Full Length Article

Endophytic Fungal Community of *Pinus densiflora* Infected by Different Incidences of *Sphaeropsis sapinea* in a Mixed Coniferous Forest

Xian Xie[1], Jun Liang[1,2*], Ming Zhang[1], Ruirui Hu[1], Yuan Cheng[1] and Xingyao Zhang[1,2]
[1]*Institute of Forest Ecological Environment and Protection, Chinese Academy of Forestry, Key Laboratory of Forest Protection of National Forestry and Grassland Administration, Beijing 100091 China*
[2]*Kunyushan Forest Ecosystem Research Station, Yantai 264100, China*
*Correspondence: liangjun@caf.ac.cn

Abstract

Shoot blight of pine caused by *Sphaeropsis sapinea* is a serious threat to the health of *Pinus densiflora*. However, little is known about the effect of *S. sapinea* on the structure of endophytic fungal communities in needles of *P. densiflora*. In this study, the diversity and structure of the endophytic fungal communities associated with asymptomatic and symptomatic needles of *P. densiflora* were investigated. Needles of *P. densiflora* were sampled from mixed coniferous forests, and high through-put sequencing was used to determine the diversity and community structure of endophytic fungi. The results demonstrated that endophytic fungal abundance increased when the lesion length was longer, and the endophytic fungal diversity showed an upward trend as the infection of *S. sapinea* worsened. The dominant genera of endophytic fungi in asymptomatic needles of *P. densiflora* in mixed coniferous forest was *Paraconiothyrium* and *Selenophoma*. *Cenangium* is considered to have a certain association with *S. sapinea* infection in the endophytic fungal community. The results showed that *S. sapinea* infection affected the endophytic fungal community in needles of *P. densiflora* in a mixed coniferous forest. © 2020 Friends Science Publishers

Keywords: Community structure; Endophytic fungi; *Pinus densiflora*; *Sphaeropsis sapinea*

Introduction

Sphaeropsis sapinea Dyko & Sutton (syn. *Diplodia sapinea* (Fr.) Fuckel, *Diplodia pinea* (Desm.) Kickx.) causes shoot blight and canker diseases throughout the world on conifers (Blodgett *et al.* 2005; Ye and He 2011). Disease occurrence and its pathogenicity are economically important, affecting many coniferous species, in particular *Pinus* spp. (Stanosz and Cummings 1996; Vornam *et al.* 2019). According to CABI database, *S. sapinea* is one of the most common fungal phytopathogen in more than 65 countries in the world. *S. sapinea* now is known to be widely distributed in the natural ranges of pines in the Northern Hemisphere and where these trees have been introduced in the Southern Hemisphere (Smith and Stanosz 2006). In China, it was widely distributed in coniferous forest in thirteen provinces, which had caused great economic and ecological losses (Ye and He 2011). The pathogen can invade the host from wound and stomata of needles (Ye and He 2011). Through the experiment of isolation and culture of fungi in tissue of *Pinus* spp., Liu and Ye (2003) found that the colonization of pathogen on the asymptomatic shoots in winter may cause the incidence of shoot blight on spring shoots in the coming year. Factors reducing the vigor of latently infected trees, for example, when the host tree experiences environmental stress such as drought, have been shown to trigger the latent infection becoming pathogenic, thereby causing severe tip blight symptoms (Stanosz *et al.* 2001).

Endophytic fungi live in various tissues and organs of plants at a certain stage or all stages of their life history, establishing harmonious associations with plants. Some endophytes secrete antifungal and antibacterial metabolites at low concentrations, thus inhibiting competitors (both endophytic and pathogenic bacteria and fungi) and maintaining a balance of antagonism with the competitors (Schulz *et al.* 2015; Suryanarayanan *et al.* 2016). Multiple symbiosis between endophytic fungi and host plants might result in reduced pathogen growth, as their growth will be limited by secary metabolites of the endophytic fungi (Schulz *et al.* 2015). According to Martinez *et al.* (2016), 36 genera of endophytic fungi with antagonistic activity or metabolites have been isolated, but endophytic fungi that are difficult to cultivate or are unculturable still account for a high proportion. Most studies concerning endophyte communities have been based on pure cultures isolated on artificial media, and it is difficult to determine the extent to which their results are representative of natural infections in terms of species abundance and occurrence.

In this study, whether *S. sapinea* can affect the intact endophytic fungal community in needles of *P. densiflora* was focused. The endophytic fungal diversity and community structure in asymptomatic and symptomatic needles of *P. densiflora* infected by *S. sapinea* were analyzed and determined by high-throughput sequencing based on ITS (internal transcribed spacer) rRNA (ribosomal Ribonucleic Acid) gene. The community structure and diversity of endophytic fungi were analyzed, which provide a theoretical basis for the regulation of disease microbial community structure. The followed assumptions were hypothesized: (i)- endophytic fungal diversity in needles of *P. densiflora* differs with different levels of disease; and (ii)- the community structure of endophytic fungi is affected by *S. sapinea* infection.

Materials and Methods

Study area and sampling site

The Kunyushan Mountains are in the Jiaodong Peninsula in Shandong Province in eastern China (121°41'34"–121°48'04" E; 37°11'50"–37°17'22" N). The Kunyushan Mountains are the original habitat and natural distribution center of *P. densiflora* in China. In this region, *P. densiflora* and other coniferous species form a zonal natural secary forest vegetation. Three standardized plots of 30 m × 30 m plots were selected. The geographical coordinates of the plots were 37°16'3.06" N, 121°45'32.60" E; 37° 15'57.94'N, 121°45'33.16" E; 37°15'52.17" N and 121°45'37.55" E. The site conditions were all mid-slope, the slope is 23±3°, and the elevation was 360 m ± 30 m. Stand structure was a mixed coniferous forest, composed of *P. densiflora* and *P. thunbergii*.

Sample collection

Field sampling of this study was carried out in three plots in September 2018, from which four types of needle were collected: needles of asymptomatic *P. densiflora* (CZ1) and asymptomatic needles of infected *P. densiflora* (CZ2), needles with a lighter level of disease (where the length of the lesion was less than half of the length of the needles; CZ3), and needles with heavier level of disease (where the length of the lesion was more than half of the length of the needles; CZ4). The samples were collected by a five-point sampling method (four vertices and the center point of the plot), after which they were rinsed and dried. They were then immersed in 75% ethanol for 1 min and rinsed with sterile water, soaked in 0.5% sodium hypochlorite solution for 2 min, rinsed with sterile water, and stored at -80°C.

Molecular detection of needle-associated fungal community

Sample DNA (deoxyriboNucleic acid) was extracted by the CTAB (cetyltrimethylammonium Ammonium Bromide) method, and genomic DNA was extracted and detected by 1% agarose gel electrophoresis (Bullington and Larkin 2015). The DNA sample was thawed on ice, centrifuged and thoroughly mixed. Then, a Nanodrop ND-2000 (Thermo) was used to detect the sample quality, and 30 ng was taken for PCR (polymerase chain reaction) amplification. The PCR amplification system was as follows: DNA sample, 1 μL; forward primer (5 μM), 1 μL; reverse primer (5 μM), 1 μL; BSA (Albumin from bovine serum) (2 ng μL^{-1}), 3 μL; 2×Taq Plus Master Mix, 12.5 μL; ddH$_2$O, 6.5 μL. The amplification primer sequences of the ITS1 region were (5'-CTTGGTCATTTAGAGGAAGTAA-3') and (5' - TGCGTTCTTCATCGATGC-3').

PCR was carried out using TransGen AP221-02 with TransStart Fastpfu DNA polymerase. The PCR products of the same sample were mixed, detected by 2% agarose gel electrophoresis, and cut with an AxyPrep DNA Gel Recovery Kit (AXYGEN). The PCR product was recovered by gel, eluted with Tris_HCl, and detected by 2% agarose electrophoresis. Amplification products were constructed using the Illumina Miseq PE300 platform to construct the Miseq library and to perform sequencing.

Statistical analyses of the fungal community

OTU (Operational Taxonomic Unit) is artificially set to a certain classification unit (strain, genus, species, group) in order to facilitate analysis in phylogenetic or population genetics research. All sequences were clustered according to 97% similarity with uclust (Version 1.2.22), and the OTU of the singleton was removed, then the representative sequence and OTU table were obtained (Youssef *et al.* 2009). OTU clustering was performed according to 97% similarity sequence with usearch (excluding single sequence), and the representative sequence was obtained. The entire sequence was mapped out, according to 97% similarity, to form the OTU list (Hess *et al.* 2011). Abundance and diversity index calculations of OTU were performed using QIIME (v1.7.0) software to obtain species richness and uniformity information within the sample. The PCA maps were drawn using R (v2.15.3) software. All read sequences were deposited in the Sequence Read Archive (SRA) of the National Coalition Building Institute (NCBI). The number of data in SRA is SUB5584140.

Results

Fungal community diversity and rarefaction curve

Following the sequencing of needle samples for the entire fungal community, a total of 470375 high-quality sequences were obtained, which were clustered into 1045 OTUs in 12 samples. The OTU rarefaction curve of each sample tended to be smooth (see Fig. 1). The results of the coverage sequencing depth index of the sample are shown in Table 1 (endophytic fungi). The results show that the coverage for

the collected needle samples was greater than 99.0%, indicating that the fungal species information in the samples was fully tested and that the test could represent the level of endophytic fungi in the needles.

Analysis of community structure of endophytic fungi in needles of *P. densiflora*

Based on the number of OTUs, a Venn diagram (Fig. 2) and community structure histograms (Fig. 3 and 4) were constructed to analyze the composition in four samples. According to the Venn diagram, there were 429 identical OTUs in all samples, accounting for 41.05% of the total number of OTUs.

The richness and Shannon diversity of endophytic fungi were shown in Table 1. According to Chao1 index, CZ4 has the highest OTU number, followed by CZ3, CZ1, and CZ2. As the disease worsened, the endophytic fungal richness showed an upward trend. The endophytic fungal diversity index of CZ1 and CZ2 was similar, higher than CZ3, and the diversity index of CZ4 was the highest. The results show that endophytic fungal abundance and diversity increased with a longer lesion length.

The 1045 OTU were classified into 29 phyla, 65classes, and 160 genera. At the level of endophytic fungi in four types of conifers (CZ1, CZ2, CZ3 and CZ4), Ascomycota accounted for the highest proportion, reaching 93.86, 89.95, 95.99 and 96.51%, respectively, followed by Basidiomycota, which accounted for 3.69, 4.70, 1.86 and 1.85%, respectively. The studied endophytic fungal communities were largely dominated by Ascomycota.

At the class level (Fig. 3), endophytic fungi in the four types of conifers (CZ1, CZ2, CZ3, and CZ4) were dominated by Dothideomycetes (39.63, 50.01, 35.97 and 28.90%, respectively), where the sec relative abundance was that of Eurotiomycetes (22.60, 11.83, 29.01 and 20.11%, respectively). Sordariomycetes, Arthoniomycetes and Leotiomycetes accounted for a relatively high abundance in the four samples, and the relative abundance of Sordariomycetes (4.80, 4.67, 3.75 and 3.69%, respectively) and Arthoniomycetes (5.73, 5.84, 8.87 and 3.68%, respectively) were similar in different samples, while the relative abundance of Leotiomycetes (5.42, 3.01, 3.98 and 30.57%, respectively) in CZ4 was significantly higher than the other three samples. In addition to the above fungi, the other classes were different for each sample. In CZ1 and CZ2, the proportion of Tremellomycetes was 2.73 and 2.92%, respectively, while its relative abundance was less than 0.1% in CZ3 and CZ4. The relative abundances of Orbiliomycetes and Taphrinomycetes were only higher than 1% in CZ2 and were lower than 1% in other samples.

At the genus level (Fig. 4), the dominant genera were different from each other for every single sample. In CZ1, there were 12 genera with abundance higher than 1%, the dominant genera were *Paraconiothyrium* (9.03%), *Selenophoma* (7.39%), and *Trichomerium* (7.37%). In CZ2,

Table 1: Abundance and diversity of endophytic fungi in needles of *P. densiflora*

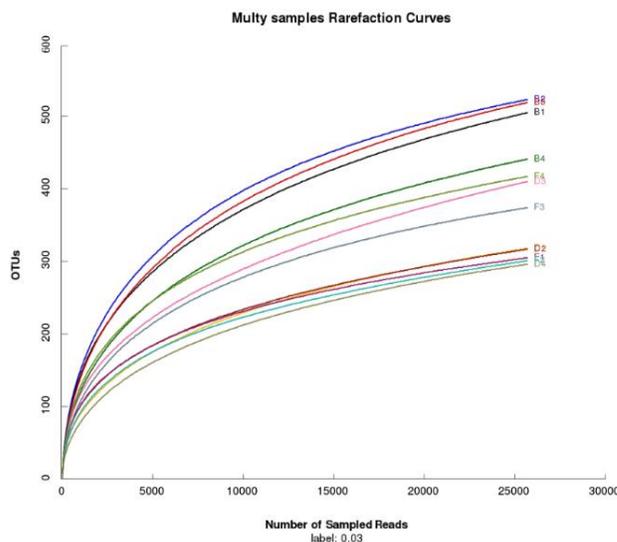| Treatment	Chao1	Shannon	Coverage
CZ1	491.656	5.059	99.5%
CZ2	491.068	5.059	99.5%
CZ3	507.608	4.881	99.4%
CZ4	549.363	5.231	99.5%

Fig. 1: OTU-based rarefaction curve of endophytic fungal communities in needles of *P. densiflora*
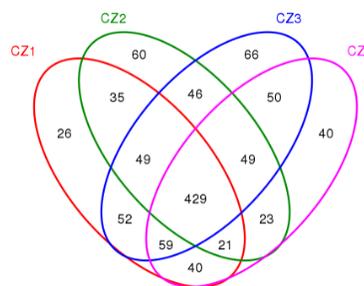

Fig. 2: Venn diagram of OTU distribution of endophytic fungi in needles of *P. densiflora*

there were 15 genera with abundance higher than 1%; the dominant genera were *Sclerostagonospora* (13.08%), *Paraphaeosphaeria* (7.26%), *Phaeococcomyces* (5.84%), and *Selenophoma* (5.25%). In CZ3, there were 9 genera with abundance higher than 1%; the dominant genera were *Phaeococcomyces* (8.87%), *Trichomerium* (5.82%), *Lapidomyces* (5.07%), and *Selenophoma* (2.67%). In CZ4, there were 10 genera with abundance higher than 1%; the dominant genera were *Cenangium* (19.00%), *Lophodermium* (10.32%), *Trichomerium* (5.85%), and *Selenophoma* (4.87%). *Selenophoma*, *Trichomerium* and *Phaeococcomyces* occupied a certain proportion in all samples. *Cenangium* and *Lophodermium* were only detected in CZ4.
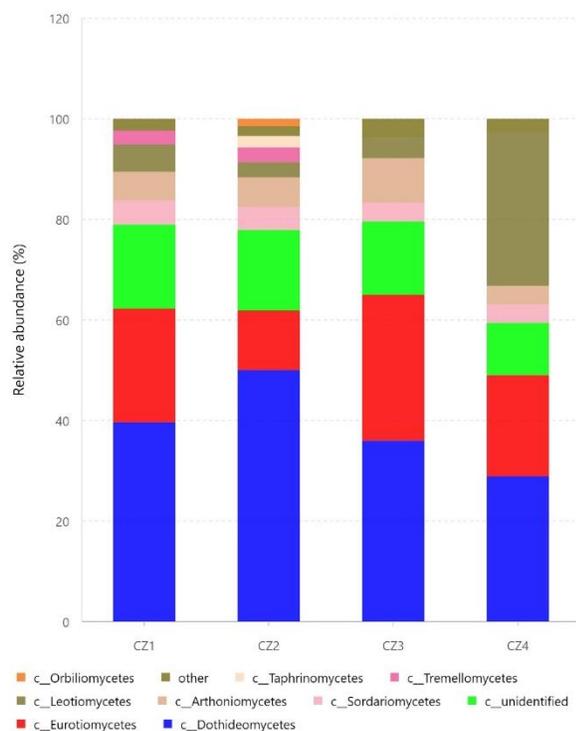
Fig. 3: Dominant fungal classes from endophytic fungi in needles of *P. densiflora* (the color of the column represents the different classes, and the length of the column represents the proportion size of the class. Sequences that could not be identified were designated as "unidentified". Genera making up less than 1% of total composition in each sample were classified as "other")

Beta diversity of endophytic fungi in needles of *P. densiflora*

Based on the principal component analysis, the differences in endophytic fungi from *P. densiflora* under different infection conditions were evaluated (Fig. 5). The results show that the contribution rates of principal component 1 (PC1) and principal component 2 (PC2) were 26.26 and 19.84%, respectively. Across the three plots, the distance between the asymptomatic needles in the same plot was relatively close, and the samples which were seriously infected by pathogens (CZ4) were concentrated, indicating that the endophytic fungi in the needles of *P. densiflora* differed between the plots, but the infection of *S. sapinea* tended to make the community structure of endophytic fungi consistent.

Discussion

High-throughput sequencing was used to analyze the diversity and community structure of endophytic fungi of *P. densiflora* in mixed coniferous forests. The results show that the diversity of endophytic fungi in *P. densiflora* needles was rich. In the present study, the dominant fungi were

Ascomycota and Basidiomycota. Ascomycota and Basidiomycota are very common and have been reported as the dominant endophytic fungi of various plant species (Deng *et al*. 2019; Yang *et al*. 2019).

Shoot blight of pine is one of the most common and widely distributed diseases in conifers. Recent research on shoot blight of pine has included the pathogens, transmission, infestation, prevention, and treatment (Lu 2017; An *et al*. 2018), as well as the diversity, pathogenicity, and biological characteristics of the pathogens (Liu *et al*. 2018; Chen *et al*. 2019). Control of the disease by antagonistic bacteria has also progressed (Liu *et al*. 2012; Wang and Ye 2016), and it has been confirmed that microbial regulation of host plants can be achieved to prevent shoot blight of pine. Endophytic fungal communities in asymptomatic tissues are more stable than that in symptomatic tissues, which inhibits development of the pathogen. Zhang *et al*. (2011) compared the differences of endophytic fungal communities between asymptomatic and susceptible leaves in different seasons through a culture-dependent method and analyzed the relationship between gray spot disease and endophytic fungi diversity in *Camellia sinensis* and the results showed that the number, diversity, and evenness of endophytic fungi in asymptomatic leaves were higher than those in infected leaves, and the level of disease had significant effects on the diversity of endophytic fungi. Studies have also shown that Ascomycota were found to be the most common fungal endophytes among all plant samples of the two varieties (*Rosa multiflora* Thunb and *R. multiflora* var. carnea Redouté and Thory), and the *Podosphaera pannosa* (Wallr.) de Bary infection can influence the fungal endophytic community, and some of the endophytes may play a role in resistance (Zhao *et al*. 2018). The endophytic fungi of the lacquer-infected branch were cultured, and the diversity of fungi in different parts was compared, and the results showed that the endophytic fungal diversity of asymptomatic branches was significantly higher than that of infected branches (Takemoto *et al*. 2014). In the current study, the diversity of endophytic fungi showed first a trend of decreasing and then increasing, and the highest diversity was observed in the heavily infected samples. And there were differences in the diversity and community structure of asymptomatic and infected needles. Endophytic fungi diversity in asymptomatic needles was higher than in less infected needles, which is consistent with previous research (Liu *et al*. 2016). When the pathogen invades the tissue, the host plant's defense mechanism and its internal endophyte balance are destroyed, therefore, other pathogenic fungi and saprophytes are more likely to then enter the plant, which increases the internal fungal diversity. In the study of endophytic fungi in cotton roots infected with *Verticillium* wilt, the endophytic fungi diversity of the roots after infection with *Verticillium* wilt was higher than that of healthy plants, indicating that the pathogens infection increased the fungal diversity and affected its community structure (Liu *et al*. 2016). This may be due to invasion of
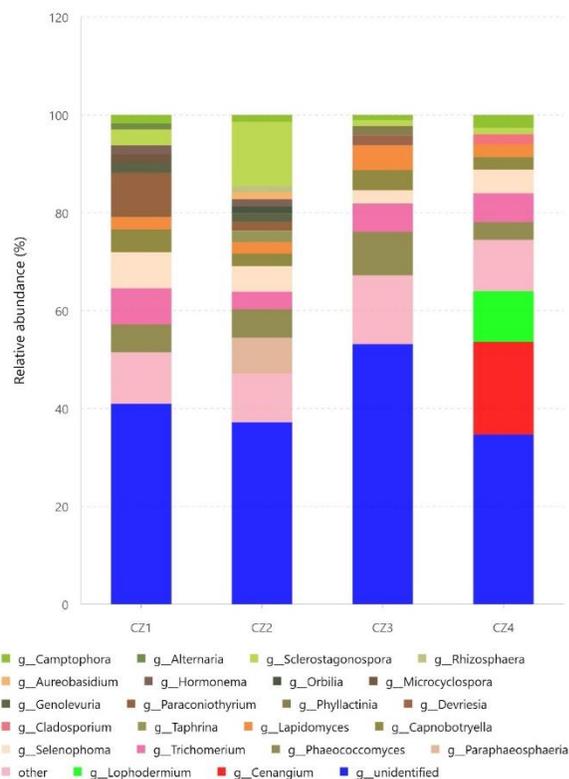
Fig. 4: Dominant fungal genera from endophytic fungi of needles of *P. densiflora* (the color of the column represents the different classes, and the length of the column represents the proportion size of the class. Sequences that could not be u identified were designated as "unidentified". Genera making up less than 1% of total composition in each sample were classified as "other")
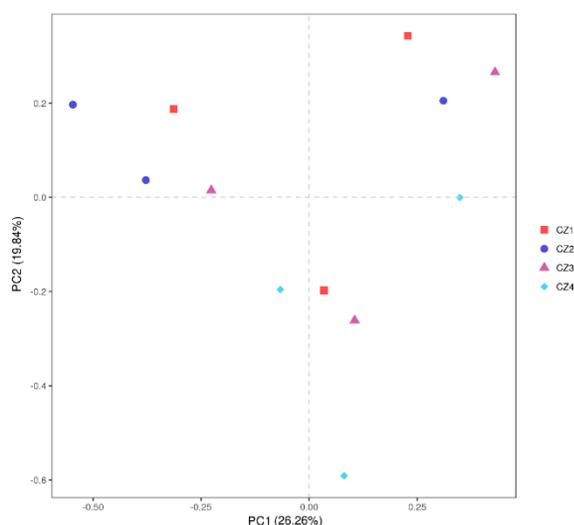

Fig. 5: Principal component analysis of endophytic fungi in needles of *P. densiflora*

the diseased tissue by other pathogens or saprophytic fungi (Arnold 2007) or as a result of the observed pattern of the fungal colonies that can trigger the latent infestation of

pathogens (Steinrucken *et al.* 2016).

Most studies on the endophytic microbes of *Pinus* have been carried out by pure culture methods. The endophytic fungi of *P. sylvestris* and *P. koraiensis* have been isolated and cultured. The diversity of endophytic fungi in pine needles and the factors affecting endophytic fungal diversity, such as the age of coniferous leaves, were initially studied (Dai and Lu 2012; Wang *et al.* 2017). In the study of endophytic fungi in *P. densiflora*, multiple dominant strains of *Lophodermium complex*, *Sydowia polyspora*, *Hymenula* spp., *Sistotrema brinkmannii*, *Septoria pini-thunbergii*, *Earliella* spp. and *Lophodermium* spp. have been isolated (Gil *et al.* 2009; Eo *et al.* 2013, 2018). Next generation sequencing technology has been frequently used in the study of fungal diversity. To study the interaction between endophytic fungi in coniferous species, Bullington and Larkin (2015) inoculated pathogens onto needles of *P. monticola*, and measured changes in endophytic fungi diversity and community structure using new generation of high-throughput sequencing methods. Interspecific competition and symbiotic patterns between the inoculated fungi and the potential pathogens were also confirmed (Lu 2017).

The dominant species of endophytic fungi in needles of *P. densiflora* in mixed coniferous forest are Dothideomycetes and Eurotiomycetes. At the genus level, *Paraconiothyrium* and *Trichomerium* are common endophytic fungi found in *Taxus baccata* (Somjaipeng *et al.* 2015) and *Coffea Arabica* (Maharachchikumbura *et al.* 2018), and intensive studies have been carried out on the metabolites produced by *Paraconiothyrium* spp., which showed moderate antibacterial activity and restoration of the growth of a mutant yeast strain inhibited by hyperactivated Ca^{2+} signaling (Suzuki *et al.* 2019). *Selenophoma* spp. is a common pathogen in ornamental plants (Sandoval *et al.* 2015) and crops (Kamlesh and Kuldeep 2006), and its role in *P. densiflora* is still unclear. *Cenangium* and *Lophodermium* were only found in the infected needles, both of which have been found as pathogens in coniferous species, such as *P. sylvestris* (Reignoux *et al.* 2014) and *P. koraiensis* (Ryu *et al.* 2018). *Cenangium* is a common genus of pine disease and is a common pathogen in pine. This genus has been shown to be closely related to *S. sapinea* infection (Milijašević and Karadžić 2004).

The fungal community may be affected by the specific composition of tree species, which may contribute to alterations in the microenvironment (Nguyen *et al.* 2016). Endophytic communities of conifer species can vary, even between the needles of one tree (Deckert and Peterson 2000). Beta diversity analyses showed that there was a difference among the asymptomatic needles of the three mixed forest samples. After *S. sapinea* infection, the community structure of endophytic fungi tended to be consistent. For phytopathogenic fungi, non-host tree species may act as barriers to spore transmission, resulting in a 'dilution effect' for fungal inoculum, thus, reduced fungal

species richness (Fernandez *et al*. 2019). For generalist fungi, several tree species may act as alternative hosts, increasing the probability of successful establishment and leading to differences between fungal community structure in mixed forests.

Conclusion

Results showed that endophytic fungal diversity and community structure in *P. densiflora* needles were affected by the *S. sapinea* infection in the mixed coniferous forest. As the *S. sapinea* infection worsened, the endophytic fungal richness showed an upward trend. The dominant endophytic fungi in *P. densiflora* needles from the mixed coniferous forest are Dothideomycetes and Eurotiomycetes. *Cenangium* was considered to have a certain association with the *S. sapinea* infection. Future research should focus on the resistance of endophytic fungi to pathogens during infection.

Acknowledgments

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