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Infestation by *Ips amitinus* (Eichhoff, 1872), Its Associated Fungi, and Butt Rots in Stands of *Pinus sibirica* in South-Western Siberia

Igor N. Pavlov [1], Rimvydas Vasaitis [2,*], Yulia A. Litovka [3], Anton A. Timofeev [4] and Audrius Menkis [2]

[1] Independent Researcher, Private P.O. Box, Karaulnaya St. 38–222, 660043 Krasnoyarsk, Russia; forester024@gmail.com
[2] Department of Forest Mycology and Plant Pathology, Uppsala BioCenter, Swedish University of Agricultural Sciences, P.O. Box 7026, SE-75007 Uppsala, Sweden; audrius.menkis@slu.se
[3] Independent Researcher, Private P.O. Box, Karaulnaya St. 43/2-212, 660020 Krasnoyarsk, Russia; litovkajul@rambler.ru
[4] Independent Researcher, Private P.O. Box, Parashutnaya St. 11A–28, 660121 Krasnoyarsk, Russia; timofeyev95@gmail.com
* Correspondence: rimvys.vasaitis@slu.se

Abstract: In 2019, the bark beetle *Ips amitinus* (native to central Europe) was identified in south-western Siberia at a distance exceeding 2500 km east of its previously known easternmost location in the European part of Russia. In Siberia, its invasive populations are characterised by high abundance and harmfulness. Here, *I. amitinus* accomplishes primary attacks on standing vital trees of *Pinus sibirica* with a lethal outcome. This invasion has already resulted in massive dieback in stands of pine over a large geographic territory. By, 2021, the invaded area was estimated to cover at least 31,200 km^2. The objectives of this study were to investigate fungi associated with/vectored by *I. amitinus* in its invasive area in south-western Siberia and wood decay fungi that cause root and butt rots to *P. sibirica*. This led to the following conclusions: (i) DNA analysis of sixty adult beetles of *Ips amitinus* collected from *P. sibirica* in south-west Siberia revealed the presence of 143 fungal taxa; (ii) species richness was significantly higher in beetles collected from dead branches than from (more recently infested) dying branches; (iii) fungal communities were >90% dominated by yeasts, among which the most common were *Nakazawaea holstii*, *Kuraishia molischiana*, and *N. ambrosiae*; (iv) entomopathogenic *Beauveria bassiana s.l.* was the most common fungus isolated from dead/mycosed beetles of *I. amitinus*, followed by *Lophium arboricola* and four *Ophiostoma* spp.; and (v) *Heterobasidion parviporum* was the most common decay fungus detected, which was causing heart rot in stems of *P. sibirica*.

Keywords: *Ips amitinus*; *Pinus sibirica*; bark-beetle-associated fungi; biological invasions; root and butt rot; south-west Siberia

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1. Introduction

The small spruce bark beetle, *Ips amitinus* (Eichhoff, 1872) (Coleoptera: Curculionidae: Scolytinae) is a European species native to the middle latitudes of the continent, from the utmost west (the Netherlands) to the east (Turkey) [1,2]. In its native range, the beetle colonises dying and suppressed coniferous trees, primarily Norway spruce (*Picea abies* (L.) H. Karst.), thus being a secondary pest of insignificant economic importance [3–5]. In 2019, *I. amitinus* was identified in south-western Siberia, in the Tomsk and Kemerovo regions [6], at a distance exceeding 2500 km east of its previously known easternmost location in the European part of Russia [5]. The proximity of the finding to the Trans-Siberian Railway provides a strong indication that the pathway for the invasion has been the long-distance transportation of wood [2].

Notably, in the newly invaded areas of south-western Siberia, the beetle almost exclusively colonises a new host, Siberian pine (*Pinus sibirica* Du Tour) [7], while being only

occasionally observed on adjacent trees of Siberian spruce (*Picea obovata* Ledeb.) [6]. Yet, the latter is a subspecies of Norway spruce [8], which is otherwise the preferred host of *I. amitinus* at its native range in Europe [3,4]. Apparently, at the invasive range the host preference of *I. amitinus* has changed. A similar observation has been previously reported for mountain bark beetle (*Dendroctonus ponderosae*, Hopkins 1902) in North America, where the beetle, upon expanding its invasive range, switched from "historical" to "novel" hosts in new territories [9,10].

Moreover, on the "novel" host (Siberian pine) in the invasive range, *I. amitinus* exhibits strikingly different behaviour to that observed on its "historical" host (Norway spruce) in the native range. In Siberia, its invasive populations are characterised by high abundance and harmfulness [7]. By contrast, here *I. amitinus* accomplishes primary attacks on standing vital trees with a lethal outcome. From the start, it colonises the upper part of the trunk with thin bark and the branches. During the first year, the upper crown dies off, while the lower branches remain green; in the second year, the whole tree dies [6]. Consequently, the invasion has already resulted in a massive dieback of *P. sibirica* stands over a large geographic territory. By 2021, the invaded area was estimated to cover at least 31,200 km^2 [7]. The native range of Siberian pine covers an immense area of over 400,000 km^2, stretching from north-west Ural to south-east Altai [11]; thus, apparently up to 10% of its whole area has already been infested by *I. amitinus*. The invasion is expanding farther at "dramatic rates" and could be deemed an ongoing "natural catastrophe" [5,7].

Bark beetles are known to be associated with numerous fungi representing a wide range of taxonomic, functional, and ecological groups, and *I. amitinus* in this respect is not an exception. Yet, published papers on fungal associates of *I. amitinus* are relatively few, targeting beetles collected almost exclusively from its preferred host, Norway spruce, at the native range in central Europe. Moreover, the studies focused either on ophiostomatoid [12–14] or entomopathogenic [15–18] fungi, and each of the cited studies was based on pure culture isolations. However, isolations of fungi from environmental samples (e.g., beetles) are biased towards in vitro fast-growing species, thus are unlikely to reveal the whole spectrum of the fungi. To a certain extent, this can be resolved by the direct analysis of fungal DNA from an insect. Direct sequencing of fungal DNA from environmental samples has proved to be a sensitive method for the detection of potentially all fungi, in particular species that are usually overlooked by isolation, including latent pathogens, slow-growing endophytes, and unculturable species [19].

Another group of fungi, namely, root and butt rot pathogens, is known to be important in predisposing trees to bark beetle attacks. Thus, Lewis and Lindgren [20] reviewed the results from thirteen North American studies, showing that infections by rot-causing fungi such as *Armillaria*, *Heterobasidion*, *Inonotus*, or *Phellinus* increased the vulnerability of a wide range of conifers (*Abies*, *Picea*, *Pinus*, *Pseudotsuga*) to attacks by several species of bark beetles (e.g., *Dendroctonus*, *Scolytus*). Moreover, another North American study has demonstrated that *Armillaria*-infected pine was more prone to attacks by *Dendroctonus* and *Pityogenes* [21]. On the other hand, in this respect different results were reported from Europe, where in *Picea abies* stands no correlations were found between the attacks by *Ips typographus* L. and the incidence of butt rot caused either by *Armillaria* [22] or *Heterobasidion* [23].

To date, the information regarding the causal agents of root and butt rots of *Pinus sibirica* accessible to the international reader is very limited. This is not surprising as the native range of this pine is almost exclusively (99%) confined within Russian Siberia, and only 1% of the total area of its forests is found on the territory of Mongolia [11]. To date, only a single review is available, in which root and butt rots caused by *Armillaria* and *Heterobasidion* were reported to play important roles in the massive dieback of *Pinus sibirica* in the Sayan Mountains, south Siberia [24]. In the literature published in Russian, more extensive data are available on rot-causing fungi colonising Siberian pine. Using the sporocarp inventory of thousands of uprooted, broken, living trees and stumps, it was found that the most common fungi causing wood rot (apart from *Armillaria* and *Heterobasidion*) belong to the genera *Coniophora*, *Serpula*, *Postia* (*Oligoporus*), *Phaeolus*, *Porodeadalia*, *Lentinus*, and *Fomitopsis* [25].

However, on living trees sporocarps of those fungi are seldom produced. For example, in the cited study only up to 7% of approx. 3000 examined living trees showed sporocarps of *Coniophora* spp., and those fungi were the species most often observed.

Knowledge related to the invasion of *I. amitinus* and its associated fungi into the new geographic range in Siberia, as well as on decay fungi colonising living trees of Siberian pine, is relevant not only from the point of view of forest health and ecology, but also from the point of view of potential/eventual exports of the infested roundwood to China. The exports, therefore, would clearly involve a risk of the farther long-distance spread of the invasive organisms, both the beetle and its associated fungi. The objectives of this study were to investigate fungi associated with/vectored by *I. amitinus* in its invasive area in south-western Siberia and wood decay fungi that cause root and butt rots to *P. sibirica*.

2. Materials and Methods

2.1. Study Sites and Collecting Beetles

Four study sites were located in Tomsk Region, south-western Siberia (Figure 1), representing 120–160-year-old stands of *Pinus sibirica* subjected to attacks by *Ips amitinus*. On the sites, since 2019 approx. 25%–40% of trees have been attacked or killed, or are dying. In the forest cover, share in species composition of *P. sibirica* comprised 70%–100%, with an admixture of *Abies sibirica* Ledeb., *Picea obovata*, *Pinus sylvestris* L., *Populus tremula* L., and *Betula pendula* Roth. In the undergrowth, the dominating species were *Padus avium* Mill., *Sorbus sibirica* Hedl., and *Rubus idaeus* L., and the groundcover was mostly composed of *Carex macroura* Meinsh., *Calamagrostis obtusata* Trin., and *Oxalis acetosella* L.

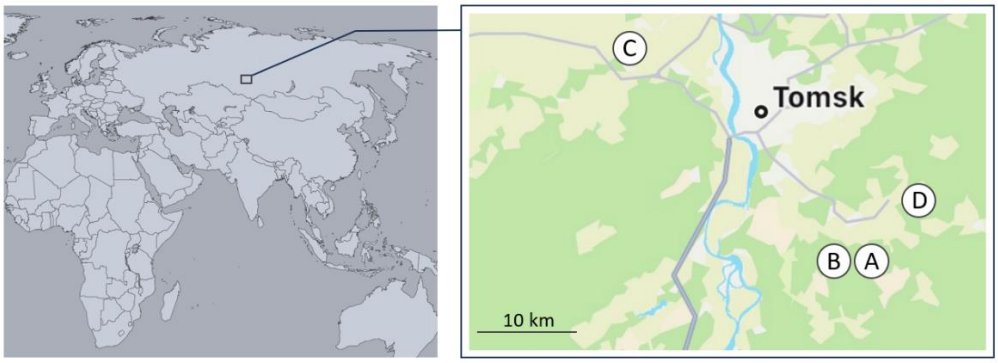

Figure 1. Map showing sampling sites denoted as A, B, C, and D. Map to the left was adapted from https://commons.wikimedia.org/wiki/File:World_blank_map_(grey_%26_white).png (accessed on 7 September 2023). Map to the right was modified from Apple Maps (accessed on 7 September 2023).

Beetles of *Ips amitinus* were collected from a total of six standing trees of *Pinus sibirica*. Sampling was carried out on 11–14 July 2021 from two trees at site A, two at B, one at C, and one at D. The geographic coordinates of the sites and condition of the examined trees are presented in Table 1. The age of the trees varied between 120 and 160 years, height between 24 and 27 m, and the stem diameter at approx. 1.3 m height (DHB) between 38 and 46 cm. Four of these trees (A1, B1, C1, D1) had been dead and infested for approx. 1–2 years. The crown of each tree was 100% dead, and the upper part partly defoliated (Figure 2). Sampled branches were located at the lower part of the crowns, apparently attacked by the beetle during the previous vegetation season. The branches retained brown foliage and showed entrance holes on the bark. Two other sample trees (A2, B2) were living, but classified as "dying". The trees exhibited crown discoloration and occasional occurrence of dead twigs/branches at the upper part of the crowns, so-called "crown top dieback" [7]. Branches sampled from those trees showed foliage discoloration and the entrance holes of the beetle.

Table 1. Generated high-quality ITS2 rDNA sequences and detected diversity of fungal taxa associated with *Ips amitinus* on six *Pinus sibirica* trees at four sites in south-western Siberia. Letters correspond to sites as indicated in Figure 1.

Site/Tree	Tree/Branch Condition	Geographic Position	Sequences (No.)	Taxa (No.)	Shannon Diversity Index
A1	dead	56.3099, 85.1733	1445	46	1.6
A2	dying	"–"	1657	32	1.3
B1	dead	56.3102, 85.1362	1466	44	1.3
B2	dying	"–"	1772	26	1.3
C1	dead	56.5389, 84.7366	1483	45	1.6
D1	dead	56.3855, 85.2599	1599	68	2.7
All			9422	143	

Figure 2. *Pinus sibirica* killed by *Ips amitinus* in south-western Siberia.

The beetles were collected from attacked branches in the crowns at the height of 18–20 m. This included climbing up to a crown (by a professional climber), then selecting a skeletal branch (diameter at the base: 8–10 cm) with symptoms of *Ips amitinus* attack, tying the branch up with a rope, cutting it off the stem, and bringing it down to the ground. After removing the bark from the section of the branch with the entry holes, five to seven galleries were sampled per branch. The insects were collected using sterilised forceps.

For subsequent molecular analyses of the fungal community (see Section 2.2), ten living beetles from each tree were pooled and placed into separate 1.5 mL centrifugation tubes (one tube per tree, six in total). For pure culture isolations of the fungi (Section 2.4), ten dead (mycosed) beetles per tree were collected. These were placed into individual tubes (one per tube, sixty in total). Upon sampling in the field, the tubes were placed into an icebox and transferred to the laboratory. Tubes with the beetles designated for DNA analysis were stored at −20 °C and beetles for fungal culturing at −4 °C.

2.2. DNA Isolation, PCR Amplification, Sequencing, and Bioinformatics

A total of six samples, representing each tree, were used for isolation of DNA. The procedures of the subsequent work with the DNA and the bioinformatic analyses were similar to those conducted in our previous study [26]. Amplification of ITS2 rDNA region was performed using the fungal-specific primer gITS7 [27] and universal primer ITS4 [28], both containing sample identification barcodes.

2.3. Statistical Analyses

Rarefaction analysis was carried out using Analytical Rarefaction v.1.3, (http://www.uga.edu/strata/software/index.html, accessed on 1 September 2023). The difference in richness of fungal taxa on *Ips amitinus* from dead and dying trees was compared using the non-parametric chi-square test [29]. Fungal community composition was analysed using the Shannon diversity index, Sørensen qualitative similarity index, and nonmetric multidimensional scaling (NMDS) based on the Bray–Curtis similarity index in Canocco v.5.12 [30,31].

2.4. Isolation and Molecular Identification of Fungal Cultures

Isolations from beetles (60 in total, each collected dead) were carried out within less than seven days after collection. The beetles were sterilised in 75% ethanol for 45 s, washed (rinsed three times) in sterile water, and dried in between two sterile paper filters. The isolations were carried out on malt-extract agar (MEA) and on MEA + 0.5% tannin media in Petri dishes by placing and slightly inserting each beetle into the medium using sterilised forceps. The dishes were incubated at +24 °C in the dark and checked daily for fungal growth. Outgrowing morphologically distinct mycelia (Figure 3a) were sub-cultured on separate dishes containing MEA, microscopically examined (Nikon Eclipse Ci microscope, Japan), and grouped according to mycelial morphotype.

Figure 3. Pure culture isolations of fungi (**a**) from dead beetles of *Ips amitinus*; (**b**) from wood samples of *Pinus sibirica*.

For isolations from the wood, all six trees were sampled by inserting an increment borer approx. 10 cm deep into the stem at two different heights: 0.2 m and 1.2 m. In addition, at each of the four sites, five dying trees, all attacked by *Ips amitinus*, were sampled in a similar manner. Thus, in total, wood cores were obtained from a total of twenty-six trees and at two different stem heights. Each core was individually placed into a sterile 15 mL centrifugation tube. In the laboratory, these were extracted from the tubes using sterile forceps (surface sterilised under a flame), placed into Petri dishes with MEA media (Figure 3b), and processed further as described for the beetles above.

For species identification, representative cultures from each morphological morphotype were subjected to sequencing of ITS rDNA. Isolation of DNA, amplification, and sequencing followed the methods described by Menkis et al. [32]. Amplification by PCR was performed using primers ITS1F [33] and ITS4 [28]. The GenBank database was used to determine the identity of the ITS rDNA sequences. The criteria used for identification were sequence coverage of >80%, similarity to species level of 98%–100%, and similarity to genus level of 94%–97%. Generated sequences were deposited in GenBank.

3. Results

A total of 9422 high-quality ITS2 rDNA sequences were generated by PacBio sequencing, representing 143 fungal taxa. The number of fungal taxa was higher in beetles collected from "dead" trees/branches than from the "dying" trees/branches (Table 1). This difference was statistically significant. For both datasets, plotting the fungal taxa vs. the cumulative number of sequences resulted in rarefaction curves that approached asymptotes (Figure 4).

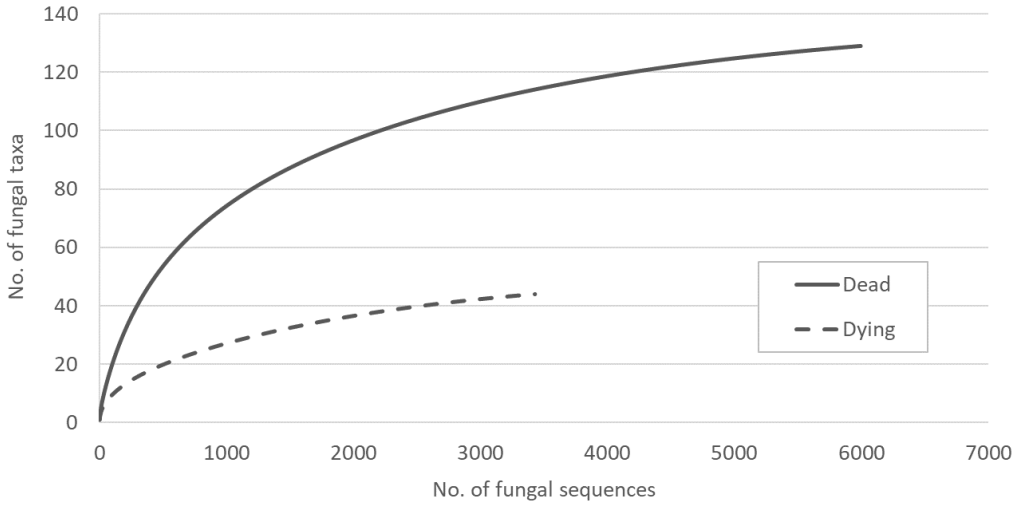

Figure 4. Rarefaction curves showing the relationship between the cumulative number of fungal taxa and the number of ITS2 rDNA sequences detected in *Ips amitinus* beetles collected from dying and dead *Pinus sibirica* trees/branches in south-western Siberia. The chi-square test showed that the richness of fungal taxa was significantly higher in *Ips amitinus* from dead trees/branches than from dying ones ($p < 0.003$).

In the NMDS analysis, fungal communities derived from beetles collected on adjacent sites/trees (A and B) were ordinated closer together (Figure 5). In these communities, the highest Sørensen similarity index values (0.40–0.51) were also detected (Table 2). In the beetle samples collected from all trees, the Shannon diversity index varied between 1.3 and 1.6, except for D1, where it was 2.7 (Table 1).

Table 3 presents the list of the most common and some of the more conspicuous fungal taxa that were detected during the study. The presented taxonomy should be taken with a certain reservation, as it is based only on short ITS2 rDNA sequences. Nevertheless, Table 3 should be considered informative, as GenBank sequence references, bp similarities, and source descriptions are also provided. The latter as a rule refer to the associations of a given fungus with the bark beetle or its activity, as well as the geographic location of the finding. The last rows under "Some other identified wood- and bark-inhabiting fungi (lichens)" might be of interest from the mycological point of view. As evident from Table 3, the prevailing majority of detected taxa (92.9%) comprised "Bark-beetle-associated yeasts". Among these, the most common were *Nakazawaea holstii* (59.0%), *Kuraishia molischiana* (13.0%), and *Nakazawaea ambrosiae* (10.1%). In total, these three species comprised 82.1% of the community. The proportion of ophiostomatoid fungi was 2.0%.

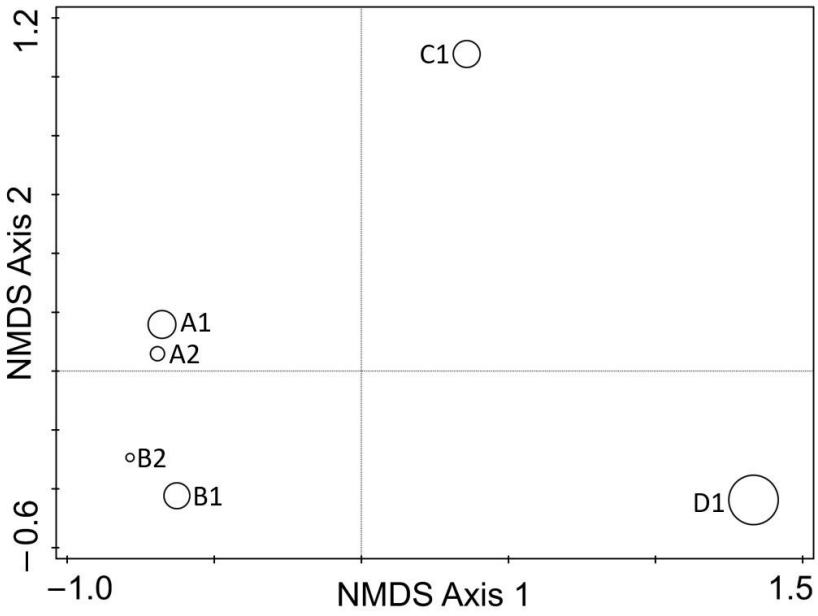

Figure 5. Ordination diagram based on nonmetric multidimensional scaling of fungal communities associated with *Ips amitinus* on *Pinus sibirica* at four different sites in south-western Siberia. The size of each plot shows the relative richness of fungal taxa. Letters correspond to sites as indicated in Figure 1.

Table 2. Similarities between fungal communities associated with *Ips amitinus* (the Sørensen qualitative similarity index) detected in different study sites (indicated by different letters, as in Figure 1). The index comparing the communities detected in beetles collected from trees on adjacent sites (A and B) is shown in bold.

Site/Tree	A1	B1	C1	D1	A2	B2
A1	-	**0.48**	0.40	0.20	**0.51**	**0.41**
B1		-	0.35	0.27	**0.43**	**0.40**
C1			-	0.36	0.38	0.27
D1				-	0.21	0.20
A2					-	**0.45**
B2						-

Table 3. Relative abundance of fungal taxa (% of all generated high-quality ITS2 rDNA sequences) detected in *Ips amitinus* beetles infesting *Pinus sibirica* in south-western Siberia.

Taxon	Sequence Reference		Similarity		GenBank Source	Abundance of Sequences, %						
	This Study	GenBank	bp	%	Description	A1	B1	C1	D1	A2	B2	All
Bark-beetle-associated yeasts												
Nakazawaea holstii	OM614738	KY104368	286/286	100	Frass on *Picea rubens*, Quebec (CBS:2091) [a]	57.2	61.6	58.2	38.4	70.6	66.9	59.0
Kuraishia molischiana	OM614742	KY103921	274/276	99	Rotten wood of *Fagus sylvatica*, Hungary [a,b]	17.4	22.6	6.5	5.2	17.2	9.9	13.0
Nakazawaea ambrosiae	OM614743	NR_165549	295/296	99	*Ips typographus* gallery, *Picea abies*, Moscow	13.0	7.8	18.5	7.5	4.6	9.8	10.1
Ogataea pini	OM614767	HQ413282	280/289	99	*Dendroctonus brevicomis*, *Pinus ponderosa*, Arizona	0.6	0.2	0.5	0.6	1.1	0.3	0.6
Ogataea neixiangensis	OM614788	NR_173264	275/280	98	Rotten wood, central China	0.2	0.6	-	-	0.1	0.2	0.2
Cryptococcus sp. 58	OM614753	MH697750	280/280	100	*Dendroctonus micans* gallery, *Picea* sp., Moscow	0.1	-	0.1	-	0.1	-	0.1
Myxozyma melibiosi	OM614830	KY037840	243/245	99	*Dendroctonus monticolae*, *Pinus ponderosa*, California	0.2	0.1	-	-	-	0.2	0.1
All yeasts						88.7	92.9	83.8	51.7	93.6	87.3	92.9
Ophiostomatoid fungi												
Ophiostoma bicolor	OM614758	HE866712	315/316	99	*Pityogenes chalcographus*, *Picea abies*, Slovenia	0.3	1.0	0.1	-	0.8	5.2	1.3
Ophiostoma sp. 1	OM614762	MW460002	326/329	99	*Dendroctonus armandii*, *Pinus armandii*, China	1.2	0.3	2.2	0.8	0.5	1.1	1.0
Ophiostoma hongxingense	OM614777	MK748194	321/323	99	*Ips subelongatus*, *Larix gmelinii*, northeast China	0.3	0.7	0.5	-	0.1	0.6	0.4
Leptographium truncatum	OM614847	AB907609	295/297	99	*Pinus densiflora* snag, Japan [c]	0.2	-	-	-	-	-	0.03
All ophiostomatoid fungi						2.0	2.0	2.8	0.8	1.4	6.9	2.0
Some other identified wood- and bark-inhabiting fungi (lichens)												
Lophium arboricola	OM614739	MK159395	250/251	99	*Abies koreana*, Republic of Korea	-	0.1	1.7	-	-	-	0.3
Hypoxylon serpens	OM614784	MG098299	258/258	100	Twig of *Pinus sylvestris*, Germany	1.3	-	-	-	-	-	0.2
Orbilia vinosa	OM614792	KT215265	286/289	99	Branch of *Pinus sylvestris*, France	-	-	0.3	0.6	-	-	0.2
Claussenomyces sp. 22	OM614746	KY633581	239/242	99	*Picea rubens*, east Canada	0.2	-	0.3	-	-	-	0.1
Stereum sanguinolentum	OM614831	KF996533	293/294	99	Wood of *Picea abies*, Italy	-	-	-	-	0.3	-	0.1
(*Parmelia sulcata*)	OM614750	MN387112	244/244	100	Lichen, Poland	-	-	0.1	1.3	-	-	0.2
(*Cyphelium lucidum*)	OM614798	EF551165	251/251	100	Lichen, Sweden	-	-	-	0.8	-	-	0.1
Sydowia polyspora	OM614744	MK762617	256/256	100	Pine, Tennessee, USA	-	0.07	-	-	0.06	-	0.02
Sarea resinae	OM614747	MN720382	249/249	100	Bark of *Larix kaempferi*, Japan	-	-	0.13	-	-	-	0.02
Celosporium laricicola	OM614779	FJ997287	249/253	98	Twigs of *Larix lyallii*, Alberta, Canada	-	-	0.13	-	-	-	0.02
All other identified wood fungi						1.5	0.1	2.4	2.7	0.3	-	1.2
Total no. of generated sequences						1445	1466	1483	1599	1657	1772	9422
Sequences of taxa presented in the table, no.						1405	1393	1320	883	1579	1669	8249
%						97.2	95.0	89.0	55.2	95.3	94.2	87.5
All detected taxa, no.						46	44	45	68	32	26	143
Taxa presented in the table, no.						15	11	14	8	11	9	23
%						32.6	25.0	31.1	11.8	34.4	34.6	16.1

[a] In China, associated with *Dendroctonus valens* killing *Pinus tabuliformis* [34]. [b] In western USA, associated with *Dendroctonus jeffreyi* on *Pinus jeffreyi* [35]. [c] In Ontario, vectored to pine by *Tomicus piniperda* [36].

Pure culture isolations from the beetles yielded a total of 33 isolates representing nine taxa (Table 4). The taxa are presented sensu lato as a single ITS marker has been used that cannot discriminate the species in more detail, in particular for ophiostomatoids. The most commonly isolated fungus was *Beauveria bassiana*, represented by 15 isolates, thus comprising 45.5% of all isolates and detected in 25.0% of the beetles subjected to isolations. *Ophiostoma canum* and *Lophium arboricola* followed, each represented by four isolates, corresponding to 12.1% of all isolates and 6.7% of the beetles, respectively. *Lophium arboricola* was also detected in the beetles by direct sequencing, as this was also the case for *Ophiostoma bicolor* and *O. hongxingense* (Table 3).

Table 4. Fungi isolated from beetles of *Ips amitinus* occurring in phloem of dead (previous season attacks) and dying (current season attacks) skeletal branches of *Pinus sibirica* in south-western Siberia. Upon collection, the beetles were dead, and, prior to the isolations, they were surface-sterilised.

Fungal Species Sensu Lato	GenBank Access. No.	Number of Beetles Harbouring Respective Fungus In Branches/Trees [a]						
		Dead			Dying			All
		A1	B1	C1	D1	A2	B2	(%)
Beauveria bassiana	OR584254	5	-	-	1	9	-	15 (25)
Lophium arboricola	OQ536234	-	1	2	-	-	1	4 (7)
Ophiostoma canum	OR574404	-	1	-	2	-	1	4 (7)
Ophiostoma bicolor	OR592582	-	-	-	-	1	2	3 (5)
Ophiostoma hongxingense	OR588874	-	1	1	-	-	-	2 (3)
Ophiostoma sp. 2	OR589101	2	-	-	-	-	-	2 (3)
Chaetomium crispatum	OR584257	-	-	-	1	-	-	1 (2)
Fusarium avenaceum		1	-	-	-	-	-	1 (2)
Trichocladium griseum	OR589102	-	-	-	1	-	-	1 (2)
All fungi		8	3	3	5	10	4	33 (55)
Number of beetles subjected to isolation		10	10	10	10	10	10	60

[a] Letters/numbers correspond to the sites/trees as indicated in Figure 1 and Table 1.

Each stem subjected to sampling for pure culture isolations from wood was visually sound or, in other words, not exhibiting any external symptoms of rot. In total, fungi were isolated from 21 out of 26 sampled stems (81.0%). The isolations yielded a total of 29 pure culture isolates representing five wood-decaying basidiomycete species. In Table 5, they are listed in order of frequency of occurrence. The most common was *Heterobasidion parviporum*, detected in twelve (46.2%) of the examined stems and comprising 41.4% of all isolates. The fungus was found exclusively at a stem height of 0.2 m, and not at 1.2 m. In four stems (15.4% of samples), the fungi were detected at a height of 1.2 m, and these were *Fomitopsis pinicola* and *Porodaedalea pini* (Table 5).

Table 5. Wood decay fungi in twenty-six stems of *Pinus sibirica* attacked by *Ips amitinus* in south-western Siberia. Age of the trees: 120–160 years, d.b.h.: 38–46 cm.

Fungus	GenBank Access. No.	No. of Stems (%)	Of Those, at Height	
			0.2 m	1.2 m
Heterobasidion parviporum	OR574397	12 (46)	12 (46)	-
Phaeolus schweinitzii	OR512100	8 (31)	8 (31)	-
Fomitopsis pinicola	OR512102	4 (15)	2 (8)	2 (8)
Porodaedalea pini	OR574401	3 (12)	1 (4)	2 (8)
Armillaria borealis	OR575851	2 (8)	2 (8)	-
No. of all isolates [a]		29	25 (96)	4 (15)

[a] Fungi were isolated from 21 stems. From two stems, three fungi were isolated, in the following combinations: (i) *H. parviporum* + *A. borealis* + *F. pinicola*; (ii) *H. parviporum* + *P. schweinitzii* + *F. pinicola*. From four stems, two fungi were isolated: (iii) *H. parviporum* + *F. pinicola*; (iv) *H. parviporum* + *P. pini*; (v) *H. parviporum* + *P. schweinitzii*; (vi) *P. schweinitzii* + *P. pini*.

4. Discussion

In this study, sixty *I. amitinus* beetles collected from six trees at four localities yielded 143 fungal taxa. Despite the relatively high richness of the taxa, the detected overall diversity of taxa was low. This was demonstrated by the low values of the Shannon diversity index observed in five trees (excluding D1). The latter implies that the evenness of taxa distribution in the communities was low, indicating that the communities were dominated by a single taxon or a few taxa. This indeed was the case, as the share of just three taxa of yeast, *Nakazawaea holstii*, *Kuraishia molischiana*, and *Nakazawaea ambrosiae*, comprised over 80% of the community. Notably, the richness of taxa was significantly higher in dead than in dying branches, which can be explained by the longer time span since the beetle attack, thus a longer exposure of dead woody substrate to the secondary colonisers. The similarities between the fungal communities were greater among samples collected from the four trees at adjacent sites (A and B). This is somewhat surprising, as those trees differed in their condition (dead and dying), thus exhibited significant differences in taxa richness (high and low, respectively).

Previous studies using ITS2 rDNA as a marker had also revealed common associations of those yeasts with conifer bark beetles from the genus *Ips*, yet within their native range in Europe. Thus, *Nakazawaea* was the most frequently identified yeast in the gut of *I. acuminatus* (Gyllenhal, 1827) infesting pine, and *Kuraishia* in the gut of *I. cembrae* (Heer, 1836) infesting larch [37]. Interestingly, the taxon of *Nakazawaea holstii* has also been detected in the uncolonised phloem of pine in the vicinity of larval galleries in stems infested by the pine shoot beetle *Tomicus piniperda* (Linnaeus, 1758) [38]. Lou et al. [34] presented an extensive review of the possible roles of the yeast associates in bark beetle biology. According to the references provided in [34], certain yeasts may contribute to bark beetle development or fecundity, interact with filamentous fungi, affect the phytochemicals of tree tissues, counteract host plant defence, be involved in bark beetle chemical communication, and attract bark beetle enemies. More specifically, regarding the taxa of yeasts detected during the present study (*Nakazawaea*, *Kuraishia*, *Ogataea*), the following information is provided on their ecological and biological functions: they assimilate nitrate, assimilate cellobiose and D-xylose, covert verbenol to verbenone, enhance the attractiveness of a mixture of attractants for the bark beetle, and affect fungal growth [34].

Four ophiostomatoid taxa were occasionally (and regularly) detected by sequencing of ITS2 rDNA. Although the precise species identification here is not possible, it is obvious that each of the fungi has certain associations with bark beetles and conifers in Asia, Europe, and North America. Similarly, four taxa (in this case species sensu lato) were detected by pure culture isolations and ITS rDNA sequencing, and two of these, *Ophiostoma bicolor s.l.* and *O. hongxingense s.l.*, were also detected by ITS2 rDNA sequencing. *O. bicolor s.l.* has been previously reported as an associate of *I. amitinus* on *Picea abies* in Europe [12–14]. Yet, the most common fungus isolated was the entomopathogen *Beauveria bassiana s.l.* Using a similar methodological approach, the fungus was exclusively detected in mycosed *I. amitinus* beetles in its native range in central Europe (Slovakia) [18]. By contrast, in studies in which the insect body was dissected and its internal organs inspected using a light microscope, *B. bassiana* has not been detected [18–20]. Instead, the genera of entomopathogens such as the ascomycete *Metschnikovia*, sporozoan *Gregarina*, zygomycete *Chytridiopsis* [15,16,39], and fungi-related spore-forming unicellular parasite *Microsporidium* [17] have been reported. Among more conspicuous fungi, it is worth mentioning the wood-inhabiting ascomycete *Lophium arboricola* that was detected using both methods: pure culture isolations from the surface of sterilised dead beetles and sequencing directly from their bodies.

Given that for the fungal isolations from the wood *I. amitinus*-attacked trees were exclusively sampled, the study was not designed to investigate the impact of root and butt rot on beetle attack. Nevertheless, despite the relatively small sample size, the data are of interest, as this study presents for the first time information on the occurrence of root and butt rot fungi in sound-looking stems of *Pinus sibirica*, and is the first of its kind in the whole of Siberia. The handful of previous related studies in this immense geographic

area were limited to observations of trees, exhibiting externally visible symptoms such as sporocarps/rhizomorphs [24,27]. In this study, the five species of fungi detected are common root-, butt-, and stem-rot-causing basidiomycetes, well presented in textbooks on forest pathology. It must be noted that all these fungi must have been present in the investigated stems for years (most likely for decades) prior to the invasion of the beetle.

The most common fungus isolated from the wood was the rot-causing pathogen *Heterobasidion parviporum*. It belongs to the *Heterobasidion* species complex known to cause "the most economically important diseases of conifers in Northern Hemisphaere" [40]. The finding of *H. parviporum* on pine is surprising, as the species is known to be "relatively strictly specialized" for spruce [41]. On the other hand, this can be explained by the fact that the investigated sites were characterised by an admixture of spruce (Section 2.1), and all were situated in the vicinity of a village. In Siberia, on such sites the selective harvesting of spruce and preservation of pine is a usual practice [11].

Thus, it is likely that cut spruce stumps created a pathway for primary infections of *H. parviporum* to the root systems. Subsequently, the pathogen apparently accomplished secondary infections in adjacent pines via root contacts. In general, in species characterised by a resinous heartwood, such as (European and North American) species of pine, *Heterobasidion* attacks sapwood, often causing mortality [41]. Therefore, another unexpected outcome of the study was the observation of *H. parviporum* as the causal agent of extensive heart rot at the butt of *Pinus sibirica* (Figure 6).

Figure 6. Heart rot caused by *Heterobasidion parviporum* in butt of *Pinus sibirica*.

Phaeolus schweinitzii and *Porodaedalea pini* are typical attributes of temperate and boreal old-growth conifer forests of Eurasia and North America, where they cause decay in living stems but also continue to develop on dead wood following tree death [42,43]. In Europe, they are regarded as indicator species for forests of high value for nature conservation [44]. *Fomitopsis pinicola* is primarily known as a common and widespread decomposer of dead wood of a broad range of woody species, yet in old natural conifer forests it occurs in living stems and is known as a mortality agent in pristine forests of northern Eurasia [45]. *Armillaria* is an opportunistic root pathogen that normally colonises dead woody substrates (both by airborne basidiospores and soilborne rhizomorphs) but is also able to attack and to cause massive mortality to weakened/stressed trees [46]; as a group of species, it has been described as "definitely the most studied fungus in the world" [47].

5. Conclusions

The present study led to the following conclusions: (i) DNA analysis of sixty adult beetles of *Ips amitinus* collected from *Pinus sibirica* in south-western Siberia revealed the presence of 143 fungal taxa; (ii) the species richness was significantly higher in beetles collected from dead branches than from (more recently infested) dying branches; (iii) fungal communities were >90% dominated by yeasts, among which the most common were *Nakazawaea holstii*, *Kuraishia molischiana*, and *N. ambrosiae*; (iv) entomopathogenic *Beauveria bassiana s.l.* was the most common fungus isolated from dead/mycosed beetles of *I. amitinus*, followed by *Lophium arboricola* and four *Ophiostoma* spp.; and (v) *Heterobasidion parviporum* was the most common decay fungus detected, which was causing heart rot in stems of *Pinus sibirica*.

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Data Availability Statement: Fungal ITS rDNA sequences generated during this study have been submitted to the GenBank database, and their accession numbers are provided in Tables 3–5.

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