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The great spruce bark beetle (*Dendroctonus micans* Kug.) (Coleoptera: Scolytidae) in Lithuania: occurrence, phenology, morphology and communities of associated fungi

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Abstract

We studied the occurrence, morphology and phenology of *Dendroctonus micans* in Lithuania and the fungi associated with the beetle at different developmental stages. The occurrence of *D. micans* was assessed in 19 seed orchards (at least 40 years old) of *Picea abies* (L. Karst.) situated in different parts of the country. Bark beetle phenology was studied in two sites: a seed orchard of *P. abies* and a plantation of *Picea pungens* (Engelm.). *D. micans* morphology was assessed under the dissection microscope using individuals at different developmental stages that were sampled during phenology observations. Communities of fungi associated with *D. micans* were studied using both fungal culturing methods and direct high-throughput sequencing from *D. micans*. Results showed that the incidence *D. micans* was relatively rare and *D. micans* was mainly detected in central and eastern Lithuania. The life cycle included the following stages: adult, egg, I–V developmental stage larvae and pupa. However, development of *D. micans* was quicker and its nests larger under the bark of *P. pungens* than of *P. abies*, indicating the effect of the host species. Fungal culturing and direct high-throughput sequencing revealed that *D. micans* associated fungi communities were species rich and dominated by yeasts from a class Saccharomycetes. In total, 319 fungal taxa were sequenced, among which *Peterozyma toletana* (37.5% of all fungal sequences), *Yamadazyma scolyti* (30.0%) and *Kuraishia capsulate* (17.7%) were the most common. Plant pathogens and blue stain fungi were also detected suggesting their potentially negative effects to both tree health and timber quality.

Keywords: spruce, climate change, fungi, symbiosis, forest pests, microorganisms, bark beetle

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Introduction

Climate change is increasing the number of natural disasters (e.g., storms, droughts, high temperatures) that are able to damage the shallow root system of Norway spruce (*Picea abies* L. Karst.) (Klavina *et al.*, 2015). Consequently, trees are weakened and exposed to attacks by bark beetles, which are

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Table 1. Seed orchards of *P. abies* used to evaluate occurrence of *D. micans* in different parts of Lithuania.

Site	Forest enterprise	Position	Area (ha)	Year of establishment
1	Biržų	N56°10′E24°15′	5.68	1966–1971
2	Dubravos	N54°51′E24°03′	5.76	1961–1965
3	Jurbarko	N55°05′E22°50′	10.93	1967–1978
4	Kaišiadorių	N54°55′E24°23′	2.80	1975–1979
5	Kretingos	N55°55′E21°20′	27.40	1968–1977
6	Kuršėnų	N56°00′E22°55′	21.79	1968–1975
7	Marijampolės	N54°38′E23°20′	8.63	1971–1975
8	Mažeikių	N56°10′E23°41′	47.31	1970–1979
9	Pakruojo	N56°00′E23°45′	3.80	1969–1973
10	Panevėžio	N56°45′E24°20′	3.00	1967–1971
11	Radviliškio	N55°20′E23°20′	5.10	1967–1972
12	Raseinių	N55°20′E23°10′	4.96	1968–1971
13	Rietavo	N55°42′E22°44′	5.90	1968–1971
14	Rokiškio	N56°01′E25°39′	14.68	1967–1975
15	Šakių	N54°55′E23°05′	9.50	1968–1977
16	Šilutės	N55°20′E21°30′	5.82	1967–1973
17	Tauragės	N55°15′E22°20′	4.50	1967–1970
18	Telšių	N56°55′E21°50′	2.87	1968–1973
19	Tytuvėnų	N55°34′E 23°06′	42.87	1970–1980

among the most common damaging agents to these trees (Christiansen & Bakke, 1988). The great spruce bark beetle (*Dendroctonus micans* Kug.) is among the largest sized bark beetles and the only representative of this genus in Europe. It often attacks older stands and clonal seed orchards of *P. abies*, especially following natural disasters or when trees have been weakened by other insects. During outbreaks, *D. micans* is able to destroy entire stands. The trees are killed mostly by girdling when several broods develop in the same tree (Evans *et al*., 1984). The life cycle of many bark beetles is dependent on many microorganisms, especially fungi. These beetles are anatomically and morphologically adapted to vector and transmit these microorganisms from one generation to the next that can infect the attacked trees (Vega & Blackwell, 2005). Among the European bark beetles, the interaction between the European spruce bark beetle (*Ips typographus* L.) and *Ophiostoma* (Ascomycota) fungi is a well-known example. Depending on the *Ophiostoma* species that are vectored by the beetle, there may be variable effects, including antagonism, commensalism or mutualism (Vega & Blackwell, 2005). In contrast, symbioses of *D. micans* and fungi are less well studied, revealing the need for better understanding of associated fungal communities and their potential effects on insect and attacked tree species. For example, Lieutier *et al*. (1992) showed that only *O. canum* was cultured from *D. micans* or from its galleries with a significant frequency of association, while other species, including *O. penicillatum*, *O. minus* and other unidentified *Ophiostoma* species were found only very occasionally.

The aim of the present study was to assess occurrence, phenology and morphology of *D. micans* in Lithuania and the fungi associated at different developmental stages.

Material and methods

Assessing the occurrence of D. micans

The assessment was carried out in July 2013. The study sites were selected based on the information provided by the Lithuanian State Forest Service and included 19 seed orchards of *P. abies*, which were at least 40 years old and as such were suitable for *D. micans* reproduction (table 1, fig. 1). The occurrence of *D. micans* in each seed orchard was evaluated by visual inspection of 100 randomly selected trees of *P. abies*. Research Randomizer (available at https://www.randomizer.org/) was used to select the trees in each seed orchard. As all trees in each seed orchard are numbered, the random numbers generated by Research Randomizer were used to select the trees. Figure 2 shows a schematic map of a seed orchard (Site 15, table 1) with a random distribution of selected trees. Characteristic symptoms of damage by *D. micans* (Francke-Grosmann & Ruhm, 1954; Forestry Commision, 2015) were assessed on the stems (from the ground level and up to a height of 2 m) and the damage symptoms were divided into three categories: fresh with living beetles, fresh without living beetles (buried in resin) and old (of the previous or earlier years).

Determining the phenology of D. micans

The phenology of *D. micans* was investigated during April–September 2014. This was done at two sites and included two tree species: a *P. abies* seed orchard (Site 15, table 1) and a plantation of *Picea pungens* that was located at the Ukmergė forest nursery (N55.24, E24.88) (fig. 1). The distance between the sites was ca. 110 km. Average temperatures for each site and observation period were obtained from the local meteorological stations of the Lithuanian Hydrometeorological Service (Vilnius, Lithuania) and are presented in fig. 3. In total, five trees of *P. abies* and five of *P. pungens* were selected. The selected trees were characterized by a high colonization and activity of *D. micans* in the lower part of their stems, which were easily accessible for regular observations. The relatively low number of selected trees was due to low incidences of *D. micans* in 2014. The observations were performed regularly at 2-week intervals. During each observation, the bark was carefully removed from the investigated trees and between 10 and 20 eggs and/or individual *D. micans* at different developmental stages were extracted each time from the nests. Special care was taken not to damage extracted individuals and not to disturb the nests. After each extraction of *D. micans*, the bark was carefully returned and fastened to the trees in

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Fig. 1. Map of Lithuania showing visited seed orchards of *P. abies* and detected (filled circles) and undetected (open circles) incidences of *D. micans*. Seed orchards are numbered as in table 1. The *P. pungens* plantation is noted with a triangle.

order to preserve the remaining eggs/individuals of *D. micans* and to minimize the negative effects of disturbance that may interfere with their further development. The collected *D. micans* eggs/individuals were placed separately in 1.5 ml centrifugation tubes in 70% ethanol solution and transported to the laboratory for morphological assessment. The length and width of collected eggs, larvae, pupae and adults of *D. micans*, as well as width of larval heads was measured at the accuracy of 0.01 mm using a dissection microscope (Stemi 2000, Carl Zeiss, Oberkochen, Germany). It is known that heads of larvae at different developmental stages differ in width and this feature can be used for their classification (Voolma, 1980).

Culturing of fungi from D. micans

Fungal culturing was carried out from the 25 larvae, ten pupae and ten adults of *D. micans* sampled at each site of phenology observations. Eggs were not used for culturing. Two nutrient media were used to culture the fungi associated with *D. micans*, malt extract agar medium and yeast extract agar medium (Atlas, 1997) supplemented with 0.5% of each antibiotic chloramphenicol and streptomycin. Before the beetles were placed in 9 cm diameter Petri dishes, these were surface sterilized for 2 min in 5% hypochlorite and rinsed twice in sterile distilled water. One individual was placed per Petri dish. Prepared dishes with insects were stored for 1 month at room temperature (ca. 21°C) in the dark. The Petri dishes were inspected every 2–3 days and any outgrowing mycelium was immediately transferred to new Petri dishes with malt agar medium. Fully developed fungal colonies were divided into morphological groups and representative cultures per each group were taxonomically identified based on morphological characteristics of fungal mycelia and reproductive structures, which were examined using a light microscope (Axiostar plus, Carl Zeiss, Oberkochen, Germany).

Direct sequencing of fungi from D. micans

For the identification of fungal communities, larvae and adults of *D. micans* were sampled during field surveys in the

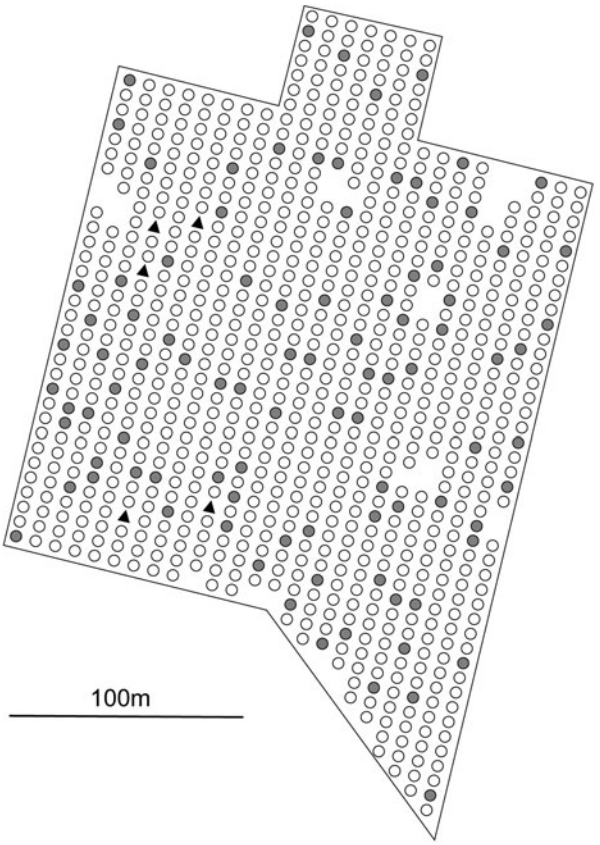

Fig. 2. A schematic map of the Šakiai seed orchard (Site 15, table 1) of *P. abies* showing random distribution of selected trees, which were used to assess incidents of *D. micans*. The *D. micans*-infested trees are shown as triangles, non-infested trees are shown as filled circles and non-assessed trees are shown as open circles. Gaps in the seed orchard indicate missing trees.

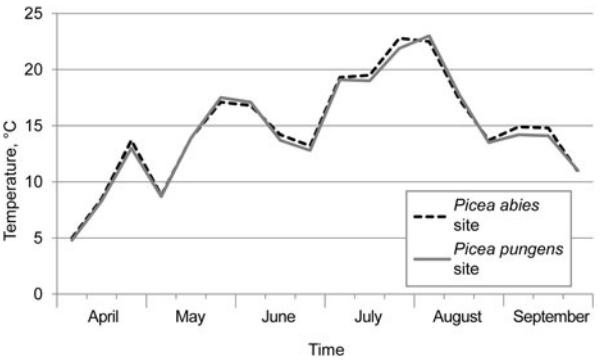

Fig. 3. Average temperatures for April–September 2014 recorded at the nearest hydrometeorological station for each *P. abies* seed orchard and *P. pungens* plantation, which were used for phenology observations of *D. micans*. Measurements are averaged and presented for every 10 days of each month.

P. abies seed orchards (table 1) and in a plantation of *P. pungens*. In trees with signs of beetle attack, the bark was carefully removed using a knife, and adults and larvae were placed into

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1.5 ml centrifugation tubes and transported to the laboratory. Collected insects were stored at −20°C until used for DNA extraction and sequencing of fungi. Genomic DNA was isolated from 125 larvae (25 larvae were collected in each of five sites: 2, 7, 14, 15 and 16 as in table 1) and from 25 adults (collected in Ukmergė forest nursery during phenology observations) of *D. micans*. Prior to isolation of DNA, larvae and adults were placed separately in 2-ml screw-cap centrifugation tubes and freeze-dried at −60°C for 2 days. No surface sterilization was carried out. Isolation of DNA, amplification and sequencing was carried out as described by Menkis *et al.* (2015). Briefly, material from larvae and adults were homogenized separately using a FastPrep shaker (Precellys 24; Bertin Technologies, Rockville, MD, USA) and subjected to isolation of genomic DNA using the CTAB method (Menkis *et al.*, 2015). Following DNA isolation, concentration of genomic DNA was determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Diluted (1–10 ng μl^{-1}) genomic DNA samples were amplified separately by PCR using the primer pair ITS9 (5′-GAACGCAGCRAAIIGYGA-3′) (Ihrmark *et al.*, 2012) and ITS4 (5′-xxxxxxxxTCCTCCGC TTATTGATATGC-3′) (White *et al.*, 1990) containing 8 base pairs (bp) sample identification barcodes denoted by ×. The concentration of purified PCR products was determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and an equimolar mix of all PCR products was used for Ion Torrent sequencing. The sequencing library construction and sequencing using a 316 chip as a part of the larger sample were carried out by SciLifeLab (Uppsala, Sweden).

Bioinformatics

The sequences generated were subjected to quality control and clustering in the SCATA NGS sequencing pipeline (http://scata.mykopat.slu.se). Quality filtering of the sequences included the removal of short sequences (<200 bp), sequences with low read quality, primer dimers and homopolymers, which were collapsed to 3 bp before clustering. Sequences that were missing a tag or primer were excluded. The primer and sample tags were then removed from the sequence, but information on the sequence association with the sample was stored as meta-data. The sequences were then clustered into different taxa using single-linkage clustering based on 98.5% similarity. The most common genotype (real read) for each cluster was used to represent each taxon. For clusters containing two sequences, a consensus sequence was produced. Fungal taxa were taxonomically identified using RDP pipeline classifier available at https://pyro.cme.msu.edu/index.jsp (Center for Microbial Ecology, Michigan State University, Michigan, USA). Sequences showing <80% similarity to phylum level were considered to be of non-fungal origin and were excluded from further analyses. Taxonomic names identified using RDP pipeline classifier are listed in online Supplementary table 1 and represent the closest match as compared to the reference sequence at a particular level of similarity. The following similarity levels should be used for reliable identification of fungal taxa: taxon level 98–100%, genus level 94–97% and unidentified <94% (online Supplementary table 1). In addition, the 20 most common fungal taxa were identified using GenBank (NCBI) database and the Blastn algorithm. The criteria used for identification were: sequence coverage >80%; similarity to taxon level 98–100%, similarity to genus level 94–97%. Sequences not matching these criteria were considered unidentified and were given unique names as shown in table 2.

Statistical analyses

Differences in relative abundance (estimated from the total number of sequences for each sample) of dominant fungal taxa detected in larvae and in adults of *D. micans* was compared by non-parametric chi-square (χ^2) test (Magurran, 1988). Shannon diversity index and quantitative Sorensen similarity index were used to characterize diversity and composition of fungal communities in larvae and adults of *D. micans* (Shannon, 1948; Magurran, 1988). Clark–Evans nearest neighbour method (Clark & Evans, 1954) was used to estimate whether the spatial distribution of attacked trees in the Šakių seed orchard (Site 15) of *P. abies* and in a plantation of *P. pungens* (both used for phenology observations) were random ($R = 1$), even ($R > 1$) or clustered ($R < 1$). The statistical test of significance of the nearest neighbour statistic provides the degree to which the distribution of individuals on a given area differs from that of a random distribution, and is estimated as the ratio of expected and observed mean value of the nearest neighbour distances.

Results

Occurrence of D. micans

Inspection of different seed orchards of *P. abies* showed that the occurrence *D. micans* was relatively rare and in many cases was not detected in western Lithuania (fig. 1). Recent and characteristic signs of damage, as well as, viable individuals of *D. micans* were detected in seed orchards of Dubravos (Site 2), Marijampolės (Site 7), Rokiškio (Site 14), Šakių (Site 15) and Šilutės (Site 16) forest enterprises (table 1). In the remaining seed orchards, the majority of *D. micans* occurrences were from the previous year or earlier years. In these sites, there were a number of adult females that were buried in the resin, and thereby failed to colonize the trees. In addition to the seed orchards of *P. abies*, *D. micans* was also detected in a plantation of *P. pungens* situated in mid-eastern Lithuania (fig. 2). Analysis of the spatial distribution of attacked trees in the Šakių seed orchard (Site 15) of *P. abies* and in the plantation of *P. pungens* using Clark–Evans nearest-neighbour method revealed that in both sites, there was clustered distribution of attacked trees ($R < 0.35$ and $R < 0.47$, respectively).

Phenology of D. micans

D. micans overwintered under the bark as larvae of the II and III developmental stages. Following overwintering, larvae were actively feeding and at the end of April under the bark of *P. pungens* there were larvae of both III and V developmental stages, and pupae. Their abundance was 26.0, 57.0 and 17.0%, respectively. At the same time, under the bark of *P. abies* there were larvae of II, III and IV developmental stages abundance was 14.8, 37.0 and 48.2%, respectively. In the middle of May, in *P. pungens* there were 79.0% of the V developmental stage larvae, 4.3% of pupae and 16.7% of young adults. For *P. abies* there were only larvae of III (6.0%), IV (67.0%) and V (27.0%) developmental stages. In the beginning of June, in *P. pungens* there were 24.2% of larvae of the V developmental stage, 24.2% of pupae, 30.3% of young adults and 21.3% of mature adults, while in *P. abies* we detected only larvae of the V

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Table 2. Relative abundance (%) of the 20 most common fungal taxa directly sequenced from larvae and adult beetles of *D. micans*. The number of individuals (larvae and adult beetles) sequenced is shown in the parenthesis. Sites are numbered as in table 1.

Taxon	Phylum	NCBI reference	Sequence similarity (%)[1]	Larvae						Adults (25)	All (150)
				Site 2 (25)	Site 7 (25)	Site 14 (25)	Site 15 (25)	Site 16 (25)	All (125)		
Peterozyma toletana	Ascomycota	HG329746	338/343 (99)	27.6	66.4	36.3	53.3	64.8	57.3	3.2	37.5
Yamadazyma scolyti	Ascomycota	HE612108	343/347 (99)	0.1	0.0	1.9	2.8	0.2	1.0	80.3	30.0
Kuraishia capsulata	Ascomycota	NR_119643	338/343 (99)	33.7	23.0	44.6	27.9	17.4	25.8	3.7	17.7
Unidentified sp. 2183_3	Ascomycota	KP898035	332/343 (97)	11.3	4.2	1.2	2.2	5.5	4.3	8.1	5.7
Nakazawaea holstii	Ascomycota	KM065944	344/348 (99)	2.2	4.2	0.6	0.0	–	2.3	0.0	1.5
Candida sp. 2183_5	Ascomycota	JX965188	327/342 (96)	1.3	0.5	5.8	4.6	1.1	2.0	0.1	1.3
Unidentified sp. 2183_6	Ascomycota	FJ824642	283/327 (87)	0.0	–	–	4.5	–	1.4	–	0.9
Rhodotorula sp. 2183_7	Basidiomycota	EU484321	351/369 (95)	2.7	0.1	1.6	2.6	0.0	1.2	0.2	0.8
Cladosporium herbarum	Ascomycota	KP939079	302/306 (99)	0.4	0.0	0.1	0.0	0.4	0.1	1.6	0.6
Candida sp. 2183_8	Ascomycota	HQ623551	339/349 (97)	–	0.0	0.2	1.0	0.0	0.3	1.2	0.6
Unidentified sp. 2183_11	Ascomycota	KP897167	330/330 (100)	2.6	0.4	0.1	0.1	1.2	0.6	0.1	0.4
Phoma herbarum	Ascomycota	JX160061	308/311 (99)	4.4	0.0	0.1	0.0	0.4	0.5	0.0	0.3
Myxozyma sp. 2183_12	Ascomycota	AF530607	288/307 (94)	1.8	0.0	–	0.5	–	0.3	–	0.2
Leptographium lundbergii	Ascomycota	KF748121	350/358 (98)	0.1	0.0	2.0	0.1	0.0	0.1	0.4	0.2
Unidentified sp. 2183_13	Basidiomycota	KC588788	354/359 (99)	2.7	–	0.1	–	–	0.3	–	0.2
Umbelopsis isabellina	Mucoromycotina	KJ028792	348/353 (99)	0.2	0.3	–	0.0	–	0.1	–	0.1
Candida sp. 2183_20	Ascomycota	JX965188	324/342 (95)	–	–	–	–	–	–	0.2	0.1
Zalerion arboricola	Ascomycota	FR837917	306/313 (98)	0.6	0.1	–	0.0	0.1	0.1	0.0	0.1
Aureobasidium pullulans	Ascomycota	KP985736	308/311 (99)	0.5	0.0	–	0.0	0.4	0.1	0.0	0.1
Unidentified sp. 2183_22	Basidiomycota	NR_073327	299/376 (80)	–	–	–	0.1	–	0.0	0.1	0.1
Total of 20 taxa				92.0	98.9	94.5	99.7	91.5	97.9	99.2	98.4

[1]The sequence similarity column shows the base pair comparison between the query sequence and the reference sequence at NCBI database, with the percentage of sequence similarity in the parenthesis.

developmental stage. By mid-June, in *P. pungens* we observed the beginning of the flying season of mature adults and new entry holes on the trees, while under the bark there were 34.5% of young adults and 65.5% of mature adults of *D. micans*. At this time, in *P. abies* there were only larvae of the V developmental stage. At the end of June, under the bark of *P. pungens* we detected a new generation of eggs of *D. micans*, while in *P. abies* there were larvae of the V developmental stage (44.4%) and pupae (55.6%). In the beginning of July, in *P. pungens* we recorded a period of intensive flying of mature adults of *D. micans*, numerous new entry holes in the same trees and in the neighbouring trees, and extensive egg laying under the bark. Eggs were laid in the cambium and each female produced up to 300 eggs, which were laid in groups of 50–80 in interconnected chambers. At the same time, in *P. abies* under the bark there were still larvae of the V developmental stage and pupae, but all of them were dead as these were destroyed by microbial parasites and/or predating insects. Thus, further observations in *P. abies* were not possible. Observations in *P. pungens* were continued and in the beginning of August there were 56.9% of eggs and 20.0% of larvae of the I developmental stage and 23.1% of the II stage under the bark. In September, we detected larvae of both II (60.0%) and III (40.0%) developmental stages, and later observations showed that larvae of these two stages remained under the bark for overwintering. In both sites, the average temperatures were similar during the entire period of phenology observations (fig. 3).

Morphology of D. micans

Morphological assessment showed that the head width of larvae of the I developmental stage was 0.48 ± 0.02 mm, and the body length was 2.20 ± 0.01 mm; of the II stage it was 0.68 ± 0.03 and 2.90 ± 0.04 mm, respectively; of the III stage it was 0.98 ± 0.03 and 4.79 ± 0.11 mm, respectively; of the IV stage it was 1.26 ± 0.02 and 5.39 ± 0.03 mm, respectively; and of the V stage it was 1.71 ± 0.01 and 8.18 ± 0.15 mm, respectively. The body length of pupae was 7.06 ± 0.24 mm. The body length of young adults was 7.10 ± 0.25 mm and width was 2.84 ± 0.06 mm, and remained similar at maturity. The young adults were light brown in colour, and as they matured, the colour darkened to brown and black. Eggs of *D. micans* were white, oval and 0.61 ± 0.01 mm in width and 1.10 ± 0.03 mm in length.

Fungi cultured from D. micans

Fungal culturing resulted in 115 pure cultures, and based on mycelial morphology were divided into 10 groups potentially representing different taxa. Morphological identification showed that in a majority of the cases, fungal culturing resulted in yeasts of the genus *Saccharomyces*, which were growing out from 70.0% of larvae but not from pupae or adult beetles. In contrast, fungi from the genus *Penicillium*, that represented 6.1% of cultures, were cultured from different developmental stages of *D. micans*. *Leptographium* sp. represented 22.0% of the fungal cultures. This species was also cultured from different developmental stages of *D. micans*. A single culture of *Graphium* sp. was cultured from an adult beetle.

Fungi directly sequenced from D. micans

Ion Torrent sequencing from 125 larvae and 25 adults of *D. micans* and subsequent quality filtering showed the presence of 139,097 high-quality ITS rDNA sequences (mean length of 347 bp). Clustering of these sequences resulted in 376 non-singleton contigs (at 98.5% similarity representing different taxa) and in 1167 singleton contigs, which were excluded from the further analyses. Taxonomic identification using the RDP pipeline classifier showed that 319 taxa were of fungal origin (all non-singleton fungal sequences are provided in online Supplementary table 1) and 57 of non-fungal origin, which were later excluded from further analyses. In this study, the detected fungi were 82.3% Ascomycota, 16.8% Basidiomycota and 0.9% Mucoromycotina. The absolute richness of fungal taxa was higher in larvae (277 taxa out of 86,784 sequences) than in adults (95 out of 50,188), and the χ^2 test showed that taxa richness between these datasets differed significantly ($P < 0.0001$). Information on the 20 most common fungal taxa representing 98.4% of all fungal sequences is shown in table 2.

Among these, however, five taxa representing 7.2% of fungal sequences could not be identified to taxon or genus level and remained unidentified (table 2). The most common fungal taxa were *Peterozyma toletana* (37.5%), *Yamadazyma scolyti* (30.0%) and *Kuraishia capsulate* (17.7%). The plant pathogen *Leptographium lundbergii* (0.2%) was the fourteenth most common fungus, though at low abundance, was detected in both larvae and beetles (table 2). The χ^2 test showed that among the 20 most common fungal taxa, the relative abundance of *P. toletana*, *K. capsulate*, Unidentified sp. 2183_3, *Nakazawaea holstii*, *Candida* sp. 2183_5, *Rhodotorula* sp. 2183_7, Unidentified sp. 2183_11, *Phoma herbarum*, *Zalerion arboricola* and *Aureobasidium pullulans* was significantly higher in larvae than in beetles. On the other hand, the relative abundance of *Y. scolyti*, *Cladosporium herbarum*, *Candida* sp. 2183_8, *L. lundbergii* and Unidentified sp. 2183_22 was significantly higher in beetles than in larvae. The remaining 299 fungal taxa were very rare and their relative abundances varied between 0.001 and 0.04% (online Supplementary table 1). The Sorensen index of similarity of fungal communities was low (0.23) when these were compared between larvae and beetles. The Shannon diversity index of fungal communities was 2.1 in larvae and 1.3 in beetles.

Discussion

During outbreaks, *D. micans* can cause severe damage to stands of *Picea* spp. (Bevan & King, 1983). At the time of this study, the population density of *D. micans* appeared to be at the minimum, and the beetles caused relatively mild damage to trees of *P. abies* in central and eastern parts of Lithuania. Clark–Evans nearest-neighbour analysis of spatial distribution showed that *D. micans* likely disperses over relatively short distances and attacks primarily the neighbouring trees, and thus resulting in the clustered distribution of damaged trees (fig. 2). The surveys showed that at the same time point within a site there were individuals of *D. micans* of different developmental stages. Moreover, it appeared that the development of *D. micans* was greatly dependent on the host tree species as temperatures in both sites were similar during the period of observations (fig. 3). However, the possibility should not be excluded that multiple factors have influenced the development of *D. micans*, suggesting that further studies might be needed to address this issue. Moreover that changing environments may have a major effect on the population dynamics and phenology of insects (Chaves *et al.*, 2012). Nevertheless, in the present study, development of *D. micans* was quicker and

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D. micans had larger nests under the bark of *P. pungens* than of *P. abies*. This is further supported by Wainhouse & Beechgarwood (1994) who showed that development of *D. micans* larvae differed in different conifer trees species. Additionally, the association of *D. micans* with *P. pungens* and its local outbreaks were also reported from Czech Republic (Lukasova *et al*., 2014). Lukasova *et al*. (2014) concluded that it may constitute a new threat to urban and forest trees of *P. pungens*, and that special attention should be given to these trees while monitoring population dynamics of *D. micans*. In the present study, the development of *D. micans* under the bark of *P. abies* was prolonged and often unsuccessful due to activity of microbial parasites and/or predating insects. Sevim *et al*. (2010) showed that pathogenic efficacy of entomopathogenic fungi against *D. micans* was 83–100% for larvae and 23–100% for beetles. *Rhizophagus grandis*, the specific predator of *D. micans*, was shown to have up to 84% effectiveness against *D. micans* when co-occurring in the galleries (Alkan Akinci *et al*., 2003). Furthermore, our study confirmed that in Lithuania, *D. micans* exhibits the following developmental stages: adult, egg, I–V developmental stage larvae and pupa. These developmental stages and morphological characteristics were similar to those reported from other European countries (e.g., Forestry Commision, 2015) indicating that information acquired in the present study has broader application elsewhere with similar environmental conditions.

Although the bacterial flora associated with *D. micans* has been relatively well investigated (Yilmaz *et al*., 2006), the fungal communities have received less attention. The fungal studies have been mainly focused on the isolation and testing of fungal pathogens for biocontrol (Sevim *et al*., 2010). The present study, by using both fungal culturing methods and high-throughput sequencing, revealed that *D. micans* is associated with species-rich fungal communities that was dominated by the yeasts (table 2). Among these, *P. toletana* has been mainly isolated from insect frass in fir and spruce trees or from wood products. This suggests that the primary habitat of, *P. toletana* is in association with wood-boring insects and their larvae (Kurtzman *et al*., 2011). The second most common fungus of this study, *Y. scolyti*, was also commonly isolated from tree-boring insect frass (Kurtzman *et al*., 2011). Interestingly, Boone *et al*. (2008) reported that parasites and dipteran predators may exploit volatiles of fungal symbionts including that of *Y. scolyti* to locate bark beetles. *K. capsulata*, the third most common fungus, has similar ecology to that of *P. toletana* and *Y. scolyti*. However, *K. capsulata* produces extracellular polysaccharides that cause fungal cells to adhere to the bark beetles, suggesting that the fungus has likely co-evolved with its beetle host, and thereby has specifically adapted to dispersal by the beetles (Wicherham *et al*., 1970). Similar to what has been shown for *I. typographus* (Persson *et al*., 2009), the present study reports the occasional occurrence of several basidomycetes wood-decay fungi including *Fomitopsis pinicola*, *Sistotrema brinkmannii* and *Ganoderma applanatum* (online Supplementary table 1). Among these, *F. pinicola* was previously detected on several *Dendroctonus* species in North America including: *D. pseudotsugae*, *D. brevicomis*, *D. ponderosae* and *D. valens* (Castello *et al*., 1976; Pettey & Shaw, 1986; Lim *et al*., 2005). Our study also revealed the presence of several plant pathogens including *L. lundbergii* (table 2). The genus *Leptographium* include important forest pathogens and blue stain fungi (Jacobs *et al*., 2005), which are commonly found in association with bark beetles (Linnakoski *et al*., 2012). Although Lieutier *et al*. (1992) were able to culture several ophiostomatoid fungi, with *O. canum* being most common, in the present study only *O. bicolor* was detected occasionally in the larvae of *D. micans* by the sequencing method (online Supplementary table 1). For *I. typographus*, Persson *et al*. (2009) have also detected a number of ophiostomatoid fungi including *O. bicolor* using both fungal culturing and molecular methods. Together, this demonstrates that *O. bicolor* is associated with different species of beetles. Furthermore, these studies highlight that fungal culturing and sequencing methods are both necessary and required to obtain a more complete picture of fungal communities associated with beetles (Lim *et al*., 2005; Giordano *et al*., 2012). Taken together, our study demonstrated that *D. micans* associates with different functional groups of fungi, including pathogens, and that some of these fungi may have negative effects on both the health of forest trees and timber quality.

Conclusions

At the time of the study, the population of *D. micans* was at its minimum with individuals mainly detected in central and eastern parts of Lithuania. *D. micans* is an annual univoltine species but development of individuals is largely dependent on the host tree species and/or the specific environmental factors of a site. *D. micans* is associated with a species-rich community of fungi, whereby the majority belong to yeasts from a class Saccharomycetes.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0007485316001048.

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