BioEdit Sequence Alignment Editor v. 7.0.0 (Hall, 1999). Sequence data generated from this study is available from GenBank under accession number KU163449.

2.7. Statistics

Statistical analyses of data conformity for testing prevalence of *M.* cf. *typographi* at all sampled locations were performed by using a general linear model. No conformity could be proven as some locations had a small sample size and two showed no *M.* cf. *typographi* infections. As such, a valid model to compare all data could not be established, and it was decided to perform statistical analyses of the beetles sampled in Weikersdorf 2009 only, where the most animals (288 beetles in total) were collected and dissected in this study. The number of males and females were tested for significant differences using a chi-square test. A possible effect of age or sex of the sampled beetles on *M.* cf. *typographi* or *G. typographi* infections was evaluated with a logistic regression model. All statistical analyses were performed in R studio (Version 0.99.473).

3. Results

3.1. Collection of bark beetles

In total, 646 *I. sexdentatus* were dissected (242 male; 404 female): 593 (227 male; 366 female) from three locations in Austria (Weikersdorf 2009, 2010, 2012, and 2015; Lanzenkirchen 2009, Baden 2012) and 53 (15 male; 38 female) from two locations in Poland (Dąbrowa and Białowieża in 2009) (Table 2).

3.2. Light and electron microscopy

3.2.1. Light microscopy

The ascomycetous yeast *M.* cf. *typographi* (Wegensteiner et al., 2007), was found only in *I. sexdentatus* from Austria. Each year, the infection started in the furthermost gastric caeca (Fig. 1). Measurements of needle-shaped ascospores (2 × 13–20 μm) were almost within the range as described in the type host, *I. typographus*.

Dissection procedure did not facilitate to detect the primal position of the asci outside the broken gut cells, but they were suspected flowing out only of broken cells of the midgut epithelium, which were ruptured during removal of the gut from the insect (Fig. 1).

In histological sections of infected *I. sexdentatus* midguts asci were seen as both longitudinal and transverse section (Figs. 3 and 4).

In addition, asci were found progressing into midgut diverticuli (Fig. 5) and around midgut regeneration crypts of *I. sexdentatus* (Fig. 6).

Asci shape of *M.* cf. *typographi* is shown in interference contrast in Fig. 7.

Gregarina typographi was found in the gut of beetles (Fig. 2) from all locations in Austria and Poland during each collection year. Infection rates were always higher with *G. typographi* than with *M.* cf. *typographi* (Table 2).

Infection rates varied between male and female beetles. In most cases, more males were infected with *M.* cf. *typographi* than females. Infection rates with *G. typographi* were similar to *M.* cf. *typographi* with some exceptions; however, sample size must be taken into consideration (Table 2).

Different results were found by comparing the infection rates of beetles cut out of the phloem in the field than with beetles allowed to emerge in the laboratory: Higher infection rates with *M.* cf. *typographi* were seen in beetles cut out of the phloem than in beetles that emerged in the laboratory. In contrast, *G typographi* infection rates were higher in laboratory-emerged beetles (in total and in both sexes) (Table 3).

Furthermore, comparing infection rates of emerging parental beetles with emerging offspring beetles brought evidence of higher *M.* cf. *typographi* infection rates in parental beetles than in offspring beetles. Similar to the difference in cut-out and emerged beetles, *G. typographi* infection rates were higher in emerged offspring beetles than parental beetles (Table 3).

Detailed statistical analyses of the samples collected from Weikersdorf in 2009 showed a significantly lower number of males ($n = 110$) than females ($n = 178$) ($p < 0.0001$). No significant difference ($p = 0.139$) was found in infections by *M.* cf. *typographi* (females 12.3%; males 17.3%) when comparing the impact of the

Table 2
Metschnikowia cf. *typographi* and *G. typographi* infected total number, male and female *I. sexdentatus* from different locations in Austria and Poland in different years, and numbers of dissected (total N) and of dissected male and female beetles.

Location	Year	*Metschnikowia* cf. *typographi*			*Gregarina typographi*			N		
		Total	Male	Female	Total	Male	Female	Total	Male	Female
Weikersdorf	2009	41	19	22	207	90	117	288	110	178
Weikersdorf	2010	15	7	8	12	6	6	56	21	35
Weikersdorf	2012	5	4	1	28	11	17	50	14	36
Weikersdorf	2015	4	1	3	31	16	15	110	53	57
Lanzenkirchen	2009	11	4	7	31	8	23	47	16	31
Baden	2012	4	2	2	33	10	23	42	13	29
Dąbrowa	2009	–	–	–	11	5	6	16	5	11
Białowieża	2009	–	–	–	20	4	16	37	10	27

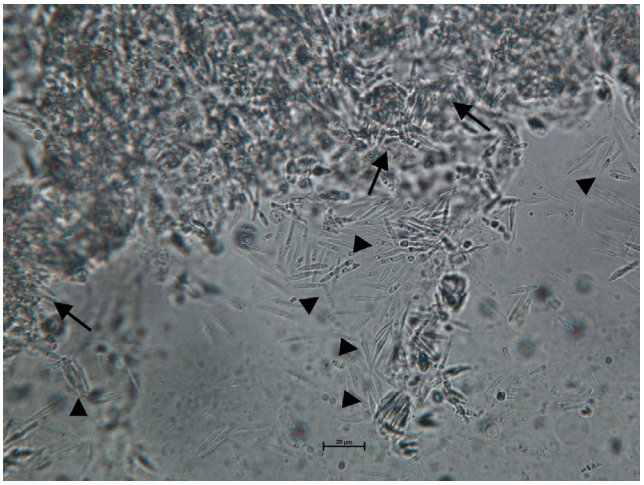

Fig. 1. *Metschnikowia* cf. *typographi* asci in cells (arrows) and outside of broken midgut epithelial cells (arrowheads) of *I. sexdentatus.*

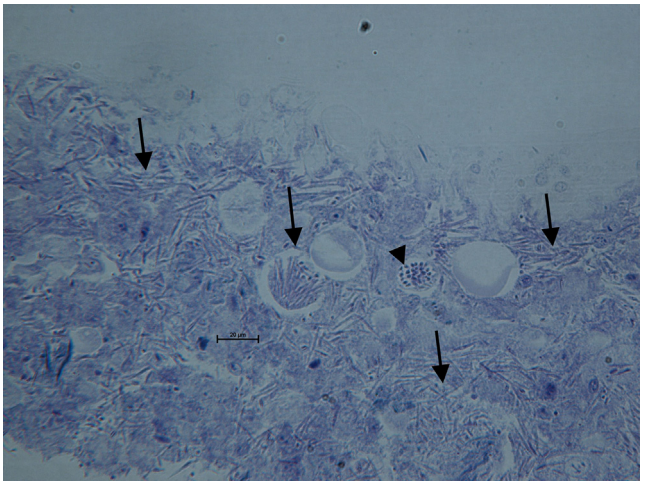

Fig. 3. *M.* cf. *typographi* asci longitudinal (arrows) and transversal (arrowhead) in cells of the midgut epithelium of *I. sexdentatus*.

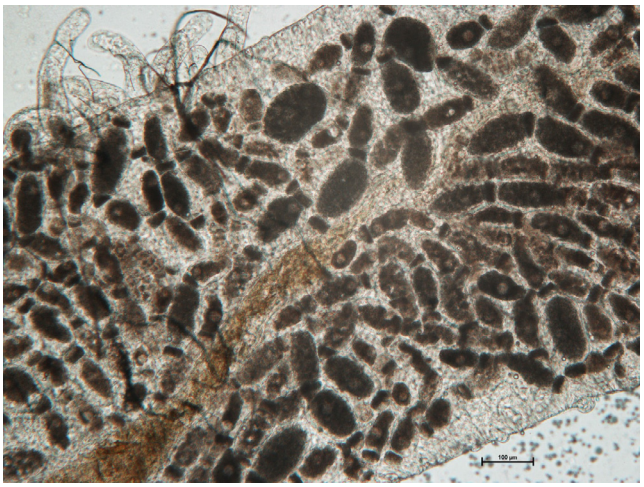

Fig. 2. Numerous *G. typographi* in the midgut lumen of *I. sexdentatus.*

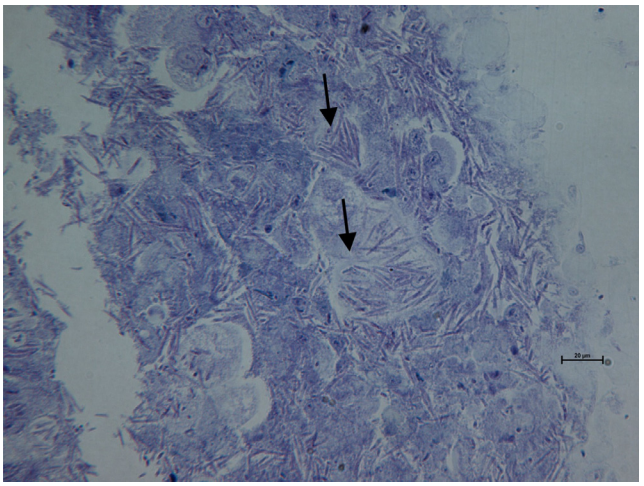

Fig. 4. *Metschnikowia* cf. *typographi* asci in cells of the midgut epithelium (arrows) of *I. sexdentatus.*

sex on the pathogen appearance, whereas there was a significantly higher infection rate ($p = 0.0095$) of *G. typographi* in males (82.7%) compared to females (65.2%). When comparing young beetles and old (parental) beetles, a significantly higher rate ($p < 0.0001$) of old beetles (27.3%) were infected by *M.* cf. *typographi* than young ones (1.4%). The opposite case was noted with *G. typographi* infections, as there was a significantly higher rate ($p < 0.0001$) of young beetles (92.4%) infected by *G. typographi* compared to parental beetles (51.1%).

3.2.2. Electron microscopy

Ultrathin sections of infected *I. sexdentatus* midguts elucidated the ultrastructure of *M.* cf. *typographi*. Typical yeast like vegetative cells were found, sometimes building branched chains (Fig. 8A) and showing budded growth (Figs. 8A and B). Developing stages of asci and ascospores were observed in Figs. 8C–F and 9A–C. Mature ascospores, still enveloped by the ascus of this *Metschnikowia* species, are shown in Fig. 9D. Each ascus harbored two ascos-

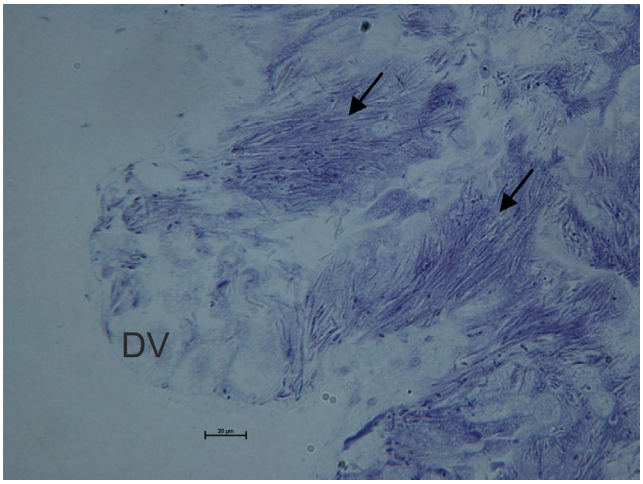

Fig. 5. *Metschnikowia* cf. *typographi* asci (arrows) in epithelial cells of *I. sexdentatus* midgut diverticuli (DV).

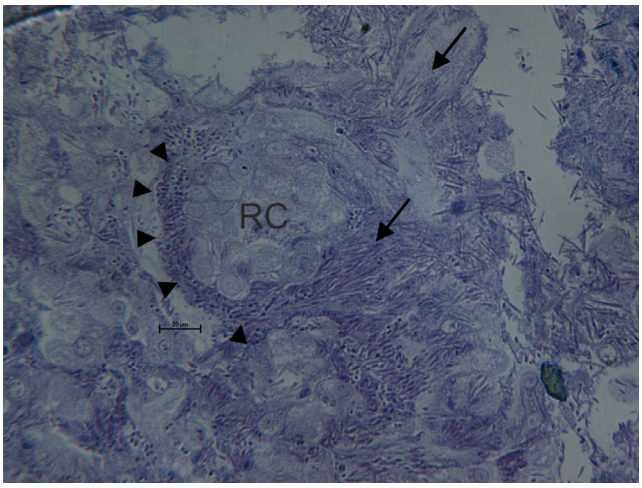

Fig. 6. *Metschnikowia* cf. *typographi* asci (arrows and arrowheads) in cells around a regeneration crypt (RC) in the midgut epithelium of *I. sexdentatus.*

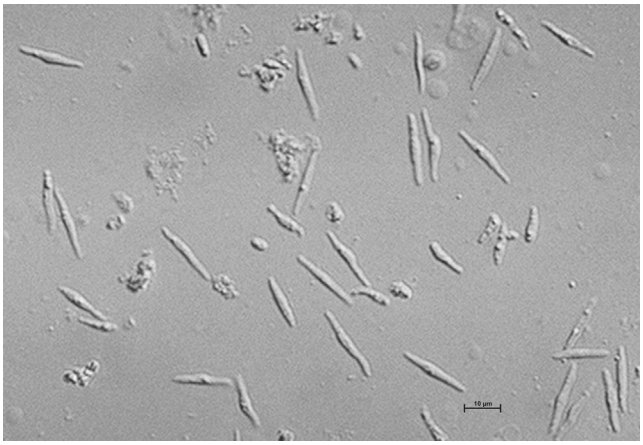

Fig. 7. *Metschnikowia* cf. *typographi* asci of *I. sexdentatus* in interference contrast.

pores. In neighbouring gut cells, all asci were often found in one direction. Therefore, only transverse or longitudinally and oblique cut groups of asci were typically observed in electron micrographs (Figs. 8C and D, respectively). Longitudinal sections of ascospores revealed an electron dense protoplast appearing less dense within the attenuating tube-like ends of the ascospore (Figs. 8F and 9C). Along the attenuating parts of the ascospore, bilateral flattened flanks like alar rims could be observed (Fig. 8E). The ultrastructure of developing ascospores in an ascus is shown in Fig. 9B. Ascus and ascospores were each enveloped by a two layered spore wall, a thin electron dense outer wall and a thicker electron translucent inner wall (Fig. 9B). The plasma membrane was attached to the inner wall, bordering the protoplast. Often, plasma membranes displayed two or three layers with invaginations, and several mictochondria could be observed (Fig. 9B). During growth and maturation of the ascospores, the protoplast of the ascus increasingly lost density (Figs. 9C and D).

3.3. Molecular investigations of M. cf. typographi

26S rRNA sequence of *M.* cf. *typographi* was successfully amplified with 343 bp covering D1 variable region. Phylogenetic analysis showed that *M.* cf. *typographi* resided in the *Metschnikowia* clade and formed a separate monophyletic group (ML support value = 91) with *M. agaves* and *Candida wancherniae*. Sequence similarity was highest with *C. wancherniae* (93.5%), followed by *M. agaves* (90.7%) (Fig. 10).

3.4. Identification of a hyphal ascomycete fungus

Besides the two detected pathogens, fungal growth (mycelium and conidia) was observed on numerous beetle cadavers collected at the Weikersdorf site (Austria). The fungus was morphologically identified as *Beauveria bassiana*. The isolate was characterized microscopically by conidiophores consisting of whorls and dense clusters of sympodial, short and globose conidiogenous cells with an apical zig-zag appearance of the rachis and one-celled spherical conidia. Conidial length and width were 2.24 ± 0.26 µm and 1.87 ± 0.34 µm respectively, with length/width ratio of 1.20. Colonies on SDA were normally white to pale yellow and the average diameter of a colony after 10 days of growth at 22 °C was 24.3 mm.

The sequence obtained with ITS primers is 574 base pairs. Aligned against sequences in GenBank, identity with five records was shown. All were identified as DNA of *Beauveria.* One sequence was only specific to the genus level, and others were identified as *B. bassiana*. All *Beauveria* strains were isolated from insects: in Canada, from emerald ash borer *Agrilus planipennis* (Coleoptera: Buprestidae) (Johny et al., 2012), eastern spruce budworm *Choristoneura fumiferana* (Lepidoptera, Tortricidae), and *Lygus* sp. (Homoptera, Miridae); in Belgium from the Rhizophagidae beetle *Tomicus piniperda* (Coleoptera, Scolytinae) (Rehner and Buckley, 2005). At the time of writing, only one sequence of *B. bassiana* isolated from *I. sexdentatus* in Spain (DQ449648) was available in the NCBI (Romon et al., 2008, unpublished data). The sequence obtained during the current study differs from the above sequence by two substitutions and one deletion/insertion.

4. Discussion

This study was an attempt to elucidate some basic details of *M.* cf. *typographi* and other pathogens found in *I. sexdentatus*. Bark beetle species (e.g. *I. typographus* and *I. amitinus*) inhabiting the same tree species may transmit pathogens from one species to another through contact, for example, by crossing their galleries (Händel et al., 2003). It is unclear how a pathogen might be transferred between beetle species feeding on different tree species. *I. typographus* primarily found on *Picea abies* (Altenkirch et al., 2002), but in rare cases may inhabit *Pinus sylvestris* (Pfeffer et al.,

Table 3
Metschnikowia cf. *typographi* and *G. typographi* infected total number, male and female *I. sexdentatus* cut out of their galleries or emerged from the log section (parental and offspring beetles) from the location Weikersdorf (2009) in Austria.

	Metschnikowia cf. *typographi*			*Gregarina typographi*			N		
	Total	Male	Female	Total	Male	Female	Total	Male	Female
Cut out	15	8	7	29	19	10	49	25	24
Emerged	26	11	15	178	71	107	239	85	154
Emerged parental	24	11	13	37	10	27	79	19	60
Emerged offspring	2	0	2	141	61	80	160	66	94

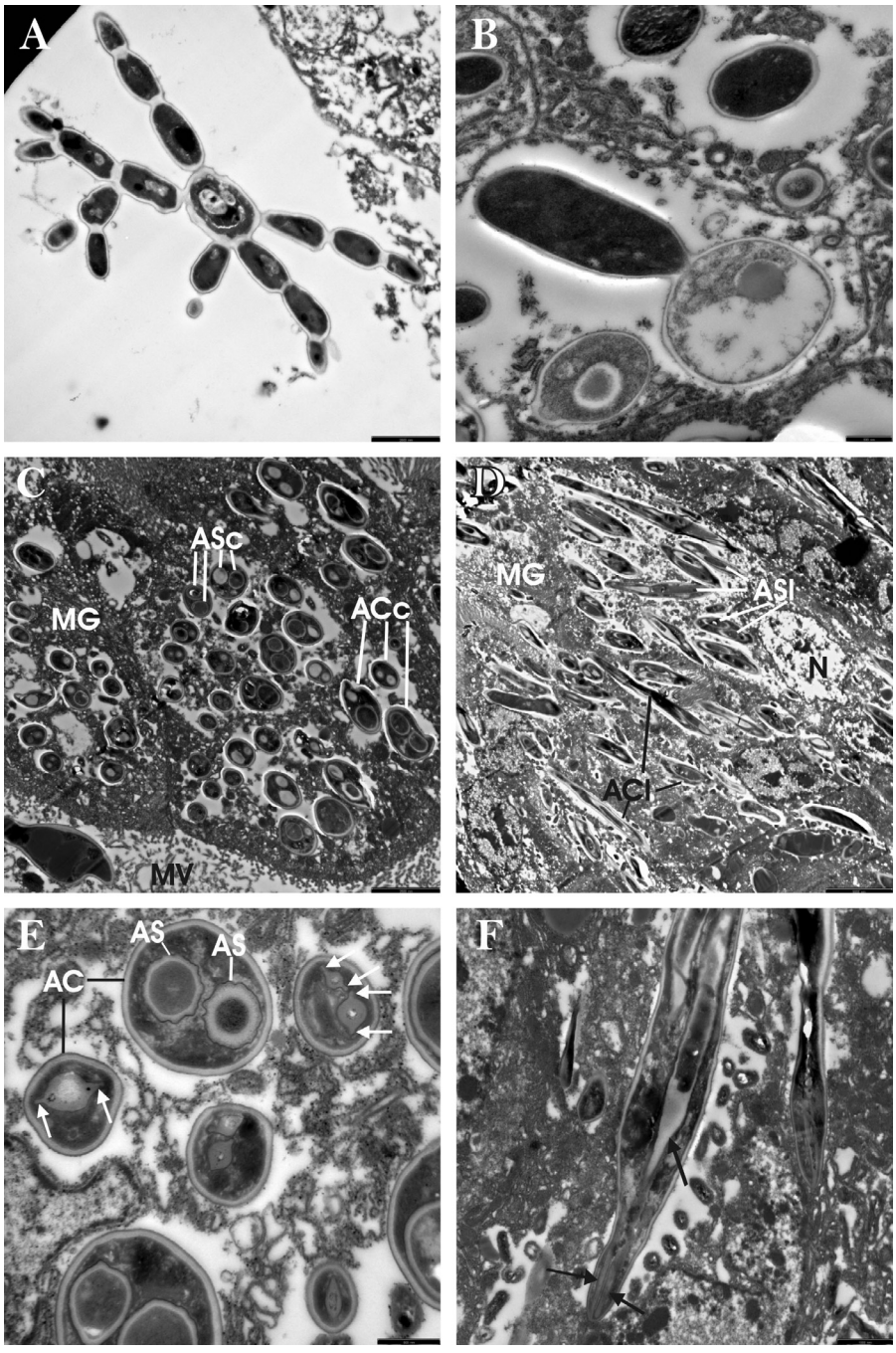

Fig. 8. Electron micrographs of midguts of *I. sexdentatus* infected by *M.* cf. *typographi*. (A) Vegetative cells in the midgut lumen with branched chains and budded growth, bar = 2.500 nm. (B) Single vegetative cells; the one in the centre displays binary fission by hyphal cell budding, bar = 500 nm. (C) Midgut cells, heavily infected with *M.* cf. *typographi*, all in cross section, MG = midgut, MV = microvilli, ACc = Ascus, cross cut, ASc = Ascospore, cross cut, bar = 2.500 nm. (D) Midgut cells, heavily infected with *M. typographi*, all in longitudinal section; MG = midgut, N = nucleus, ACl = Ascus, longitudinal cut, ASl = Ascospore, longitudinal cut, bar = 5.000 nm. (E) Cross sections of asci = AC each with developing ascospores = AS of *M.* cf. *typographi*. Note: bilateral flattened flanks like alar rims along the attenuating parts (arrows), bar = 500 nm. (F) Longitudinal section of an ascus in the centre with two ascospores each revealing an electron dense protoplast appearing less dense within the attenuating tube-like ends of the ascospore (arrows), bar = 1.000 nm.

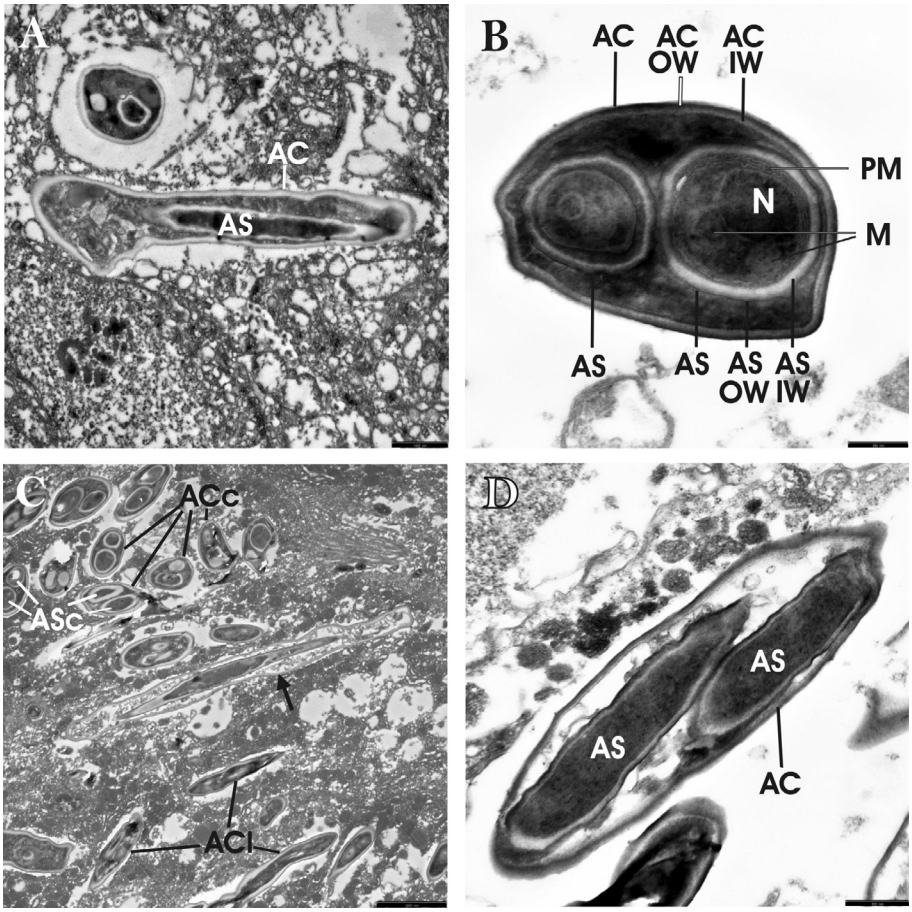

Fig. 9. Electron micrographs of midguts of *I. sexdentatus* infected by *M.* cf. *typographi*. (A) Young ascus = AC in longitudinal section showing the enlarged end (left) and one developing ascospore = AS, bar = 1.000 nm. (B) Young ascus = AC in cross section harbouring two developing ascospores = AS. Note: three layered plasma membrane = PM in the right ascospore; OW = electron dense outer wall, IW = electron lucent inner wall, N = nucleus, M = mitochondrium, bar = 250 nm. (C) Cross (ACc) and longitudinal (ACl) sections of *M.* cf. *typographi* in midgut cells. In the centre, a longitudinally cut ascus with remainders of protoplasm enveloping a mature ascospore is shown (arrow), bar = 2.500 nm. (D) Two mature ascospores = AS in a nearly protoplasm empty ascus = AC, bar = 500 nm.

1995). This poses the question of whether the *Metschnikowia* sp. and *Gregarina* sp. findings in different species of bark beetle represents a single or different congeneric pathogen species.

Until now, *M. typographi* has been found only in *I. typographus* from Austria and Finland (Händel et al., 2003; Weiser et al., 2003), and was characterized as a non-cultivable, obligate parasite. The unclear status of this species is also mentioned by Lachance (2011). The mechanism of yeast transfer is not clear for bark beetles, but oral ingestion of spores seems plausible. For example, ingestion of faecal pellets of an infected individual by healthy conspecifics could be possible. Alternatively, beetles may come into contact with yeast spores when infesting a tree, as *Metschnikowia* spp. have been observed in both tree exudates and rotten wood (Lachance, 2011; Hui et al., 2013). However, there have been no findings of *Metschnikowia* sp. on *Picea* sp.

Obtention of any *M. typographi* infected specimen of *I. typographus* or *I. amitinus* for comparison was not possible, however, the outcome of our study is consistent with earlier results, where rates of *M. typographi* infection were consistently higher in male *I. typographus* and *I. amitinus* than in female (Weiser et al., 2003). In most cases, this also stands true for *I. sexdentatus* (Wegensteiner et al., 2007).

Six out of eight sampling events found *M.* cf. *typographi* infections. Five of these showed a higher number of *M.* cf. *typographi* infections in males than females. The difference in the Weikersdorf (Austria) 2009 sampling was statistically insignificant – it nevertheless suggests that bark beetle males are generally more susceptible to pathogen infections than females. This is further supported by the significantly higher infection rates of male bark beetles with *G. typographi*.

The comparison of the pathogen appearance in juvenile and parental beetles also yielded novel and interesting data. Young beetles were found to have very little *M.* cf. *typographi* infections, but very high *G. typographi* abundances. For beetles of the parental generation, the opposite was true (higher *M.* cf. *typographi*, lower *G. typographi*). It could be hypothesized that, once consumed, it takes *M.* cf. *typographi* time to establish in the gut and to generate visible symptoms, whereas *G. typographi* cysts are more or less immediately ready to proliferate when consumed and introduced into the digestion system.

Until now, little is known about pathogenesis of *Metschnikowia* spp. in their hosts (Lachance et al., 1976). Several *Metschnikowia* spp. were found on the surface of insects or in their intestine (Nguyen et al., 2006; Lachance, 2011). Ingestion of *M. bicuspidata* by *Daphnia magna* in water has been reported (Ebert et al., 2000), as has transfer of unharmed spores to *Daphnia dentifera* via ingestion of fish faeces (Duffy, 2009). *M. bicuspidata* was detected penetrating the gut wall, progressing into the haemolymph and destroying the ovary of *D. magna* (Ebert et al., 2000). Only living *I. sexdentatus* were dissected in the present study, and while the infection could be clearly assigned to the midgut epithelium, it can be reasonably assumed that this yeast is also able to penetrate

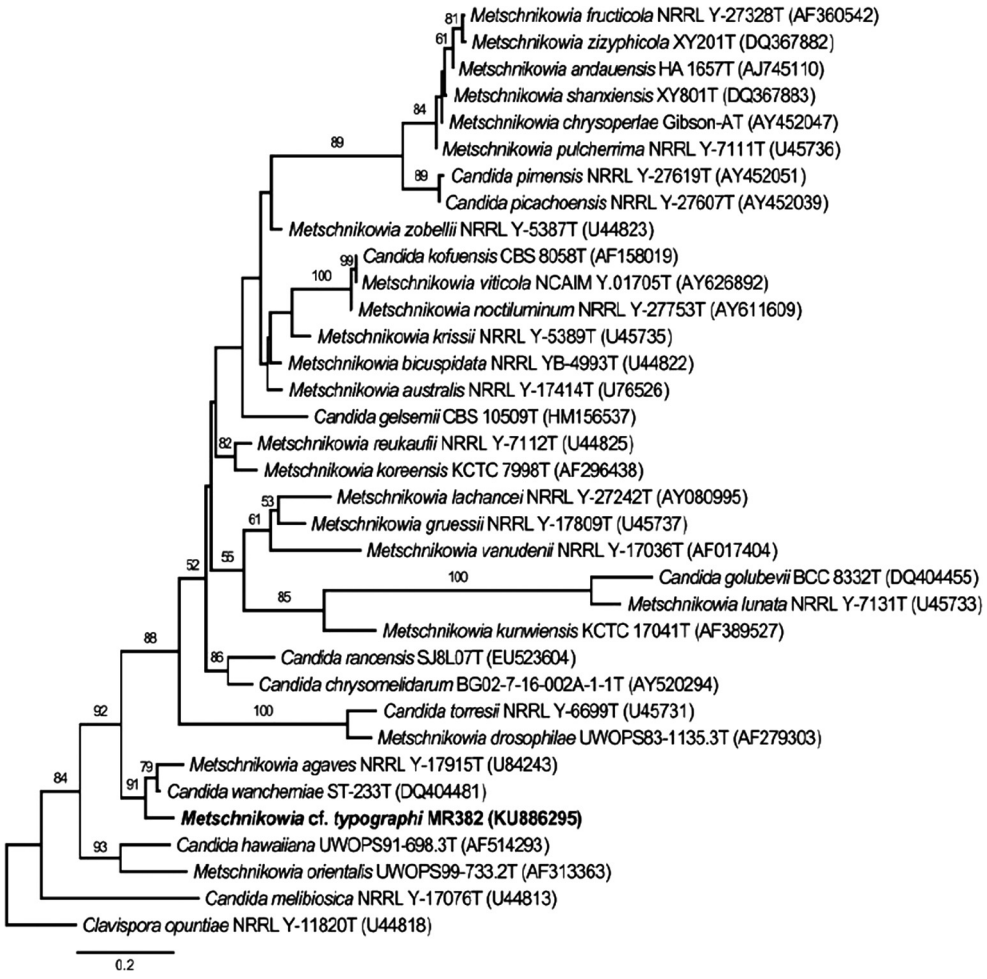

Fig. 10. Maximum likelihood tree inferred from the partial 26S rRNA sequence for *M.* cf. *typographi* and allied species.

the gut wall and develop further on into the haemolymph and other organs.

Ultrastructural details of the studied *M.* c.f. *typographi* are shown in this work for the first time. Vegetative growth was similar to other yeast fungi as shown by Osumi (1998) who described their general ultrastructure, growth, and propagation. Talens et al. (1973a, 1973b) described bud formation of *Metschnikowia krissii* and studied the ultrastructure of asci and ascospores by transmission and scanning electron microscopy. In the asci of *M. krissii* only one ascospore was produced, while in *M.* c.f. *typographi*, there are two ascospores in each ascus. Measurements of needle-shaped ascospores (2 × 13–20 μm) and their spore wall are in a similar range as is described in the type host, *I. typographus* (Weiser et al., 2003). With transmission electron microscopy of the present work, it could not be clearly seen if the outer spore wall of the ascospores possessed any surface sculptures, as was seen in *Metschnikowia continentalis* var. *borealis* by Lachance et al. (1998) using scanning electron microscopy. Notably, the bilateral flattened flanks like alar rims at both sides of the attenuating tube-like ends of the ascospores seen in the described *Metschnikowia* species and in the type host *I. typographus* are features that have not been previously reported in any yeast species.

Phylogenetic analysis of the 26S rRNA region showed similar topology to a phylogeny using multiple protein coding genes (Guzmán et al., 2013). However, *M.* cf. *typographi* changed the phylogenetic position of *M. agaves* that was formerly considered to be the early lineage of group 4 (Guzmán et al., 2013). In the present analysis, *M.* cf. *typographi* formed a sister clade of *M. agaves* and *C. wancherniae*, and this clade was distinct from the group 4 described by Guzmán et al. (2013). This result suggests that *C. wancherniae*, *M. agaves*, and *M. typographi* may form a separate phylogenetic group with the other *Metschnikowia* species. *C. wancherniae* was isolated from insect frass and plant leaves in Thailand (Nakase et al., 2009). Since ascospores of *C. wancherniae* were not observed in pure culture, morphological characteristics of *C. wancherniae* and *M.* cf. *typographi* could not be compared directly. Phylogenetic analyses based on multiple genes and common morphological characters are needed for further clarification of the phylogenetic relationships of these species.

The occurrence of *B. bassiana* from *I. sexdentatus* had thus far been reported with only morphological and cultural data (Jankevica, 2004; Wegensteiner et al., 2015b). This work presents the first time that a *B. bassiana* strain infecting *I. sexdentatus* was investigated using molecular tools.

Until now, the occurrence of *B. bassiana* has been reported in *I. sexdentatus* from Bulgaria (Draganova et al., 2007; Takov et al., 2012), Austria, and France (Wegensteiner et al., 2015b). In the same beetle population of France, another *Beauveria* species, *B. caledonica*, was also observed.

Beauveria bassiana is a well known entomopathogenic fungus that has a wide range of insect hosts (Feng et al., 1994; Inglis et al., 2001; Zimmermann, 2007). It is also known to infect a number of bark beetle species in their galleries (Novák and Samšiná ková, 1967; Wulf, 1983; Lutyk and Świeżyńska, 1984;

Wegensteiner and Weiser, 1996; Wegensteiner, 2000; Wegensteiner et al., 2015b). This common natural occurrence obtrudes it as one of the most promising candidates for the biological control of three species of bark beetle, *I. typographus* (Wegensteiner, 1992; Kreutz et al., 2004), *Polygraphus poligraphus* (Wegensteiner, 2000), and *I. sexdentatus* (Draganova et al., 2007; Steinwender et al., 2010).

Phylogenetical studies on *B. bassiana* showed that morphological features, such as conidia shape, did not reflect the phylogenetical position, and some strains originally identified as *Beauveria brongniartii* should be re-classified as *B. bassiana*. Moreover, the work by Rehner and Buckley (2005) indicated that isolation by distance has been an important factor in the evolutionary diversification of this genus. The *B. bassiana* strain found in the present work showed an overwhelming dominance of North American sequences in the cluster to which the here obtained sequence belongs. This could be an indicator of the origin of the strain. The abovementioned article also supports the assumption that this species is not host specific.

5. Conclusion

The present study gives an overview on occurrence and spectrum of natural antagonists of the bark beetle species *I. sexdentatus* in Austria and Poland. *M.* cf. *typographi* and *G. typographi* were the most frequently found species, but little information is available on the role of these antagonists as natural mortality factors. In particular, the role of the fungus *M.* cf. *typographi* is not yet well investigated. This study shows details of its ultrastructure and its phylogenetic position for the first time that place it close to *C. wancherniae* and to *M. agaves*. Further studies in other European countries will contribute to a better understanding of possible natural microbial control agents against *I. sexdentatus* and other related bark beetle species, and to the ecological functions of the *Metschnikowia* species.

Compliance with ethical standards

This study was funded by Universität für Bodenkultur Wien (Austria) and by Siedlce University of Natural Sciences and Humanities (Poland) (Grant No. PL 09/2009) in the Scientific and Technological Cooperation programme Austria-Poland.

Conflict of interest

All authors declare that they have no conflict of interest.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

This article does not contain any studies with human participants performed by any of the authors.

Author contribution statement

RW designed and conducted research. RW, CT and BMS collected *I. sexdentatus* from different sites and were responsible for identification of pathogens. RGK conducted transmission electron microscopy and described the ultrastructure of *Metschnikowia* cf. *typographi*. YWL is responsible for the molecular data of *M.* cf. *typographi*, and MW of *Beauveria bassiana*. BMS analysed the data statistically. All authors wrote parts of the manuscript.

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