The fungal phytobiome of *Searsia lancea* (karee) trees with Karee Malformation Disease in South Africa

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Introduction

Fungi form one of the important components of the plant microbiome and occur either as endophytes or epiphytes. Fungi play different roles within their community and can be either saprobic, parasitic and symbiotic[1-6]. Community structures are expected to form due to host specificity mechanisms while the roles these fungi play in the community determine succession. Succession rests upon the assumption that a community can be viewed as an organismic entity that arises, grows, matures and dies[4]. *Searsia lancea* (karee) is a common native tree in South Africa. A new disease called Karee Malformation Disease (KMD) consists of hypertrophic malformations occurring mostly on vegetative and floral tissues[2]. This disease partially resembles Mango Malformation Disease that is caused by *Fusarium* species. The causal agent of KMD has not yet been identified but previous studies showed significant changes occurring in fungal communities in the malformation symptoms[2], based on isolations. The aim of this study is to use, culture-independent methods such as next generation sequencing to characterize the mycobiomes found in the floral and vegetative tissues of *Searsia lancea* to determine if there are differences and a level of succession between communities in malformed and healthy tissues. A baseline of how a typical fungal phytobiome of karee would be structured, was also established for more accurate comparisons with diseased tissues. Minibarcodes using the Internal Transcribed Spacer 2 region of the ribosomal operon, was generated with Illumina sequencing and analysed in a bioinformatics pipeline.

Materials and Methods

SUB-SAMPLING AND PREPARING AMPLICON LIBRARIES

- Sampling was done in Bloemfontein (Free State), Christiana (North West) and Pretoria (Gauteng). A set of healthy and malformed samples from *Searsia lancea* trees were divided into leaves, twigs, stems, seed or inflorescences and subjected to a serial process of surface sterilization (bleach-distilled water-96% ethanol-distilled water).
- The samples were lyophilized and ground separately according to their substrates to avoid contamination.
- The plant material (40mg) was processed for DNA extraction using the NucleoSpin Plant II Midi extraction kit (MACHEREY-NAGEL) according to the manufacturer protocol.
- The amplicon library was prepared from the ITS2 region using primers ITS3F and ITFS4R with overhang Illumina adapters and KAPA HiFi ready mix Taq.
- Sequencing of the amplicons was done following the Illumina Miseq 16S Metagenomic sequencing protocol at the NGS facility of the University of the Free State.

BIOINFORMATICS

- The ITS sequence data was analysed using QIIME according to Caporaso et al. (2010).
- Pre-processing of the reads was done to check quality of the reads using PrinSeq-lite v 0.20.4.
- Trimming was done to obtain an average quality score of ≥ 20 using a 7nt window with a 4nt step, sequences shorter than 200bp were filtered out and paired end reads merged using PEAR 0.96
- Chimeric sequences were identified using usearch 6.1.544 as the chimeric detection method against the RDP Gold database and filtered out of the quality trimmed reads using identify_chimeric_seqs.py and filter_fasta.py commands.
- In QIIME OTU picking was done using the pick_open_reference_otu.py script at 97% sequence identity against the SILVA 119 database.
- Microbial diversity was analysed using Principle Coordinate Analysis (PCoA), performed in R (www.r-project.org) using plot _ordination in the Phyloseq package using Bray-curtis distance.

Results and Discussion

- Community differences were observed in the healthy trees between different tissue types, and between young and old tissues (Fig. 2-5). A total of 58 Operational Taxonomic Units (OTUs) were found in healthy plants and a total of 113 OTSs were found in malformed plants.
- Thirty two fungi in the healthy plants were identified up to genus level with the majority of the population being *Aureobasidium* (50%) and *Microdiplodia* (8%). In malformed plants 88 fungi were identified up to genus level with populations of *Cladosporium* (45%), *Cytospora* (2%) and *Filobasidiella* (2%) prominent.
- Clustering (Fig. 3) within the healthy samples (right) was vertically spread confirming the successive change of community populations between the young and old tissues.

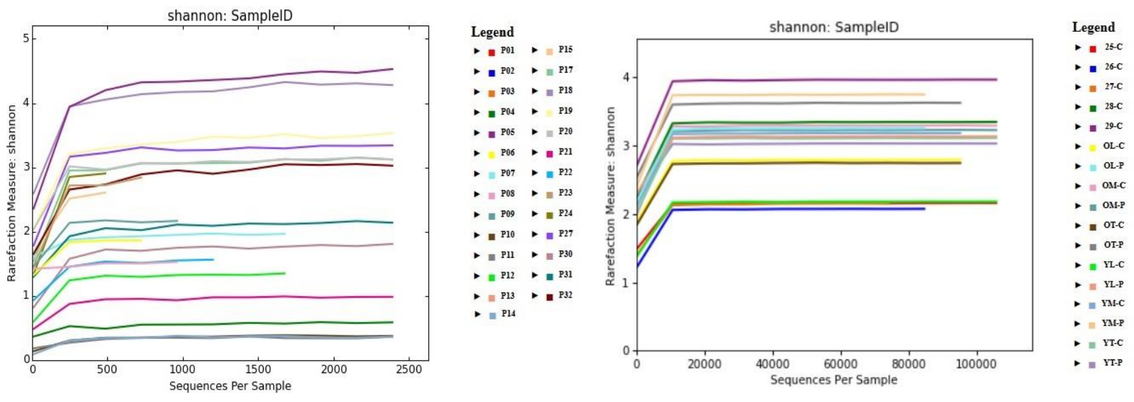

Fig.1. Shannon Diversity index for each plant from healthy trees (left) and malformed trees (right).

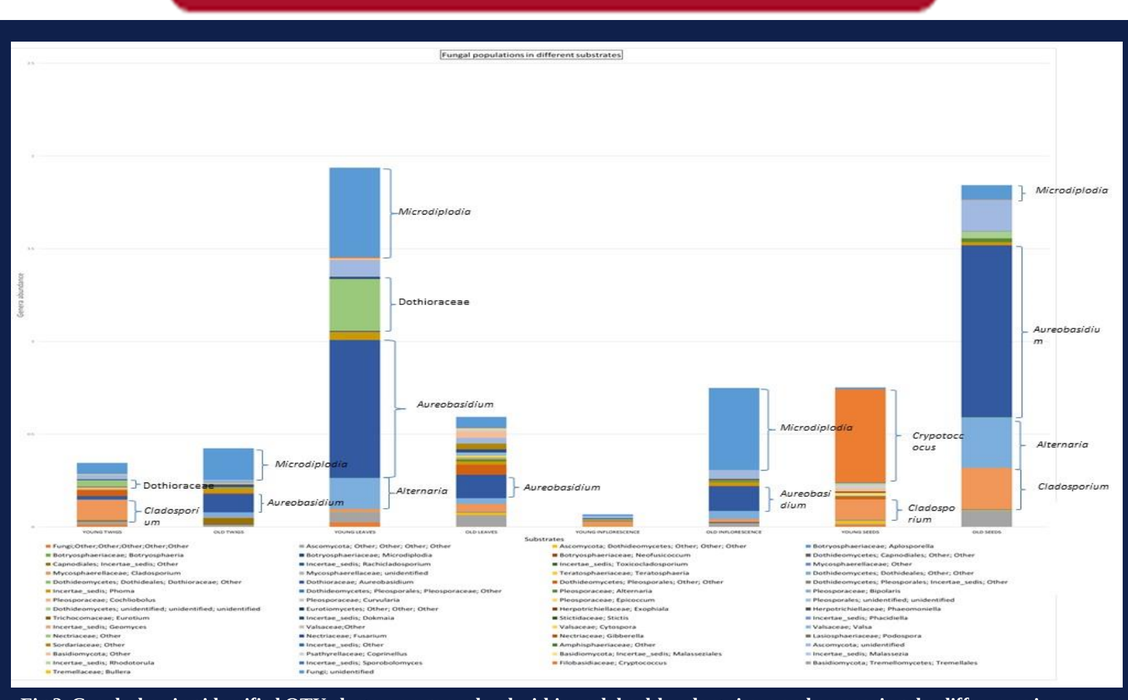

Fig.2. Graph showing identified OTU clusters on genus level within each healthy plant tissue and comparing the differences in diversity between young and old.

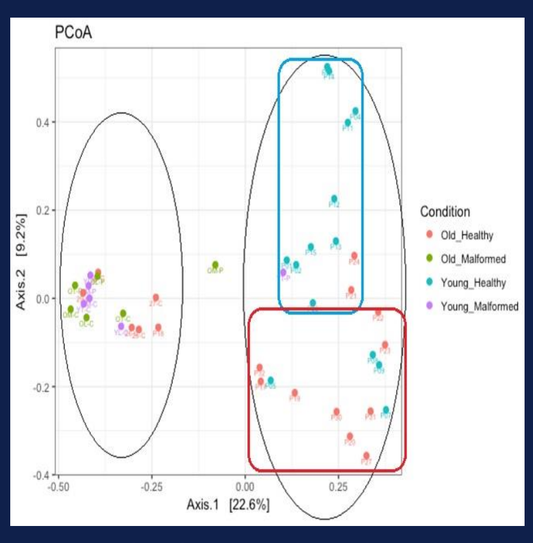

Fig.3. PCoA plot with Bray-Curtis distance showing that the malformed samples (left) clustered separately from the healthy samples (right).

- Fungal communities in malformed tissues differed from those in healthy counterpart tissues (Fig. 3-5).
- There was a change in community structure depicted by a significant change in the dominating populations (Fig. 4-5).
- As consistent with the latent pathogen life cycle of many pathogens, genera such as *Alternaria, Botryosphaeria* and *Valsa* were present in all tissues (Fig. 3-4, highlighted with green broader).
- No dominant fungal group that could be the cause of the disease were detected, thus confirming previous studies concluding that fungi most likely is not the cause of the disease.

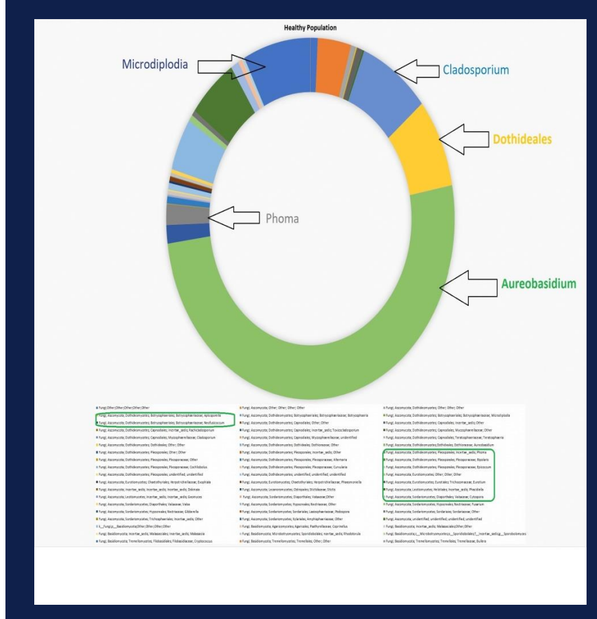

Fig.4. Pie chart showing dominant populations at genus level in the healthy samples.

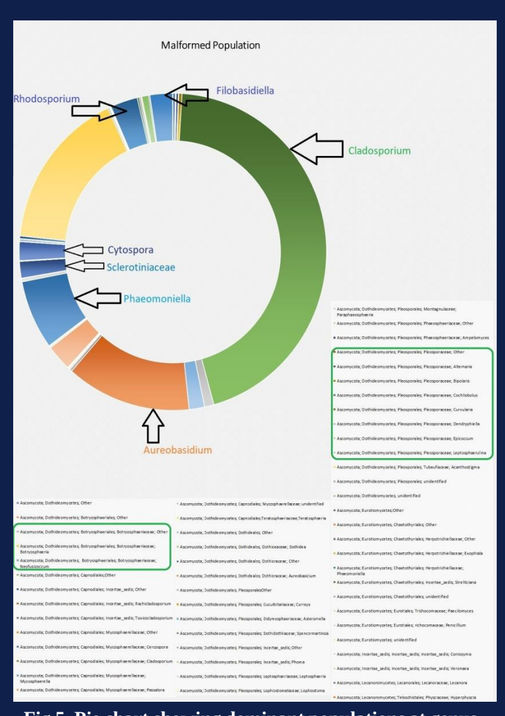

Fig.5. Pie chart showing dominant populations at genus level in malformed samples.

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