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ORIGINAL RESEARCH PAPER

# Diversity of wood-inhabiting fungi in woodpecker nest cavities in southern Poland

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## Abstract

Globally, tree-holes are important ecological component of forest and woodlands. Numerous microorganisms rely on cavities, both natural and those excavated by primary cavity nesting birds, mainly by woodpeckers, for their survival and reproduction. However, the fungi occurring in cavities are not well characterized. Specifically, very little is known about the fungal communities inhabiting the woodpecker nest cavities. Therefore, in this study, we investigated the fungal diversity of cavities in southern Poland. The samples were collected from freshly excavated woodpecker nest cavities using a nondestructive method (ND). The spatial distribution of fungal communities within the cavities was evaluated by sampling different parts of a single cavity using a destructive method (D). We detected 598 fungal isolates that included 64 species in three phyla and 16 orders using the ND method. Most of the fungi isolated from the cavities represented the phylum Ascomycota (73.9% of the isolates) with 11 orders, and Microascales was the predominant order (30% of the isolates). The most common species detected was *Petriella musispora*, which was isolated from 65% of the cavities. A total of 150 isolates (25%) were members of Basidiomycota, with Hymenochaetales being the dominant order (16% of the isolates). The basidiomycetous fungi were isolated from 55% of the cavities. Several taxa closely related to the pathogenic fungi and associated with secondary animal infections were detected in the wood of cavities. We identified different fungal communities in the three cavity parts using the D method. The cavity entrance had more number of species than the middle and bottom parts. The results of this study advanced our current knowledge on the mycobiota in woodpecker nest cavities and provided preliminary evidence for tree cavities being the hotspot for fungal diversity.

## Keywords

Basidiomycetes; cavity; Microascales; wood-inhabiting fungi; wood-decay fungi; woodpeckers; tree-hollow

## Introduction

Globally, tree-holes are important ecological component of forest and woodlands. Tree-holes are formed by three major mechanisms: natural formation, where the fungi decompose the wood over time; excavation by birds, mainly by woodpeckers, which are often considered as habitat engineers that play a key role in forest ecosystems [1];

wood-boring insects that attack and damage the bark and wood of trees [2]. More than thousand vertebrate species across the globe rely heavily on tree cavities for reproduction and roosting [3]. The cavities created by primary excavators are later used by a large group of secondary cavity nesters, which are species that are unable to independently excavate tree-holes [4].

Fungi, especially the wood-decay species, can assist avian species to excavate cavities in the stems and branches of trees by softening the wood [5–8]. Most Holarctic woodpecker species are reported to select trees that are softened by fungi, including the sections of tree bole that contain heart rot for cavity construction [6,7,9,10]. The trees selected for cavity excavation by woodpeckers may be inhabited either by specific fungal species [11] or by distinct host fungal communities [8,12]. Red-cockaded woodpecker (*Picoides borealis*) prefers trees infected with the heart rot fungus, *Porodaedalea pini* for cavity excavation [6,8]. Recently, Jusino et al. [13] reported that *Picoides borealis* shares a symbiotic relationship with the fungi, where the fungi may help excavators by softening the wood and the excavators may facilitate the fungal dispersal.

Woodpeckers excavate nest cavities in live trees, snags, dead parts of living trees, or within the decaying limbs of living trees [10]. Most woodpeckers usually excavate a new nest cavity each year. Additionally, most woodpecker species excavate cavity only 2–6 weeks before nesting. Cavity excavation in softwood (*Populus* spp. and *Salix* spp.) takes about 2 weeks, while that in hardwood (*Fagus* spp. and *Quercus* spp.) takes about 3–4 weeks. However, large woodpecker species such as black woodpecker (*Dryocopus martius*) may excavate a cavity within 5–6 years and reuse it for several years [14,15].

Tree cavities offer a specific and more stable microclimate when compared to the ambient conditions. The internal temperature of cavities change at a lower rate compared to the outside temperature. Consequently, daily temperature extremes are reduced and typically lag several hours behind the ambient temperature [16–18]. The mean daily relative humidity in tree cavities is high (typically exceed 90%) and stable throughout the day, which is in contrast to a much lower and highly fluctuating ambient humidity [19,20]. Compared to the majority of secondary-cavity nesting birds, woodpeckers do not use any external materials to fill the nest cup and lay their eggs directly on the cavity bottom, which only contains small wood debris, such as wood scrapes or rotten wood fragments [21].

Generally, woodpeckers remove fecal material from the cavity while nesting [22,23]. Consequently, the nests of primary-cavity nesting birds remain fairly clean and rarely have nonwooden organic material. However, during the breeding event, which includes incubation, hatching, offspring feeding, and molting of adults, some small portions of organic debris may be deposited and accumulated at the cavity bottom. Small fraction of feces and other organic materials such as parts of eggshells, single feathers, or even dead offspring could remain in the cavity bottom. The other unique trait of a bird nest, including those located in cavities, is its thermal properties induced by the presence of warm-blooded parents or nestlings [24], which promote the growth of thermophilus fungi [25]. Therefore, the specific climatic and nutritional conditions in the tree-cavities may influence the composition of fungal communities and promote ultra-rich and specific fungal diversity.

Compared to the fungi inhabiting bark wounds on trees [26,27], the mycobiome of tree cavities has been poorly studied. Most studies have reported a positive association between wood-decay fungi and cavity excavating birds [3,6,7,10,28,29]. These studies have relied only on visual observation of fungal fruiting bodies. This technique could lead to a poor measure of association because many fungi inhabit a tree for decades without fruiting [30,31]. Recently, Jusino et al. [8] reported that specific fungal communities inhabit the living pine cavity excavated by red-cockaded woodpeckers using molecular methods.

Although several studies have explored the cavity ecology, very little is known on the fungal composition of cavities excavated by woodpeckers. Therefore, in this study we identified the diverse of wood-inhabiting fungi in the cavities excavated by European woodpeckers using DNA-based techniques. We excluded old cavities as their age and the presence of secondary-nesting species might influence the fungal community composition and dynamics. Therefore, only the new cavities excavated in a given year were used in this study.

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## Material and methods

### Study site

The study was conducted in southern Poland in 2014–2015 at two study regions (Krakow region 50°05′ N, 19°55′ E and Western Carpathians 49°40′ N, 19°30′ E). Krakow region represents a human-modified landscape type. The area is characterized by a broad urbanization gradient from a densely built-up city center to the suburbs with a moderate number of buildings and from the scattered buildings, typical of a rural landscape to farmlands with minor fraction of tree vegetation (urban greenery, orchards, and woodlots). The Western Carpathians represent forest-dominated landscape type. Various plant communities dominate, including the fertile Carpathian beech forest (*Dentario glandulosae-Fagetum*), fir-spruce forest (*Abieti-Piceetum*), and the upper subalpine acidophilous Carpathian spruce forest (*Plagiothecio-Piceetum*). The climate is temperate, transitional from maritime to continental. The mean annual temperature is 7°C (maximum 17°C in July, minimum −3°C in January) and the total annual precipitation is 800 mm.

### Collection of samples and fungal isolations

The study regions were surveyed from early spring to identify the breeding territories of woodpeckers. We search for the cavities at the stage of excavation from these breeding territories. This approach enables us to study only the cavities that were excavated in a given breeding season and exclude the older cavities. This standardization was necessary as the composition of fungal community may potentially change with time. Moreover, cavities were sampled only if they were completed, successful broods were recorded, and the offspring had left the nest. We used these criteria as the interior of unfinished cavities may have a different microclimate than the completed cavities, which may in turn influence the fungal community. Moreover, the lack of breeding event may affect thermal and trophic conditions within a cavity as incubating parents and sitting offspring may potentially increase the thermal properties of a cavity interior. Additionally, continuous feeding may potentially provide external nutrients to a cavity interior.

Upon completion of breeding event, the wood samples from the cavity were collected. To capture maximal fungal communities in the cavity, we analyzed 20 cavities (C1 to C20), representing seven woodpecker species, sampled from eight tree species (Tab. 1). We collected the samples from the interior of woodpecker cavities using a nondestructive method (ND) as described previously by Jusino et al. [32]. The wood samples were collected using a specially designed tool, which was a 40-cm-long steel pipe with 2-cm diameter. The edge at one end was fabricated to form a chisel-like blade. The sample collection did not cause extensive destruction of the cavity, as the sample was a puck wood of ca. 2 × 2 cm size. From each cavity, three wood fragments were collected from the central part of the hole for the isolation of fungi. The sampling tool was flame-sterilized between each sampling location. As some of the cavities were located very high in the trees, trained and certified arborists surveyed the trees using ladders or by climbing.

During 2014–2015, fungi were isolated from 60 wood fragments collected from the interior of cavities. The wood fragments were placed individually in sterile plastic containers and stored at 4°C for 1–2 days until fungal isolation. Each wood fragment was surface sterilized in 96% ethanol for 15 s. The fragment was dried using sterile filter paper and the wood surface was removed using a sterile scalpel. Each wood fragment was divided into 12 pieces (4 × 4 mm) and placed in 9-cm Petri dishes containing the following culture medium: malt extract agar [MEA; 20 g malt extract (Biocorp Polska Sp. z.o.o., Poland), 20 g agar (Biocorp Polska Sp. z.o.o., Poland), 1,000 mL sterile water, and 50 mg/L tetracycline (Polfa S.A., Poland)] to isolate the general mycobiota; malt extract agar with cycloheximide [CMEA, MEA with 200 mg/L cycloheximide (Sigma-Aldrich, St. Louis, Co. LLC.)] to isolate *Ophiostoma*/*Leptographium* spp. [33]; or malt extract agar with benomyl [BMEA, MEA with 8 mg/L benomyl (Sigma-Aldrich)] to isolate basidiomycetes [34]. We used 720 wood pieces for fungal culturing.

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The fungal communities inhabiting the woodpecker nest cavity were detected using a total destructive method (D). In fall 2015, a European beech (*Fagus sylvatica*) tree with a cavity excavated by white-backed woodpecker (*Dendrocopos leucotos*) (C16) was felled after the breeding event. A section including the whole cavity (150 cm long) was excised out from the tree and transported to the laboratory. The next day, the section was cut along the center of the trunk axis to expose the interior of the cavity. The wood fragments were disinfected for approximately 15 s using 96% ethyl alcohol and dried on filter paper. The fungi were isolated from the wood surrounding the cavity entrance, and from the wood layers underneath the entrance to a depth of 5, 15, and 25 cm till the cavity bottom. The wood fragments (about 4 × 4 mm), were cut using a sterile chisel, and placed on the culture medium. We collected 360 wood fragments for isolating the fungus.

## Fungal identification

The cultures were incubated at room temperature (22–25°C) in the dark for 16 weeks. The cultures were purified by transferring small pieces of mycelium or spore masses from the individual colonies to fresh MEA. The purified cultures were grouped into morphotypes based on the morphological characteristics of asexual and sexual structures, and anverse and reverse colony color reported in the literature [35–41] using a Nikon Eclipse 50i microscope (Nikon Corporation, Tokyo, Japan) fitted with an Invenio 5S digital camera (DeltaPix, Maalov, Denmark) and linked to the COOLVIEW 1.6.0 software (Precoptic, Warsaw, Poland). Depending on the size of the morphological group, one to nine representative strains of each morphotype were further subjected to molecular identification based on internal transcribed spacer (ITS) and 28S large ribosomal subunit (LSU) sequence comparison. We selected 146 isolates for molecular identification (Tab. S2). Additionally, the protein coding genes (β-tubulin or the elongation factor 1-α) were sequenced to identify the Ophiostomatales order, *Fusarium* spp., *Neonectria* spp., and *Trichoderma* spp.

The isolates were subjected to DNA extraction, PCR amplification, and sequencing following the methods used by Jankowiak et al. [42]. See Tab. S1 for primers used to sequence ITS region (ITSI-5.8S-ITSII), LSU, and elongation factor 1-α (TEF 1-α).

The sequences (Tab. S2) were deposited in the GenBank of the National Center for Biotechnology Information (NCBI) database. The sequences were aligned with those available in GenBank using the BLASTn algorithm. Only a 99–100% match with a reliable source (ex-type sequences, published taxonomic studies) was accepted as proof of identification. The sequences were considered to belong to the same species when sequences exhibited ≥99.0% similarity with the ITS or LSU region (400–500 bp). Additionally, the β-tubulin or the TEF 1-α sequences were compared with the sequences available in GenBank to identify *Fusarium* spp. and *Trichoderma* spp. All the sequenced isolates are deposited in the Culture Collection of Fungi of the Laboratory of Department of Forest Pathology, Mycology and Tree Physiology, University of Agriculture in Krakow, Poland (Tab. S2).

For the identification of Microascales, the most dominant order in this study, the individual data sets for the ITS and LSU gene regions were used for phylogenetic analysis. The data sets were compiled and edited in MEGA ver. 6.06 [43]. Sequence alignments were performed using the online version of MAFFT ver. 7 [44]. The ITS and LSU data sets were aligned using the E-INS-i strategy with a 200PAM/κ=2 scoring matrix, a gap opening penalty of 1.53, and an offset value of 0.00. For maximum likelihood (ML) and Bayesian (BI) analyses, the best-fit substitution models for each data set were estimated using the corrected Akaike information criterion (AICc) in jModelTest ver. 2.1.10 [45,46]. Phylogenetic analyses were performed for each of the data sets using two different methods: ML and BI. ML searches were conducted in PhyML 3.0 [47] using the Montpelier online server (http://www.atgc-montpellier.fr/phyml/) with 1,000 bootstrap replicates. BI analyses based on a Markov chain Monte Carlo (MCMC) were performed using MrBayes ver. 3.1.2 [48]. The MCMC chains were run for 10 million generations using the best-fit model. The trees were sampled every 100 generations, resulting in 100,000 trees from both the runs. The burn-in value for each dataset was determined in TRACER ver. 1.4.1 [49].

**Tab. 1** Characterization of woodpecker nest cavities in Poland.

| No. of cavity | Species of woodpecker | Species of cavity-tree | Tree height (m) | Tree DBH (cm) | Height of cavity location (m) | Entrance exposition | Tree health | Cavity location | Fruiting body present | Habitat type | Date of collection | Latitude (N) | Longitude (E) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C1 | *Picus viridis* | *Salix fragilis* | 19 | 36 | 2.0 | SE | Live | Stem | - | Riparian woodlot | 2014-07-16 | 49°58′ | 20°11′ |
| C2 | *Picus viridis* | *Salix fragilis* | 19 | 36 | 3.0 | SE | Live | Stem | - | Riparian woodlot | 2014-07-16 | 49°58′ | 20°11′ |
| C3 | *Dendrocopos medius* | *Malus domestica* | 7 | 24 | 1.5 | SE | Live | Dead branch | - | Orchard | 2014-07-22 | 50°26′ | 20°09′ |
| C4 | *Picus viridis* | *Salix fragilis* | 24 | 38 | 8.0 | NW | Live | Stem | + | Riparian woodlot | 2014-10-15 | 49°43′ | 19°29′ |
| C5 | *Picus viridis* | *Salix fragilis* | 24 | 38 | 8.0 | NW | Live | Stem | + | Riparian woodlot | 2014-10-15 | 49°43′ | 19°29′ |
| C6 | *Dendrocopos major* | *Alnus incana* | 22 | 32 | 4.0 | SW | Live | Stem | - | Riparian forest | 2014-10-18 | 49°24′ | 20°45′ |
| C7 | *Dendrocopos leucotos* | *Salix fragilis* | 15 | 35 | 8.0 | E | Live | Stem | - | Riparian woodlot | 2014-10-18 | 49°24′ | 20°45′ |
| C8 | *Dendrocopos major* | *Fagus sylvatica* | 4 | 45 | 3.0 | SE | Snag | Stem | + | Riparian forest | 2014-11-27 | 49°38′ | 19°39′ |
| C9 | *Picoides tridactylus* | *Picea abies* | 25 | 40 | 4.0 | S | Snag | Stem | - | Coniferous forest | 2014-11-28 | 49°35′ | 19°31′ |
| C10 | *Dendrocopos major* | *Fagus sylvatica* | 11 | 25 | 8.0 | SE | Snag | Stem | + | Mixed forest | 2014-11-28 | 49°36′ | 19°31′ |
| C11 | *Dryocopus martius* | *Abies alba* | 45 | 87 | 18.0 | SE | Live | Stem | - | Coniferous forest | 2014-12-04 | 49°34′ | 19°35′ |
| C12 | *Dendrocopos leucotos* | *Fagus sylvatica* | 17 | 38 | 13.0 | S | Live | Stem | + | Deciduous forest | 2014-12-04 | 49°38′ | 19°39′ |
| C13 | *Dendrocopos major* | *Acer pseudoplatanus* | 12 | 50 | 6.0 | E | Live | Stem | + | Mixed forest | 2014-11-28 | 49°36′ | 19°28′ |
| C14 | *Picus viridis* | *Salix fragilis* | 17 | 35 | 4.5 | E | Live | Stem | + | Riparian woodlot | 2015-11-17 | 49°42′ | 19°27′ |
| C15 | *Dendrocopos major* | *Fagus sylvatica* | 32 | 48 | 6.0 | NE | Live | Stem | - | Deciduous forest | 2015-11-17 | 49°36′ | 19°31′ |
| C16 | *Dendrocopos leucotos* | *Fagus sylvatica* | 11 | 34 | 8.0 | E | Snag | Stem | + | Deciduous forest | 2015-11-17 | 49°36′ | 19°31′ |
| C17 | *Picoides tridactylus* | *Picea abies* | 35 | 72 | 9.0 | SE | Live | Stem | - | Coniferous forest | 2015-11-17 | 49°35′ | 19°31′ |
| C18 | *Dendrocopos minor* | *Prunus domestica* | 4 | 26 | 3.0 | NE | Live | Dead branch | + | Orchard | 2015-11-22 | 49°43′ | 19°05′ |
| C19 | *Dendrocopos medius* | *Salix fragilis* | 15 | 45 | 6.0 | SW | Live | Live branch | + | City park | 2015-12-19 | 49°59′ | 19°57′ |
| C20 | *Dendrocopos major* | *Salix fragilis* | 22 | 38 | 7.0 | S | Live | Stem | - | Riparian woodlot | 2015-12-19 | 50°01′ | 19°58′ |

### Statistical analyses

The Shannon [50] and Simpson [51] diversity indices were estimated for each cavity (*DM*) and each cavity site (*D*). The fungal dominance was determined by Camargo's index (1/*S*), where *S* represents species richness. A species was defined as dominant if $P_i > 1/S$, where $P_i$ is the relative abundance of species *i*, which is defined as the number of competing species present in the community [52].

The chi-square test was performed to evaluate the difference among the proportions, followed by the Marascuilo procedure for pairwise comparison of the proportions, using StatTools.net software (http://www.statstodo.com/). These procedures were performed to determine the difference in the frequency of a fungus among the cavity sites.

A principal component analysis (PCA) was used to understand the correlation between the abundance of fungal species in the woodpecker species and the different tree species. The data were log transformed prior to the analysis. This statistical analysis was conducted using PAST 3.18 [53].

## Results

### Collection of isolates and fungal identification

We obtained 742 fungal isolates from 1,080 wood fragments of woodpecker nest cavities using two sampling techniques. The isolates included 182 Basidiomycota, 554 Ascomycotina, and six Mucoromycotina (Tab. 2 and Tab. 3). The isolates were separated into 52 morphotypes based on the preliminary morphological investigation. As the initial morphological survey of the isolated cultures and ITS sequence data revealed that our morphotypic criteria were not stringent, the morphotypes were grouped into 69 species based on the ITS and other gene sequence analysis (Tab. S2).

The ITS and LSU sequence analyses within the order Microascales revealed that 43 isolates resided in two major phylogenetic clades: Graphiaceae and Microascaceae (Fig. 1 and Fig. 2). Within Graphiaceae, two isolates named as *Graphium* sp. 1 were unknown species that are closely related to *Graphium penicillioides*, while four other isolates named as *Graphium* sp. 2 were phylogenetically related to *Graphium madagascariense* (Fig. 1 and Fig. 2). Within Microascaceae, the ITS and LSU trees identified *Parascedosporium putredinis* (three isolates), *Petriella musispora* (nine isolates), *Petriella guttulata* (five isolates), and *Petriella sordida* (one isolate). Additionally, four unidentified isolates that are closely related to *Lophotrichus fimeti* (*Lophotrichus* sp.) were also identified (Fig. 1 and Fig. 2). The ITS and LSU sequence analysis revealed that some isolates resided in the *Scopulariopsis* and *Acaulium* genera. Among them, six isolates were identified as *Scopulariopsis candida*, two isolates were closely related to *Scopulariopsis soppii* (*Scopulariopsis* cf. *soppii*), three isolates were identified as *Acaulium albonigrescens*, and two isolates, named as *Acaulium* sp. represented species that were closely related to *Acaulium acremonium*. This family was also represented by *Cephalotrichum stemonitis* and *Wardomyces inflatus* (Fig. 1 and Fig. 2).

### Diversity of fungal species isolated from different cavities by ND method

Among the 720 wood pieces (collected from woodpecker nest cavities) used for fungal culturing, we obtained fungal growth from 418 (58.1%) wood pieces. Among these 418 wood pieces, we obtained between one and three different fungal cultures from each wood piece. In total, we obtained 598 cultures. We did not observe any fungal growth from the samples obtained from the cavity excavated by the great-spotted woodpecker (*Dendrocopos major*) on sycamore (*Acer pseudoplatanus*) (C13). The 598 fungal isolates included 64 fungal species that were assigned to three phyla, and 16 orders. Within the phylum Basidiomycota, we isolated members belonging to the orders Agaricales, Hymenochaetales, and Polyporales. Within the phylum Mucoromycotina, we isolated the members belonging to the orders Mortierellales and Mucorales. Most of the fungi isolated from the cavities represented the phylum Ascomycota (73.9% of total). They

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**Tab. 2** Wood-colonizing fungi and their diversity in different woodpecker nest cavities.

| Taxon | Number of isolates obtained* | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | C13 | C14 | C15 | C16 | C17 | C18 | C19 | C20 | Total isolated |
| **Basidiomycota** | | | | | | | | | | | | | | | | | | | | | |
| *Fomes fomentarius* | | | | | | | | | | 6 | | 10 [A] | | | | | | | | | 16 [A] |
| *Fomitiopora punctata* | | | | | | | 18 [A] | | | | | | | | | | | | | | 18 [A] |
| *Inonotus obliquus* | | | | | | | | | | | | | | | 2 | 19 [A] | | | | | 21 [A] |
| *Ischnoderma benzoinum* | | | | | | | | | | | | | | | | | | 14 [A] | | | 14 [A] |
| *Phellinus alni* | | | | | | 18 [A] | | | | | | | | | | | | | | | 18 [A] |
| *Phellinus igniarius* | 18 [A] | 21 [A] | | | | | | | | | | | | | | | | | | | 39 [A] |
| *Phellinus* cf. *igniarius* | 1 | | | | | | | | | | | | | | | | | | | | 1 |
| *Trametes suaveolens* | | | | | | | | | | | | | | | | | | | | 1 | 1 |
| *Trametes versicolor* | | | 22 [A] | | | | | | | | | | | | | | | | | | 22 [A] |
| **Ascomycota: Microascales** | | | | | | | | | | | | | | | | | | | | | |
| *Acaulium* sp. | | | | | | | | | | | | | | 1 | | | | | | | 1 |
| *Cephalotrichum stemonitis* | | | | | | | | | | | | | | | 2 | | | | | 1 | 3 |
| *Graphium* sp. 1 | | | | | | | | | | | | 2 | | 1 | | | | | | | 3 |
| *Graphium* sp. 2 | | 2 | | | | | | | | | | | | 2 | | | | | | | 4 |
| *Lophotrichus* sp. | | | | 6 [A] | 6 [A] | | | | | | | | | 12 [A] | | | | | | | 24 [A] |
| *Parascedosporium putredinis* | | 2 | | | | | | | | | | | | | | | | | | 2 | 4 |
| *Petriella guttulata* | | 1 | | | | 2 | | | | | | 5 | | | 17 [A] | | | | | | 25 [A] |
| *Petriella musispora* | 2 | 7 [A] | | 12 [A] | 18 [A] | 18 [A] | 3 | 1 | | 5 | | 16 [A] | | 12 [A] | 1 | | | 3 | | 1 | 99 [A] |
| *Petriella sordida* | | | | | 1 | 1 | | | | | | | | | | | | | | | 2 |
| *Scopulariopsis candida* | | | | | | | | | | | 3 | | | | | | 10 [A] | | | | 13 [A] |
| *Scopulariopsis* cf. *soppii* | | | | | | | | | | | | | | | | | | | 1 | 1 | 2 |
| *Wardomyces inflatus* | | 1 | | | | | | | | | | | | 1 | | | | | | | 2 |
| **Ascomycota: other** | | | | | | | | | | | | | | | | | | | | | |
| *Alternaria arborescens* | | | | | | | | | | | | | | | | 1 | | | | | 1 |
| *Alternaria* sp. | | | | | | | | | | | | | | | | | 1 | | | | 1 |
| *Arthrobotrys oligospora* | 4 | | | | | | | | | | | | | | | | | | | | 4 |
| *Arthrobotrys* sp. | | | | | | 6 | | | | | | | | 1 | | | | | | | 7 |
| *Cadophora malorum* | | | | | | | | | | | | | | | 1 | | | | | | 1 |
| *Chaetomium angustispirale* | | | | | | | | | | | | | | | 1 | | | | | | 1 |

**Tab. 2** Continued

| Taxon | Number of isolates obtained* | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | C13 | C14 | C15 | C16 | C17 | C18 | C19 | C20 | Total isolated |
| *Chaetomium* sp. 1 | | | | | | | 1 | 1 | | | | | | | 1 | | | | | | 3 |
| *Chaetomium* sp. 2 | 4 | | | | | 1 | | | | | | | | 10 [A] | 1 | | | 12 [A] | 6 [A] | 1 | 35 [AA] |
| *Chaetomium* sp. 3 | | | | | | | | | 1 | 15 [A] | 7 | | | | 1 | | 2 | | | | 26 [A] |
| *Clonostachys rosea* | | 2 | | | | | | | | | | | | | 1 | | | | | | 3 |
| *Clonostachys* sp. | 1 | | | | | | | | | | | | | 9 [A] | | | | | | | 10 [A] |
| *Cosmospora viridescens* | 1 | 1 | | | | | | | | | | | | | | | | | | | 2 |
| *Cosmospora* sp. | | 1 | | | | | | | | | | 1 | | | | | | | | | 2 |
| *Duddingtonia flagrans* | | | | | | 1 | | | | | | | | | | | | | | | 1 |
| *Epicoccum nigrum* | | | | | | | | | | | | | | | | 1 | | | | | 1 |
| *Fusarium solani* s. stricto | | 1 | | | | | | | | | | | | | | | | | | | 1 |
| *Fusarium* sp. 1 (FSSC complex) | | 1 | | 1 | 1 | | | | | | | | | | | | | | | | 3 |
| *Fusarium* sp. 2 (FSSC complex) | | 1 | | | | | | | | | | | | | | | | | | | 1 |
| *Fusarium* sp. 3 (FSSC complex) | | | | | | 6 | | | | | | | | | | | | | | | 6 |
| *Galactomyces geotrichum* | | | | | | | | | | | | | | | | | | | | 1 | 1 |
| *Geotrichum* sp. | | | | 5 [A] | | | | | | | | | | | | | | | | | 5 |
| *Humicola fuscoatra* | | | | | | | | | | | | | | | | | | | 1 | | 1 |
| *Neobulgaria* sp. | 1 | | | 1 | | | | | | | | | | | | 1 | | | | 5 [A] | 8 |
| *Neonectria* sp. | | | | | | | | | | | | | | | 1 | | | | | | 1 |
| *Ophiostoma flexuosum* | | | | | | | | | | | 14 [A] | | | | | | | | | | 14 [A] |
| *Paracremonium* sp. | 10 [A] | 12 [A] | | 6 [A] | 7 [A] | | | | | | | | | | | | | | | | 35 [A] |
| *Penicillium brevicompactum* | | | | | | | | | 1 | 6 | 4 | | | | | | | | | | 11 [A] |
| *Phialemonium* sp. | | | 1 | | | | | | | | | | | | | | | | | | 1 |
| *Phialophora* sp. | | | 7 | | | | | | | | | | | | 1 | | | | | | 8 |
| *Phoma* sp. | | | 1 | | | | | | | | | | | | | | | | | | 1 |
| *Podospora* sp. | | 1 | | | | | | | | | | | | | | | | | | | 1 |
| *Pseudocosmospora vilior* | | | | | | | | | | | | 1 | | | | | | | | | 1 |
| *Pseudogymnoascus pannorum* | | | | | | | | | | | | 3 | | 2 | | | | | | 5 [A] | 10 [A] |
| *Sporothrix* sp. | | | 3 | | | | | | | | | | | | | | | | | 5 [A] | 8 |
| *Trichocladium* cf. *asperum* | | | | | | | | | | | | | | | | | | 1 | | | 1 |
| *Trichoderma harzianum* | 1 | | 12 [A] | 1 | | | | 1 | | | | | | | | | | | | 10 [A] | 25 [A] |
| *Trichoderma longibrachiatum* | | | | | | | | | | | | | | | 1 | | | | | | 1 |

**Tab. 2** Continued

| Taxon | Number of isolates obtained* | | | | | | | | | | | | | | | | | | | | Total isolated |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | C13 | C14 | C15 | C16 | C17 | C18 | C19 | C20 | |
| *Trichoderma paraviridescens* | | | | | | | | | | | | | | | | 6 [A] | | | | | 6 |
| *Trichoderma trixiae* | | 1 | | | | | 11 [A] | | | | | | | | | | | | | | 12 [A] |
| **Mucoromycotina** | | | | | | | | | | | | | | | | | | | | | |
| *Mortierella* cf. *hyalina* | | | | 1 | | | | | | | | | | | | | | | | | 1 |
| *Mortierella zychae* | | | | 1 | | | | | | | | | | 2 | | | | | | | 3 |
| *Mucor hiemalis* | | | | | 1 | | | | | | | | | | | | | | | | 1 |
| *Mucor piriformis* | | | | 1 | | | | | | | | | | | | | | | | | 1 |
| No. of total fungal isolates | 43 | 55 | 46 | 35 | 34 | 53 | 33 | 3 | 2 | 32 | 28 | 38 | 0 | 53 | 31 | 28 | 13 | 30 | 8 | 33 | 598 |
| Species richness (*S*) | 10 | 15 | 6 | 10 | 6 | 8 | 4 | 3 | 2 | 4 | 4 | 7 | 0 | 11 | 13 | 5 | 3 | 4 | 3 | 11 | 64 |
| Camargo's index (1/*S*) | 0.100 | 0.067 | 0.167 | 0.100 | 0.167 | 0.125 | 0.250 | 0.333 | 0.500 | 0.250 | 0.250 | 0.143 | 0 | 0.091 | 0.077 | 0.200 | 0.333 | 0.250 | 0.333 | 0.091 | 0.016 |
| Simpson's index (*D*) | 0.252 | 0.217 | 0.325 | 0.202 | 0.356 | 0.259 | 0.418 | 0.333 | 0.500 | 0.315 | 0.344 | 0.274 | 0 | 0.173 | 0.320 | 0.510 | 0.621 | 0.389 | 0.594 | 0.170 | 0.055 |
| Simpson's index of diversity [*SID*; *SID* = (1 – *D*)] | 0.749 | 0.784 | 0.675 | 0.798 | 0.644 | 0.741 | 0.582 | 0.667 | 0.500 | 0.686 | 0.656 | 0.726 | 0 | 0.827 | 0.681 | 0.490 | 0.379 | 0.611 | 0.406 | 0.830 | 0.945 |
| Shannon index of diversity (*H*) | 1.726 | 1.979 | 1.334 | 1.859 | 1.279 | 1.575 | 1.021 | 1.099 | 0.693 | 1.273 | 1.210 | 1.529 | 0 | 1.959 | 1.791 | 0.950 | 0.687 | 1.066 | 0.736 | 2.025 | 3.392 |

* Results obtained overall from selective and nonselective media. A – dominant species.

were distributed in 11 orders, with Microascales being the most abundant order (30% of the isolates) (Tab. 2). The genus *Petriella* was the most abundant (21%), which was isolated from 80% of the woodpecker nest cavities. Additionally, the predominant species was from the Hypocreales order (15 species). We often isolated the members of Microascales on benomyl MEA medium, which promotes the growth of basidiomycetes. A total of 150 isolates (25% of total) were classified to the Basidiomycota division, mainly to the Hymenochaetales order (16% of the isolates). The basidiomycetous fungi were isolated from 55% of the cavities. The members of the Mucoromycotina were sparsely represented (Tab. 2).

The predominant species was *Petriella musispora* (16.5% of the total number of fungal isolates), which was isolated from 65% of the cavities (Tab. 2). The other species were rarely isolated from the cavities, although *Chaetomium* sp. 2, *Paracremonium* sp., and *Phellinus igniarius* comprised 5.8–6.5% of the total isolates and detected in 10%, 30%, and 20% of the cavities, respectively (Tab. 2).

The number of isolates obtained from cavities excavated by woodpeckers ranged from 0 (C13) to 55 (C2). The species richness (*S*) for all cavities was 64 and ranged from 0 (C13) to 15 (C2). The fungal diversity values varied widely among the cavities (Tab. 2). The fungal community associated with the cavities C20 (*D* = 0.83) and C14 (*D* = 0.82) exhibited the highest diversity, while that associated with the cavities C13 (*D* = 0.00) and C17 (*D* = 0.37) exhibited the lowest diversity (Tab. 2).

The PCA analysis separated the samples along the first axis (22.3%) mainly based on the prevalence of *Petriella musispora*, *Paracremonium* sp., *Lophotrichus* sp., and *Phellinus igniarius* in the cavities of *Salix fragilis* excavated by *Picus viridis* woodpecker. The fungal communities associated with the cavity excavated by *Dendrocopos medius*, *Dryocopus martius*, and *Piocoides tridactylus* were the most distant from those associated with the cavity excavated by *Picus viridis*. The second PCA axis revealed a variability of 19.6% and separated the fungal communities associated with the cavity excavated by woodpeckers that generally use *Salix fragilis* from those associated with the cavity excavated by woodpeckers that use other tree species. We observed a strong correlation between *Chaetomium* sp. 2 and samples of *Dendrocopos medius* nest cavity, while *Chaetomium* sp. 3 was strongly correlated with the samples of *Picoides tridactylus* nest cavity (Fig. 3).

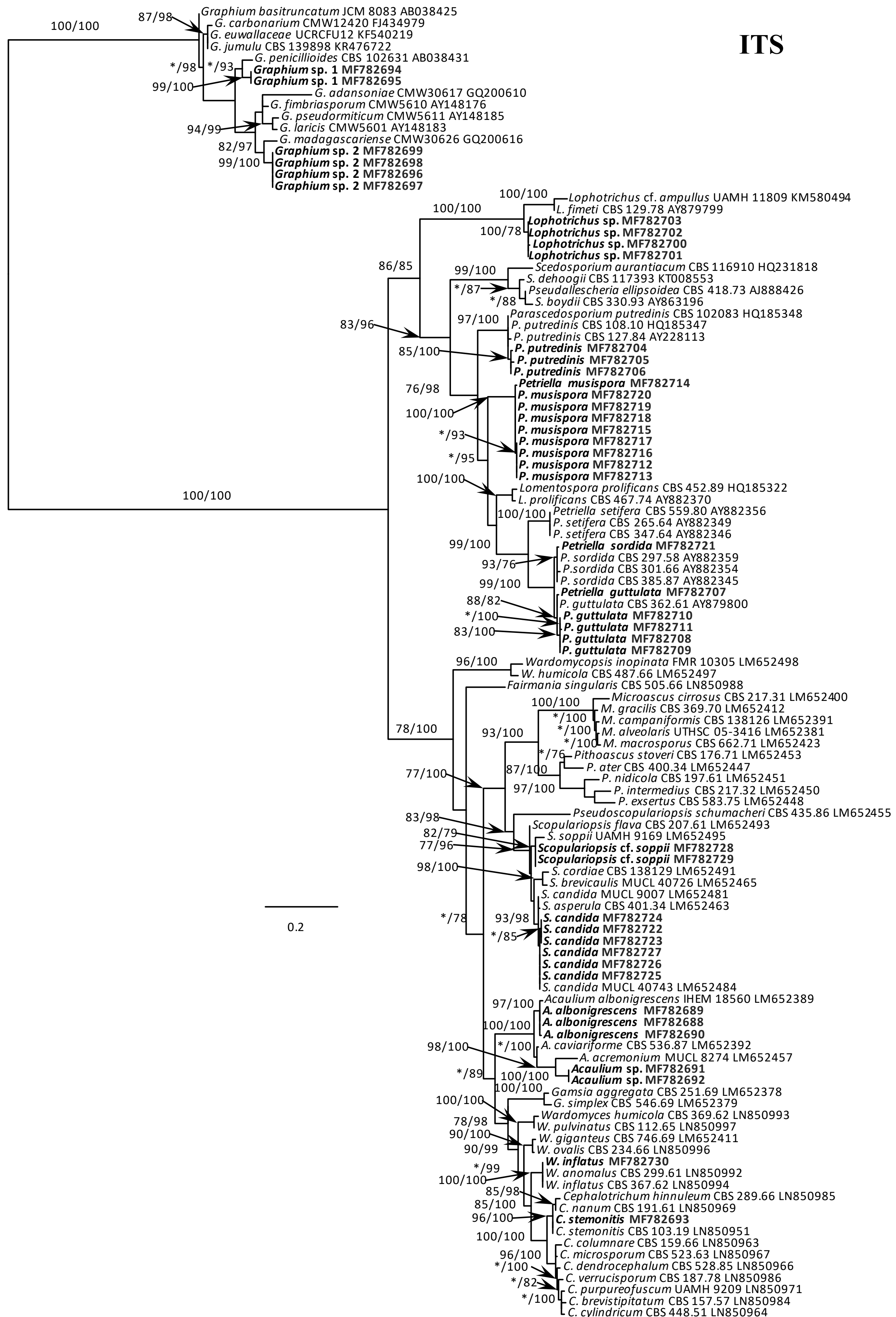


**Fig. 1** Phylogram obtained from maximum likelihood (ML) analysis of the internal transcribed spacer (ITS) region. The phylogram shows the placement of isolates obtained from woodpecker nest cavities representing the order Microascales. Sequences obtained during this study are presented in boldface. Bootstrap values >75% for ML and posterior probabilities >75% obtained from Bayesian (BI) analysis are presented at nodes as follows: ML/BI. * Bootstrap values <75%.

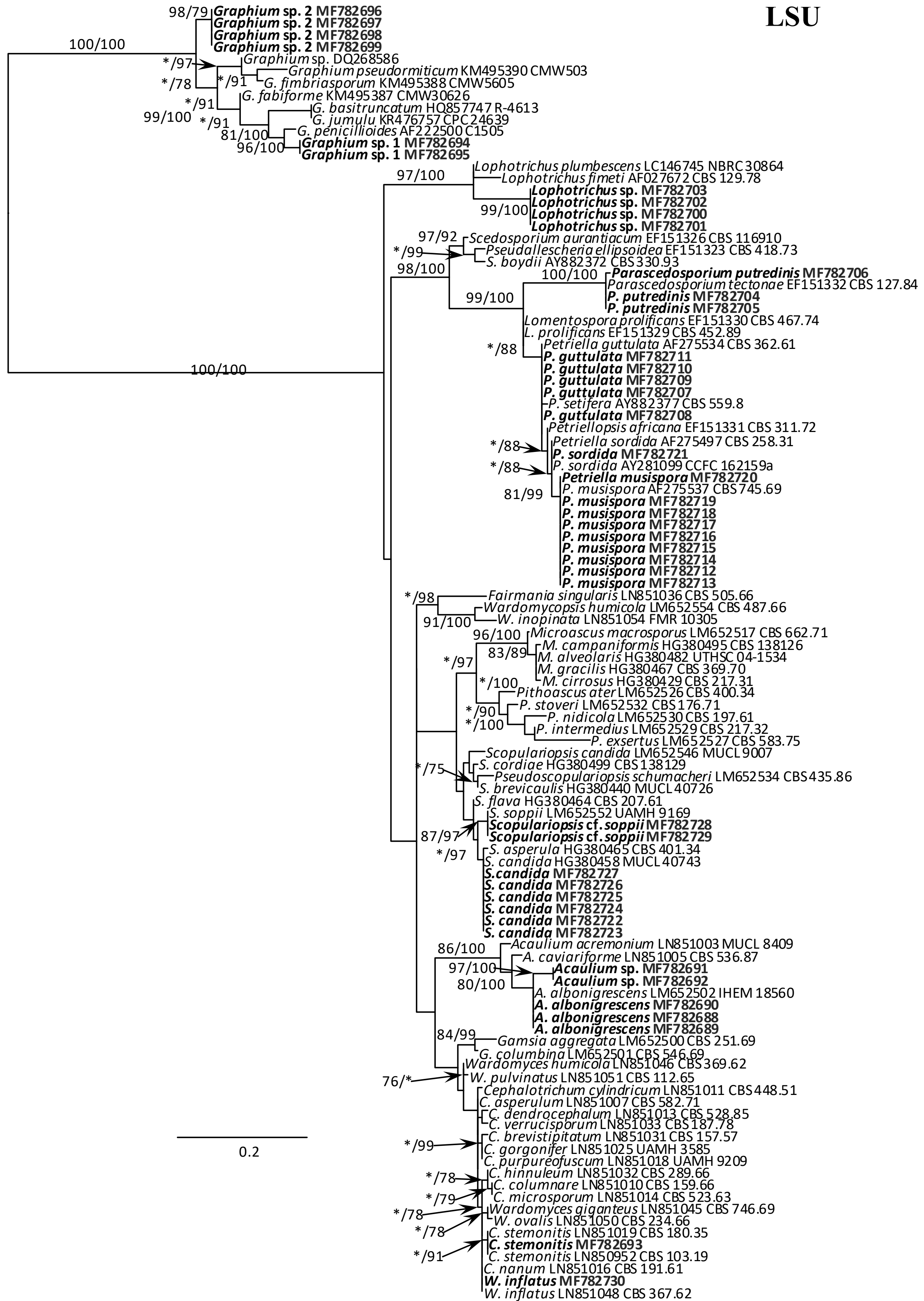


**Fig. 2** Phylogram obtained from maximum likelihood (ML) analysis of the 28S large ribosomal subunit (LSU) region. The phylogram shows the placement of isolates representing the order Microascales. Sequences obtained during this study are presented in boldface. Bootstrap values >75% for ML and posterior probabilities >75% obtained from Bayesian (BI) analysis are presented at nodes as follows: ML/BI. * Bootstrap values <75%.

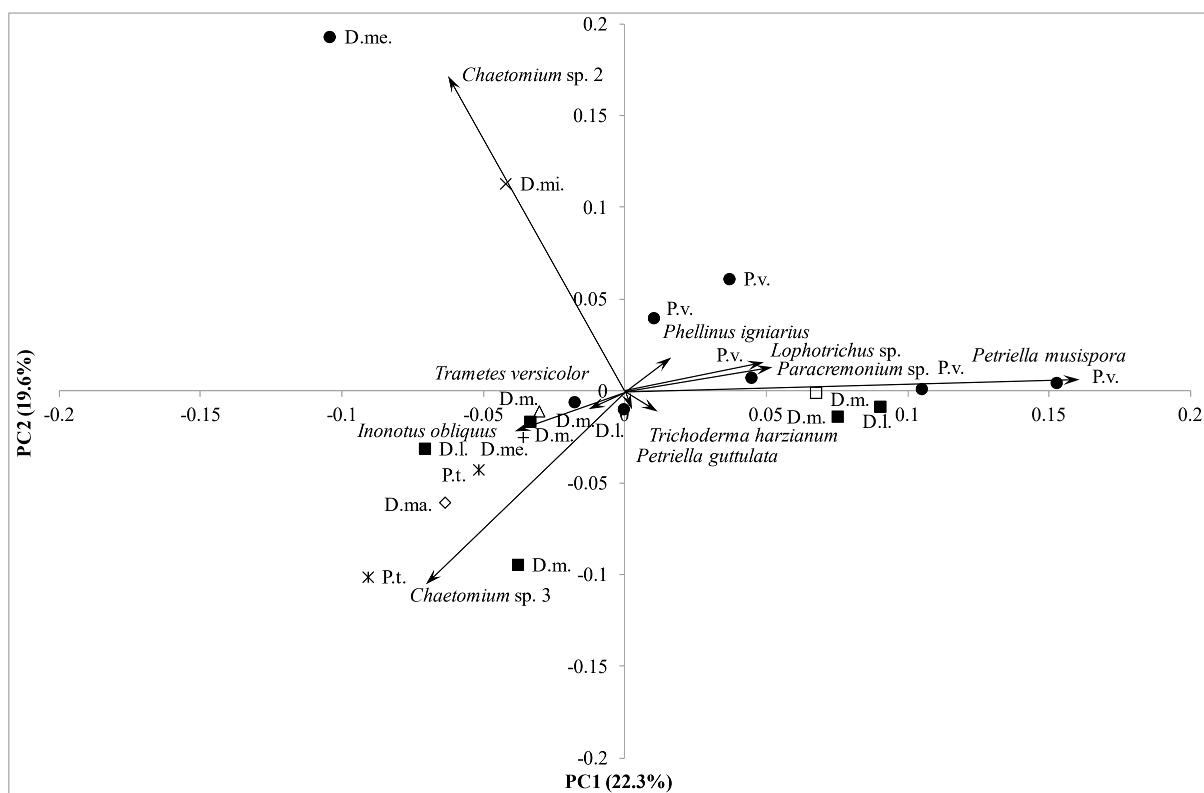


**Fig. 3** Biplot of the principal components analysis (PCA) with log-transformed frequencies of fungal species, woodpecker species (D.l. – *Dendrocopos leucotos*; D.m. – *Dendrocopos major*; D.ma. – *Dryocopus martius*; D.me. – *Dendrocopos medius*; D.mi. – *Dendrocopos minor*; P.t. – *Picoides tridactylus*; P.v. – *Picus viridis*) and cavity trees (diamond – *Abies alba*; triangle – *Acer pseudoplatanus*; square – *Alnus incana*; fill square – *Fagus sylvatica*; plus – *Malus domestica*; star – *Picea abies*; x – *Prunus domestica*; dot – *Salix fragilis*). Principal components were calculated from a covariance matrix for those fungal species for which the total sample size exceeded 20 isolates.

### Spatial distribution of fungal communities within the cavity excavated by white-backed woodpecker (D method)

We obtained 144 fungal isolates from wood samples collected from different sites of the cavity. We isolated 30 fungal species: five species of Basidiomycota, six species of Microascales, and 19 species of other fungi order. Some of the fungi isolated in this study, particularly Basidiomycota and Microascales, were not detected with the ND method. Among the basidiomycetes, we could not detect *Bjerkandera adusta*, *Cylindrobasidium evolvens*, *Ischnoderma benzoinum*, and *Trametes versicolor* in the cavities using the ND method. Additionally, two species of the Microascales order (*Acaulium albonigrescens* and *Acaulium* sp.) were not detected in the same cavity using the ND method. The most dominant species was *Inonotus obliquus* with an average isolation frequency of 20.8%. The second-most dominant species was *Acaulium albonigrescens*, which was isolated from 16.7% of the wood samples. *Scopulariopsis candida* was also commonly isolated (15.8%) (Tab. 3).

We detected considerable variation in the fungal diversity upon comparison of the data from the five different sites of the cavity. The highest species richness was found directly in the entrance and under the entrance of the cavity, followed by the central parts of the cavity. The lowest species richness and diversity were at the cavity bottom (Tab. 3). Most basidiomycete species were found at the cavity entrance (*Bjerkandera adusta*, *Ischnoderma benzoinum*, *Trametes versicolor*), although they were isolated at low frequencies (Tab. 3). The cavity entrance was dominated by various ascomycetes, particularly by *Phoma* sp., *Pseudocosmospora rogersonii*, *Pseudogymnoascus pannorum*, and *Trichoderma* spp. (Tab. 3). The wood underneath the entrance was most commonly colonized by the basidiomycete species *Inonotus obliquus*, which was isolated from 62.3% of the wood samples. Besides *Inonotus obliquus*, *Petriella* spp. and *Phoma* sp. were also frequently detected from the wood of the cavity (Tab. 3). The number of

**Tab. 3** Number of isolates and frequency of fungi (in parentheses*) isolated from the *Fagus sylvatica* cavity excavated by *Dendrocopos leucotos*.

| Fungal species | Cavity site | | | | | Total |
|---|---|---|---|---|---|---|
| | Entrance | Under entrance | Central | Under central | Bottom | |
| **Basidiomycota** | | | | | | |
| *Bjerkandera adusta* | 2(8.3) $^{A}_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 2(1.7) |
| *Cylindrobasidium evolvens* | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 2(8.3) $_{a}$ | 2(1.7) |
| *Inonotus obliquus* | 0 $_{c}$ | 15(62.5) $^{A}_{a}$ | 8(33.3) $^{A}_{ab}$ | 2(8.3) $_{bc}$ | 0 $_{c}$ | 25(20.8) $^{A}$ |
| *Ischnoderma benzoinum* | 1(4.2) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 1(0.8) |
| *Trametes versicolor* | 2(8.3) $^{A}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 2(1.7) |
| **Ascomycota: Microascales** | | | | | | |
| *Acaulium albonigrescens* | 0 $_{b}$ | 0 $_{b}$ | 0 $_{b}$ | 11(45.8) $^{A}_{a}$ | 9(37.5) $^{A}_{a}$ | 20(16.7) $^{A}$ |
| *Acaulium* sp. | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 1(4.2) $_{a}$ | 1(0.8) |
| *Cephalotrichum stemonitis* | 0 $_{a}$ | 1(4.2) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 1(0.8) |
| *Petriella guttulata* | 0 $_{a}$ | 2(8.3) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 2(1.7) |
| *Petriella musispora* | 0 $_{a}$ | 2(8.3) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 2(1.7) |
| *Scopulariopsis candida* | 0 $_{b}$ | 0 $_{b}$ | 0 $_{b}$ | 16(66.7) $^{A}_{a}$ | 3(12.5) $_{b}$ | 19(15.8) $^{A}$ |
| **Ascomycota: other and Mucoromycotina** | | | | | | |
| *Alternaria arborescens* | 1(4.2) $_{a}$ | 1(4.2) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 2(1.7) |
| *Alternaria* sp. | 2(8.3) $^{A}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 2(1.7) |
| *Cadophora malorum* | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 1(4.2) $_{a}$ | 0 $_{a}$ | 1(0.8) |
| *Chaetomium* sp. 1 | 0 $_{a}$ | 0 $_{a}$ | 1(4.2) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 1(0.8) |
| *Chaetomium* sp. 2 | 0 $_{a}$ | 1(4.2) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 1(0.8) |
| *Chaetomium* sp. 3 | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 2(8.3) $_{a}$ | 0 $_{a}$ | 2(1.7) |
| *Epicoccum nigrum* | 0 $_{a}$ | 1(4.2) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 1(0.8) |
| *Humicola fuscoatra* | 0 $_{a}$ | 0 $_{a}$ | 1(4.2) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 1(0.8) |
| *Mortierella* cf. *hyalina* | 1(4.2) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 1(4.2) $_{a}$ | 0 $_{a}$ | 2(1.7) |
| *Mucor hiemalis* | 1(4.2) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 1(0.8) |
| *Neonectria* sp. | 0 $_{a}$ | 1(4.2) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 1(0.8) |
| *Penicillium brevicompactum* | 2(8.3) $^{A}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 2(1.7) |
| *Phialemonium* sp. | 0 $_{a}$ | 1(4.2) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 1(0.8) |
| *Phoma* sp. | 7(29.2) $^{A}_{a}$ | 7(29.2) $^{A}_{a}$ | 0 $_{b}$ | 0 $_{b}$ | 0 $_{b}$ | 14(11.7) $^{A}$ |
| *Pseudocosmospora rogersonii* | 4(16.7) $^{A}_{a}$ | 1(4.2) $_{ab}$ | 1(4.2) $_{ab}$ | 0 $_{b}$ | 0 $_{b}$ | 6(5.0) $^{A}$ |
| *Pseudogymnoascus pannorum* | 8(33.3) $^{A}_{a}$ | 0 $_{b}$ | 7(29.2) $^{A}_{a}$ | 0 $_{b}$ | 0 $_{b}$ | 15(12.5) $^{A}$ |
| *Trichoderma longibrachiatum* | 0 $_{a}$ | 1(4.2) $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 1(0.8) |
| *Trichoderma olivascens* | 7(29.2) $^{A}_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 7(5.8) $^{A}$ |
| *Trichoderma trixiae* | 6(25.0) $^{A}_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 0 $_{a}$ | 6(5.0) $^{A}$ |
| No. of total fungal isolates | 44 | 34 | 18 | 33 | 15 | 144 |
| Species richness (*S*) | 13 | 12 | 5 | 6 | 4 | 30 |
| Camargo's index (1/*S*) | 0.08 | 0.08 | 0.20 | 0.17 | 0.25 | 0.03 |
| Simpson's index (*D*) | 0.12 | 0.25 | 0.36 | 0.36 | 0.42 | 0.10 |
| Simpson's index of diversity [*SID*; *SID* = (1 − *D*)] | 0.88 | 0.75 | 0.64 | 0.64 | 0.58 | 0.90 |
| Shannon index of diversity (*H*) | 2.29 | 1.85 | 1.21 | 1.27 | 1.08 | 2.73 |
| Percentage of sterile fragments MEA/CMEA/BMEA | 38/100/83 | 50/100/38 | 71/100/67 | 25/100/92 | 67/100/92 | 48/100/55 |
| No. of investigated samples MEA/CMEA/BMEA | 24/24/24 | 24/24/24 | 24/24/24 | 24/24/24 | 24/24/24 | 120/120/120 |

* The isolation frequency = (No. of hole fragments, from which a particular fungus was isolated / Total No. of hole fragments) × 100. The isolation frequency was calculated using isolation results obtained individually from selective media: CMEA for *Ophiostoma*/*Leptographium* spp., BMEA for basidiomyceteous fungi, and MEA for other fungi. A – dominant species. Within rows, values with different subscript (small letters) are statistically different ($p < 0.05$) according to post hoc multiproportions test using the Marascuilo procedure.

fungal species in the central part of the cavity was considerably lower than that in the entrance and underneath the entrance of the cavity. The wood of the central parts of the cavity was most frequently colonized by *Inonotus obliquus* and *Pseudogymnoascus pannorum* (Tab. 3). The microascalean fungi was abundant in the wood surrounding the lower part of the cavity along with the two species of basidiomycetes, *Cylindrobasidium evolvens* and *Inonotus obliquus* (Tab. 3). The central and the bottom parts of the cavity were most commonly colonized by *Acaulium albonigrescens*, which was isolated from 45.8% and 37.5% of samples, respectively. *Scopulariopsis candida* was also commonly obtained from the wood located underneath the central part of the cavity (Tab. 3).

The fungal diversity values varied widely along the cavity sites. The fungal community associated with the cavity entrance exhibited the highest diversity ($D = 0.88$), while that associated with the cavity bottom exhibited the lowest diversity ($D = 0.58$). The highest species-richness values were associated with the fungal community at the entrance ($S = 13$ species), while the lowest species-richness values were associated with the fungal community at the bottom of the cavity ($S = 4$ species) (Tab. 3).

## Discussion

The results of this study provide preliminary evidence that the woodpecker nest cavity serves as a fungal diversity hotspot in the temperate forests. In this study, we surveyed seven woodpecker species cavities in eight different host tree species. Our data revealed the presence of complex fungal communities in this niche. We isolated 69 fungal species representing at least 12 orders of Ascomycota, Basidiomycota, and Mucoromycotina. Ascomycota was the predominant phylum, comprising 74% of the isolates and represented by 51 species. Among the 51 species, *Petriella musispora* was the most common species. Another group of fungi that commonly colonize the woodpecker nest cavities were basidiomycetes, which were represented by nine taxa. Both ascomycetes and basidiomycetes are frequently detected in the dead wood and are wood-decaying or saprophytic fungi [30,54–57].

Our data revealed that woodpeckers preferentially excavate cavities in trees having decay caused by basidiomycetes, although these fungi were present in only half of the studied cavities. The correlation between wood-decaying fungi and woodpeckers has been demonstrated in several studies [6–8]. Our investigation revealed that European woodpeckers excavate nest cavities in wood exhibiting clear signs of decay. Recently, Jusino et al. [13] indicated that interactions between fungi and primary woodpecker species are likely to be more complex. The study demonstrated that red-cockaded woodpecker may facilitate the dispersal of Basidiomycota, which helps the excavator by softening the wood. The basidiomycetous species found in our study are common rot fungi inhabiting various hardwood species in Poland [58,59].

The cavities were also commonly colonized by members of the Microascales order belonging to eight genera within the Graphiaceae family (*Graphium*) and the Microascaceae family (*Acaulium*, *Cephalotrichum*, *Lophotrichus*, *Parascedosporium*, *Petriella*, *Scopulariopsis*, and *Wardomyces*). The Microascaceae family includes many ecologically important species, comprising saprobic fungi mostly found in air, soil, plant material, and urban environment. Some species of the Microascaceae are opportunistic pathogens of animals, including humans [60–65]. The members of Microascaceae isolated from the woodpecker nest cavity represented species with different ecological roles and interactions. Among them, the *Graphium* species represent wound-inhabiting saprobes. The vectors of *Graphium* species are insects [66–68] and it is likely that the two unknown species found in this study could have been introduced into the cavities by different insects. Moreover, woodpeckers could transfer the *Graphium* species into the cavity. As these birds forage commonly on bark and wood-boring beetles during the breeding season, they may transfer insects (which carry fungal spores) as a food for the offspring. While feeding some food items could accidentally fall out of the bill, or spores could be transmitted through excrements into the cavity.

In this study, the dominant species detected in the cavities was *Petriella musispora*. This species was strongly associated with the *Salix fragilis* cavities excavated by *Picus viridis*. The presence of *Petriella musispora* and the two other representatives of the

genus *Petriella* (*Petriella guttulata* and *Petriella sordida*) may be transferred through the bird digestive system and feces. The members of this genus tend to grow on dung or in environments enriched by animal feces [69]. Although the parent woodpeckers remove the offspring's feces from cavities [22,23], our study demonstrated that nestlings probably live in environment contaminated by bird excrements, which can be utilized by *Petriella* fungi. It is possible that other Microascales, including *Lophotrichus*, *Microascus*, and *Scopulariopsis* may also utilize bird feces in cavities. However, excrements are a source of potential pathogenic microorganism, which may play an important role in breeding success and survival [70]. Some species of the Microascales can cause diseases in young nestlings [41,63,64,71]. Some species of *Scopulariopsis*, including *Scopulariopsis candida*, are known as opportunistic pathogens, mainly causing superficial tissue infections, and are associated with nondermatophytic onychomycosis [37,64]. Additionally, *Lophotrichus* and *Petriella* species have been reported from superficial tissue [63] and can infect humans and other animals. Our study demonstrated that potentially pathogenic fungi can grow in the cavities and probably survive until the following breeding season. It appears that these fungi can be particularly dangerous in secondary cavity nesting birds. However, many aspects of the woodpeckers–pathogenic fungi association are poorly understood, which require further studies.

Interestingly, we detected the occurrence of two members of the Ophiostomatales in the cavities. Due to their morphological and ecological similarities, fungi from the orders Ophiostomatales and Microascales have been designated as ophiostomatoid fungi [72]. One of the species reported in this study, *Ophiostoma flexuosum*, has been reported in only one previous study in Poland. Jankowiak [73] detected this species from the galleries of *Ips typographus* on Norway spruce (*Picea abies*). The presence of *Ophiostoma flexuosum* in silver fir (*Abies alba*) is reported here for the first time, suggesting that this species may have a wider host distribution. A second ophiostomatoid species was tentatively identified as an unknown *Sporothrix* species, which is closely related to *Sporothrix polyporicola*.

The fungal isolations using a destructive method (D) in this study were limited to a single cavity, and thus, provide only preliminary information. Additionally, we revealed some variations in the spatial composition of fungal communities in the cavity excavated by white-backed woodpecker. In our study, *Alternaria*, *Penicillium*, *Mucor*, *Mortierella*, and *Trichoderma* species, known as typical invaders of wood [74,75], were mainly detected in the wood surrounding the entrance to the cavity. The wood surrounding the entrance to the cavity was also dominated by basidiomycetous species, such as *Bjerkandera adusta*, *Ischnoderma benzoinum*, and *Trametes versicolor*, which did not occur in deeper parts of the cavity. This suggested that these decay fungi are not involved in the wood decay of the cavity. The wood was also often colonized by *Penicillium brevicompactum*, *Pseudocosmospora rogersonii*, *Pseudogymnoascus pannorum*, and *Trichoderma* spp. The occurrence of *Pseudocosmospora rogersonii* was unexpected, as it is reported to be a parasite of only *Eutypella* sp. (Ascomycota: Xylariales) [76]. In this study, we reported the presence of *Pseudocosmospora rogersonii* for the first time outside of the USA. The middle part of the cavity was dominated by *Inonotus obliquus*. This indicates that this fungus is mainly responsible for the wood decay of the cavity. Interestingly, in this part of the cavity, the wood was also often colonized by microascalean species. The dominant species that colonized the deepest parts of the cavity were *Acaulium albonigrescens* and *Scopulariopsis candida*. *Acaulium albonigrescens* is a well-circumscribed species detected in soil, dung, and wood in Scandinavia, northern North America, and Japan [60,61]. We detected this species in Central Europe for the first time. The abundant presence of *Acaulium albonigrescens* and *Scopulariopsis candida* may be associated with specific and a nutrient-rich microhabitat in the bottom of the cavity.

The difference in the composition of fungal communities was highly dependent on the sampling method. We extensively studied only one cavity using the D method (C16). However, the number of fungal species obtained in this method was markedly higher than that obtained by the ND method. We detected 30 fungal species using the D method, while only five species were detected by the ND method. We believe that the ND sampling is extremely imprecise to detect the mycobiota of woodpecker nest cavities. Our study clearly determined the spatial composition of fungal mycobiota in the woodpecker nest cavities. The use of a special tool to excise a single sample from the interior of cavity resulted in the omission of majority part of the cavity. Further

comparative studies are required to determine the efficacy of determining the fungal composition of wood cavities between nondestructive and destructive sampling method.

PCA analysis revealed that there was no correlation between the woodpecker species and the fungal community structure in the cavities, although species such as *Petriella musispora*, *Paracremonium* sp., *Lophotrichus* sp., and *Phellinus igniarius* were strongly associated with the *Salix fragilis* cavities excavated by *Picus viridis*. The fungal communities associated with the cavities excavated by *Dendrocopos medius*, *Dryocopus martius*, and *Piocoides tridactylus* were distant from those associated with the cavities excavated by *Picus viridis*. However, we evaluated only a small number of cavities in each category (woodpecker species / tree species) in this study. Therefore, we could not evaluate various aspects of woodpecker nest cavity–fungi association, which requires further studies.

In conclusion, the results of this study provided insight into the fungal communities associated with woodpecker nest cavities in Poland. Some of the detected species are reported to be wood-decay fungi, but several species remain unidentified. Our results indicated that Microascales and Basidiomycota dominate the wood-inhabiting fungal communities of woodpecker nest cavities. Additionally, our study clearly demonstrated that the fungi exhibit a differential spatial distribution within the woodpecker nest cavity.

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## Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/am/rt/suppFiles/am.1126/0:

**Tab. S1** Information on loci used in the phylogenetic analyses.

**Tab. S2** Fungi isolated from woodpecker holes.

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