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Comparative Analysis of Microbial Communities in Adult Trees and Seedlings of Douglas fir †

Jens-Ulrich Polster [1,*], Kristin Morgenstern [1], Birgit Reiche [1], Patrick Schützel[2], Imke Hutter[3] and Doris Krabel[1]

[1] Technische Universität Dresden, Institute of Forest Botany and Forest Zoology, working group "Molecular Physiology of Woody Plants", Pienner Straße 7, 01737 Tharandt, email: jens-ulrich.polster@tu-dresden.de
[2] Biomasse Schraden e. V., Straße zum Stützpunkt 15, 04932 Großthiemig
[3] Institut für Pflanzenkultur e. K., Solkau 2, 29465 Schnega
* Correspondence: jens-ulrich.polster@tu-dresden.de

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Abstract: The composition and complex interaction of microorganisms in forest trees is still unknown. However, current studies on cultivated plants indicate that the microbiome can have a significant influence on plant development, vitality and susceptibility to pathogens.

In the project "TreeLAMP", the composition of the microbial community of ten adult trees and nine seedlings of Douglas-fir was characterized in an exploratory study using sequence-based methods. Approximately 9.5 million fungal sequences (ITS1) and approx. 4.3 million bacterial sequences (16S rRNA gene) were generated over all samples by Illumina MiSeq sequencing. In a first step the quality of the sequence data was checked and sequences with low quality were filtered with dada2. The taxonomic classification was carried out with the program kaiju (database NCBI nr+euk).

For the characterization of the fungal community, between 13,817 and 410,464 sequences per sample were analyzed. In total 83 fungal species were identified. However, on average 0.5 % of the sequences could not be classified and 48.1 % could not be determined to species level. In the bacterial community, with 899 to 11,807 sequences significantly less DNA was detected per sample. But in comparison 79.4 % of the sequences could be determined to species level, 15.2 % of the sequences were assigned to at most a genus, and 5.3 % could not be classified. In total 142 bacterial species were identified. In summary, the composition of the fungal and bacterial species community differs, as expected, between adult trees and seedlings. However, differences were also observed within the group of adult trees and seedlings, whereby the bacterial community was generally more heterogeneous than the fungal community.

Keywords: Douglas-fir; Microbiota; Fungi; Bacteria; ITS; 16S; Illumina MiSeq

1. Introduction

The Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) is the only species of the genus *Pseudotsuga* with a wide distribution area and high economic importance. It is a dominant tree species in large parts of western North America and thus an important timber source.

After introduction of the first seeds to Great Britain by David Douglas in 1827, the species spread fast in botanical gardens throughout Europe. First systematic cultivation trials in Germany by members of the Union of German Forest Experiment Stations were initiated in 1881. Encouraged by the success of these first trials, Douglas-firs were planted all over Germany and became the economically most important foreign tree species in Germany today. Due to its high growth rate and

good wood quality, the species was cultivated throughout Europe and later in many countries worldwide.

However, forestry use of Douglas-fir often experiences serious setbacks caused by fungal infections, especially in monoculture stands. The pathogens that cause the most severe damage are Rhabdocline needle cast (*Rhabdocline pseudotsugae*) and Swiss needle cast (*Nothophaeocryptopus gaeumannii*), which not only result in loss of growth or the death of single trees, but also of entire forest stands if infections occur repeatedly.

It is known that plants serve as hosts for a multitude of different microorganisms. A joint evolution over millions of years [1,2] has led to the formation of complex communities and interactions between plants and the microorganisms that colonize them, often based on a mutualistic symbiosis for the provision of necessary metabolic products [3,4]. When investigating these relationships, a differentiation is made between key species, the core microbiome, i.e. species essential for healthy plant development, ubiquitous species and pathogens. Additionally, individual species can switch between mutualistic and pathogenic lifestyles, which is often associated with a disturbance of the biocoenosis by external factors [5]. The identification of key microbial species as well as the comparison of the core microbiome of healthy-looking plants and plants infested with pathogens can help to identify species that are essential for a vital plant.

The described complexity of the interdependencies is also the reason why the majority of microbial species cannot be cultivated with conventional approaches. They are currently detectable only by molecular genetic methods, whereby two methods are commonly used to study the community as a whole. The first is "Metagenome Sequencing", in which species-specific genes are identified [6], and the other is "Targeted Amplicon Sequencing" (or "DNA-barcoding") [7], in which highly variable species-specific sections of microbial DNA are used to determine the species.

In both methods, the sequenced DNA fragments are compared to known reference patterns in a database (e.g. NCBI or EMBL) and assigned to species or higher taxonomic units using bioinformatic methods [8,9]. The abundance of individual species and their distribution in the samples allows the description and further comparative analysis of the microbiome with samples from other populations.

However, such studies have not yet been carried out on the Douglas-fir, like on most other forest trees. This is certainly associated with the high costs of DNA sequencing in the past. With the advent of NGS technologies, these approaches become available for forestry studies as well.

In the research project “TreeLAMP”, which primarily aimed at the development of detection methods for the needle-cast pathogens on Douglas-fir, the microbiome of selected Douglas-firs was examined in a first explorative sequencing experiment. The aim of this study was to obtain first basic insights on the composition of the microbiome when comparing trees and seedlings.

2. Materials and Methods

In 2018, six Douglas-firs from a roughly 25-year old stand of a controlled cross-breeding trial in Tharandter Wald near Hetzdorf (Saxony; 50° 58' N, 13° 28' E), four pure varieties of Douglas-fir from the Tharandt Botanic Garden and Arboretum and nine seedlings of Douglas fir from different areas of origin in Germany together with one seedling of the Chinese Douglas fir (*P. sinensis*) were investigated.

The seedlings of *P. menziesii* were cultivated and provided by the project partner Biomasse Schraden e.V. and the seeds used for cultivation came from four selected areas of origin. Therefore, seeds of two areas with high *R. pseudotsugae* infestation in the seeds (Billenhagen and Mittweida) and two areas with no detectable *R. pseudotsugae* infestation in the seeds (Dillenburg and Grillenburg) were selected for cultivation. Two seedlings were then randomly selected from each group for sequencing. The seedling of the Chinese Douglas fir was provided by the Forest Research and Competence Center Gotha (FFK Gotha) of the Thuringian Forest Administration (ThüringenForst AöR). An overview of the samples used is shown in Table 1.

Table 1. Plant material used for microbial analysis with information on (sample) name, (species), (location) of samples or seed origin and (age class).

Sample	Species	Location	Age class
816-2	*P. menziesii* var. *glauca*	Tharandt [1]	pre-thicket
818-13	*P. menziesii* var. *glauca*	Tharandt [1]	pre-thicket
839-1	*P. menziesii* var. *viridis*	Tharandt [1]	pole stage
839-2	*P. menziesii* var. *viridis*	Tharandt [1]	pole stage
Th16-15	*P. menziesii* var. *glauca x viridis*	Hetzdorf [2]	timber stage
Th21-2-4	*P. menziesii* var. *glauca x caesia*	Hetzdorf [2]	timber stage
Th30-1-4	*P. menziesii* var. *glauca x caesia*	Hetzdorf [2]	timber stage
Th35-25	*P. menziesii* var. *viridis x glauca*	Hetzdorf [2]	timber stage
Th36-10	*P. menziesii* var. *glauca x viridis*	Hetzdorf [2]	timber stage
Th40-9	*P. menziesii* var. *glauca x viridis*	Hetzdorf [2]	timber stage
BH-6	*P. menziesii* var. *viridis*	Billenhagen	seedling
BH-13	*P. menziesii* var. *viridis*	Billenhagen	seedling
9123 (BH-15)	*P. menziesii* var. *viridis*	Billenhagen	seedling
MW-1	*P. menziesii* var. *viridis*	Mittweida	seedling
MW-8	*P. menziesii* var. *viridis*	Mittweida	seedling
DB-1	*P. menziesii* var. *viridis*	Dillenburg	seedling
DB-9	*P. menziesii* var. *viridis*	Dillenburg	seedling
GB-1	*P. menziesii* var. *viridis*	Grillenburg	seedling
GB-8	*P. menziesii* var. *viridis*	Grillenburg	seedling
Nr.7-a	*P. sinensis*	Prov. Guizhou, China	seedling

[1] Tharandt Botanic Garden and Arboretum

[2] Tharandter Wald, Saxony

The samples were arranged in order to compare old trees with different levels of *R. pseudotsugae* infestation, seedlings from different areas of origin and seedlings with older trees to achieve a maximum of differentiation in one sequencing experiment with at least two individuals in each group for comparison reasons.

Selected shoots from the older trees and the complete seedlings were used as samples and no surface sterilization was applied to collect as much information as possible about microbial species on and in the plants. After collection, all samples were stored at -40 °C and then sent together cooled on dry ice to the sequencing service provider Starseq GmbH (Mainz, Germany). There the DNA was extracted from individual needles of each sample using the DNeasy Power Soil Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's manual. In a subsequent PCR, the V4-V5 region of the 16S rRNA gene was amplified to identify bacterial and other procaryote species. In parallel, the ITS1 region of the ribosomal DNA was amplified in another PCR to identify fungi in the same sample tissue. All amplicons were then sequenced on an Illumina MiSeq system (Illumina Inc., San Diego, USA) with paired-end reads.

After quality control and filtering steps using the implementation of the “dada2” software package [10] in R, the taxonomic classification of the remaining sequences was performed in the program “Kaiju”[11] using the NCBI BLAST nr Database with additional fungi (nr_euk). Subsequent bioinformatic analyses were made using the “vegan” [12] and “ggdendro” [13] libraries in R.

2. Results

In total, approx. 9.5 million fungal sequences (ITS1) and approx. 4.3 million bacterial sequences (16S rRNA gene) were generated for all 20 samples. After a quality control, all sequences that were too short or of low quality were removed. This left about 5.7 million fungal sequences and about 90 thousand bacterial sequences for further analysis. These sequences were merged into Operational Taxonomic Units (OTUs) and classified taxonomically with the program "Kaiju" with the microbial subset of the NCBI BLAST non-redundant protein database *nr* including fungi and microbial eukaryotes as reference.

The results of the taxonomic classification are summarized in Figure 1 for fungi and Figure 2 for bacteria. Each diagram shows the cumulated absolute abundance as well as the proportion of the

recorded species per sample. The dendrogram describes the similarity of species composition between the samples based on the relative frequency of each species in the respective samples. The species lists for fungal and bacterial microorganisms are available as supplementary files.

For the characterization of the fungal microorganisms, between 13,817 sequences (sample Nr.7-a) and 410,464 sequences (sample Th36-10) were analyzed per sample. On average 0.5 % of the sequences could not be classified (Fig. 1 diagram: dark grey) and 48.1 % of the sequences could not be determined to the species level (Fig. 1 diagram: light grey). A total of 83 fungal species were identified. The number of species varies between 29 (sample Nr.7-a) and 58 (sample Th36-10). The sample of *P. sinensis* (No.7-a) differs considerably from the other samples of *P. menziesii*, both in the abundance of the sequences and in the number of fungal species. In the comparison between trees (Hetzdorf and Tharandt) and the seedlings (without *P. sinensis*), a slightly higher number of species was found in the trees with an average of 53.3 fungal species than in the seedlings with an average of 47.8 fungal species. Differences can also be seen in the composition of the species. The dendrogram (Fig. 1) shows two main clusters, where the first main cluster contains the negative control (negC) and the sample of *P. sinensis* (Nr.7-a) as well as the seedlings from the origin Mittweida (MW-1; MW-8) and the sample 839-1 (*P. menziesii* var. *viridis*). In the second main cluster, five seedlings (DB-1, 9123, GB-8, BH-6, GB-1) form a sub-cluster with sample 839-2 (*P. menziesii* var. *viridis*). The trees of the Hetzdorf experimental area, sample number 816-2 and 818-31 (*P. menziesii* var. *glauca*) and the seedlings BH-13 and DB-9 are grouped in a second sub-cluster. Differences in fungal communities between the groups of trees and seedlings were to be expected. Unexpected was, that the varieties *viridis* (839-1; 839-2) and *glauca* (816-2; 818-31) from the Tharandt Botanical Garden are clearly different from each other. The variety *glauca* clusters with the trees from Hetzdorf and the variety *viridis* is more similar to the seedlings. At present, no clear correlation between plant health and accompanying fungal communities can be identified.

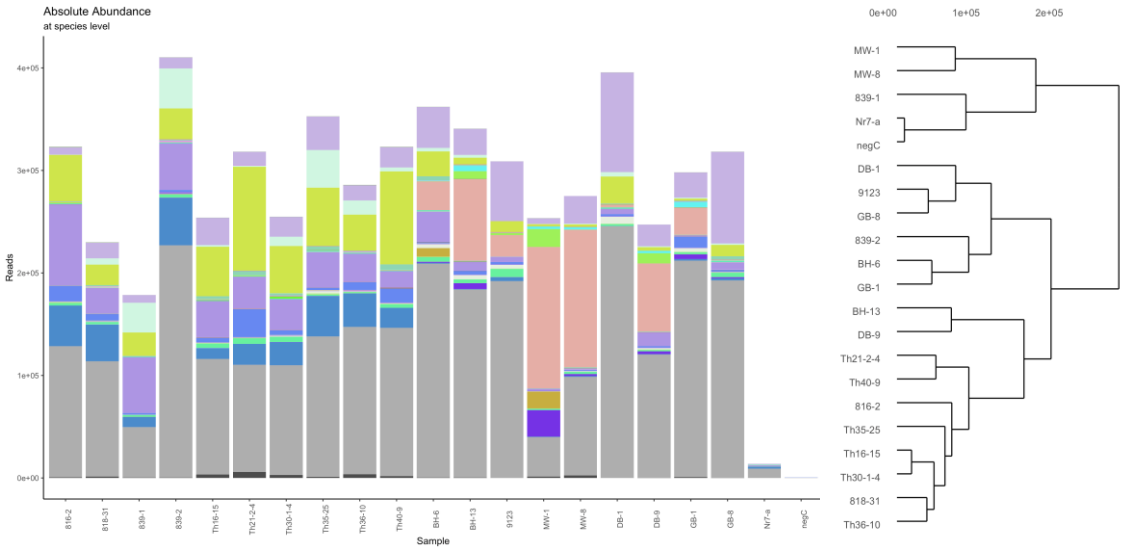

Figure 1. Taxonomic classification of the fungal microbiome of 19 different *P. menzesii*, 1 *P. sinensis* and 1 negative control (left to right): The diagram shows for each sample the cumulated absolute abundance of the sequences; each color corresponds to one species; dark gray = sequences that could not be classified; light gray = sequences that could not be determined to the species; sample Nr7-a is *P. sinensis*.

The bacterial microorganisms are more heterogeneous than the fungal community (Fig. 2). With 899 (sample DB-1) to 11,807 sequences (sample BH-13) per sample, significantly less bacterial DNA sequences were detected. Nevertheless 79.4 % of the sequences could be determined to the species, 15.2 % of the sequences were at least assigned to the genus (Fig. 2 diagram: light grey) and 5.3 % could not be classified (Fig. 2 diagram: dark grey). A total of 142 bacterial species were identified. Within the samples the number of species varies between 32 species (sample 839-2) and 91 species

(sample BH-13). In the group of seedlings (without *P. sinensis*) the number of species is with an average of 63 species per sample slightly higher than in the group of older trees with 58.4 species per sample. In general, the composition of the bacterial species differs less between the samples than the fungal species. Nevertheless, a similar clustering of the samples can be observed in the dendrogram. The seedlings (with the exception of BH-13) and the samples of *P. menziesii* var. *viridis* (839-1; 839-2) as well as the trees from Hetzdorf (with the exception of Th35-25) and the samples of *P. menziesii* var. *glauca* (816-2; 818-31) each form a separate cluster. At present, no clear correlation between plant health and accompanying fungal communities can be identified.

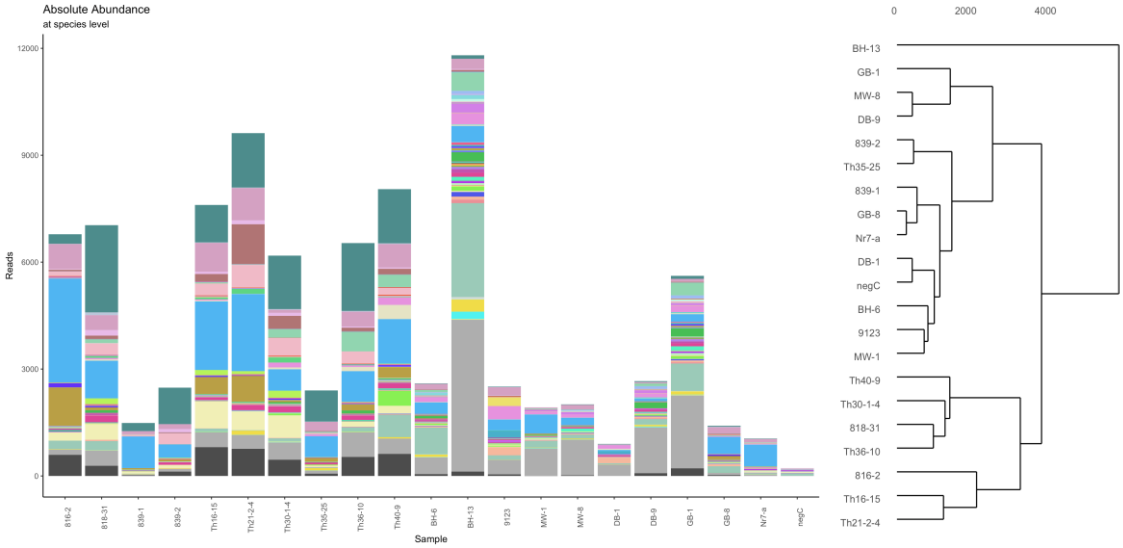

Figure 2. Taxonomic classification of the bacterial microbiome of 19 different *P. menzesii*, 1 *P. sinensis* and 1 negative control (left to right): The diagram shows for each sample the cumulated absolute abundance of the sequences; each color corresponds to one species; dark gray = sequences that could not be classified; light gray = sequences that could not be determined to the species; sample Nr7-a is *P. sinensis*.

2. Discussion

The aim of this study was to provide a first insight into the abundance and composition of microbial fungi and bacteria in Douglas-firs of different ages and from different origins. Since there were no indicators for the number of species to be expected prior to this work, only a small sample size was chosen. This unfortunately does not allow a significant statistical evaluation with community ecological methods. However, this study provides the basis for further, targeted research into the species relationships in Douglas-firs. This is particularly important in the context of the investigation on pathogens of these trees.

In order to obtain the most comprehensive characterization of the species composition, no surface disinfection of the samples was applied. Consequently, no differentiation between random adhesions and a real epiphytic or endophytic colonization of the needles can be determined. Compared to the classical cultivation of microorganisms, DNA-based methods allow a more comprehensive survey of microbial communities in plants. However, an influence of the methods used on the observed result cannot be excluded even with these methods [14]. The used extraction methods or the selection of PCR primers may give preference to individual species or make their detection more difficult. The sequencing technology used as well as the selection and length of the sequenced DNA fragments can also bias the results [15,16]. This is most likely the reason why individual OTUs could not be determined to the species level. Another reason for this observation together with OTUs that could not be assigned at all, lies in the reference database used, which may not contain references for all existing species.

Rarefaction analyses were performed on all samples to estimate the influence of these factors. Most of the constructed rarefaction curves reached not the asymptote, suggesting that the fungal and bacterial communities were not completely identified. Individual rarefaction curves reached the asymptote for some samples but not for all (results not shown), suggesting that a deep-sequencing approach with long-read technologies could be useful to identify more of the extremely rare species in these communities. Even if the result of the sequencing is not exhaustive, it provides initial insights into important factors affecting the composition of microbial communities in Douglas-fir needles.

The results of the presented study show differences in species composition between seedlings and mature trees despite the small sample size. The absolute quantity of microorganisms as well as the number of identified species differ only negligibly. The observed influence of the plant age on the species composition in the microbiome was to be expected and was for example also described on the Corsican pine (*Pinus nigra* subsp. *laricio*) [17]. However, the cluster analysis showed an additional differentiation in the tree samples from the Tharandt Botanic Garden and Arboretum. The trees of the variety *viridis* appear similar to the seedlings (also var. *viridis*), the trees of the variety *glauca* on the other hand are more closely related to the trees from Hetzdorf (controlled cross-breeding with var. *glauca*). This indicates a considerable influence of the host plants' genotype, as already described for Norway spruce [18] and Aspen [19]. The expected influence of the habitat [20,21] or origin of the seed is not significant.

A surprising result was also the very low microbial colonization of the Chinese Douglas fir, since this plant was cultivated outdoors like the other seedlings. Whether the low microbial colonization is a coincidental finding, a sequencing artifact or due to the characteristics of the species cannot be judged at the moment.

In conclusion, the results presented indicate a genetic component of the host plant that affects the composition of the microbial species. Due to the small sample size, the importance of other factors, such as habitat or vitality of the plants cannot be evaluated adequately at this stage. Further, specific investigations are necessary for this purpose. In the "TreeLAMP" project, the variation of microbial composition in Douglas-fir stands is currently being investigated with focus on the genotype and vitality of the host plant.

Supplementary Materials: The species lists are available at:
https://cloudstore.zih.tu-dresden.de/index.php/s/3xg4XXqq2XCM4Nr

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