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ORIGINAL ARTICLE

Fungi associated with *Ips acuminatus* (Coleoptera: Curculionidae) in Ukraine with a special emphasis on pathogenicity of ophiostomatoid species

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Abstract. Conifer bark beetles are well known to be associated with fungal complexes, which consist of pathogenic ophiostomatoid fungi as well as obligate saprotroph species. However, there is little information on fungi associated with *Ips acuminatus* in central and eastern Europe. The aim of the study was to investigate the composition of the fungal communities associated with the pine engraver beetle, *I. acuminatus*, in the forest-steppe zone in Ukraine and to evaluate the pathogenicity of six associated ophiostomatoid species by inoculating three-year-old Scots pine seedlings with these fungi. In total, 384 adult beetles were collected from under the bark of declining and dead Scots pine trees at two different sites. Fungal culturing from 192 beetles resulted in 447 cultures and direct sequencing of ITS rRNA from 192 beetles in 496 high-quality sequences. Identification of the above revealed that the overall fungal community was composed of 60 species. Among these, the most common were *Entomocorticium* sp. (24.5%), *Diplodia pinea* (24.0%), *Ophiostoma ips* (16.7%), *Sydowia polyspora* (15.1%), *Graphilbum* cf *rectangulosporium* (15.1%), *Ophiostoma minus* (13.8%) and *Cladosporium pini-ponderosae* (13.0%). Pathogenicity tests were done using six species of ophiostomatoid fungi, which were inoculated into Scots pine seedlings. All ophiostomatoid fungi tested successfully infected seedlings of Scots pine with varying degrees of virulence. *Ophiostoma minus* was the only fungus that caused dieback in inoculated seedlings. It is concluded that *I. acuminatus* vectors a species-rich fungal community including pathogens such as *D. pinea* and *O. minus*. The fungal community reported in the present study is different from that reported in other regions of Europe. Pathogenicity tests showed that *O. minus* was the most virulent causing dieback in seedlings of Scots pine, while other fungi tested appeared to be only slightly pathogenic or completely non-pathogenic.

INTRODUCTION

Bark beetles (Coleoptera: Curculionidae) are among the most destructive insects in forest ecosystems and may cause huge economic losses (Ploetz et al., 2013). For example, the European spruce bark beetle, *Ips typographus* L. (Coleoptera: Curculionidae: Scolytinae), is capable of killing trees in large numbers, which makes it the most important and destructive bark beetle in both economic and ecological terms in coniferous forests in the palaearctic region (Christiansen & Bakke 1988; Öhrn, 2012). A well-known characteristic of bark beetles is their association with specific fungi, most commonly with ophiostomatoid fungi (Wingfield et al., 1993; Kirisits, 2004; Linnakoski et al., 2012), which include genera that are morphologically similar, although not phylogenetically closely related (De Beer & Wingfield, 2013). The group includes genera such as *Ophiostoma*, *Ceratocystiopsis*, *Graphilbum*, *Raffaelea* and *Leptographium* in the Ophiostomatales, and *Ceratocystis* sensu stricto, *Chalaropsis*, *Endoconidiophora* and *Graphium* in the Microascales (De Beer et al., 2013, 2014). Moreover, fungi belonging to the genus *Geosmithia* in the Hypocreales (Ascomycota) and producing dry-spores, are also known as associates of conifer-infesting bark beetles (Kolařík & Jankowiak, 2013; Jankowiak et al., 2014; Dori-Bachash et al., 2015).

The ophiostomatoid fungi include some of the best-known tree pathogens (Kirisits, 2004), which are responsible for discolouration of wood, serious tree diseases and high rates of tree mortality (Wingfield et al., 1993; Kirisits, 2004; Linnakoski, 2011). For example, the pathogenic fungus and primary invader, *Endoconidiophora polonica* (Siemaszko) Z.W. de Beer, T.A. Duong & M.J. Wingf. (previously known as *Ceratocystis polonica*), which can be an aggressive pathogen and kill trees (Solheim, 1988; Kirisits,

2010), is associated with the spruce bark beetle, *Ips typographus*. Some bark beetles of the family Scolytinae vector the Dutch elm disease pathogens, *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier (Menkis et al., 2016), which during the last 100 years have destroyed billions of elm trees (*Ulmus* spp.) worldwide (Kirisits, 2013).

Bark beetles and fungi form complex and dynamic associations, which have been shaped during long periods of co-evolution (Six & Wingfield, 2011; Villari et al., 2013). Although a number of studies have investigated bark beetle-fungus interactions since they were first recognized in the 19th century (Harrington & Wingfield, 1998; Six & Wingfield, 2011; Kirisits, 2013), some interactions and beetle-fungus associations are still poorly understood (Six & Wingfield, 2011). This can probably be explained by the fact that most previous studies mainly focused on the most economically important bark beetle species (Lieutier et al., 2009; Villari et al., 2012; Jankowiak & Bilanski, 2013).

One such bark beetle, that is still poorly investigated, is *Ips acuminatus* Gyll. (Coleoptera: Curculionidae), the pine engraver beetle. This species infests the thin bark of Scots pine (*Pinus sylvestris* L.) throughout Europe (Villari et al., 2013). For many years *I. acuminatus* was considered of minor significance, however, recently *I. acuminatus* is reported as commonly causing extensive damage to young plantations and stands of Scots pine (Lieutier et al., 1991; Villari, 2012; Siitonen, 2014). Scots pine is among the most common and important tree species with a wide distribution in hemiboreal forests in Europe.

Several of the species of *Ophiostoma* associated with *Ips acuminatus* can cause reduction in growth, crown thinning, chlorosis or even death of trees (Colombari et al., 2012; Villari, 2012). Among the most common fungal species associated with *I. acuminatus*, *Ophiostoma clavatum* Math. is the most consistently reported (Mathiesen, 1950, 1951; Rennerfelt, 1950; Francke-Grosmann, 1952, 1963; Mathiesen-Käärik, 1953; Käärik, 1975). *O. brunneo-ciliatum* Math.-Käärik, although morphologically similar to *O. clavatum*, is reported in a few studies (Lieutier et al., 1991; Guérard et al., 2000; Villari et al., 2012) as an associate of *I. acuminatus*.

There is a general lack of information about fungi associated with *I. acuminatus* in central and eastern Europe as previous studies have mainly been conducted in other regions of Europe, including Sweden (Mathiesen, 1950, 1951; Mathiesen-Käärik, 1953; Käärik, 1975a, 1980), Germany (Francke-Grosmann, 1952, 1963), Finland (Linnakoski et al., 2012), Norway (Waalberg, 2015), Italy (Villari et al., 2012) and France (Lieutier et al., 1991). As damage caused by *I. acuminatus* is increasingly recorded (Villari, 2012; Siitonen, 2014), the investigation of fungal communities associated with *I. acuminatus* and assessment of the pathogenicity of ophiostomatoid fungi to host trees is of particular interest. Such information can be of particular practical importance for forest health, yet they have never been investigated in Ukraine, which is at the south-eastern edge of the distribution of Scots pine in Europe.

The aim of this study was to investigate the composition of fungal communities associated with the pine engraver beetle, *Ips acuminatus*, in the forest-steppe zone in eastern Ukraine. We also evaluated the pathogenicity of six associated ophiostomatoid by inoculating three-year-old seedlings of Scots pine with these species of fungi.

MATERIALS AND METHODS

Study sites and sampling

The sites studied were two forest stands located in the Luhansk (48°43′N, 39°05′E) and Kharkiv (49°10′N, 37°14′E) regions, which are in the forest-steppe zone in eastern Ukraine. Stands at both sites were ca. 50–70 year-old plantations of Scots pine. Sampling of *I. acuminatus* was carried out in May 2012. At each site, 192 adults of *I. acuminatus* were collected from stems of 38 randomly selected declining or dying trees attacked by bark beetles, resulting in a total of 384 individuals. Selected declining and dying trees were heavily defoliated due to the extensive damage caused by pine sawflies (*Neodiprion serifier* Geoff. and *Diprion pini* L., (Hymenoptera: Diprionidae) in the same season. The bark beetle entry holes were most common at the top of stems (between ca. 12 and 26 m from the base). The time of sampling coincided with emergence of *I. acuminatus* beetles from their galleries and their flight period. Each beetle was collected from a separate gallery; in total, between 2 and 8 individuals (5.1 on average) per tree were collected.

The trees were felled, bark was carefully removed and *I. acuminatus* beetles were sampled using sterilized forceps, individually placed in sterile 1.5 ml centrifugation tubes, labelled and transported to a laboratory. Half of the beetles from each site (Luhansk and Kharkiv) were stored at 4°C for fungal culturing and the other half at –20°C for direct DNA sequencing of fungi.

Fungal isolates and molecular identification of species

One to seven days after collection, 96 dead frozen beetles from each site (192 in total) were placed separately (without surface sterilisation and crushing) in Petri dishes containing ca. 30 ml of Hagem agar medium supplemented with the antibiotic chloramphenicol (0.5%) and incubated at room temperature (ca. 21°C) in the dark (Persson et al., 2009). Petri dishes were checked daily for two months and outgrowing fungal mycelia were subcultured in new Petri dishes with fresh medium. Fungal cultures were divided into groups based on their morphology, and for species identification representative cultures from each group were subjected to sequencing of the internal transcribed spacer regions of the fungal ribosomal RNA (ITS rRNA). Isolation of DNA, amplification and sequencing followed the methods described by Menkis et al. (2006). Amplification by PCR was done using primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White et al., 1990). In addition, the β-tubulin gene and partial elongation factor 1-alpha (EF1-a) gene were amplified and sequenced for fungi of the genera *Ophiostoma*, *Graphilbum* and *Grosmannia*. The β-tubulin gene was amplified using the primers Bt2a and Bt2b (Glass & Donaldson, 1995) and the EF1-a gene the primers EF1F and EF2R following the protocols of Jacobs et al. (2004).

Each PCR reaction contained 200 µM deoxyribonucleotide triphosphates, 0.2 µM of each primer, 0.03 U/µl Thermo Green Taq polymerase with reaction buffer Green, and 2.75 mM final concentration of $MgCl_2$. The thermal cycling was carried out using an Applied Biosystems GeneAmp PCR System 2700 thermal cycler (Foster City, CA, USA).

For ITS primers an initial denaturation step at 95°C for 5 min was followed by 35 amplification cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for

30 s. The thermal cycling was ended by a final extension step at 72°C for 7 min. PCR conditions for EF1-a amplification were the same as those for ITS except the annealing temperature, which was 60°C. Thermal cycling conditions for the β-tubulin gene were 2 min at 95°C followed by 35 cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 45 s, with a final extension of 72°C for 5 min.

PCR products were separated on 1% agarose gels and visualized under UV light. The PCR products were purified with Qiagen DNA extraction PCR M kit (Qiagen, Hilden, Germany). Sequencing was carried out by Macrogen Inc., Korea. Raw sequence data were analyzed using the SeqMan Pro version 10.0 software from DNASTAR package (DNASTAR, Madison, WI, USA). Sequences were quality-filtered and assembled into different contigs (of ≥ 98% similarity representing different fungal species), which were used for analysis. Databases at GenBank (Altschul et al., 1990) and at the Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, were used to determine the identity of ITS rRNA sequences. The criteria used for identification were: sequence coverage > 80%; similarity to species level 98–100%, similarity to genus level 94–97%. All generated sequences were deposited in GenBank and accession numbers are provided in Table 1.

Sequencing of fungi from the beetles

Isolation of DNA (without surface sterilisation) from the beetles (beetles were frozen, crushed and DNA was isolated separately from each individual), amplification and sequencing of fungal ITS rRNA were carried out as described by Persson et al. (2009). Amplification by PCR was done in two steps: firstly using fungal specific primers NLC2 (GAGCTGATTCCCAAACAACTC) and NSA3 (AAACTCTGTCGTGCTGGGGATA) (Davydenko et al., 2014), and then in a second (nested) PCR using primers ITS1F and ITS4 as above. PCR products were separated on 1% agarose gels and visualized under UV light. If only one DNA band was present on the gel per sample, following nested PCR, the PCR product was used for sequencing. Multiple-banded PCR products were separated on 2.0% agarose gels, individual bands were excised and re-amplified using the universal primers ITS1 and ITS4 (White et al., 1990). Resulting single-band products were sequenced in both directions using the same primers as for PCR amplification. Sequencing and analysis of sequencing data were performed as described above. All generated sequences were deposited in GenBank (Table 1).

Pathogenicity tests

To investigate the effect of the ophiostomatoid fungi isolated from *I. acuminatus* infesting Scots pine, three-year-old seedlings of Scots pine produced in a nursery were inoculated with the fungi. Seedlings were grown outdoors in containers and watered as required. Stem diameters at the inoculation site were between 10.4 mm and 13.2 mm (12.2 mm on average). Six species of ophiostomatoid fungi, that were commonly cultured from *I. acuminatus*, were used in the pathogenicity tests: *Graphilbum* cf. *rectangulosporium*, *O. ips*, *O. minus*, *O. pallidulum*, *O. piceae* and *Grosmannia olivacea.* Fungi were maintained on 2% malt-extract agar (MEA) medium and actively growing 15-day-old fungal mycelia were used for inoculation. In May 2012, 120 seedlings of Scots pine were inoculated with selected fungal species (20 seedlings for each species). In addition, twenty plants were inoculated with sterile 2% MEA as controls. Inoculations were done by cutting out a piece (ca. 4 × 8 mm in size) of the bark on the stem ca. 40 cm above the root collar with a sterile scalpel, placing fungal inoculum on the exposed sapwood and covering it up with the bark and wrapping with Parafilm, as described by Krokene & Solheim (1998). These plants were monitored weekly for 20 weeks. A seedling was considered dead when the bark and needles above the inoculation point were discoloured and dead. After 20 weeks, all plants were harvested and the bark was removed around the inoculation site. The length of the necrotic lesion on the sapwood and the depth of blue-stain were measured to estimate the area colonized. In addition, two pieces of wood tissue ca. 1 cm in length from each side of the infection zone were removed and plated on Hagem agar medium containing antibiotics (0.8% cycloheximide and 0.2% streptomycin sulphate, CSMA) in order to re-isolate the inoculated fungi. In total, 280 wood pieces were used for re-isolation of fungi. Plates were stored for five weeks at 20°C in the dark and checked daily for growth of the inoculated fungi.

Statistical analyses

Richness of fungal species detected in beetles from trees of different health status (living vs. dead), at different sites (Luhansk vs. Kharkiv) and detected by different methods (culturing vs. sequencing) was compared using chi-squared tests (Mead & Curnow, 1983). The relative abundance of fungal species was calculated from actual numbers of observations (presence/absence data) as a percentage of the observations (fungal cultures/sequences) for the total fungal community. Shannon diversity indices and quantitative Sorensen similarity indices were used to characterize the diversity and composition of fungal communities (Shannon, 1948; Magurran, 1988). The Simpson diversity index (Simpson, 1949) was used to estimate the dominance in fungal diversity while taking into account both richness and evenness. Fungal dominance was determined using Camargo's index (Camargo, 1993) and dominant species are marked with "d" in the table. A species was defined as dominant if Pi > 1/S. The data from the inoculation test were analyzed using analysis of variance (ANOVA). Significant treatment differences were further evaluated by Fisher's (LSD) test in STATISTICA® 7.0 (StatSoft, Inc., Tulsa, OK, USA).

RESULTS

Fungi vectored by *Ips acuminatus*

Of the 192 beetles of *I. acuminatus* used for fungal culturing, 183 individuals (95.3%) yielded fungal cultures (Table 1). There were between one and five different fungal cultures from each beetle (2.3 on average), resulting in 447 cultures in total. The sequencing of representative fungal cultures revealed the presence of 42 distinct fungal species among which 37 (88.1%) could be identified to species level, four (9.5%) to genus level and one (2.4%) remained unidentified (Table 1). All of the 192 beetles used for direct amplification and sequencing of the fungal ITS rRNA region resulted in successful amplification. There were between one and three fungal amplicons (2.6 on average) from each. Separation and sequencing of individual amplicons resulted in 496 high-quality sequences for 54 distinct fungal species among which 39 (72.6%) were identified to species level, ten (18.5%) to genus level and five (9.2%) remained unidentified (Table 1). A chi-squared test revealed no statistically significant difference in the richness of fungal species detected by culturing vs. direct sequencing ($p > 0.05$). The Sorensen index of similarity of fungal communities was high (0.59) when the results of culturing vs. direct sequencing were compared. Pooling the results from culturing and sequencing revealed

Table 1. Frequency (%) of fungal species cultured / directly sequenced from adults of *Ips acuminatus* that were collected from *Pinus sylvestris* growing at Luhansk and Kharkiv in eastern Ukraine.

Fungal species	Genbank access. no.	Luhansk			Kharkiv			Total
		Sequencing	Culturing	All	Sequencing	Culturing	All	
Ascomycota (ophiostomatoid species)								
Graphilbum cf. *rectangulosporium* Ohtaka, Masuya & Yamaoka [d]	KU663982	–	11.5	5.7	13.5	35.4	24.5	15.1
Grosmannia olivacea (Math.-Käärik) Zipfel, Z.W. de Beer & M.J. Wingf.	KU663987	–	3.1	1.6	–	–	–	0.8
Ophiostoma ips (Rumbold) Nannfeldt	KU663983	–	23.9	11.9	12.5	30.2	21.4	16.7
Ophiostoma minus (Hedgc.) Syd. & P. Syd [d]	KU663984	12.5	16.7	14.6	4.2	21.9	13.0	13.8
Ophiostoma pallidulum Linnak., Z.W. de Beer & M.J. Wingf.	KU663985	–	7.3	3.7	2.1	27.1	14.6	9.1
Ophiostoma piceae (Münch) Sydow & P. Sydow	KU663986	–	7.3	3.7	–	19.8	9.9	6.8
Other Ascomycota								
Alternaria alternata (Fries) Keissler	KU663948	1.0	–	0.5	8.3	5.2	6.8	3.7
Anthostomella pinea Crous	KU663949	–	–	–	6.3	–	3.1	1.3
Apiospora montagnei Sacc.	KU663950	–	–	–	–	9.4	4.7	2.3
Aspergillus pseudoglaucus Blochwitz	KU663951	–	–	–	–	6.3	3.1	1.6
Aspergillus versicolor (Vuill.) Tirab.	KU663952	–	–	–	2.1	2.1	2.1	1.1
Beauveria bassiana (Balsamo-Crivelli) Vuillemin	KU663953	2.1	6.3	4.2	1.0	3.1	2.1	3.1
Bionectria ochroleuca (Schwein.) Schroers & Samuels	KU663954	–	–	–	5.2	–	2.6	1.3
Bionectriaceae sp. Samuels & Rossman	KU663955	–	–	–	1.0	–	0.5	0.3
Chaetomium globosum Kunze ex Fries	KU663957	–	–	–	1.0	–	0.5	0.3
Chaetomium sp. Kunze	KU663958	–	5.2	2.6	–	14.6	7.3	4.9
Chalara sp. (Corda) Rabenhorst	KU663959	–	–	–	2.1	–	1.0	0.5
Cladobotryum dendroides (Bull.) W. Gams & Hooz	KU663960	–	–	–	1.0	3.1	2.1	1.0
Cladobotryum mycophilum (Oudem.) W. Gams & Hooz	KU663961	6.3	9.4	7.8	–	9.4	4.7	6.3
Cladosporium cladosporioides (Fresen.) G.A. de Vries	KU663962	4.2	–	2.1	3.1	2.1	2.6	2.3
Cladosporium pini-ponderosae K. Schub [d]	KU663963	22.9	-	11.5	17.7	11.5	14.6	13.1
Cladosporium sp. Link	KU663964	–	–	–	4.2	5.2	4.7	2.3
Cyclaneusma niveum (Pers.) DiCosmo, Peredo & Minter	KU663965	–	–	–	3.1	–	1.6	0.8
Dactylonectria macrodidyma (Halleen, Schroers & Crous) L. Lombard, van der Merwe & J.Z. Groenew. & Crous	KU663971	–	–	–	3.1	–	1.6	0.8
Gibberella avenacea R.J. Cook	KU663968	2.08	1.1	1.6	–	–	–	0.8
Isaria farinosa (Holmskjold) Fries	KU663972	–	2.1	1.1	1.1	15.6	8.3	4.7
Lophodermium seditiosum Minter, Staley & Millar	KU663973	2.08	–	1.1	–	5.2	2.6	1.8
Mariannaea elegans (Corda) Samson	KU663974	–	6.3	3.1	2.1	3.1	2.6	2.9
Metapochonia bulbilosa (W. Gams & Malla) Kepler, Rehner & Humbe	KU663975	–	–	–	1.1	2.1	1.6	0.8
Nakazawaea holstii (Wick.) Y. Yamada, K. Maeda & Mikata	KU663978	–	–	–	4.2	8.3	6.3	3.1
Neocatenulostroma germanicum (Crous & U. Braun) Quaedvl. & Crous	KU663979	–	–	–	2.1	–	1.1	0.5
Ogataea henricii (Wick.) Y. Yamada, K. Maeda & Mikata	KU663981	6.3	–	3.1	7.3	–	3.7	3.4
Ogataea neopini Nagatsuka, S. Saito & Sugiyama	KU663980	–	–	–	7.3	–	3.7	1.8
Penicillium citreonigrum Dierckx	KU663988	4.2	–	2.1	1.1	3.1	2.1	2.1
Penicillium roqueforti Thom	KU663990	4.2	–	2.1	–	7.3	3.4	2.9
Pezicula eucrita (P. Karst.) P. Karst	KU663991	2.1	–	1.1	–	5.2	2.7	1.8
Phoma macrostoma Montagne	KU663993	–	–	–	1.1	2.1	1.6	0.8
Phomopsis sp. Sacc. & Roum	KU663994	6.3	–	3.1	6.3	3.1	4.7	3.9
Pleosporales sp.	KU663989	2.1	–	1.1	2.1	–	1.1	1.1
Rhizoctonia sp. DC	KU663995	–	–	–	5.21	–	2.60	1.30
Diplodia pinea (Fr.) Dyko & B. Sutton [d]	KU663996	39.6	14.6	27.1	28.1	13.5	20.8	23.9
Sydowia polyspora (Brefeld & Tavel) E. Müller	KU663997	16.7	1.1	8.9	19.8	22.9	21.4	15.1
Talaromyces minioluteus (Dierckx) Samson, N. Yilmaz, Frisvad & Seifert	KU663998	2.1	–	1.1	–	3.1	1.6	1.3
Talaromyces purpureogenus Samson, Yilmaz, Houbraken, Spierenb., Seifert, Peterson, Varga & Frisvad	KU663999	–	–	–	1.1	7.3	4.2	2.1
Trichoderma asperellum Samuels, Lieckfeldt & Nirenberg	KU664000	4.2	–	2.1	–	2.1	1.1	1.6
Truncatella sp. Steyaert	KU664001	–	1.1	0.5	3.1	5.2	4.2	2.3
Unidentified Ascomycota175244	KU664003	8.3	–	4.2	20.8	–	10.4	7.3
Unidentified Helotiales HH79	KC768103	–	–	–	3.1	–	1.6	0.8
Unidentified Pezizales	KU664004	–	–	–	3.1	–	1.6	0.8
Basidiomycota								
Bjerkandera adusta (Willdenow) P. Karsten	KU663956	2.1	–	1.1	3.1	2.1	2.6	1.8
Entomocorticium sp. H.S. Whitney, Bandoni & Oberw [d]	KU663966	62.5	–	31.3	35.4	–	17.7	24.5
Fomitopsis pinicola (Swartz) P. Karsten	KU663967	14.6	–	7.3	11.5	5.2	8.3	7.8
Hebeloma sp. (Fr.) P. Kumm.	KU663969	6.3	–	3.1	–	7.3	3.7	3.4
Hyphoderma setigerum (Fr.) Donk	KU663970	–	–	–	7.3	9.4	8.3	4.2
Phlebiopsis gigantea (Fr.) Jülich	KU663992	4.2	–	2.1	–	3.1	1.6	1.8
Unidentified Basidiomycota FG139	KU664005	–	–	–	1.1	4.2	2.6	1.3
Mucoromycotina								
Mucor sp. P. Micheli ex L.	KU663977	2.1	–	1.1	–	–	–	0.5
Mucor fragilis Bainier	KU663976	–	–	–	–	6.3	3.1	1.6
Umbelopsis isabellina (Oudemans) W. Gams	KU664002	–	–	–	5.2	–	2.6	1.3
Unidentified culture *Mucor*-like		–	–	–	5.2	–	2.6	1.3
Total		24.3	11.8	36	28.3	35.6	63.9	100
No. of species		25	15	35	43	39	56	60
Shannon-Weaver diversity index		2.6	2.4	2.9	3.2	3.3	3.6	3.5
Simpson diversity index		0.11	0.1	0.1	0.1	0.04	0.03	0.04

Dominant species are marked with “d” in Table 1.

that there were 35 different fungal species recorded for Luhansk and 56 for Kharkiv (chi-square test, difference significant at $p < 0.0001$) (Table 1). Thirty five (58.3%) fungal species were common to both sites. As a result, the Sorensen index of similarity of fungal communities was 0.37 between the sites (Luhansk and Kharkiv). For different datasets (detected by different methods and at different sites), the Shannon diversity index varied between 2.4 and 3.59, while the Simpson diversity index was between 0.03 and 0.11 (Table 1).

The overall fungal community (detected by both methods and at both sites) was composed of 80% Ascomycota, 11.7% Basidiomycota and 8.3% Mucoromycotina. The most commonly detected fungi were *Entomocorticium* sp. (24.5%), *Diplodia pinea* (24.0%), *Ophiostoma ips* (16.7%), *Sydowia polyspora* (15.1%), *G.* cf. *rectangulosporium* (15.1%), *Ophiostoma minus* (13.8%) and *Cladosporium pini-ponderosae* (13.0%). The relative abundance of each of the remaining fungal species was lower than 10% (Table 1). Six ophiostomatoid fungi were detected and their identity confirmed using ITS rRNA, EF1-a and β-tubulin sequencing. *Ophiostoma ips* was the most common ophiostomatoid fungus (Table 1). While *G.* cf. *rectangulosporium* and *O. minus* were relatively highly abundant, *O. pallidulum*, *O. piceae* and in particular *G. olivaceum* were less common (9.11%, 6.8% and 0.78%, respectively) (Table 1). Interestingly, *O. pallidulum* was relatively common at Luhansk, while *G. olivaceum* was found only at Kharkiv (Table 1). *Entomocorticium* sp. was the most common species at both sites, where it was detected only by direct DNA sequencing. However, the number of beetles with an association with *Entomocorticium* sp. at Luhansk was almost twice as high as at Kharkiv.

A non-ophiostomatoid species, *Diplodia pinea* (Fr.) Dyko & B. Sutton, was also frequently detected with a 28.1% abundance at Kharkiv and 24.0% at Luhansk (Table 1).

Pathogenicity tests

Inoculation with the six ophiostomatoid fungi commonly resulted in the formation of necrotic wounds and resin flow from the stem, and revealed that the virulence and colonization patterns of the tissues of the different fungal species differed (Table 2). While only *O. minus* caused the death of the inoculated seedlings, both *O. minus* and *G.* cf. *rectangulosporium* caused seedling decline (Table 2). Dead and declining seedlings showed symptoms of stem necrosis, and discolouration and loss of needles. No dieback or decline was recorded in the control seedlings.

All inoculated fungi were able to infect plant tissues and cause lesions of different sizes (Table 2). With the exception of *G. olivacea*, all fungi caused dark-brown lesions. The lesions were generally covered with resin and extended vertically in both directions from the point of inoculation. *O. minus* caused significantly larger necrotic lesions than *G.* cf. *rectangulosporium* ($p < 0.05$) and both caused significantly larger necrotic lesions than *O. ips*, *O. pallidulum* and *O. piceae*, which caused similar sized lesions. In contrast to the other species, the size of the lesions caused by *G. olivacea* did not differ significantly from those of controls (Table 2). The inoculated fungi were successfully re-isolated from 70–100% of the plants (Table 2).

DISCUSSION

Fungal community

The results demonstrate that *I. acuminatus* in the Ukraine is associated with a species rich community of fungi, which includes 39 different genera and 60 different species (Table 1). This study also revealed site-specific differences in the fungal communities with that recorded at Kharkiv significantly more species-rich than that at Lugansk (Table 1). Difference between the fungal communities at Kharkiv and Luhansk may be due to differences in microclimate conditions at these two sites. For example, higher temperatures (even up to 45°C) throughout the vegetative season in the last decades and lower levels of precipitation at Luhansk than at Kharkiv might account for the lower abundance of fungi at Luhansk. Moreover, temperature fluctuations during the vegetative season can limit dispersal of some ophiostomatoid and other symbiotic fungi, which have lower range of optimal temperatures (Six & Bentz, 2007).

As mentioned above, the Sorensen index of similarity of the fungal communities was high (0.59) when comparing culturing vs. direct sequencing. Similarly, there was no significant difference between Shannon's diversity index ($p = 0.02$) among the fungal identification methods. Despite this, there were some significant differences in the fungal species detected using only one of these methods. *Grosmannia olivacea* was only found using the isolation method whereas *Entomocorticium* sp., several yeasts and other fungal species only by direct sequencing. Moreover, a recently discovered phytopathogen in Ukraine *Neocatenulostroma germanicum* (Markovskaja et al., 2016) was also

Table 2. Effect of inoculation of three-year-old *Pinus sylvestris* seedlings with ophiostomatoid fungi associated with *Ips acuminatus*. Values for the depth to which the blue-stain extended and lesion length are means ± one standard error.

Fungal species	Strain	Origin of strains	Depth of blue-stain (mm)	Length of the lesion (mm)	Dead plants (%)	Declining plants (%)	Re-isolation success (%)
G. cf *rectangulosporium*	AB41	Kharkiv	1.3 ± 0.12^{d}	11.6 ± 0.2^{c}	0^{a}	10^{c}	95
G. olivacea	AK77	Luhansk	0^{a}	0.9 ± 0.1^{a}	0^{a}	0^{a}	70
O. ips	AK188	Kharkiv	0.2 ± 0.04^{b}	3.9 ± 0.4^{b}	0^{a}	0^{a}	100
O. minus	AB14	Kharkiv	1.6 ± 0.11^{c}	19.6 ± 0.9^{d}	45^{b}	25^{b}	100
O. pallidulum	AK127	Kharkiv	0.2 ± 0.4^{b}	2.9 ± 0.2^{b}	0^{a}	0^{a}	80
O. piceae	AN1	Kharkiv	0.2 ± 0.03^{b}	3.2 ± 0.1^{b}	0^{a}	0^{a}	85
Control			0^{a}	0^{a}	0^{a}	0^{a}	0

Within columns, values followed by the same letter are not significantly different at $p = 0$.

found by direct sequencing. The latter demonstrates that the use of the two different detection methods provided complementary information about the fungal communities associated with *I. acuminatus* in Ukraine.

The present study, as well as providing new information for the south-eastern part of the Scots pine distribution in Europe, corroborates previous studies (Kirisits, 2004; Linnakoski et al., 2010) that fungi of the genera *Ophiostoma*, *Graphilbum* and *Grosmannia* are among the most common symbionts of bark beetles (Table 1).

Despite their common occurrence, the richness of species of *Ophiostoma* was relatively low and included only *O. ips, G.* cf. *rectangulosporium, O. minus, O. pallidulum, O. piceae* and *G. olivacea* (Table 1). Among these, *O. minus* is one of the most well studied and widespread ophiostomatoid fungi commonly associated with different species of bark beetle and pine-infesting weevils (Kirisits, 2004; Jankowiak, 2013). However, there are only three reports of it's association with *I. acuminatus*: Sweden (Mathiesen-Käärik, 1960), Finland (Linnakoski et al., 2012) and Norway (Waalberg, 2015). In the present study, *G.* cf. *rectangulosporium* was commonly associated with *I. acuminatus*. This fungus was also isolated earlier in Ukraine from *Hylurgus ligniperda* Fabricius (Coleoptera: Curculionidae) (Davydenko et al., 2014). Previous studies in Poland and Spain show that *G. rectangulosporium* is associated with weevils and bark beetles (Romon et al., 2007; Jankowiak & Kolařík 2010; Jankowiak & Bilański 2013). Moreover, in 2006 *G. rectangulosporium* was also described from Japan in association with the bark beetle infesting *Abies* spp. (Ohtaka et al., 2006) and in 2009 from China in association with *Dendroctonus valens* Le Conte (Coleoptera: Curculionidae) infesting *Pinus tabuliformis* Carr. (Lu et al., 2009).

Ophiostoma ips has a worldwide distribution and is associated with a number of different bark beetles (Kim et al., 2003, Zhou et al., 2007). In the present study, *O. ips* was isolated from *I. acuminatus* collected at both sites. Earlier reports indicate this fungus occurs at a low frequency in association with *H. lignipeda* in Ukraine (Davydenko et al., 2014) and is also found in Sweden (Mathiesen-Käärik, 1953), Finland (Linnakoski et al., 2012) and Norway (Waalberg, 2015).

Surprisingly, both *O. brunneo-ciliatum* and *O. clavatum* (or *O. clavatum* species complex), which are commonly associated with *I. acuminatus* in Italy (Villari et al., 2013) and Sweden (Mathiesen-Käärik, 1953, 1960), were not detected. A recent publication (Linnakoski et al., 2016) confirms that *O. clavatum* is closely associated with *I. acuminatus*, while *O. brunneo-ciliatum* appears to be mainly associated with another beetle, *Ips sexdentatus*. It is possible that high temperatures and low humidity during the vegetative period has limited the spread of these species into eastern Ukraine (Six & Bentz, 2007). In contrast, our results show that the ophiostomatoid community associated with *I. acuminatus* in Ukraine is similar to that of *Ips sexdentatus* (Börner), (Coleoptera: Curculionidae) in Poland (Jankowiak, 2012).

Interestingly, a mycangial fungus *Entomocorticium* sp., which was the most commonly detected fungus in the present study, has previously only been reported in association with *Dendroctonus ponderosa* Hopkins (Coleoptera: Curculionidae) (Whitney et al., 1987) and *Dendroctonus frontalis* Zimmermann (Coleoptera: Curculionidae) (Klepzig et al., 2004). *Entomocorticium* sp. may provide nutritional benefits to larvae of bark beetles, whereas *O. minus* results in poorly developed larvae, which often fail to reach the adult stage (Klepzig et al., 2004; Six, 2012). *Entomocorticium* sp. may provide some protection for larvae of bark beetles against the negative effect of *O. minus* (Kirisits, 2007). In contrast, *Ambrosiella macrospora* (Franke-Grosm.), which is primarily a food source for larvae and thought to be non-pathogenic, is commonly associated with *I. acuminatus* in Italy (Villari et al., 2012), but was not detected in the present study. The latter examples demonstrate that *I. acuminatus* may vector rather different fungal communities in different parts of Europe.

Diplodia pinea is a widely distributed pathogen of conifers causing Diplodia tip blight and stem canker disease (Oliva et al., 2013). Previously, associations between *D. pinea* and *Tomicus piniperda* L. (Coleoptera: Curculionidae), *Hylastes attenuatus* Erichson, (Coleoptera: Curculionidae), *Hylurgops palliates* (Gyll.) (Coleoptera: Curculionidae), and *Xyleborus dispar* (F.) (Coleoptera: Curculionidae) beetles were reported from northern Spain (Goldazarena et al., 2012). Also, a possible interaction between the exotic insect *Leptoglossus occidentalis* Heidemann (Hemiptera: Coreidae) and *D. pinea* is postulated because both these species damage pine cones in Italy (Luchi et al., 2012). Moreover, *D. pinea* is effectively vectored by *Hylastes ater* (Paykull) (Coleoptera: Curculionidae), *Hylastes opacus* Erichson (Coleoptera: Curculionidae) and *H. ligniperda* in Poland (Jankowiak & Bilański, 2013). The present study, therefore, provides evidence, for the first time, that *D. pinea* is commonly associated with *I. acuminatus* in Ukraine. As disease symptoms caused by *D. pinea* were often observed on Scots pine at the time of sampling, the possibility should not be excluded that in addition to the negative effect of *I. acuminatus* and ophiostomatoid fungi, *D. pinea* has also contributed to the decline and dieback of Scots pine, however this requires more specific investigation.

Interestingly, *Geosmithia* spp. are not detected in many studies on the fungal species associated with *I. acuminatus*. Anamorphic fungi of the genus *Geosmithia* (Ascomycota: Hypocreales) are associated with 33 species of bark beetles (Kolařík et al., 2008; Kolařík & Jankowiak, 2013). However, most isolations of species of *Geosmithia* are from bark beetle galleries (Jankowiak, 2006; Kolařík et al., 2008). All seven species of *Geosmithia* principally inhabit bark beetle galleries, but are rarely in insect-free wood (Kolařík et al., 2008).

Pathogenicity test

All the ophiostomatoid fungi tested infected seedlings of Scots pine with varying degrees of virulence. The pathogenicity results were generally consistent with pre-

viously described inoculation experiments, with similar lesion morphology and patterns of plant tissue colonization by different ophiostomatoid species (Krokene & Solheim, 1998; Solheim & Krokene, 1998; Jankowiak, 2013). *Ophiostoma minus* caused dieback in Scots pine seedlings thereby confirming the results of previous studies (Jankowiak, 2006, 2013). In addition, following mass inoculation, *O. minus* also kills large Scots pine trees (Långstrom et al., 1993; Solheim et al., 1993, 2001). In dead and declining seedlings, *O. minus* colonized sapwood and produced a substantial blue-stain in the area of the inoculation. The ability to invade sapwood and phloem are considered critical for the pathogenic colonization (Krokene & Solheim, 1998). *O. minus* is the most aggressive pathogen of Scots pine seedlings and may also affect large trees; however, this remains to be quantitatively tested (Långstrom et al., 1993; Solheim et al., 1993, 2001). In addition to *O. minus*, *G.* cf. *rectangulosporium* caused the decline of Scots pine seedlings, which indicates this species is moderate or weakly pathogenic, as reported previously (Jankowiak, 2012; Dori-Bachash et al., 2015).

Inoculation with *O. ips, O. pallidulum, O. piceae* and *G. olivacea* resulted in relatively small lesions indicating that these species were significantly less virulent than *O. minus* and *G.* cf. *rectangulosporium*. Although inoculation with *O. ips*, *O. pallidulum* and *O. piceae* resulted in significantly longer and deeper lesions compared to controls, these fungi are probably weak pathogens. In contrast to our results, *O. ips* isolated from *I. sexdentatus* in Poland causes dieback of ca. 30% of the Scots pine seedlings inoculated. Such contradictory results can probably be explained by differences in virulence of different *O. ips* isolates from Poland and Ukraine. Moreover, *O. minus* isolated from *I. sexdentatus* in Poland (Jankowiak, 2012) causes dieback of 100% of the Scots pine seedlings inoculated compared to the 45% of seedlings recorded in the present study. It appears that ophiostomatoid fungi are an important factor determining the aggressiveness of bark beetles (Krokene & Solheim, 1998) and may account for *I. acuminatus* being less aggressive than *I. sexdentatus*. The associated fungi may play key roles in overcoming tree defence through pathogenic colonization of sapwood and phloem and facilitate the establishment of bark beetles.

CONCLUSIONS

This study revealed that *I. acuminatus* vectors a species-rich fungal community, which is dominated by tree pathogens. This study also revealed that the community of ophiostomatoid fungi associated with *I. acuminatus* in Ukraine appears to be rather different from that reported in other regions of Europe (Guérard et al., 2000; Linnakoski, 2011; Villari et al., 2013). The association between *D. pinea* and *I. acuminatus*, which is reported here for the first time, is of considerable practical importance for forest health and requires further attention. Pathogenicity tests on six ophiostomatoid fungi showed that *O. minus* is the most virulent and causes dieback in seedlings of Scots pine, while other fungi tested appeared to be only slightly pathogenic or completely non-pathogenic to trees.

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