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Mycobiota of *Ips sexdentatus* (Börner, 1776) (Coleoptera, Curculionidae: Scolytinae) in Belarus

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Abstract—The fungal component of the microbiome associated with the six-toothed bark beetle *Ips sexdentatus* (Börner, 1776) in the territory of Belarus was studied in order to clarify the role of this xylophage as a vector of dangerous phytopathogenic fungi. More than 360 pure fungal cultures isolated from the surface and hemocoel of *I. sexdentatus* were analyzed by morphological and molecular genetic methods, and 35 species from 25 genera, 17 families, and 3 divisions of fungi were identified. All these species were conventionally classified into three groups based on their impact on wood: (1) fungi that cause wood discoloration (mold, blue stain, etc.), (2) fungi that cause rot in living trees and cut wood, and (3) fungi whose effect on harvested timber remains unclear. Five species of *Ophiostomataceae* fungi associated with blue rot were identified: *Leptographium* Lagerb. & Melin sp., *Ophiostoma ips* (Rumbold) Nannf., *O. minus* (Hedgc.) Syd. & P. Syd., *O. canum* (Münch) Syd. & P. Syd., and *O. piceae* (Münch) Syd. & P. Syd. The root rot agent *Heterobasidion annosum* (Fr.) Bref. was detected in 7.8% of the studied fungal samples. The fairly high occurrence of *I. sexdentatus* confirms its participation in the development of complex pine dieback foci in Belarus. A considerable level of infection with four species of entomopathogenic fungi (11.4%) was recorded in the *I. sexdentatus* population by molecular genetic methods.

Keywords: *Ips sexdentatus*, *Pinus sylvestris* L., fungi, species diversity

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Belarus is among the ten European states with the largest forest resources, as indicated by such parameters as the relative forested area, the total territory of forests, and the growing timber reserve per head of population (Lesnoi fond …, 2022). The Scots pine *Pinus sylvestris* comprises 40.1% of the woodland. The condition of pine forests have deteriorated during the last decade, so that drying stands occupied 144 530 ha, or 3.9% of the total area of pine forests in Belarus by the end of 2021.

The main factors of weakening of pine stands are infection with the root rot fungus *Heterobasidion annosum* and damage caused by trunk pests, among which the dominant species are the pine engraver beetle *Ips acuminatus* Gyll. and the six-toothed bark beetle *I. sexdentatus* (Börner, 1776) (*Obzor* …, 2022).

The insect-vectored fungal complexes associated with the xylophagous pests causing large-scale pine dieback have not been previously studied in Belarus. Our study of the fungal microbiome of the six-toothed bark beetle is the first step in our research of the fungal flora associated with the insect pest community in Scots pine stands.

Ips sexdentatus often forms complex foci (Fig. 1) together with *I. acuminatus* and the fungus *H. annosum* (Mashnina, 1963; Dushin, 1981; Sazonov et al., 2017;

Fig. 1. A complex focus of pine dieback. Photo by M.O. Ramanenka.

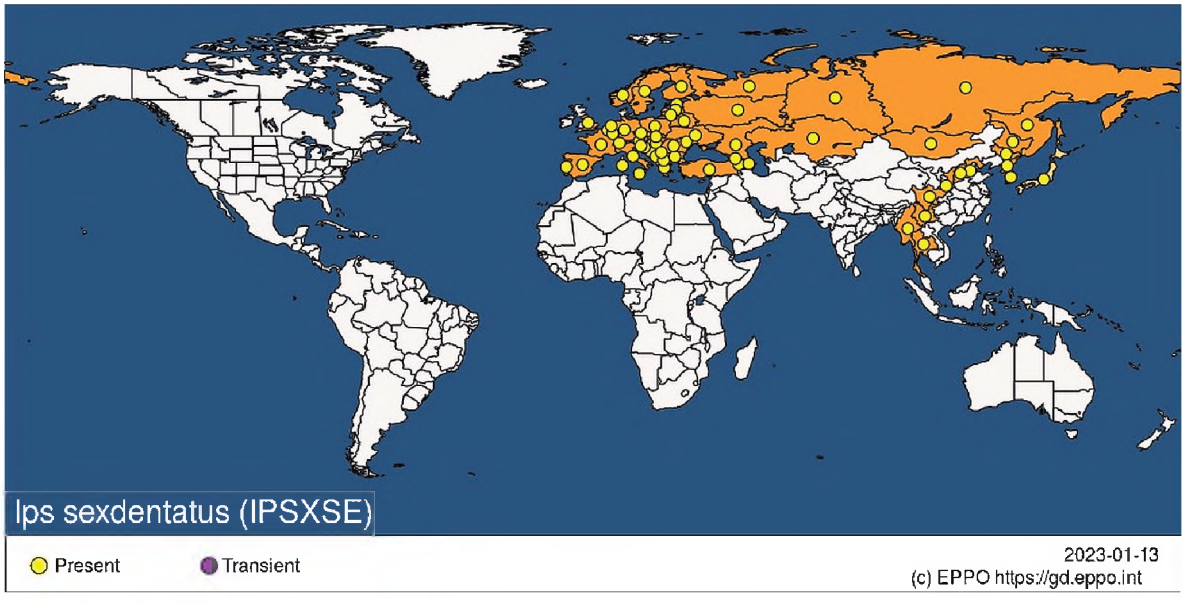

Fig. 2. Distribution of *Ips sexdentatus* (Börner) based on the EPPO database as of August 2017 (after Jeger et al., 2017).

Fig. 3. Galleries of *Ips sexdentatus* (Börner) under the pine bark. Photo by M.O. Ramanenka.

Obzor ..., 2022). By the beginning of 2019, such complex foci were detected in Belarusian pine stands with a total area of 5.7 thousand hectares (*Obzor* ..., 2022), and during 2016–2021, pine stands were damaged by trunk pests in an area of 826.1 thousand hectares. The required sanitation felling amounted to 38.1 million m^3 of wood.

The six-toothed bark beetle is a Palaearctic xylophagous species that is widespread throughout Europe (Fig. 2) (Stark, 1952; Mozolevskaya et al., 2010; López and Goldarazena, 2012; Alonso-Zarazaga et al., 2017; Jeger et al., 2017). This is the largest of the bark beetles found on pine trees in Belarus (Aleksandrovich et al., 1996). It differs from other species within the genus in a large body size (6–8 mm long) and the number of teeth along the margins of the apical elytral declivity. The egg galleries of this species are also quite remarkable, as they reach up to 40 cm in length and 3–4 mm in width and can be made in both thick and thin bark (Fig. 3).

The flight of adults begins in April–May and may continue until September in years with high summer temperatures (Jactel and Gaillard, 1991; López and Goldarazena, 2012). The beetles fly at a speed of 1.3 m/s and can cover a distance of 5 to 45 km in a day (López and Goldarazena, 2012).

The main host plants of this bark beetle are pines: *Pinus sylvestris*, *P. pinaster* Aiton, *P. radiata* D. Don, *P. leucodermis* Antoine, and *P. nigra* J.F. Arnold. The pest can also infest various species of spruce (*Picea abies* (L.) H. Karst. and *P. ajanensis* Lindl. et Gordon), fir (*Abies alba* Mill., *A. nordmanniana* (Steven) Spach,

A. sibirica Ledeb., *A. holophylla* Maxim., *A. nephrolepis* (Trautv. ex Maxim.) Maxim., and *A. sachalinensis* F. Schmidt), and larch (*Larix decidua* Mill., *L. sibirica* Ledeb., *L. gmelinii* Rupr., and *L. olgensis* A. Henry) (Stark, 1952; Jeger et al., 2017; European Environment Agency, 2022).

In the territory of Belarus, *I. sexdentatus* forms two complete generations a year. The beetles act as a vector of plant pathogens. Colonizing weakened and sometimes even apparently healthy trees together with *I. acuminatus* (Gyllenhal, 1827), they introduce fungal and bacterial infections under the bark and cause sapwood discoloration (Fig. 4).

More than 30 species of ophiostomatoid fungi vectored by *I. sexdentatus* have been described (Davidson, 1955; Bueno, 2010; Linnakoski et al., 2010; Jankowiak, 2012; Aas et al., 2018; Davydenko et al., 2021). The number of species of the fungal genera *Ophiostoma* and *Leptographium* known to be associated with this bark beetle is increasing annually due to progress in molecular genetic methods. The isolated ophiostomatoid fungi vary in virulence, but all of them cause wood discoloration (log blue and other sapwood stains), even after artificial inoculation of felled wood (Davydenko et al., 2021). Information on other pathogenic fungi included in the *I. sexdentatus* microbiome is scanty, since only the damage due to ophiostomatoid fungi is recorded in many cases.

This paper reports the results of investigation of the the fungal component of the *I. sexdentatus* microbiome in the territory of Belarus.

MATERIALS AND METHODS

During the field season of 2020–2021, we collected first-generation adults of *I. sexdentatus* from egg galleries on pine trees of IV and V categories in pine dieback foci within the territories of seven experimental forest farms: Uzdenskii, Lelchitskii, Zhlobinskii, Slonimskii, Baranovichskii, Kobrinskii, and Negorelskii. The sampling sites are mapped in Fig. 5. According to the *Sanitary Rules in the Forests of the Republic of Belarus* (Ob utverzhdenii ..., 2016) and the Standard Practice Technical Code 634–2019 for forest protection measures (Poryadok provedeniya ..., 2019), the pine dieback foci were classified as active root rot foci with a low damage level. The population parameters of *I. sexdentatus* were not calculated, as this was beyond the scope of our study.

The collected beetles were placed separately in labeled 1.5-mL Eppendorf sample tubes and transported to the laboratory. The specimens to be used for isolation and cultivation of fungi were stored at 4°C for no more than 3 days. Some beetles were preserved at −20°C in an Arctiko ULTF 220 ultra low temperature freezer for further molecular genetic analysis. The identified pure fungal cultures are currently kept in a Memmert IPP55 cooling incubator.

In the laboratory, the bark beetles were rinsed with sterile distilled water and placed without rubbing on the surface of wort agar or MEA medium in Petri dishes. The media contained no antibiotics (Bilai, 1982; Davydenko et al., 2017). Incubation was carried out at 22°C in a KhT-3 temperature-controlled chamber.

To isolate the intestinal fungal symbionts from the hemocoel of bark beetles, the specimens were sterilized externally with 15% hydrogen peroxide for 1 min, rinsed with sterile water, dried on a clean cloth, and placed in Petri dishes on nutrient medium that contained streptomycin (0.025 g per 250 mL) to prevent bacterial growth. All the fungi that germinated were considered to have been carried in the hemocoel, since the spores of the ophiostomatoid fungi vectored by beetles are located on the integuments and are removed during intensive surface sterilization.

The Petri dishes were checked daily for three weeks; after the appearance of mycelia, the fungi were subcultured on fresh medium in new Petri dishes. The fungi were identified and their taxonomic diversity was assessed at the stage of well-developed conidial sporulation, using Olympus and Leica DMLB microscopes operating in transmitted and reflected light. The species were identified using published keys (Nobles, 1965; Barnett and Hunter, 1972; Alexopoulos et al., 1996; Watanabe, 2002); their taxonomic position is given according to the *Index fungorum* (2022).

DNA was extracted from fungal mycelium using the CTAB method (Padutov et al., 2007). The main requirements for the technique were (1) obtaining nucleic acid fragments longer than 20 000 bp and (2) absence of DNA degradation and PCR inhibitors (A260/A280 and A260/A230 ratios higher than 1.8) (Gardes and Bruns, 1993; Glasel, 1995; Tataurov et al., 2008).

Fig. 4. Log blue transmitted by *Ips sexdentatus* (Börner): (*a*) young adults on pine wood and log blue spreading over the sapwood; (*b*) log blue visible at the butt ends of logs. Photo by A.A. Sazonov.

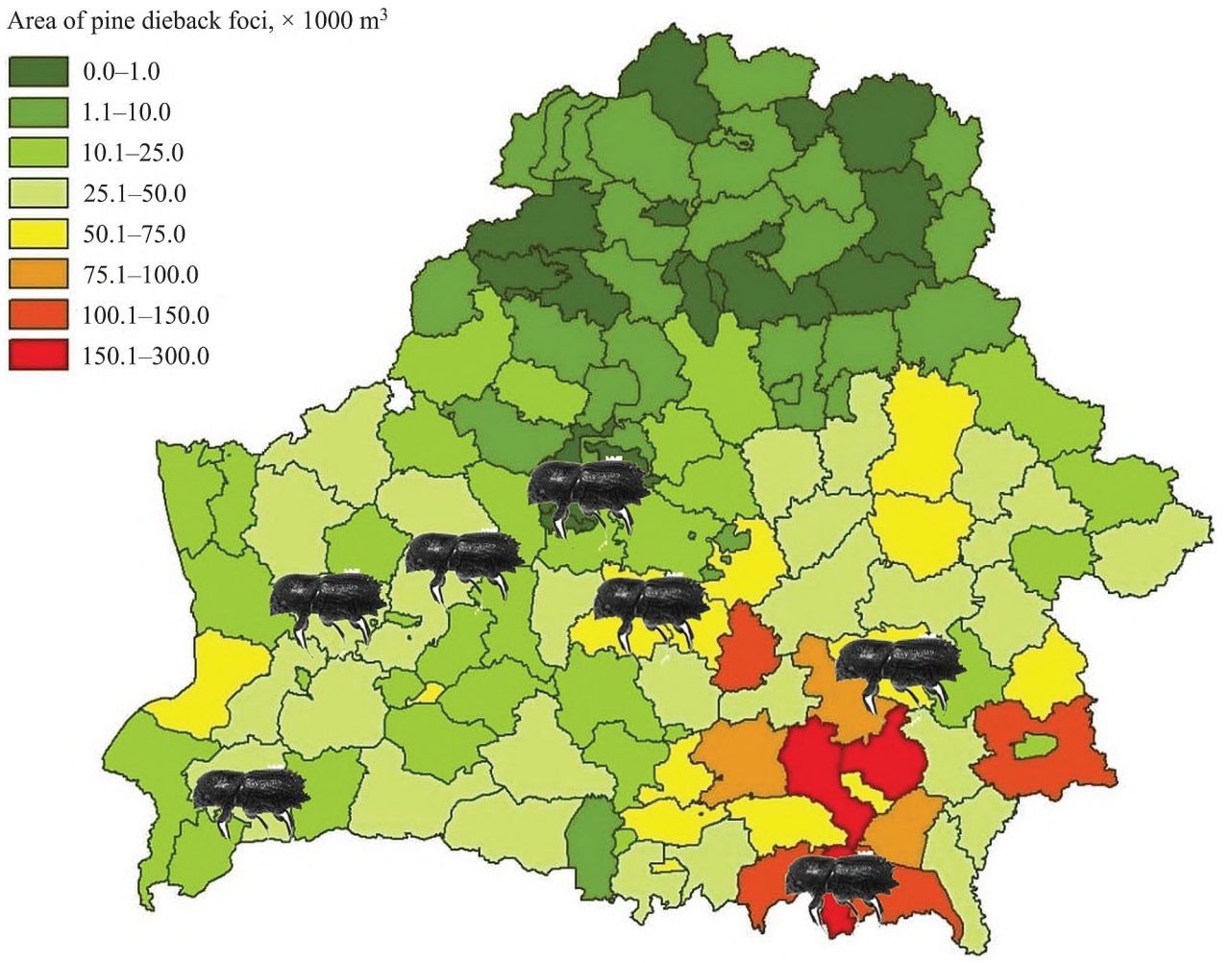

Fig. 5. Sampling sites of *Ips sexdentatus* (Börner) in the pine dieback foci in Belarus.

The PCR was performed using the ArtMix Phoresis 2× kit (ArtBioTech, Belarus) according to the manufacturer`s protocol. The marker locus for species identification was 18S rDNA-ITS1-5.8S rDNA-ITS2-28S rDNA. This locus was amplified using ITS1F/ITS4 primers (White et al., 1990; Gardes and Bruns, 1993) under the following conditions: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation (95°C, 20 s), annealing (55°C, 20 s), and elongation (72°C, 45 s), and final cooling down to 4°C.

The PCR products were separated by electrophoresis in 1.5% agarose gel with 1 × TBE buffer according to the manufacturer`s protocol. The visualized amplicons were purified with the AMPure XP kit (Beckman Coulter, USA).

Sequencing was performed by the chain termination method (Sanger et al., 1977), based on incorporation of terminating deoxynucleoside triphosphates. We used the BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) and the following conditions: initial denaturation (96°C, 1 min) followed by 40 cycles of denaturation (96°C, 10 s), annealing (50°C, 5 s), and elongation (60°C, 3 min), and final cooling down to 4°C. Electrophoretic analysis and detection of the sequencing products were performed in an ABI Prism 310 genetic analyzer, using the following conditions: 30 μL sample, POP-4 polymer, P4StdSeq (1ml) E.md4, rapid sequencing (28 min per sample). The results were interpreted using the Sequencing Analysis Software 5.1.1 and NCBI BLAST database (National Center for Biotechnological Information, 1922).

RESULTS AND DISCUSSION

In 2020–2022, we processed 230 specimens of *I. sexdentatus* and isolated over 360 pure fungal cultures. The frequency of detecting fungal material was 65.3%; in other cases the nutrient medium in Petri dishes was abundantly overgrown with bacteria, and such samples were discarded.

All the fungal species were conventionally divided into three groups by their relative occurrence: sporadic species (occurrence up to 1%), rare species (occurrence from 1.1 to 2.0%), and those commonly detected in the microbiome (occurrence 2.1% and higher). Our samples yielded 35 species of fungi whose biological material was present on the exoskeleton or in the hemocoel of six-toothed bark beetles (Table 1).

As the result of PCR analysis performed for detecting fungal DNA in insect tissues, we obtained an electrophoretic spectrum of amplicons containing a number of fractions of varying intensity. The dominant fungal species and several co-occurring ones could be detected simultaneously in *I. sexdentatus* adults, the total number of transferred species varying from 3 to 9. The alternative dominant amplicon variants were selected for sequencing.

For comparison, it should be noted that the mycobiome of *I. sexdentatus* and wood from its galleries described by K. Davydenko and co-authors (Davydenko et al., 2021) included 56 species from 39 genera; 6 species were not recorded in the NCBI BLAST database, and 16 species (28%) were common to the territories of Belarus and Ukraine.

The isolated fungi included some cosmopolitan species that occur on a wide range of substrates: decaying plant and animal remains, wood, and soil (Barnett and Hunter, 1972; Watanabe, 2002). Fungi of the genera *Alternaria*, *Cladosporium*, *Botrytis*, and *Fusarium*, often isolated from bark beetles, can both develop as saprophytes on plant remains and actively infect woody plants. The same fungi also cause surface molding of stored timber (Fedorov, 2004; Agrios, 2005; Dean et al., 2012). Many of the isolated species, such as those of the genera *Phoma* and *Epicoccum*, are conditionally pathogenic to trees and mostly develop as endophytes.

Such saprophytic fungi as *Trichoderma*, *Penicillium*, *Umbelopsis isabellina*, and *Mucor mucedo* were also detected during other studies of *I. sexdentatus* and other pine xylophages (Linnakoski, 2011; Davydenko et al., 2021).

Three species of the division Basidiomycota were identified in our material; two of them cause trunk and root rot in living coniferous trees. The isolation of the root rot agent *Heterobasidion annosum* confirms the ability of *I. sexdentatus* to transmit this dangerous pine pathogen and its participation in the development of complex pine dieback foci in Belarus. This fungus was also recorded as a permanent associate of bark beetles in other similar studies (Persson et al., 2009; Davydenko et al., 2017, 2021).

Coniophora puteana, diagnosed in a pure culture in our material, is an important destructor of harvested timber and can occasionally infect weakened living trees (Fedorov, 2004). In the latter case, this fungus often induces latent rot that kills the tree, after which its basidiomes start growing (Bernicchia and Gorjon, 2010). This species had a low occurrence of 1.7%.

The detected mycoflora included four species of entomopathogenic fungi (11.4% of the total species number) causing disease and death of bark beetles: *Akanthomyces muscarius*, *Beauveria bassiana*, *Cephalosporium muscarium*, and *Purpureocillium takamizusanense*. The pathogenicity of these fungi to bark beetles is still insufficiently studied, except for *B. bassiana*, which was found in the *I. sexdentatus* mycobiome in Belarus with a frequency of 2.2%. The latter fungus species was also recorded in the mycoflora of *I. acuminatus*, *I. typographus*, and *Tomicus* spp. (Annila et al., 1999; Jankowiak, 2006, 2007; Davydenko et al., 2017, 2021), confirming its polyhostal associations (Burjanadze, 2010).

Our molecular genetic study of the intestinal symbionts of *I. sexdentatus* revealed ambrosia fungi and yeasts that accounted for 8.5% of the total species number; however, in our opinion, the actual number of these species may be much greater. The *Sugiyamaella* species isolated from our samples are known to ferment xylose (Urbina et al., 2013; Cheng-Feng Shi et al., 2021). The yeast species *Nakazawaea ambrosiae* was previously found in the larval galleries and larvae of *I. typographus* and *Dendroctonus micans* in Moscow Province (Crous et al., 2019). Species of the genus *Cyberlindnera* are symbiotic yeasts that occur in the guts of xylophages and play a significant role in detoxification of plant defense chemicals and metabolism of lignin (Soto-Robles

Table 1. Fungal species identified from the surface and hemocoel of *Ips sexdentatus* (Börner) by morphological and molecular genetic methods

Class	Order	Family	Genus	Species	Identification method	Occurrence, %
Division **Ascomycota**						
Sordariomycetes	Hypocreales	Cordycipitaceae	*Akanthomyces* Lebert	*A. muscarius* (Petch) Spatafora, Kepler & B. Shrestha	g	1.1
			Beauveria (Bals.-Criv.) Vuill.	*B. bassiana* (Bals.-Criv.) Vuill.	m, g	2.2
			Cephalosporium Link	*C. muscarium* Petch	g	0.8
		Ophiocordycipitaceae	*Purpureocillium* Luangsa-ard, Hywel-Jones, Houbraken & Samson	*P. takamizusanense* (Kobayasi) S. Ban, Azuma & Hiroki Sato	g	0.6
		Nectriaceae	*Fusarium* Link	*F. solani* (Mart.) Sacc.	m, g	4.7
				F. oxysporum sensu Smith & Swingle	m	5.6
				Fusarium sp.	m	1.7
		Incertae sedis	*Sarocladium* W. Gams & D. Hawksw.	*S. strictum* (W. Gams) Summerb.	g	0.6
		Hypocreaceae	*Trichoderma* Bisset	*T. viride* Schumach.	m	12.8
				Trichoderma sp.	m	3.6
		Bionectriaceae	*Clonostachys* Corda	*C. rosea* (Preuss) Mussat	g	0.3
	Ophiostomatales	Ophiostomataceae	*Leptographium* Lagerb. & Melin	*Leptographium* sp.	g	1.4
			Ophiostoma Syd. & P. Syd.	*O. ips* (Rumbold) Nannf.	m, g	5.0
				O. minus (Hedgc.) Syd. & P. Syd.	m, g	4.2
				O. canum (Münch) Syd. & P. Syd.	m, g	2.5
				O. piceae (Münch) Syd. & P. Syd.	m, g	3.3
Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium* Link	*P. bialowiezense* K.W. Zaleski	g	1.4
				Penicillium sp.	m	4.7
			Aspergillus Micheli	*A. versicolor* (Vuill.) Tirab.	m	2.5
				A. ochraceus G. Wilh.	m	2.8

Table 1. (Contd.)

Class	Order	Family	Genus	Species	Identification method	Occurrence, %
Leotiomycetes	Helotiales	Sclerotiniaceae	*Botrytis* P. Micheli ex Pers.	*B. cinerea* Pers.	m	2.2
				Botrytis sp.	m	1.7
Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium* Link	*C. cladosporioides* (Fresen.) G.A. de Vries.	m	2.5
				Cladosporium sp.	m	3.1
	Pleosporales	Didymellaceae	*Epicoccum* Link	*E. nigrum* Link	m	3.3
			Phoma Saccardo	*Phoma* sp.	m	2.5
		Pleosporaceae	*Alternaria* Nees	*A. alternata* (Fr.) Keissl.	m	1.9
Saccharomycetes	Saccharomycetales	Trichomonascaceae	*Sugiyamaella* Kurtzman & Robnett	*Sugiyamaella* sp.	g	0.6
		Incertae sedis	*Nakazawaea* Y. Yamada, K. Maeda & Mikata	*N. ambrosiae* Kachalkin, Tomashevskaya, T.A. Kuznetsova & M.V. Vecherskii	g	1.4
		Incertae sedis	*Cyberlindnera* Minter	*C. mississippiensis* (Kurtzman, M.J. Smiley, C.J. Johnson, Wick. & Fuson) Minter	g	0.8
			Division **Basidiomycota**			
Agaricomycetes	Polyporales	Fomitopsidaceae	*Fomitopsis* P. Karst.	*F. pinicola* (Sw.) P. Karst.	m, g	3.3
	Russulales	Bondarzewiaceae	*Heterobasidion* Bref.	*H. annosum* (Fr.) Bref.	m, g	7.8
	Boletales	Coniophoraceae	*Coniophora* DC.	*C. puteana* (Schumach.) P. Karst.	g	1.7
			Division **Mucoromycota**			
Mucoromycetes	Mucorales	Mucoraceae	*Mucor* Fresen.	*M. mucedo* de Bary & Woron.	m, g	3.6
Umbelopsido-mycetes	Umbelopsidales	Umbelopsidaceae	*Umbelopsis* Amos & H. L. Barnett	*U. isabellina* (Oudemans) W. Gams	m, g	2.5
			Total			
8	12	17	25*	35	–	100.0

* Including 3 genera of uncertain taxonomic position (incertae sedis); identification methods: m, morphological; g, molecular genetic.

et al., 2019; Chakraborty et al., 2020). Studying this group of fungi is difficult and requires development of special techniques.

Thus, all the fungi identified in our material can be divided into the following groups based on the nature of their impact on the forest and wood processing industry:

(1) fungi that cause wood discoloration: molds and sapwood stains;

2) fungi that cause rot in living trees and harvested timber;

3) fungi whose effect on harvested timber remains unclear.

A proportion of the *I. sexdentatus* mycoflora has evaded detection. It is possible that some species cannot be cultured on the nutrient media used or are biotrophic; some species may occur only during a specific part of the season or only in a specific generation of bark beetles. The biological characteristics of these fungi and their significance for forest pathology remain to be studied.

Our study of the *I. sexdentatus* mycoflora using DNA analysis is one of the first in Belarus. We plan to continue a similar study on other pests, in particular using the metagenomic approach.

FUNDING

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Statement on the welfare of animals. All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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