

Genetic identification of wood-destroying fungi in weakened Scots pine (*Pinus sylvestris* L.) forests using DNA barcoding

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Abstract

Weakened forest stands provide a favorable environment for the development of saprotrophic and necrotrophic fungi. Traditional identification methods for these ecological groups of fungi are not always effective. However, modern DNA barcoding methods based on sequencing DNA regions of the studied fungal specimens and comparing them with the nucleotide sequences of previously classified organisms often allow for precise species identification. Thus, to identify fungi using barcoding in weakened Scots pine (*Pinus sylvestris* L.) stands in the Central Forest-Steppe of European Russia (Voronezh, Voronezh Region), 55 mycelium and fruiting body samples were collected from conifer litter and Scots pine damaged butt part of the trunk and roots. After morphological and ecological description and systematization of the collection, DNA extraction was successfully performed, and the ITS1 and ITS2 regions were sequenced in 14 samples. Among them 11 taxa were identified – six at species and five at genus levels, including species *Trichaptum fuscoviolaceum* (Ehrenb.) Donk, *Gymnopilus penetrans* (Fr.) Murrill, and *Ganoderma applanatum* (Pers.) Pat. that infect living wood. The remaining eight representatives of the mycobiota were saprotrophic fungi involved in the mineralization of organic residues. Two phyla, Ascomycota and Basidiomycota, were identified, with the most samples belonging to Basidiomycota and the class Agaricomycetes with seven

orders. The DNA barcoding method complements the results of morphological and ecological analysis.

Keywords: DNA barcoding, pine forests, weakened stands, wood-destroying fungi

Introduction

Modern molecular genetic approaches to the identification of organisms, such as DNA barcoding, make it possible to differentiate morphologically difficult to distinguish species. These organisms include fungi that cause wood rot. DNA barcoding is currently widely used for this purpose (Xu 2016; Čelepirović et al. 2020; Guo et al. 2022). This method has several advantages: identification from a single sample, regardless of morphological features or life cycle stage, and identification accuracy. DNA barcoding makes it possible to determine the species composition of environmental samples (Valentini et al. 2009), to treat fungal diseases at early stages of their development, and to monitor the spatial and temporal patterns of fungal distributions and migrations. DNA barcoding is a potent approach for rapid identification of fungal specimens, generating novel species hypotheses, and guiding biodiversity and ecological studies (Xu 2016). The most commonly used nucleotide sequence for fungal systematics and taxonomy is the internal

transcribed spacer (ITS) sequence of nuclear DNA (Čelepirović et al. 2020), which enables species identification. The method is based on comparing the analyzed unknown DNA sequence with homologous sequences in a database. Basic Local Alignment Search Tool (BLAST) comparison and search algorithms are used for this purpose. Currently, this method is widely used in both agriculture and forestry, enabling a more accurate and complete description of fungal communities, including the identification of pathogens and their antagonists.

Disadvantages of barcoding include the presence of pseudogenes in the genome, which can contribute to misidentification (Gong et al. 2018). Due to insufficient intra- and interspecific variability, species identification can also be difficult for some taxa (Purty and Chatterjee 2016). Furthermore, the universality of primers may be limited, and they may not amplify target genes in some species. Results are verified by comparison with previously deposited sequences from publicly available databases, which are constantly being updated but possibly do not include all species (Ji et al. 2020).

Pine forests are widespread worldwide, with Scots pine (*Pinus sylvestris* L.) being the most widespread and important species of the genus *Pinus* in Eurasia and one of the most ecologically and economically important tree species in Europe (Brichta et al. 2023). According to a recent press release of the Federal Forestry Agency (Rosleshoz) of the Ministry of Natural Resources and Environment of the Russian Federation, the area covered by forest vegetation in the Russian Federation was approximately 766 million hectares, of which pine trees accounted for about 15.5 %, which is equivalent to ~118.7 million hectares (Rosleskhoz 2024). Scots pine accounts for 99 % of the conifer forests of the Central Forest-Steppe of European Russia (Musievsky 2022).

Adverse weather conditions are the primary cause of forest degradation and decline. A range of weather and climate events and their consequences impact forest ecosystems, leading to their transformation. The combination of rising surface temperatures and reduced precipitation or precipitation patterns in some regions of the country (for example, in the south of European Russia) leads to an increase in the frequency and number of droughts, as well as an increase in the incidence of forest fires and the area burned. Furthermore, the expansion of pest and disease habitats is a significant factor in forest vulnerability to climate change (Lipka et al., 2021). According to the Federal State Statistics Service of the Ministry of Economic Development of the Russian Federation (Rosstat) data summarized by Zamolodchikov et al. (2020; section 2.2.1 "The main natural forest disturbances and climate inter-linkages", page 21), damage to Russian forests in 2014–2017 was predominantly caused by fires (63 %), insects (15 %), weather conditions (11 %), diseases (10 %), and other factors such as industrial pollution (~1 %).

The development of epiphytotic of fungal diseases is facilitated by a combination of factors acting simultaneously: presence of a source or focus of infection and disease vectors,

weather, climate, and edaphic conditions favorable for the pathogen and unfavorable for the host plant, the absence of active competitors or antagonists, and anthropogenic impacts leading to the weakening of trees (Varentsova et al. 2023). The region's geographic location determines the fragmentation of forest stands, which is characteristic of the forest-steppe zone. At the southern border of forest distribution, trees experience a combination of negative factors, the most severe of which are droughts (Camarero et al. 2021). Lack of moisture not only negatively affects the activity of physiological processes in trees but also disrupts interactions with mycorrhizal fungi, while pathogen activity increases (Boczoń et al. 2021; Ryu et al. 2018). One of the most dangerous pathogens for conifer stands, including Scots pine stands, is the root fungus *Heterobasidion annosum* (Fr.) Bref. (Garbelotto and Gonthier 2013; Shen et al. 2024), and *Cenangium ferruginosum* Fr., which cause severe tree mortality (Jurc et al. 2000; Ryu et al. 2018). These pathogens cause significant damage to conifer forests worldwide.

The species composition of wood-destroying biotrophic fungi that cause rot damage (wood decay) to pine remains unchanged along the entire longitudinal gradient of pine distribution within the Russian Plain. Only the occurrence of individual wood-destroying fungi species changes significantly. The main species of wood-destroying biotrophic fungi include *Climacocystis borealis* (Fr.) Kotl. & Pouzar, *Heterobasidion annosum* (Fr.) Bref., *Phaeolus schweinitzii* (Fr.) Pat., *Porodaedalea chrysoloma* (Fr.) Fiasson & Niemelä, and *Porodaedalea pini* (Brot.) Murrill [= *Phellinus pini* (Brot.) A. Ames] (Storozhenko 2015). Currently, there are a number of scientific publications on the identification of fungal species in Scots pine stands in the Central Forest-Steppe of European Russia (e.g., Storozhenko 2015), but DNA barcoding was not used in these or similar studies yet. Therefore, the main aim of this study was the molecular genetic identification of fungi causing wood rot in Scots pine stands in weakened stands using DNA barcoding.

Materials and Methods

Fungi sampling

All studied Scots pine stands were planted in suburban and urban forests; their ages ranged from 40 to 80 years (Table 1). In total, 28 samples were collected from suburban forests and 27 from the urban "Northern Forest" (NF, Voronezh City) plantation.

Table 1

Silvicultural and forest mensuration characteristics of Scots pine forest permanent sample plot stands where mycelial samples were collected in the summer of 2024.

Permanent sample plot	Geographic latitude and longitude	Species composition	Age range, years	Density	Type of vegetation
77	51.792036, 39.340998	Scots pine trees	70, 78	0.4-0.8	Fresh pine forest
79	51.795452, 39.310147	Scots pine trees	40-66	0.6-0.8	Fresh subor
80	51.793165, 39.315866	Scots pine trees	26	0.6-0.7	Fresh subor
83	51.786626, 39.344315	Scots pine trees	83	0.6-0.8	Fresh subor
"Northern Forest" (NF)	51.713607, 39.148136	Scots pine and occasional birch, aspen, and maple trees	50 (mean)	0.6-0.8	Fresh pine forest

Note: The permanent sample plots consist of heterogeneous stands that may differ in some or all silvicultural characteristics (height, density, age, composition, etc.), so their characteristics may vary.

The study used the mycelium of sapro- and necrotrophic fungi collected from forest conifer litter (litter), roots and butt parts of Scots pine in foci of root fungus infection in weakened pine stands of the Central forest-steppe of the European part of Russia in the Voronezh region. In total, over 60 Voronezh region sample plots of Scots pine stands were analyzed by forest mensuration descriptions; 43 of them (125.1 hectares) were weakened stands, displaying a combination of root fungal infestations. Five permanent sample plots with weakened Scots pine stands (No. 77, 79, 80, 83, and NF) were selected to collect mycelium from fungi that cause wood rot (Table 1). Weakened stands included stands with the following characteristics: 1) the presence of pathological mortality, namely dried-out and wind-fallen trees with a diameter close to or above the average, with a predominance of weakened trees with a sparse crown, reduced growth, but not more than half, and individual dried branches, 2) severely weakened with a very sparse crown, light green, gray, matte or two-thirds eaten needles, weak growth (less than half of the usual), the presence of drying or dried branches, possible significant mechanical damage to the trunk, dry tops, signs of damage by diseases and pests of the trunk, root paws, branches, and needles, 3) drying with a very sparse crown, crumbling needles, gray, yellowish or yellow-green color, absence of needle development on the shoot, very weak or completely absent growth, 4) drying out of more than two-thirds of the branches, dry branches of more than 50 %, damage to the trunk and root paws by more than two-thirds, and signs of infestation by stem pests). The "Northern Forest" (NF) permanent sample plot is located in Voronezh City. The other four permanent sample plots are located in the Voronezh suburban area: No. 77, 79, 80, and 83 compartments of the Prigorodnoye forestry, the Left Bank district forestry of the Voronezh region, the educational and experimental forestry of the G.F. Morozov Voronezh State Forestry University, as well as the Northern Forest Nature Park, Voronezh (Figure 1). Sampling was conducted during a walk-through of the sample plots. At this stage of the study, no consistent quantitative sampling was performed on each substrate; samples were

collected only from the surface and base of the tree trunks. If the tree was visibly weakened, the butt portion was excavated. Scots pine stands were classified as weakened based on a preliminary assessment of the tree health. No sampling was conducted in healthy stands.

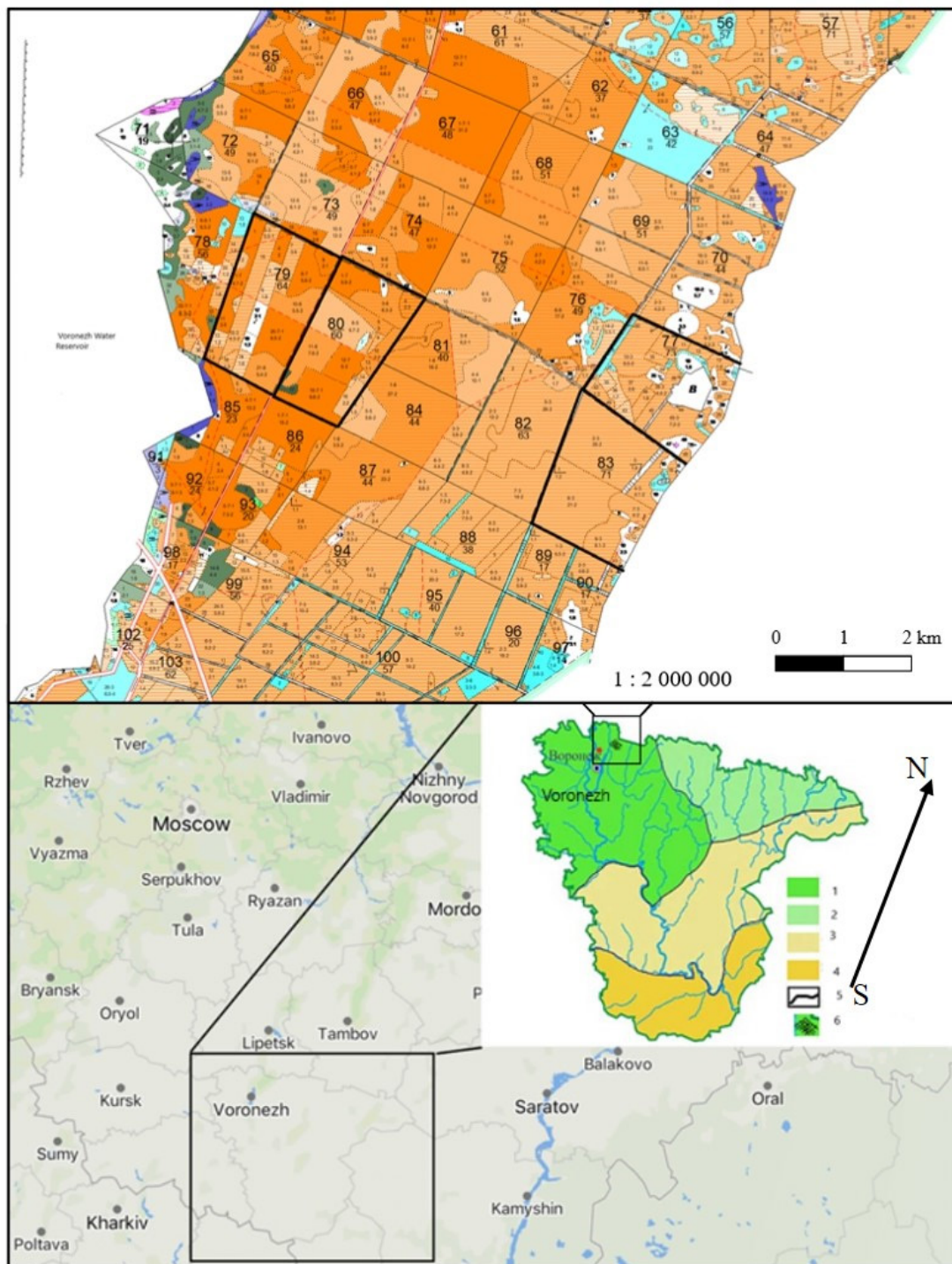


Figure 1

Location of sample plots 77, 79, 80, 83, and NF and climatic zoning in the Voronezh region: 1 - Western forest-steppe region; 2 - Eastern forest-steppe region; 3 - Northern steppe region; 4 - Southern steppe region; 5 - boundary of climatic regions; 6 - Left-bank forestry of the Voronezh region. Red dot on the map near Voronezh depicts location of the "Northern Forest" (NF) sample plot; upper part represents the map with sample plots 77, 79, 80, and 83.

Fungi mycelium was collected in the spring and summer 2024. During collection, the collection spot (litter, trunk, root or stump), morphological and ecological characteristics were recorded. In total, 55 samples were collected from all study plots. All collected samples were assigned unique ID names composed of compartment number, number of the sample, and specific spot on the tree. For example, sample ID "79/3 root" includes "79" as a compartment number, "3" as a number of the sample collected in that compartment, and "root" as a specific spot from which the sample was taken. Mycelium samples were sown on a nutrient medium to obtain pure cultures. Malt-peptone agar was used as a growth medium, with the following composition: malt extract – 30 g, peptone – 1 g, agar – 20 g, distilled water – 1 liter. The grown samples were then transferred to a new, clean growth medium. The process of multiple transfers was repeated until a pure culture was obtained. The experiment was repeated 10 times. After obtaining pure cultures of the fungal samples, they were identified. Pure fungal cultures were stored in test tubes on malt-peptone agar in the refrigerator at 4°C. The cultures were maintained by periodic subcultures, every six months. After labeling and systematization of the samples, a process of their morphological and ecological description was carried out, a general table of samples was compiled, DNA was isolated and used for barcoding. Sample purification was performed only for specimens that best matched the descriptions of *Heterobasidion* by location and morphology. We excluded contaminated samples from species-level ecological interpretation.

DNA isolation and PCR

DNA was isolated from fungal cultures using the PROBA-GS reagent kit (DNA-Technology, Moscow, Russia), according to the manufacturer's protocol. DNA concentration was determined using an F-7000 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) at a wavelength of 260 nm. The purity of the resulting preparations was assessed by the A260/A280 ratio.

The DNA extraction protocol was optimized according to the recommendations of the manufacturer of the commercial kit. Cell lysis was performed using an automatic homogenizer (Allsheng Bioprep-6, China) with 1.4 mm ceramic nanobeads. Then, the samples were re-homogenized using a mortar and pestle. This protocol optimization increased the yield of extracted DNA.

DNA regions containing internal transcribed spacer 1 (ITS1), small subunit ribosomal RNA gene, 5.8S ribosomal RNA gene and internal transcribed spacer 2 (ITS2) were amplified using PCR with universal forward ITS1 (TCCGTAGGTGAACCTGCGG) and reverse ITS4 (TCCTCCGCTTATTGATATGC) primers in the following mixture: deionized water – 16 µl, DNA – 2 µl, ITS1 – 1 µl (10 µM), ITS4 – 1 µl (10 µM), 5x ScreenMix-HS buffer (Eurogen, Russia) – 5 µl.

The PCR was performed using a MasterCycler Personal Thermocycler (Eppendorf, Hamburg, Germany) and the following program: initial heating at 95°C for 5 min, 37 cycles of denaturation at 95°C for 30 sec, primer annealing at 54°C for 30

sec, and chain elongation at 72°C for 45 sec. For PCR, we used the commercial 5X ScreenMix-HS premix (Eurogen, Russia), which includes all necessary PCR components: Hot Start *Taq* DNA polymerase, a mixture of deoxynucleoside triphosphates, Mg²⁺, and reaction buffer, as well as dyes for applying the reaction mixture to the gel during gel electrophoresis. The total PCR reaction volume was 25 µl. The PCR reaction protocol was also optimized based on the selected primers and the study object. We selected the optimal temperature and number of cycles that yielded the best PCR product. The PCR products were checked using 2 % agarose gel electrophoresis and visualized using a QuantumM-312B transilluminator (Helikon, Moscow, Russia) at 312 nm. A DNA ladder 100 bp+ (Eurogen, Moscow, Russia) was used to determine the product length. The gel fragment containing the desired DNA fragment was cut out from the agarose gel and purified using the Cleanup Standard kit (Eurogen, Moscow, Russia).

DNA sequencing and barcoding

Sequencing of purified PCR products was performed on a NANOFOR 05 genetic analyzer (Synthol, Moscow, Russia) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the ITS4 primer. Raw Sanger chromatograms were inspected and processed using Mutation Surveyor software V5.1.1. (SoftGenetics, USA). The processed nucleotide sequence reads were used to search for the most close homologs in the NCBI GenBank database (<https://www.ncbi.nlm.nih.gov>) using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the BOLD System (<https://boldsystems.org>). The best matching sequences in the BLASTn search results were considered as homologous candidates based on the highest bit score (and lowest E-value), coverage and identity values, which varied for different query sequences. Among ties, matches with the longest aligned region and complete ITS1–5.8S–ITS2 annotation were selected considering also type material or voucher specimens. The sequences annotated as "uncultured/environmental sample" were excluded. Sequences were assigned to a particular species when the similarity was ≥ 97 % with the matching reference sequence considering also full coverage. Assignment at the genus level was accepted at a similarity of 92–96 % with the matching reference sequence or with several best matching sequences in this range all representing the same genus considering also high coverage.

Negative controls were used at all stages of the study, from DNA extraction to PCR sequencing. Test tubes containing all components of the reaction mixture except the DNA template served as negative controls. Amplicon sequencing was unidirectional, using the ITS4 primer.

Results

Phytopathogen taxa names are provided according to the MycoBank on-line database (<http://www.mycobank.org> as of August 6, 2025). The taxonomic affiliation was unambiguously established only for 14 specimens presented in Table 2.

Table 2
DNA extraction, PCR, sequencing, and barcoding results of fungal samples.

Sample	ID	Extraction	PCR	Sequencing	Taxonomic identification
NF 1 forest litter	NF 1	+	+	+	-
NF 2 forest litter	NF 2	+	+	-	-
NF 3 stem	NF 3	+	+	+	<i>Trichaptum fuscoviolaceum</i>
NF 4 forest litter	NF 4	+	+	+	<i>Xenopolyscytalum pinea</i> Crous
NF 5 The lower part of the tree trunk	NF 5	+	+	-	-
NF 6 root neck	NF 6	+	-	-	-
NF 7 The lower part of the tree trunk	NF 7	+	+	-	-
NF 8 The lower part of the tree trunk	NF 8	+	-	-	-
NF 9 forest litter	NF 9	+	+	-	-
Sample	ID	Extraction	PCR	Sequencing	Taxonomic identification
NF 10 The lower part of the tree trunk	NF 10	+	+	+	-
NF 11 The lower part of the tree trunk	NF 11	+	+	-	-
NF 12 root	NF 12	+	-	-	-
NF 13 old windfallen tree	NF 13	+	+	+	<i>Xylodon</i> sp.
NF 14 The lower part of the tree trunk	NF 14	+	+	+	-
NF 15 stem	NF 15	+	+	+	-
NF 16 stem	NF 16	+	+	+	-
NF 17 forest litter	NF 17 (13)	+	+	-	-
NF 18 forest litter	NF 18	+	+	+	<i>Trechispora</i> sp.
NF 19 the core of the stump	NF 19	+	+	-	-
NF 20 forest litter	NF 20	+	+	+	<i>Trechispora</i> sp.
NF 21 butt of a tree	NF 21	+	+	+	<i>Sertulicium</i> sp..
NF 22 root of old windfallen tree	NF 22	+	+	+	<i>Gymnopilus penetrans</i>
NF 23 butt of a dead tree	NF 23	+	+	+	<i>Ganoderma applanatum</i> (Fr.) Murrill
NF 24 forest litter	NF 24	+	+	-	-
NF 25 the core of the stump	NF 25 (14)	+	+	-	-
NF 26 forest litter	NF 26 (15)	+	+	-	-
NF 27 forest litter	NF 27	+	+	-	-
77/2 root	77/2	+	-	-	-
77/2.2 root	77/2.2	+	+	-	-
79/1 root	79/1	+	-	-	-
79/2 root	79/2	+	+	-	-
79/3 root	79/3	+	+	+	-
79/4 root neck	79/4	+	-	+	-
79/5 stem	79/5	+	+	-	-
79/7 root neck	79/7	+	-	-	-
79/1 root	79/1 r	+	+	-	-
79/2 root neck	79/2 rn	+	+	-	-
79/4 forest litter	79/4 fl	+	-	-	-
79/4 root	79/4	+	+	+	<i>Dichomitius squalens</i> (P. Karst.) D.A. Reid
79/5 root	79/5 r	+	+	+	<i>Dichomitius squalens</i> (P. Karst.) D.A. Reid
79/6 root;	79/6 r	+	+	+	<i>Sistotrema</i> sp.
79/7 root	79/7 r	+	+	-	-
79/8 root	79/8 r	+	+	+	<i>Dichomitius squalens</i> (P. Karst.) D.A. Reid
79/10 root	79/10 r	+	+	-	-
79/11 root neck	79/11 rn	+	+	-	-

Sample	ID	Extraction	PCR	Sequencing	Taxonomic identification
80/0 root (on the border of the sample plot)	80/0 r	+	+	-	-
80/1 root	80/1 r	+	+	-	-
80/2 root	80/2 r	+	+	-	-
80/3 root	80/3 r	+	+	-	-
83/1 root	83/1	+	-	-	-
83/1.2 root	83/1.2	+	+	-	-
83/2 root	83/2	+	+	-	-
83/3.2 The lower part of the tree trunk	83/3.2	+	+	+	<i>Athelia sp.</i>
83/3.3 root neck	83/3.3	+	+	-	-
K3, K7 root	*	+	+	+	<i>Trametes hirsuta</i>

Note: «+» -successful, «-» - unsuccessful, * - mycelium morphologically similar to *Heterobasidion* (in vitro).

In total, 55 samples were described and systematized. Fungal identification was based on a comprehensive approach that included morphological diagnostics of the study objects, a study of the characteristic symptoms of fungal disease development in forest stands, and DNA barcoding. Of the 55 samples studied, we obtained barcodes for only 14, representing six

species and five genera. Their morphological and ecological properties and sequence accession numbers are presented in Table 3. Fourteen nucleotide sequences obtained in the study and submitted to the NCBI GenBank database are presented in Supplementary Table S1. The top five best matches among BLAST hits are presented in Supplementary Table S2.

Table 3
Brief characteristics of 11 identified taxa.

Sample	Identified species	Environmental group	Mycelium collection site	NCBI GenBank accession number
79/5 root	<i>Dichomitus squalens</i> (P. Karst.) D.A. Reid	wood saprotroph	dead wood of pine trees	PX132320
79/8 root				PX132322
79/4 root				PX132312
79/6 root; NF 16 trunk	<i>Sistotrema sp.</i>	ectomycorrhizal, wood saprotroph	dead wood and plant detritus in the forest litter upper layers	PX132321
83/3.2 root	<i>Athelia sp.</i>	wood saprotroph	dead wood and small woody plant residues	PX132313
Sample	Identified species	Environmental group	Mycelium collection site	NCBI GenBank accession number
NF 4 forest litter	<i>Xenopolyscytalum pinea</i> Crous	plant saprotroph	pine tree litter	PX132315
NF 13 old windfallen tree	<i>Xylodon sp.</i>	wood saprotroph	dead wood of pine and deciduous trees	PX218715
NF 18 forest litter	<i>Trechispora sp.</i>	wood saprotroph	dead wood of pine and deciduous trees	PX132316
NF 20 forest litter	<i>Sertulicium sp.</i>	wood saprotroph	dead wood of pine and deciduous trees	PX132317
NF 21 butt of a tree				PX218716
NF 22 root of old windfallen tree	<i>Gymnopilus penetrans</i> (Fr.) Murrill	plant pathogen, wood saprotroph	live and dead wood of pine and deciduous species	PX132318
NF 23 butt of a deadtree	<i>Ganoderma applanatum</i> (Pers.) Pat.	plant pathogen, wood saprotroph	live and dead wood of pine and deciduous trees	PX132319
K3, K7 root	<i>Trametes hirsuta</i>	plant pathogen, wood saprotroph	old stumps, deadwood, dying trunks of deciduous trees	PX132323
NF 3 stem	<i>Trichaptum fuscoviolaceum</i>	wood saprotroph	live and dead wood of pine trees	PX132314

The taxonomic position of the identified wood-decay fungi species is presented in Table 4.

Table 4
Taxonomy of identified species of wood-destroying fungi.

Phylum	Class	Order	Family	Species
Ascomycota	Leotiomycetes	Helotiales	Hamatocanthoscyphaceae	<i>Xenopolyscytatum pinea</i> Crous
Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	<i>Gymnopilus penetrans</i> (Fr.) Murrill
		Atheliales	Atheliaceae	<i>Athelia</i> sp.
		Cantharellales	Hydnaceae	<i>Sistotrema</i> sp.
		Hymenochaetales	Schizoporaceae	<i>Xylodon</i> sp.
		Sistotrematales	Sistotremastraceae	<i>Sertulicium</i> sp.
		Trechisporales	Hydnodontaceae	<i>Trechispora</i> sp.
		Polyporales	Polyporaceae	<i>Dichomitius squalens</i> (P. Karst.) D.A. Reid. <i>Ganoderma applanatum</i> (Pers.) Pat. <i>Trametes hirsuta</i> (Wulfen) Lloyd. <i>Trichaptum fuscoviolaceum</i> (Ehrenb.) Ryvarden

Bioinformatic analysis of the obtained barcodes allowed us to identify 14 samples with a high degree of accuracy, representing representatives of 11 different taxa. The nucleotide sequences of the identified fungi were deposited in the NCBI GenBank, and their sequence accession numbers are presented in Table 3, along with a brief description of the morphological and ecological properties of the identified wood-decay fungi.

Photos of mycelium and fruiting bodies with established taxonomic affiliation are presented in Supplementary Table S3.

Based on the results of identification, morphological and ecological description and taxonomy, a database “Collection of fruiting bodies and mycelium of saprotrophic and necrotrophic fungi in pine plantations of Voronezh and the Voronezh region” was compiled (Supplementary Table S3).

DNA barcoding has made it possible to systematize the following representatives of pathogenic fungi.

The species *Dichomitius squalens* (P. Karst.) D.A. Reid was identified in three samples (Table 2). Based on morphological and ecological description, its fruiting body is white with a yellowish tint, leathery, of indefinite shape, has a mushroom odor, hymenophore is tubular. The fungus is a xylosaprotroph. Usually found on conifer wood, but can also grow and destroy deciduous trees. It grows after fires on dead conifer wood – pine (*Pinus*), fir (*Abies*), juniper (*Juniperus*), larch (*Larix*), spruce (*Picea*), hemlock (*Tsuga*), Douglas-fir (*Pseudotsuga*), and arborvitae (*Thuja*). In Western Siberia, it has been recorded on Scots pine (*Pinus sylvestris*) and Siberian stone pine (*Pinus sibirica* Du Tour). It is capable of effectively decomposing cellulose and lignin. It causes a rapidly developing, white, pitted rot; in the final stage, the wood splits into fibers. This species is rare and is listed in the Red Data Book of the Republic of Karelia (López et al. 2017; Ageev and Bulkonova 2025). The ITS sequence matched the *Dichomitius squalens* sequence in the NCBI GenBank

(accession number ON088332.1) with 100% identity and 99.64 % coverage. This pathogen was detected in three samples tested.

The Genus *Sistotrema* sp. was found in two samples (Table 2). It represents saprotrophic organisms with the ability to decompose wood; this process is called white rot. According to the morphological and ecological description, it is white in color, with a yellowish or whitish-yellow tint. It has no distinct shape, can cover small areas of wood, and forms fruiting bodies in the form of a flat cap, attached laterally to the substrate. The cap is white with brown speckles on top. A black stripe runs along the edge of the cap. The hymenophore is tubular. They live on dead wood and plant detritus in the upper layers of the forest litter. They form small fruiting bodies on decaying wood and quite often on the dead fruiting bodies of other wood fungi. They cause a visually white rot; ground-dwelling species are capable of forming ectomycorrhizas. Members of the genus *Sistotrema* live on dead wood and appear to be saprotrophic, although whether they are capable of degrading wood remains an open question (Hallenberg 1984; Nagy et al. 2016). ITS nucleotide sequences identified *Sistotrema* sp. in the two studied samples with 98.28 % and 93.73 % coverage, respectively, and 99 % identity to the nucleotide sequence of *Sistotrema* sp. in the NCBI GenBank (accession number ON561591.1).

A fungus of the Genus *Athelia* Pers. was identified in the sample 83/3.2 root. According to the morphological and ecological description, its fruiting body has no distinct shape, appearing as a dry, dense, white coating; the hymenophore is not expressed. Fungi of this genus are widespread in the Northern Hemisphere; their high species diversity is observed in dark conifer taiga forests. Most representatives of the genus develop on dead wood and small woody plant debris, causing inactive white rot. Some species parasitize algae, mosses, and attack lichen thalli. For a number of representatives,

Rhizoctonia-like imperfect stages were noted (Zmitrovich 2004). The nucleotide sequence of the ITS was 96.99 % identical with 92

% coverage to the sequence of *Athelia* sp. in the NCBI GenBank (accession number KP814461.1).

The species *Xenopolyscytium pinea* Crous was identified in the sample NF 4. According to the morphological and ecological description, it has no distinct shape, appearing as a white coating, sometimes forming sparse white compactions; the hymenophore is not pronounced. It inhabits conifer forest litter (Koukol 2012). The ITS region sequenced in DNA isolated from pine needle litter was 99.60 % identical with 95 % coverage to the nucleotide sequence of *Xenopolyscytium pinea* in the NCBI GenBank (accession number MH864121.1).

The genus *Xylodon* was identified in the sample 13. Fungi of the genus *Xylodon* are found in a wide variety of locations worldwide in temperate, tropical, and subtropical regions of the Americas, Asia, and Europe. They grow on the wood of both conifers and deciduous trees, as well as on ferns such as *Cyathea*. They play an important role in the ecosystem as white rot fungi, degrading some of the most resistant macromolecules using extracellular ligninolytic enzymes used in biotechnology (Cho et al. 2021). The ITS sequence of DNA isolated from the fruiting body was 92.08 % identical with 98 % coverage to the *X. flaviporus* sequence in the NCBI GenBank (accession number PQ498256.1). However, given the low percentage of identity due to the poor quality of the sequence, we cannot unambiguously claim that it belongs to this species, but certainly to the genus *Xylodon*.

The genus *Sertulicium* sp. was identified in the sample NF 21. The sequenced ITS region was 94.99 % identical with 93 % coverage to the *S. vernale* nucleotide sequence in the NCBI GenBank (accession number NR_173909.1). The fungus is widespread, growing on dead wood of conifers and deciduous trees (Spirin et al. 2021). According to the morphological and ecological description, the fungus has no distinct shape, appears as a dense, moist layer in cracks and cavities of wood, is yellow in color, and has a tubular hymenophore. However, given the low percentage of identity due to the poor quality of the sequence, we cannot unambiguously claim that it belongs to this species, but certainly to the genus *Sertulicium*.

The species *Gymnopilus penetrans* (Fr.) Murrill was identified in the sample NF 22. According to the morphological and ecological description, the mycelium has no distinct shape, appearing as a dense, web-like white coating; the hymenophore is not pronounced. The fungus is widespread, growing on dead wood: conifer and deciduous stumps, logs, chips, fallen trees, and sawdust; it sometimes parasitizes living trees. It has a fruiting body in the form of a cap and a stem. The cap reaches 3 to 8 cm in diameter and has a highly variable shape: from rounded in young specimens to convex and even prostrate in more mature specimens. The cap is brown with a rufous tint, darker in the center. The surface is dry and smooth to the touch, becoming oily after exposure to moisture. The gills are narrow but closely spaced, gently descending along the stipe. In young fruiting bodies, they are yellow, but as the fungus grows, they change color to rusty-brown. The spore powder,

which is secreted in copious quantities by *Gymnopilus penetrans*, is also of the same color (Lincoff and Mitchel 1977; Kirk et al. 2008). Pathogen identification was based on the morphological characteristics of the fruiting body. The sequenced ITS region of DNA isolated from the fruiting body was 99.69 % identical with 99 % coverage to the *G. penetrans* sequence in GenBank (accession number AY281002.1).

The species *Ganoderma applanatum* (Pers.) Pat. was identified in the sample 23. According to the morphological and ecological description, the fruiting body was leathery, cap-shaped, attached laterally to the substrate, gray-brown in color, white in places with a dark yellow bloom, and had a tubular hymenophore (partly labyrinthine). Fruiting bodies are perennial and can persist for several years, increasing in size and forming new pore layers as they grow. *G. applanatum* is one of the most widespread polypores in the world. As a wood-destroying fungus, it causes heartwood rot in various trees. It can also grow as a pathogen on living sapwood, especially on older trees that are sufficiently moist. It can be a common cause of decay and mortality in beech and poplar, and, less commonly, in other tree genera, including alder, apple, elm, horse chestnut, maple, pedunculate oak, holm oak, walnut, willow, hemlock, Douglas-fir, and spruce. *G. applanatum* is more common on dead trees than on living ones (Biek 1984; Zmitrovich et al. 2019). The sequenced DNA region, including ITS1 and ITS2, was 98.57 % identical with 99 % coverage to the *G. applanatum* sequence in the NCBI GenBank (accession number MK351671.1).

The species *Trametes hirsute* was identified in two samples, №3 and №7r. It is a common species of Basidiomycete fungi growing on tree bark, also called white rot fungus (Begum et al. 2023). According to the morphological and ecological description, it has fruiting bodies in the form of lateral, thin caps, gray on the upper side, with stiff pubescence (Trudell and Ammirati 2009). They were initially attributed to *Heterobasidion annosum* (Fr.) Bref. based on a complex of morphological characters, but the sequence of ITS1 and ITS2 in DNA isolated from the bark of the infected branch was 98.02 % identical with 98 % coverage to the *T. hirsuta* sequence in the NCBI GenBank (accession number PV108805.1).

The sample NF 3 was identified as representing the *Trichaptum fuscoviolaceum* species. According to the morphological description, its fruiting body is a cap-shaped one, attached laterally to the substrate. The upper surface of the fruiting body is brown, and the hymenophore is greenish. The hymenophore is tubular-labyrinth-shaped. It is found on living trees and dead wood of conifers throughout the year, causing white sapwood rot with a cellular structure; decay is quite active (GBIF Secretariat 2023). The sequenced ITS region in DNA isolated directly from the fungal mycelium was 98.99 % identical with 97 % coverage to the *T. fuscoviolaceum* sequence in the NCBI GenBank (accession number MF319120.1).

Two samples (NF 18 and 20) were identified as *Trechispora* sp. According to the morphological description, it has no distinct shape, appears as a dense white coating, and has a tubular hymenophore. It participates in the decomposition of dead wood and other plant materials. It is found in forested areas

worldwide and is a saprotroph (Liu et al. 2022). The sequenced ITS region of DNA isolated from the two dead wood samples showed 98.66 % and 95.28 % identity, respectively, with 96 % coverage of the *Trechispora* sp. sequence in the NCBI GenBank (accession number JF519107.1).

Discussion

Comprehensive long-term studies of the xylotrophic fungal complex were carried out in Southern Urals (Safonov and Safonova, 2023a,b), Northern Poland (Kwaśna et al., 2016), and SE Norway (Stokland and Larsson 2011) and showed that the species composition may be significantly higher than in our study. For instance, in contrast to the 11 taxa we identified, long-term studies have recorded 138 species in a 87-year-old Scots pine forest stand in the Torzym forest in Northern Poland (Kwaśna et al., 2016) and 75 species in the steppe zone of the Southern Urals in Orenburg oblast (Safonov and Safonova, 2023a), 39 of which were collected from the Scots pine trees (Safonov and Safonova, 2023b). However, among 39 species only three species (*Fomitopsis pinicola*, *Heterobasidion annosum* (Fr.) Bref., and *Porodaedalea pini* (Brot.) Murrill) were collected from the weakened pine trees (Safonov and Safonova, 2023b). Research by Kwaśna et al. (2016) showed that dead pine wood is colonized by representatives of three classes, including Zygomycota (19 species), Ascomycota (90 species), and Basidiomycota (29 species). Stokland and Larsson (2011) found even greater diversity in SE Norway, up to 290 species on spruce and pine logs. These differences may be primarily due to the specifics of mycelial sampling: in our study, samples were collected from the forest litter and the surface of the trunk butt and roots. Importantly, the samples were primarily collected from living, weakened trees. The stand's condition, composition, and age are factors influencing the species diversity of xylotrophic fungi. It is believed that the greatest species diversity of xylotrophic fungi is achieved as mycogenic xylolysis progresses at stages III-IV, with the majority of fungi belonging to aphylliphoric fungi. Stages III-IV are characterized by the dominance of the cellulose-degrading fungus *Neorantrodia serialis*, as well as the lignin-degrading fungi *Stereum sanguinolentum* and *Trichaptum fuscoviolaceum* (Neklyayev, Larina, Seraya, 2023). Among the fungi identified on pine wood, we found *Trichaptum fuscoviolaceum*. To further determine the stage of mycogenic xylolysis, it is necessary to analyze more samples to obtain better decay stage assessments for substrates.

The species composition of wood-destroying fungi of the biotrophic complex that cause pine rot remains unchanged across the entire longitudinal gradient of pine distribution within the Russian Plain (Storogenko, 2015). The main species of wood-destroying biotrophic fungi include *Climacocystis borealis* (Fr.) Kotl. et Pouzar, *Heterobasidion annosum* (Fr.) Bref., *Phaeolus schweinitzii* (Fr.) Pat., *Porodaedalea chrysoloma* (Fr.) Fiasson et Niemela, *Phellinus pini* (Thore: Fr.) A. Ames [= *Porodaedalea pini* (Brot.: Fr.) Murrill]. The listed species were not detected in our study; two samples collected from roots and initially attributed to *Heterobasidion annosum* (Fr.) Bref. based

on a complex of morphological characters, were then identified as *Trametes hirsute* based on DNA barcoding.

We have chosen the ITS region for DNA barcoding as a well-established universal genetic marker for fungal identification (Das and Deb, 2015). The ITS region offers the highest resolution among all possible target regions for DNA barcoding of fungi (Badotti et al., 2017). Schoch et al. (2012) reported that ITS can even identify closely related species. However, to overcome the difficulties encountered in assigning some samples to the species level additional markers, such as *LSU*, *tef1-a*, and *rpb2*, should be genotyped to increase efficiency of taxonomic identification.

Only three species identified in the study can infect living wood: *Trichaptum fuscoviolaceum*, *Gymnopilus penetrans*, and *Ganoderma applanatum*. Thus, the study provided important novel data from the use of DNA barcoding to identify wood-destroying fungi in weakened pine forests. The observed specificity, as well as the low number of identified species, indicate the need to continue the study, including stands in both good and bad sanitary condition and more analysis of stumps and fallen wood. This will provide a more complete picture of the dependence of biotrophic and saprotrophic fungi on the forest condition.

Conclusions

For the area covered in this pilot study, the results obtained are the first that used molecular identification methods for wood-destroying fungi in forest stands. Further comprehensive long-term studies with a larger number of identified samples are required, taking also into account the degree of substrate decay.

The number of samples that underwent successfully barcoding was relatively low (14 out of 55, respectively). Some of the collected samples could not be subjected to DNA barcoding due to the poor quality of the collected samples, namely, low biomass, dried fruiting bodies, and old mycelium. Another complicating factor could be the impurity of DNA isolated from some samples due to its contamination by the pine wood compounds, such as polyphenols, tannins, humic acid, and lignin, that may inhibit PCR amplification, despite the DNA extraction protocol optimization. We hypothesize that the problem with sequencing of some samples could be also due to the presence of additional contaminant species in the samples, which could result in spurious sequences that hampered species identification. Nevertheless, the study identified 11 wood saprotrophic fungal taxa at the species and genus levels, three of which attack living wood: *Trichaptum fuscoviolaceum*, *Gymnopilus penetrans*, and *Ganoderma applanatum*. The remaining 8 are saprotrophic fungi involved in the mineralization of organic matter. DNA barcoding nicely complements the detailed study of the morphological and ecological characteristics of wood-destroying fungi in weakened pine forests providing jointly a more complete mycological picture of the study area and allowing us to perform more informative monitoring and

forest health assessment. This study should be continued with an examination of pine forests in different conditions.

Funding

This study was funded by the Russian Science Foundation grant № 24-26-20120.

Acknowledgments

The authors express their sincere gratitude to G. M. Melkumov (Voronezh State University) for consultations during the article preparation.

Supporting information

Supplementary materials are presented in Tables S1, S2, and S3

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