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FUNGAL MICROBIOLOGY

Comparison of the Diversity of Basidiomycetes from Dead Wood of the Manchurian fir (*Abies holophylla*) as Evaluated by Fruiting Body Collection, Mycelial Isolation, and 454 Sequencing

Yeongseon Jang[1] · Seokyoon Jang[2] · Mihee Min[2] · Joo-Hyun Hong[2] · Hanbyul Lee[2] · Hwanhwi Lee[2] · Young Woon Lim[1] · Jae-Jin Kim[2]

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Abstract In this study, three different methods (fruiting body collection, mycelial isolation, and 454 sequencing) were implemented to determine the diversity of wood-inhabiting basidiomycetes from dead Manchurian fir (*Abies holophylla*). The three methods recovered similar species richness (26 species from fruiting bodies, 32 species from mycelia, and 32 species from 454 sequencing), but Fisher's alpha, Shannon-Wiener, Simpson's diversity indices of fungal communities indicated fruiting body collection and mycelial isolation displayed higher diversity compared with 454 sequencing. In total, 75 wood-inhabiting basidiomycetes were detected. The most frequently observed species were *Heterobasidion orientale* (fruiting body collection), *Bjerkandera adusta* (mycelial isolation), and *Trichaptum fusco-violaceum* (454 sequencing). Only two species, *Hymenochaete yasudae* and *Hypochnicium karstenii*, were detected by all three methods. This result indicated that Manchurian fir harbors a diverse basidiomycetous fungal community and for complete estimation of fungal diversity, multiple methods should be used. Further studies are required to understand their ecology in the context of forest ecosystems.

Keywords Dead wood · Mycoparasitic fungi · Mycorrhizal fungi · Wood-decay fungi

✉ Jae-Jin Kim
jae-jinkim@korea.ac.kr

[1] School of Biological Sciences, Seoul National University, Seoul 151-747, Republic of Korea

[2] Division of Environmental Science and Ecological Engineering, College of Life Sciences and Biotechnology, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul 136-701, Korea

Introduction

With respect to biodiversity, dead wood is recognized as a primary resource for a wide range of organisms, such as insects, bryophytes, lichens, and fungi [3]. Among these organisms, wood-inhabiting basidiomycetes have significant ecological roles in forest ecosystems [35], with most acting as wood decayers [43], mycorrhizal fungi that have mutualistic relationships with trees [39], plant pathogens [2], or as mycoparasites [8]. The diversity and distribution of these fungi are related to the substrate quality such as tree species, tree sizes, and tree parts [14, 40, 54] and environmental factors such as exposure to sunlight, temperature, humidity, and distance to soil contact [10, 13, 46]. Lindner et al. [43] showed that the number and composition of wood-inhabiting species is not static and changes across years.

The diversity of wood-inhabiting basidiomycetes is generally determined by collecting the fungal fruiting bodies [3, 14, 33, 34, 40, 54]. However, certain species may be missed because of a lack of fruiting bodies at the time of sampling [43] or because the fruiting bodies are inconspicuous [40]. As such, to properly estimate the diversity by collecting fruiting bodies, repeated surveys and a significant investment of time are required [11]. In addition to performing a fruiting body inventory, fungal mycelia can be isolated to determine the diversity of wood-inhabiting basidiomycetes. With advances in molecular identification methods, mycelial isolation is now widely employed [22–25]. However, this process is laborious, and not all species inside wood are culturable. Moreover, discriminating basidiomycetes from other fungi is not always successful [53]. Therefore, DNA-based community fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and restriction fragment length polymorphism (RFLP), have been implemented to determine the diversity of wood-

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inhabiting basidiomycetes [17, 50]. These culture-independent methods use band patterns to differentiate fungal communities; however, each band might not represent a single species, and communities can be underestimated because these methods have a limited resolution [9]. To overcome such limitations, 454 sequencing, a recently developed next-generation sequencing system, has been introduced to this field. This high-throughput sequencing method can provide a better understanding of fungal communities inside wood [26, 42, 43]. Although this method is rapidly replacing traditional methods, 454 sequencing is not free from methodological biases. Moreover, the databases used to identify the sequences include errors, and annotation information is not included for all of the sequences [7, 32, 42]. Several attempts have been made to understand the differences in the diversities measured by the fruiting body collection, mycelial isolation and/or DNA-based methods and it was concluded that wood-inhabiting fungi are best detected through a combination of methods [1, 42, 46].

The Manchurian fir (*Abies holophylla* Maxim.) is an evergreen coniferous tree that grows to 40 m in height and 1 m in diameter at breast height [28]. According to the International Union for Conservation of Nature (IUCN) red list of threatened species, Manchurian fir is distributed in China, Korea, and Russia and has near-threatened status because of logging activities, especially in Russia [20]. This tree is distributed in mountainous regions ranging up to 1200–1500 m above sea level depending on the geographic location. Currently, Manchurian fir is used for timber as well as plywood and veneer. In South Korea, Manchurian fir is widely distributed on Mt. Odae in Odaesan National Park, which contains one of the most famous fir forests in South Korea. Although several wood-inhabiting basidiomycetes have been reported from Manchurian fir [18, 29, 30], no intensive surveys have been conducted. Thus, our knowledge of the diversity of fungi present in these forests and their ecological significance is still limited.

The goal of this study was to determine the diversity of wood-inhabiting basidiomycetes from dead wood of Manchurian fir in Odaesan National Park. To determine the basidiomycete diversity, (1) fruiting body collection, (2) mycelial isolation, and (3) 454 sequencing were performed in combination.

Materials and Methods

Sampling

Sampling was conducted at Mt. Odae in Odaesan National Park, South Korea (37° 42′–47′ N, 128° 33′–35′ E), and the fruiting bodies of wood-inhabiting basidiomycetes were collected from dead wood of Manchurian fir from October 2012 to October 2013. The morphological characteristics of the fruiting bodies were examined, and they were identified to genus or species level.

For mycelial isolation and 454 sequencing, five dead branches and five dead logs of Manchurian fir were randomly selected from July to October 2013 from the same region. Wood cores were taken in the field by inserting increment borers (5 mm in diameter). After removing the first 2~3 cm, the subsequent 2~3 cm were placed into 15-ml sterile conical tubes. In addition, wood blocks (size ~10 cm^3) were cut in the field using a hand axe or hand saw when possible, and the samples were transported to the laboratory in an icebox.

For branches (~2 m long), each was subsampled at approximately 40-cm intervals throughout its length. Depending on the length of the branch, two to five samples were obtained: one close to the trunk, zero to three from the middle of the branch, and one at the tip of the branch. In total, 17 subsamples were collected from branches. For logs (~5 m long), each was subsampled six times at approximately 1-m intervals throughout its length. In total, 30 subsamples were collected from five logs.

Next, subsamples were processed for mycelial isolation and DNA sequencing. First, bark was removed, then subsamples were sterilized by flaming to remove surface contaminants [51]. Three small fragments (~5 mm^3) from each subsample were placed into Petri dishes containing 2 % malt extract agar [20 g malt extract (Difco, Detroit, MI, USA), 15 g agar (Showa, Tokyo, Japan), 1000 ml distilled water] and 4 mg l^{-1} benomyl (Sigma-Aldrich, St. Louis, MO, USA) for mycelial isolation [52]. The remainder of each wood sample was immediately frozen at −80 °C and stored for DNA extraction. Fungal colonies were subcultured until pure cultures were isolated. All of the fruiting body specimens and isolates are preserved in the Korea University Culture Collection (KUC), South Korea.

Molecular Analysis of the Fruiting Bodies and Mycelial Isolates

DNA extraction was performed on the fruiting bodies and mycelial isolates using the Accuprep DNA Extraction Kit (Bioneer, Seoul, Korea). To obtain the sequences of internal transcribed spacer (ITS), PCR reactions were performed according to Jang et al. [16]. The Accuprep PCR Purification Kit (Bioneer, Seoul, Korea) was used to purify the PCR products, which were then sequenced with the forward or reverse primer by Macrogen Ltd. (Seoul, Korea).

DNA Library Preparation from Wood Samples and 454 Sequencing

For each branch or log, three fragments (~5 mm^3) of each subsample were taken and combined (6–15 fragments for each

branch, 18 fragments for each log) for DNA extraction. Wood samples frozen at −80 °C were combined and ground in liquid nitrogen using a mortar and pestle. DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to Rachmayanti et al. [45]. A DNA library was prepared using the FastStart High Fidelity PCR System (Roche, Penzberg, Germany) with 20 ng DNA, 1 μl of each forward and reverse primers (10 μM), 0.5 μl dNTP mix (10 mM each), 2.5 μl FastStart 10× buffer #2, 0.25 μl FastStart Hifi Polymerase (5 U μl^{-1}), and molecular biology-grade water in a 25-μl reaction. The ITS2 region was targeted for DNA library construction using the following primers: ITS3_KYO2 (5′-α-GATGAAGAACGYAGYRAA-3′)/ITS4 (5′-β-TCCTCCGCTTATTGATATGC-3′) [49], where α (5′-CCATCTCATCCCTGCGTGTCTCCGAC-3′) and β (5′-CCTATCCCCTGTGTGCCTTGGCAGTC-3′) are GS FLX Titanium primers followed by a four-digit key sequence (TCAG) and an eight-digit barcode sequence. The PCR conditions were as follows: initial denaturation step at 94 °C for 3 min, followed by 35 cycles at 94 °C for 15 s, 55 °C for 45 s, and 72 °C for 1 min, and a final extension step at 72 °C for 8 min. The size of the PCR product was verified using an Agilent 2100 Bioanalyzer with a DNA 1000 LabChip kit (Agilent Technologies, Waldbronn, Germany). The PCR product was purified with Agencourt AMpure XP (Beckman Coulter, Krefeld, USA). 454 Sequencing was performed using GS FLX Titanium platform (454 Life Sciences, Branford, CT, USA) at Macrogen Ltd. (Seoul, Korea). The sequences were read from the forward primer.

Bioinformatics

The preprocessing of sequences was performed with the program CD-HIT-OTU [31]. The sequences were sorted using the barcode sequence, and then trimmed by removing the primer, key, and barcode sequences from both ends. Errors such as homopolymers, primer dimers, and chimera sequences were also removed. After removing the erroneous sequences, 5527 sequences (>200 bp) remained for analysis. Operational taxonomic unit (OTU) clustering was performed using a greedy incremental clustering algorithm with a ≥97 % similarity threshold.

Phylogenetic Analysis

The ITS sequences generated from the fruiting bodies, mycelial isolates, and representative OTUs sequences from 454 sequencing were deposited in GenBank (Table 1). Sequences were compared with the reference sequences in GenBank using a BLASTn search (http://www.ncbi.nlm.nih.gov/BLAST/). The ITS sequences and reference sequences were aligned using MAFFT 7.130 [19] and manually verified with MacClade 4.08 [36]. A Bayesian analysis was performed with MrBayes 3.2.1 [48] with the model selected by MrModeltest 2.3 [41] according to a previously described method [16]. FigTree 1.4.0 was used to view the tree (http://tree.bio.ed.ac.uk/software/figtree/).

Statistical Analysis

Fisher's alpha, Shannon-Wiener index, Shannon evenness, and Simpson's index of diversity of fungal communities inferred from fruiting bodies, mycelial cultures, and 454 sequencing were calculated using PAST 3.04 [12]. To understand similarities of fungal communities inferred by the three different methods, unweighted pair-group method using arithmetic averages (UPGMA) clustering analysis was performed on Bray-Curtis similarities also using PAST. The reliability of the topology was tested with 1000 bootstrap resampling.

Results

Diversity of Wood-Inhabiting Basidiomycetes

From the fruiting body field surveys, 37 specimens were collected from Manchurian fir. Of these, 26 species were identified using morphological and molecular analyses: 11 polyporoid fungi, 8 corticioid fungi, 6 agarics, and 1 puffball (Table 1). These species were classified into 22 genera, 12 families, and 5 orders. The remaining six specimens could not be identified to the species level using either morphological or molecular analysis (*Ceriporia* sp. KUC20130718-42, *Galerina* sp. KUC20131001-02, Hymenochaetales sp. KUC20131001-10, *Phanerochaete* sp. 3 KUC20130808-05, *Pholiota* sp. KUC20121019-14, and *Steccherinum* sp. KUC20131001-16) (Fig. 1).

From the mycelial isolation method, 88 basidiomycetous fungal isolates were obtained and classified as 32 species, 20 genera, 13 families, and 6 orders. Based on the resolution of ITS, 20 species were fully identified, 8 species identified to genus level, 1 to family (Hydnaceae sp. KUC11065), 2 to order (Dacrymycetales sp. 1 KUC11054 and Dacrymycetales sp. 2 KUC11057), and 1 to phylum (Basidiomycete sp. 1 KUC11081) (Fig. 1, Table 1). Species not resolved to species level showed low sequence similarity to reference sequences in GenBank.

From the 454 sequencing, 5527 sequences were assigned to 81 OTUs, and the rarefaction curve reached a plateau (not shown). Thirty-two OTUs (4874 sequences) were assigned to Basidiomycota, and the remaining OTUs belonged to Ascomycota (48 OTUs of 648 sequences) and Mucoromycotina (1 OTU of 5 sequences) (Fig. 2). The basidiomycetous OTUs were classified into 20 genera, 13 families, and 10 orders. Only 12 OTUs were identified to the species level; 9 OTUs to the genus level; and 11 OTUs were identified

Table 1 The list of wood-inhabiting basidiomycetes from Manchurian fir detected by fruiting body collection, mycelial isolation, and 454 sequencing

Species	GenBank accession no. (ITS)			No. of samples		
	Fruiting body (Fb)	Mycelial culture (Mc)	454 Sequence read (Sr)	Fb	Mc	Sr
Amylostereum chailletii (Pers.) Boidin		KJ713994	KJ714029		6	53
Antrodiella semisupina (Berk. & M.A. Curtis) Ryvarden	KJ668572			1	–	–
Atractiellales sp.			KJ714035	–	–	11
Basidiomycete sp. 1		KJ713986		–	1	–
Basidiomycete sp. 2			KM245577			19
Basidiomycete sp. 3			KJ714039	–	–	9
Basidiomycete sp. 4			KJ714044	–	–	4
Bjerkandera adusta (Willd.) P. Karst.		KJ714016		–	14	–
Cantharellales sp.			KJ714036	–	–	16
Ceriporia pseudocystidiata Y.C. Dai & B.S. Jia	KJ668566			1	–	–
Ceriporia sp.	KJ668564			1	–	–
Coprinellus disseminatus (Pers.) J.E. Lange		KJ714005		–	4	–
Coprinellus micaceus (Bull.) Vilgalys, Hopple & Jacq. Johnson		KJ713992		–	1	–
Coprinellus radians (Desm.) Vilgalys, Hopple & Jacq. Johnson		KJ714004		–	2	–
Coprinellus sp.		KJ713990		–	4	–
Corticium roseum Pers.	KJ668558			1	–	–
Dacrymyces aureosporus Shirouzu & Tokum.		KJ713985		–	4	–
Dacrymyces subalpinus Kobayasi		KJ713998	KJ714025	–	1	50
Dacrymycetales sp. 1		KJ714000	KJ714031	–	3	20
Dacrymycetales sp. 2		KJ713997	KJ714043	–	1	5
Dacrymycetales sp. 3			KJ714030	–	–	34
Dacrymycetales sp. 4			KJ714023	–	–	120
Daedaleopsis confragosa (Bolton) J. Schröt.	KJ668555			1	–	–
Fibricium rude (P. Karst.) Jülich	KJ668552			1	–	–
Fomitopsis pinicola (Sw.) P. Karst.	KJ668547			3	–	–
Galerina marginata (Batsch) Kühner	KJ713984			1	–	–
Galerina sp.	KJ713983			1	–	–
Ganoderma applanatum (Pers.) Pat.	KJ668542			1	–	–
Gloeoporus dichrous (Fr.) Bres.	KJ668541			1	–	–
Gloeostereum incarnatum S. Ito & S. Imai	KJ668540			1	–	–
Gloiothele sp.		KJ713991	KJ714038	–	1	15
Hericium abietis (Weir ex Hubert) K.A. Harrison			KJ714042	–	–	5
Heterobasidion orientale Tokuda, T. Hatt. & Y.C. Dai	KF218833			5	–	–

Table 1 (continued)

Species	GenBank accession no. (ITS)			No. of samples		
	Fruiting body (Fb)	Mycelial culture (Mc)	454 Sequence read (Sr)	Fb	Mc	Sr
Hydnaceae sp.		KJ713993		–	2	–
Hymenochaetales sp.	KJ668529			2	–	–
Hymenochaete yasudae Imazeki	KJ668525	KJ713999	KJ714021	4	1	223
Hyphoderma sp.		KJ714002	KJ714024	–	1	109
Hyphodermella rosae (Bres.) Nakasone		KJ714014		–	1	–
Hypochnicium karstenii (Bres.) Hallenb.	KJ668512	KJ713989	KJ714020	1	7	735
Irpex lacteus (Fr.) Fr.			KJ714032	–	–	104
Irpex sp.			KJ714045	–	–	4
Lycoperdon pyriforme Schaeff.	KJ713982			1	–	–
Microporus vernicipes (Berk.) Kuntze		KJ714006		–	1	–
Mucronella sp.		KJ713995		–	1	–
Mycena pura (Pers.) P. Kumm.	KJ713981			1	–	–
Omphalotus japonicus (Kawam.) Kirchm. & O.K. Mill.		KJ713987		–	1	–
Panellus stipticus (Bull.) P. Karst.	KJ713980			1	–	–
Peniophora sp.		KJ713988	KJ714026	–	1	68
Peniophorella pubera (Fr.) P. Karst.		KJ714003		–	3	–
Phanerochaete carnosa (Burt) Parmasto		KJ714007	KJ714019	–	4	306
Phanerochaete sordida (P. Karst.) J. Erikss. & Ryvarden		KJ714012		–	5	–
Phanerochaete sp. 1		KJ714013		–	1	–
Phanerochaete sp. 2		KJ713996		–	1	–
Phanerochaete sp. 3	KJ668487			1	–	–
Phlebia acanthocystis Gilb. & Nakasone		KJ714010		–	1	–
Phlebia acerina Peck		KJ714015		–	3	–
Pholiota sp.	KJ713979		KJ714034	1	–	19
Pluteus plautus (Weinm.) Gillet			KJ714022	–	–	178
Polyporus alveolaris (DC.) Bondartsev & Singer	KJ668478			1	–	–
Postia sp.			KJ714033	–	–	25
Radulomyces sp.			KJ714027	–	–	58
Schizophyllum commune Fr.		KJ714011		–	1	–
Sebacinales sp.			KJ714047	–	–	4
Sistotrema brinkmannii (Bres.) J. Erikss.		KJ714008	KJ714041	–	6	6
Sistotrema oblongisporum M.P. Christ. & Hauerslev			KJ714028	–	–	31
Sistotremastrum guttuliferum Melo, M. Dueñas, Telleria & M.P. Martín			KJ714037	–	–	6

Table 1 (continued)

Species	GenBank accession no. (ITS)			No. of samples		
	Fruiting body (Fb)	Mycelial culture (Mc)	454 Sequence read (Sr)	Fb	Mc	Sr
Sistotremastrum sp. 1			KJ714018	–	–	906
Sistotremastrum sp. 2			KJ714040	–	–	6
Steccherinum sp.	KJ668452			1	–	–
Tremellales sp.			KJ714046	–	–	5
Trichaptum abietinum (Dicks.) Ryvarden	KJ668437			1	–	–
Trichaptum fusco-violaceum (Ehrenb.) Ryvarden	KJ668436		KJ714017	2	–	1720
Tricholomopsis flammula Métrod ex Holec	KJ713978			1	–	–
Tulasnella sp.		KJ714001		–	4	–
Tyromyces chioneus (Fr.) P. Karst.		KJ714009		–	1	–

Fig. 1 Bayesian phylogenetic tree inferred from the ITS2 region sequences of fruiting bodies (*black square*), mycelial isolates (*black circle*), and 454 sequencing reads (*black diamond*). The dataset is composed of 165 taxa and 629 characters. The GTR+I+G model was applied. For the consensus tree, 10,202 trees from a Bayesian analysis were used. The tree was rooted to the sequence of *Tremella aurantialba* ACCC 50219 (AY866425). *Numbers above or below branches* indicate posterior probabilities ≥0.5. GenBank accession numbers of the ITS sequences are shown in parentheses

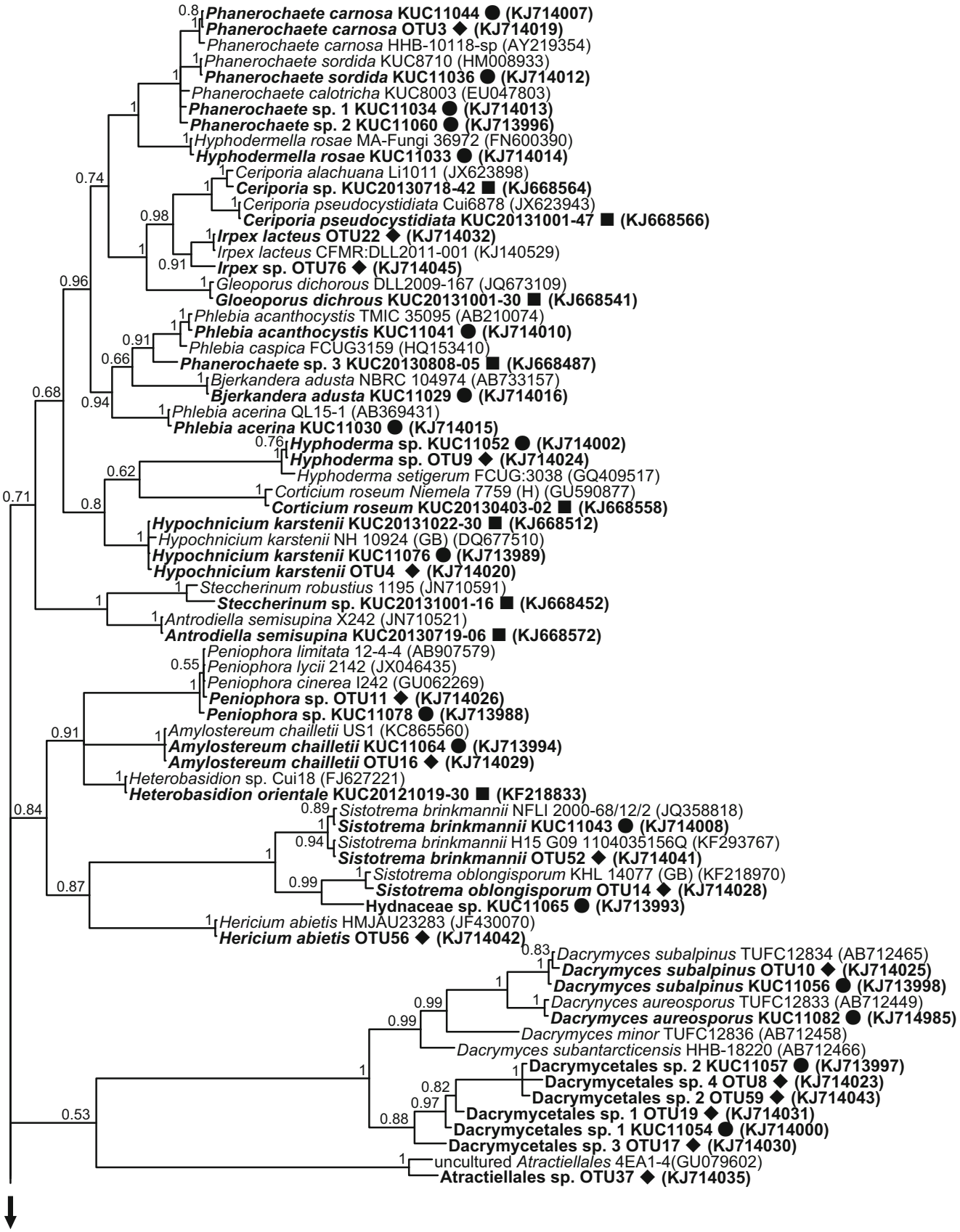
up to the order level due to the low sequence similarities to GenBank sequences (Table 1).

In total, 75 basidiomycetes species were detected from Manchurian fir using the three different detection methods (Fig. 3, Table 1). Each method yielded a similar number of species: 26 species from fruiting bodies, 32 species from mycelial isolates, and 32 species from 454 sequencing. Largely due to a difference in sample number (37 fruiting bodies, 88 mycelial isolates, and 4874 from 454 sequencing), Fisher's alpha, Shannon-Wiener, and Simpson's diversity index of fungal communities showed that fruiting body collection and mycelial isolation displayed higher diversity compared with 454 sequencing (Table 2). Shannon evenness was also higher in fruiting body collection (0.84) and mycelial isolation (0.70) than in 454 sequencing (0.27). The UPGMA clustering analysis (not presented) using Bray-Curtis similarities showed that the similarity among the communities was low (i.e., 0.003 between fruiting body collection and 454 sequencing; 0.013 between mycelial isolation and 454 sequencing) and the communities of fruiting bodies and mycelial isolates were slightly more similar (0.032).

Discussion

In this study, wood-inhabiting basidiomycetes were identified using three different methods (fruiting body collection, mycelial isolation, and 454 sequencing) to determine their diversity in Manchurian fir in Odaesan National Park. Previous studies with fruiting body collection only identified four basidiomycetous fungi from the Manchurian fir in this region: *Asterostroma cervicolor*, *Ceriporiopsis subvermispora*, *Climacocystis borealis*, and *Polyporus brumalis* [18, 29, 30]. Although those four species were not found in this study, this study revealed 75 basidiomycetous fungi which is more than two times the number of fungi detected by any single method (Table 1). Of these, 18 species were only identified to the genus level and 14 species up to the family level because of low ITS sequence similarities to the known taxa. Thus, they might represent novel taxa. At the generic level, most of the genera found in this study could also be found from other conifers such as *Picea abies* [1, 26, 44, 46].

Species detected as fruiting bodies were rarely detected by the other two methods (Fig. 3, Table 1). Similar results were


0.8 Phanerochaete carnosa KUC11044 ● (KJ714007)
1 Phanerochaete carnosa OTU3 ◆ (KJ714019)
Phanerochaete carnosa HHB-10118-sp (AY219354)
1 Phanerochaete sordida KUC8710 (HM008933)
1 Phanerochaete sordida KUC11036 ● (KJ714012)
Phanerochaete calotricha KUC8003 (EU047803)
1 Phanerochaete sp. 1 KUC11034 ● (KJ714013)
Phanerochaete sp. 2 KUC11060 ● (KJ713996)
1 Hyphodermella rosae MA-Fungi 36972 (FN600390)
Hyphodermella rosae KUC11033 ● (KJ714014)
0.74 1 Ceriporia alachuana Li1011 (JX623898)
1 Ceriporia sp. KUC20130718-42 ■ (KJ668564)
1 Ceriporia pseudocystidiata Cui6878 (JX623943)
0.98 Ceriporia pseudocystidiata KUC20131001-47 ■ (KJ668566)
1 Irpex lacteus OTU22 ◆ (KJ714032)
1 Irpex lacteus CFMR:DLL2011-001 (KJ140529)
0.91 Irpex sp. OTU76 ◆ (KJ714045)
0.96 1 Gleoporus dichorous DLL2009-167 (JQ673109)
Gloeoporus dichrous KUC20131001-30 ■ (KJ668541)
1 Phlebia acanthocystis TMIC 35095 (AB210074)
1 Phlebia acanthocystis KUC11041 ● (KJ714010)
0.91 Phlebia caspica FCUG3159 (HQ153410)
0.66 Phanerochaete sp. 3 KUC20130808-05 ■ (KJ668487)
1 Bjerkandera adusta NBRC 104974 (AB733157)
0.68 Bjerkandera adusta KUC11029 ● (KJ714016)
0.94 1 Phlebia acerina QL15-1 (AB369431)
Phlebia acerina KUC11030 ● (KJ714015)
0.76 Hyphoderma sp. KUC11052 ● (KJ714002)
1 Hyphoderma sp. OTU9 ◆ (KJ714024)
0.62 Hyphoderma setigerum FCUG:3038 (GQ409517)
1 Corticium roseum Niemela 7759 (H) (GU590877)
0.71 0.8 Corticium roseum KUC20130403-02 ■ (KJ668558)
Hypochnicium karstenii KUC20131022-30 ■ (KJ668512)
1 Hypochnicium karstenii NH 10924 (GB) (DQ677510)
Hypochnicium karstenii KUC11076 ● (KJ713989)
Hypochnicium karstenii OTU4 ◆ (KJ714020)
1 Steccherinum robustius 1195 (JN710591)
1 Steccherinum sp. KUC20131001-16 ■ (KJ668452)
1 Antrodiella semisupina X242 (JN710521)
Antrodiella semisupina KUC20130719-06 ■ (KJ668572)
Peniophora limitata 12-4-4 (AB907579)
0.55 Peniophora lycii 2142 (JX046435)
1 Peniophora cinerea I242 (GU062269)
Peniophora sp. OTU11 ◆ (KJ714026)
Peniophora sp. KUC11078 ● (KJ713988)
0.91 1 Amylostereum chailletii US1 (KC865560)
Amylostereum chailletii KUC11064 ● (KJ713994)
Amylostereum chailletii OTU16 ◆ (KJ714029)
1 Heterobasidion sp. Cui18 (FJ627221)
Heterobasidion orientale KUC20121019-30 ■ (KF218833)
0.84 0.89 Sistotrema brinkmannii NFLI 2000-68/12/2 (JQ358818)
1 Sistotrema brinkmannii KUC11043 ● (KJ714008)
0.94 Sistotrema brinkmannii H15 G09 1104035156Q (KF293767)
1 Sistotrema brinkmannii OTU52 ◆ (KJ714041)
1 Sistotrema oblongisporum KHL 14077 (GB) (KF218970)
0.87 0.99 Sistotrema oblongisporum OTU14 ◆ (KJ714028)
Hydnaceae sp. KUC11065 ● (KJ713993)
1 Hericium abietis HMJAU23283 (JF430070)
Hericium abietis OTU56 ◆ (KJ714042)
0.83 Dacrymyces subalpinus TUFC12834 (AB712465)
1 Dacrymyces subalpinus OTU10 ◆ (KJ714025)
1 Dacrymyces subalpinus KUC11056 ● (KJ713998)
0.99 1 Dacrynyces aureosporus TUFC12833 (AB712449)
0.99 Dacrymyces aureosporus KUC11082 ● (KJ714985)
Dacrymyces minor TUFC12836 (AB712458)
Dacrymyces subantarcticensis HHB-18220 (AB712466)
1 1 Dacrymycetales sp. 2 KUC11057 ● (KJ713997)
Dacrymycetales sp. 4 OTU8 ◆ (KJ714023)
0.82 Dacrymycetales sp. 2 OTU59 ◆ (KJ714043)
0.97 Dacrymycetales sp. 1 OTU19 ◆ (KJ714031)
0.53 0.88 Dacrymycetales sp. 1 KUC11054 ● (KJ714000)
Dacrymycetales sp. 3 OTU17 ◆ (KJ714030)
1 uncultured Atractiellales 4EA1-4(GU079602)
Atractiellales sp. OTU37 ◆ (KJ714035)

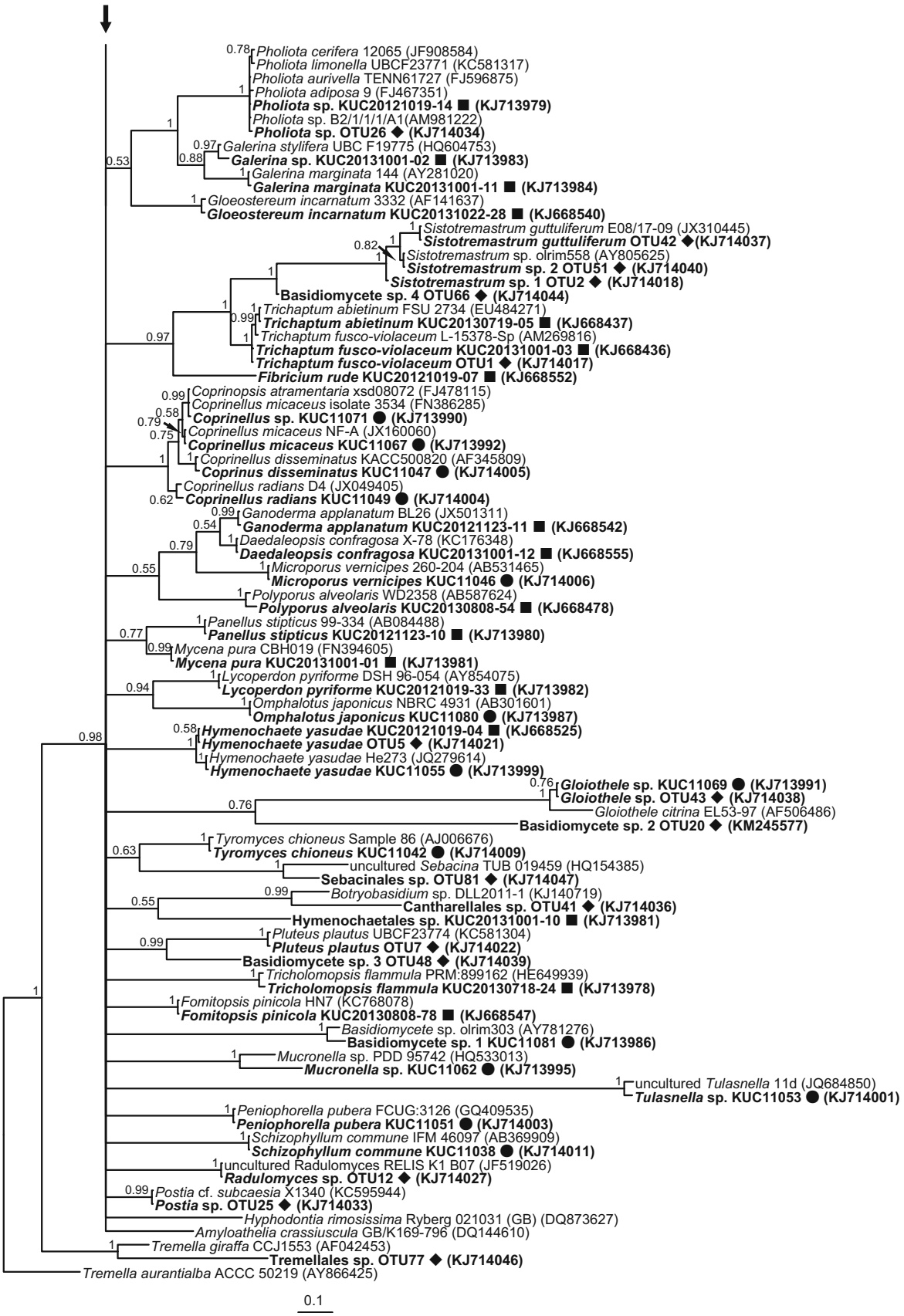


Fig. 1 (continued)

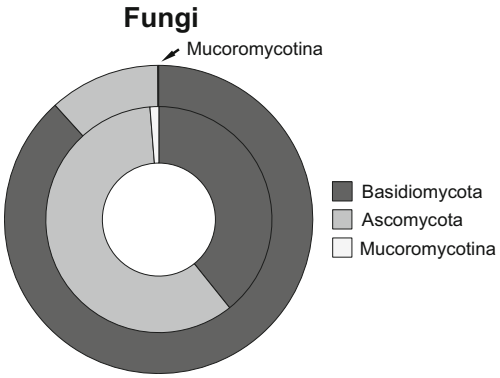


Fig. 2 Fungal diversity of Manchurian fir revealed by 454 sequencing. The ring chart illustrates the proportion of OTUs (*inner ring*) and proportion of sequences (*outer ring*) in each taxonomic group of fungi

obtained by Allmér et al. [1] in a comparison of species diversity from wood piles of Norway spruce determined by fruiting body collection, mycelial isolation, and ITS T-RFLP. In Rajala et al. [46], one third of the fungal species from Norway spruce logs identified by fruiting body collection overlapped with the species identified by a DGGE approach. The 454 sequencing method was also compared to fruiting body collection performed on spruce logs, and only five species were detected by 454 sequencing among the 15 species observed as fruiting bodies [42]. As noted by Allmér et al. [1] and Rajala et al. [46], the number of detected species is related to the sampling size. In this study, the sampling size for fruiting body collection was larger compared with the ones for mycelial isolation and 454 sequencing. Thus, a greater volume of Manchurian fir was covered by fruiting body collection, thus increasing the likelihood of detecting rarer species.

Conversely, 49 species found in this study were not detected as fruiting bodies. Fruiting body collection is only possible when the fruiting body is formed. Fruiting bodies often develop after drastic changes in environmental conditions, and this environmental signal is different from species to species [27]. In addition, ephemeral fruiting bodies of gilled mushrooms and jelly fungi only remain in the environment for a short period because of a high water content, and this behaviour is dissimilar to that of the typical fruiting bodies of polyporoid and corticioid fungi. Consequently, species with more ephemeral fruiting bodies might be missed during fruiting body collection. In this study, *Coprinellus* spp., *Dacrymyces* spp., *Tremellales* sp., and *Tyromyces chioneus*, which typically have fruiting bodies with a high water content, were detected as mycelial isolates and/or 454 sequencing reads but not by fruiting body collection. Moreover, species having small and thin fruiting bodies might be missed during surveys. Here, resupinate fungi such as *Hyphoderma* sp., *Phanerochaete* spp., *Phlebia* spp., *Sistotrema* spp., and *Sistotremastrum* spp., which typically have small and thin fruiting bodies, were also found by mycelial isolation and/or 454 sequencing but not by fruiting body collection.

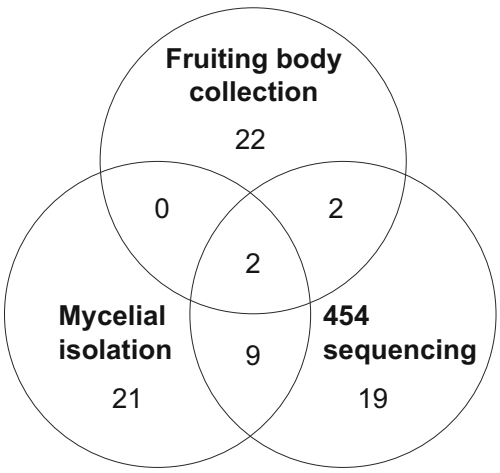


Fig. 3 The number of wood-inhabiting basidiomycetes collected by fruiting body collection, mycelial isolation, and 454 sequencing. The *numbers in the overlapping fractions* indicate the number of species identified by overlapping two or three methods

The composition of the fungal community changes during the wood decay process [13, 21]. In the early stage of decay in spruce logs, Rajala et al. [46] indicated that the wood-decay fungi that cause brown rot and white rot were dominant, whereas mycorrhizal fungi only accounted for a small proportion. In the later stages of decay, the proportion of mycorrhizal fungi increased, whereas the proportion of wood-decay fungi decreased. As stated by Rajala et al. [46], it is probably due to high competitiveness of mycorrhizal fungi against wood-decay fungi when readily accessible nutrients are scarce. The wood used in this study was relatively intact; thus, it was assumed that they were in the early stage of decay. This conclusion is indirectly supported by the mycorrhizal genera, such as *Sistotrema*, only accounting for a small proportion of the detected fungi (Table 1). The fungal community composition also varies according to whether it is in sapwood or heartwood [5, 26]. In this study, most of the detected species were sap-rot fungi. Heart-rot fungi, such as *Fomitopsis pinicola*, were only found by fruiting body collection, which might have been caused by a lack of heart rot fungi in the wood samples used for mycelial isolation and 454 sequencing or a lack of heartwood samples from the selected branches and logs of Manchurian fir.

Using 454 sequencing, 81 OTUs (5527 sequences) were detected from Manchurian fir. In Ovaskainen et al. [42], 175 OTUs (62,214 sequences) were detected in four spruce logs. Kubartová et al. [26] found 1224 OTUs in 140,000 sequences from 13 spruce logs from one site and 743 OTUs in 90,000 sequences from 12 spruce logs from another site. Although direct comparisons cannot be made with other studies because each study used different sampling strategies, the number of detected OTUs tends to increase with a greater number of analysed sequences.

Table 2 Diversity indices of wood-inhabiting basidiomycetes associated with Manchurian fir

Diversity index	Fruiting body	Mycelial culture	454 Sequence read
Number of taxa	26	32	32
Number of samples	37	88	4874
Fisher's alpha	38.89	18.09	4.59
Simpson's index of diversity	0.942	0.939	0.809
Shannon-Wiener	3.08	3.11	2.15
Shannon evenness	0.84	0.70	0.27

Comparing between phyla, both the number of species and number of sequences were significantly different (Fig. 2). Greater than half (48 OTUs, 59.3 %) were ascomycetous OTUs; however, their relative proportion among the sequences was only 11.7 %. Conversely, 32 OTUs (39.5 %) belonged to Basidiomycota; however, their relative proportion among the sequences was high (88.2 %). A similar tendency was observed in certain spruce logs studied by Kubartová et al. [26]. In their study, ascomycetes were more likely to be detected in a greater number of logs compared with basidiomycetes and have a low number of sequences, suggesting that only a small volume was occupied by ascomycetes in each log. Conversely, basidiomycetes were more likely to be detected in fewer logs compared with ascomycetes and have a high number of sequences, suggesting they occupied a large volume in each log. In this study, the top 6 basidiomycetous species constituted 73.6 % of the total sequences, suggesting that their mycelia occupied a large volume in Manchurian fir.

Of the 53 species detected by mycelial isolation and 454 sequencing, 11 species were detected by both methods; 21 species were only found by mycelial isolation and 21 species were only found by 454 sequencing (Fig. 3, Table 1). This incongruence may have been caused by several possible reasons. First, it is likely that because some subsamples are large (~10 cm^3), fragments used for mycelial isolation and 454 sequencing may be sampling a different community. Second, for 454 sequencing, the primers used to amplify the DNA may have caused biases [4, 15, 37, 49]. ITS sequences of certain taxa might have been overexpressed or underexpressed, and the DNA of certain species might not have been PCR-amplified despite occurring in the wood. Third, for mycelial isolation, certain species might not have grown on the artificial media used. We used benomyl to isolate basidiomycetous fungi and prevent the growth of other fungi. Benomyl is effective against a broad spectrum of fungi; however, certain species of basidiomycetes may not be able to tolerate it [6, 38]. Thus, certain species may not have been represented as mycelia despite occurring in the wood. On the other hand, fungi that could not be isolated with the mycelial method (dead mycelia) might have been recoverable by the DNA-based method. Rao et al. [47] found that RNA-based methods could detect species that were active in the environment, and these methods provided different results than were obtained with DNA-based methods.

We have two major conclusions from this study. First, we find that the diversity of wood-inhabiting basidiomycetes in Manchurian fir is higher than previously known. Second, 454 sequencing, the newest and most technologically advanced method we used, does not guarantee the correct estimation of the fungal diversity; each method detected a different subset of diversity, so a combination of multiple methods provides better understanding of the total biodiversity. Further studies are required to properly estimate the diversity of wood-inhabiting basidiomycetes from different stages of wood decay and to better understand their roles in forest ecosystems.

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