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Fungal Communities in Decaying Sapwood and Heartwood of a Conifer *Keteleeria evelyniana*

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Abstract Fungal communities in decaying sapwood and heartwood of *K. evelyniana* were demonstrated through construction of four 18 S rRNA gene libraries. The 210 sequenced clones were clustered into 11 subgroups, belonging to Basidiomycota (71.9%) and to Ascomycota (22.4%) and unclassified (1 subgroup; 5.7%). The heartwood displayed higher species richness than the sapwood. Basidiomycota were dominant in either the heartwood or the sapwood. Phylogenetically diverse Homobasidiomycetes were detected in the heartwood, contrary to the sapwood, where Heterobasidiomycetes were detected. Clones close to *Spongipellis unicolor* dominated in the heartwood (21 of 99 clones), while those close to *Hydnochaete olivacea* dominated in the sapwood (41 of 111 clones). The common species between the two parts were those related to *S. unicolor*, *Calocera cornea*, *Debaryomyces hansenii*, *Davidiella tassiana*, and *Nomuraea rileyi* and those from Chaetothyriomycetes.

Introduction

Wood residues are a crucial source of nutrients in forest ecosystems and their decomposition is mainly carried out by fungal communities [4]. The decomposition process varies greatly from site to site, depending on the local climate, temperature, moisture, and residue quality [11, 17]. In a given tree species, the decomposition rate between the heartwood and the sapwood is also different [21]. The sapwood is generally decomposed more rapidly than the heartwood because the latter contains more substances resistant to fungal attack, such as lignin and phenols [3, 9, 26]. Therefore, we hypothesize that fungal communities associated with heartwood are significantly different from those associated with sapwood in the same tree. However, till now there has been no report on studies in this area.

Traditionally, studies on the diversity of wood-inhabiting fungi rely mainly on the culture methods. Many wood-inhabiting fungi are unculturable in an artificial medium, therefore species recorded by these methods represent the actual communities poorly [19]. Direct identifications of fungal species based on the observation of fungal sporocarps can also fail to detect the species presented as mycelia [16, 17]. The molecular method, which generally involves using PCR to amplify target regions (rDNA or ITS) of DNA extracted directly from environmental samples and cloning and sequencing the resulting PCR products, has been successfully applied to investigate fungal communities in a variety of environments [1, 2]. In this study, we used the molecular method to evaluate the differences in fungal communities between the heartwood and the sapwood of an economically important conifer, *K. evelyniana* [24].

Materials and Methods

Collection of Samples and Microbial DNA Extraction

K. evelyniana is mainly distributed in Laos, Vietnam, and southern areas of China. In China, it is dominant in

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Yunnan, Sichuan, and the central mountains of Hainan provinces [7]. It is also an economically important plant species in these regions [24]. In January 2005, about 1 kg of decayed wood was cut from dead *K. evelyniana* on a mountain in Yunnan province, China (25°05′N, 102°46′E). These woods have less than 50% of their bark. The sapwood is defined as white-colored wood about 2–3 mm under the bark, and the heartwood is defined as wood located near the center of the tree. Samples collected with sterilized knives from three trees were pooled as the sapwood and heartwood samples, respectively. Total microbial DNA was extracted from 1 g of wood by methods described previously [15].

Construction of the rRNA Gene Clone Library and Phylogenetic Analysis

Fungal 18 S rRNA genes were amplified with a nested PCR. The first-round PCR was carried out with two pairs of universal primers, GeoA1-ART4 [22] and NS1-NS6 [25], respectively. The second round was carried out with primers SSU-0817 to SSU-1196 [5]. Purified PCR products were ligated into the pGM18-T vector (TAKARA, Dalian, China) and transformed into competent cells (*Escherichia coli* HB101). Positive clones were screened and the inserted DNA fragment with the correct size was commercially sequenced.

The sequences were edited, and primer sequences were deleted using Editseq and Seqman of the DNAstar package. These sequences were then tested for chimeras using the CHIMERA_CHECK program (RDP Database, http://rdp8.cme.msu.edu/html/analyses.html), and possible chimeras were excluded from further analysis. The closest relatives were searched and downloaded from NCBI. Phylogenetic trees were constructed using the PHYLIP package [8], with the F84 evolutionary model and neighbor-joining method. Bootstrap analysis was carried out with 1000 replicates. OTU (operational taxonomic unit) and species rarefaction curves were calculated using the DOTUR program with the farthest neighbor assignment algorithm [20]. Sampling coverage was calculated according to the previous formula [10].

The sequences reported in this paper were deposited in GenBank under accession numbers EF120720 to EF120781. Only the representative sequence of each OTU was deposited.

Results and Discussion

To our knowledge, the present study is the first to investigate the fungal community difference between the heartwood and the sapwood of the same tree. We analyzed 210 partial 18 S rRNA gene fragments (typically 400 bp in length). At 3% sequence difference, 61 OTUs were identified and half of them overlapped between the sapwood and the heartwood (Table 1). Sampling coverage for each library was >70%, with an integrated value of nearly 90%; therefore the majority of the species was sampled.

These fungal OTUs were clustered into 11 subgroups, phylogenetically belonging to Basidiomycota (71.9%) and Ascomycota (22.4%) and unclassified (one subgroup; 5.7%) (Fig. 1). Basidiomycota included Homobasidiomycetes (105 clones) and Heterobasidiomycetes (46 clones). Ascomycota included members from eight classes and most of the clones were related to Saccharomycetes, Pezizomycetes, and Chaetothyriomycetes. The phylogenetic breadth of fungal clones in *K. evelyniana* revealed by the molecular method in this study was significantly larger than of those previously observed in other woods by the traditional method. For example, sampling of fruit bodies on 75-m hazel wood lying dead in eight stands revealed a total of 140 species, all belonging to Ascomycetes (Pyrenomycetes) and Basidiomycetes (Aphyllophorales) [18]. In an investigation of fungal assemblages of spruce, pine,

Table 1 Distribution and sampling coverage of OTUs in rRNA gene clone libraries

Parameter	Heartwood (GeoA1-ART4)	Heartwood (NS1-NS6)	Sapwood (GeoA1-ART4)	Sapwood (NS1-NS6)	Integrated value
Total no. OUTs	24 (47)[a]	26 (52)	20 (55)	19 (56)	61 (210)
No. unique OTUs[b]	9 (14)	10 (16)	8 (11)	9 (12)	36 (53)
% unique OTUs	37.5	38.5	40.0	47.4	59.0
No. singletons[c]	13	15	13	10	25
% sampling coverage[d]	72.3	71.1	76.4	82.1	88.1

[a] Values in parentheses are the numbers of clones

[b] Patterns that are found in only one clone library

[c] OTUs that are represented by only a single clone in a clone library

[d] Good coverage [10]. Coverage calculated as $Cx = 1 - (Nx/n)$, where Nx is the number of unique sequences and n is the total number of sequences

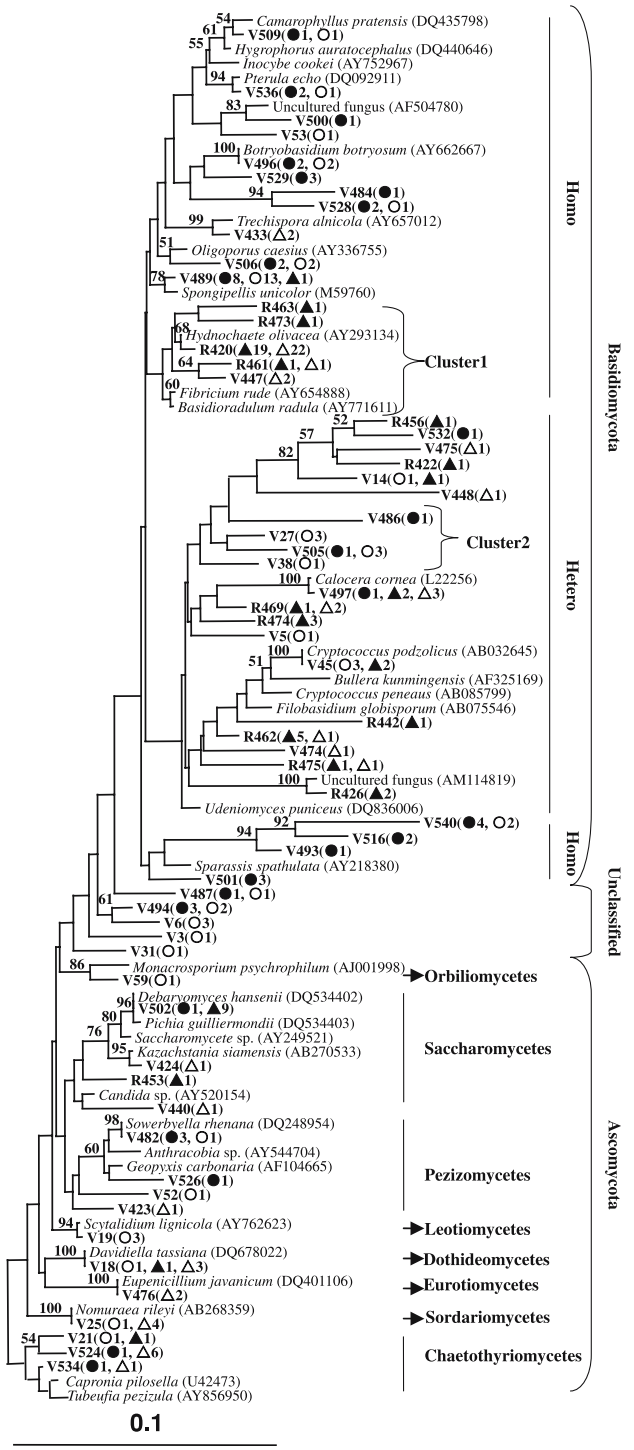

Fig. 1 Phylogenetic relationship of representative 18 S rRNA gene sequences from *K. evelyniana* wood affiliated with fungal relatives from GenBank. The occurrence of each OTU in the heartwood (circles) and the sapwood (triangles), with the number of times it occurs is indicated in parentheses. Filled and open symbols represent OTUs obtained by primer pair GeoA1-ART4 and NS1-NS6, respectively. Homo, Homobasidiomycetes; Hetero, Heterobasidiomycetes. Bootstrap values (1000 replications) ≥50% are indicated at branch nodes. GenBank accession numbers of reference sequences are shown in parentheses. The scale bar represents 10% estimated sequence divergence

and oak in northern Britain, 251 fungal species identified based on sporocarps were Basidiomycetes, including genera *Cortinarius*, *Galerina*, *Inocybe*, *Lactarius*, *Mycena*, and *Russula*, and 21 species were Ascomycetes [14].

The common clones between the heartwood and the sapwood of *K. evelyniana* were those related to *Spongipellis unicolor*, *Calocera cornea*, *Debaryomyces hansenii*, *Davidiella tassiana*, and *Nomuraea rileyi* and those from Chaetothyriomycetes (Fig. 1). In particular, OTU V489 (96% similar to *S. unicolor*) dominated in the heartwood (21 of 99 clones) and OTU V420 (99% similar to *Hydnochaete olivacea*) dominated in the sapwood (41 of 111 clones). These two OTUs could be equally detected by two primer pairs. However, they are not the dominant fungal species in other tree species. For example, on beech logs, *Eutypa spinosa, Fomes fomentarius*, and *Kretzschmaria deusta* are dominant [12]. In conifer plantations in northern Britain, spruce are characterized by large numbers of *Cortinarius* and *Inocybe*, pine tends to have greater numbers of *Russula* and *Suillus*, and oak has its own distinctive *Russula* [14]. Therefore, the fungal community in decaying *K. evelyniana* was different from those previously reported in other tree species. However, a host preference of these species for *K. evelyniana* cannot be concluded unless more woods collected from different geographical lands are investigated.

No difference in the observed OTU numbers of Ascomycota was detected between the heartwood and the sapwood (11 vs. 11), however, the species composition of Basidiomycota was obviously different between the two parts. The heartwood clones obtained from both primer pairs, GeoA1-ART4 and NS1-NS6, included phylogenetically diverse Homobasidiomycetes (14 OTUs, 55 clones). However, with the exception of OTU V433, which was close to *Trechispora alnicola* (98%), all of the sapwood clones (5 OTUs, 47 clones) formed a branch close to species *H. olivacea* (Fig. 1, Cluster 1). In contrast, in Heterobasidiomycetes, the sapwood clones were diverse, but most of the heartwood clones formed a branch that grouped with no known sequences (Fig. 1, Cluster 2). Moreover, the heartwood had higher species richness than the sapwood. When 46 sequences were sampled, approximately 20 OTUs could be observed in the heartwood, more than in the sapwood (Fig. 2). A previous report demonstrates that Homobasidiomycetes are more important in lignin degradation than Heterobasidiomycetes [13]. Microbes generally develop greater diversity in degradation-resistant than in degradation-susceptible materials [6]. Therefore, these data basically reflect the chemical differences between the sapwood and the heartwood; that is, the heartwood contains more substances resistant to fungal attack, such as lignin and phenols, than the sapwood does [3, 9, 23]. In addition, all unclassified clones were from the heartwood.

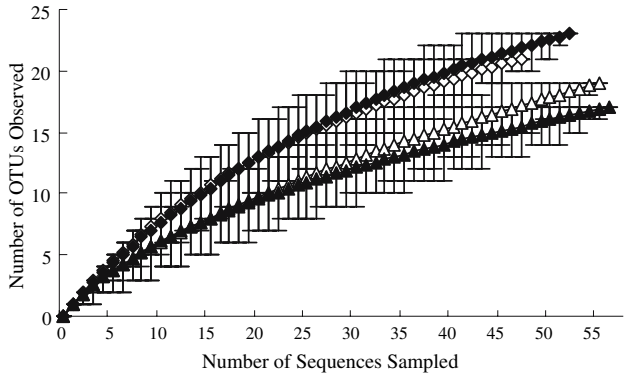

Fig. 2 Rarefaction curves from DOTUR analysis using the farthest neighbor assignment algorithm with 18 S rRNA gene libraries from heartwood (diamond) and sapwood (triangle). Open symbols represent primer pair GeoA1-ART4, and filled symbols represent primer pair NS1-NS6. Error bar represents 95% confidence interval

Although the three primer pairs used in this study are fungus-specific [5, 22, 25], weak and unstable PCR signals were obtained from DNA extracted from wood using any of the primer pairs individually (data not shown). Consequently it was very difficult to construct the clone library. In contrast, a strategy using either primer pair ART4-GeoA1 or pair NS1-NS6 in a first-round PCR, then using primer pair SSU-0817-SSU-1196 in a second-round PCR, generally yielded consistent and reproducible results. One practical problem for nested PCR was carryover contamination, and therefore negative controls were used in each PCR experiment.

The number of species detected by GeoA1-ART4 and by NS1-NS6 showed no significant difference because the 95% confidence interval of the number of observed OTUs from the two libraries always overlapped (Fig. 2), and half of the OTUs (23/61), particularly the dominant clones such as OTU V489 and R420, could also be detected by them. However, the two primer pairs could also be complementary in detecting the species represented by the minority of sequences. A number of unique OTUs, either in the heartwood or in the sapwood, detected by primer pair ART4-GeoA1 could not be detected by primer pair NS1-NS6, and vice versa (Fig. 1). The phylogenetic breadth of species detected by primer pair NS1-NS6 was also slightly greater than of those detected by GeoA1-ART4. For Ascomycota, primer pair NS1-NS6 amplified all classes, but primer pair GeoA1-ART4 amplified only Saccharomycetes, Pezizomycetes, Dothideomycetes, and Chaetothyriomycetes (Fig. 1). Therefore, the application of multiple primer pairs could reduce the primer biases toward the amplification of some taxonomic microbes [1].

The primer specificity for fungi varies greatly depending on the origin of the substrate being analyzed and the diversity of eukaryotic DNA contained within the extracted DNA pool [2]. A previous report indicated that primer pair SSU-0817-SSU-1196 is specific for fungi [5]; however, the majority of clones (70%) obtained using this primer pair is related to species of soil invertebrates [1]. In the present study, only 2.4% of clones were nonfungus sequences, close to invertebrate Alveolata and Metazoa (these sequences were excluded from analysis). Such a high fungus specificity agrees with previously reported results [5]. Although the specificity observed in this study may also result from more fungal templates being accumulated after the first round of amplification with NS1-NS6 and GeoA1-ART4, differences in the composition and richness of fungal species between the heartwood and the sapwood were observed using the current molecular methods (Fig. 1). These methods provide a fast way for further establishing the link between the succession of fungal communities in different decay stages and chemical changes in wood.

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