Analysis of fungal communities associated with grapevine wood diseases, based on fungal ITS pyrosequencing

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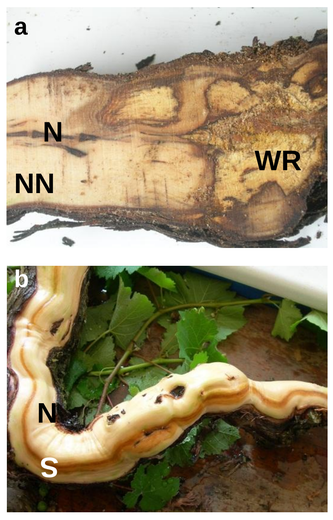
Abstract

The Grapevine Trunk Diseases (GTDs) are the most common diseases of grapevine wood inducing a slow decay leading to plant death. Due to the human and environmental impact, chemical treatments (sodium arsenite) are no longer authorized. Prophylactic methods or trunk removal are the last available control methods. Fighting against these slow evolving diseases requires a better knowledge of fungal and bacterial communities associated with GTDs. Our approach is based on fungal species identification using ITS (Internal Transcribed Spacer) sequences obtained by pyrosequencing (Roche 454) of grapevine wood samples.

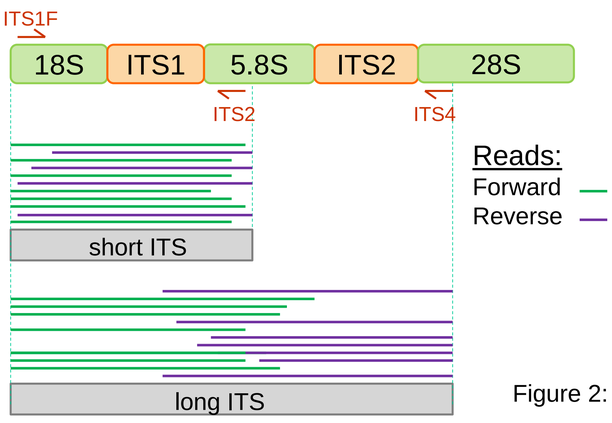
Sampling and Material



Two campaigns of samplings were conducted to collect different kinds of internal or external tissues of symptomatic and asymptomatic trunks (Fig 1). The first study was carried out on old plants and the second on 10-year-old plants. The latter includes 4 collecting dates in order to follow communities evolution over a cultural season.

Figure 1: Photographs of longitudinal sections of trunks that had expressed esca foliar symptoms.
Samplings from internal (a) or external wood (b) of Necrotic (N) or Non-Necrotic tissue (NN), White Rot (WR) or brown Stripe (S).

Methods



ITS regions were amplified with universal primers and sequenced with 454 pyrosequencing technology. Forward and Reverse primers were linked with different MID tags, that is useful for long amplicon analysis. Indeed, forward reads are used in ITS1 analysis, when reverse reads to investigate the ITS2 region (Fig 2).

Figure 2: ITS region amplicons and expected DNA sequencing reads

Bioinformatic analyses

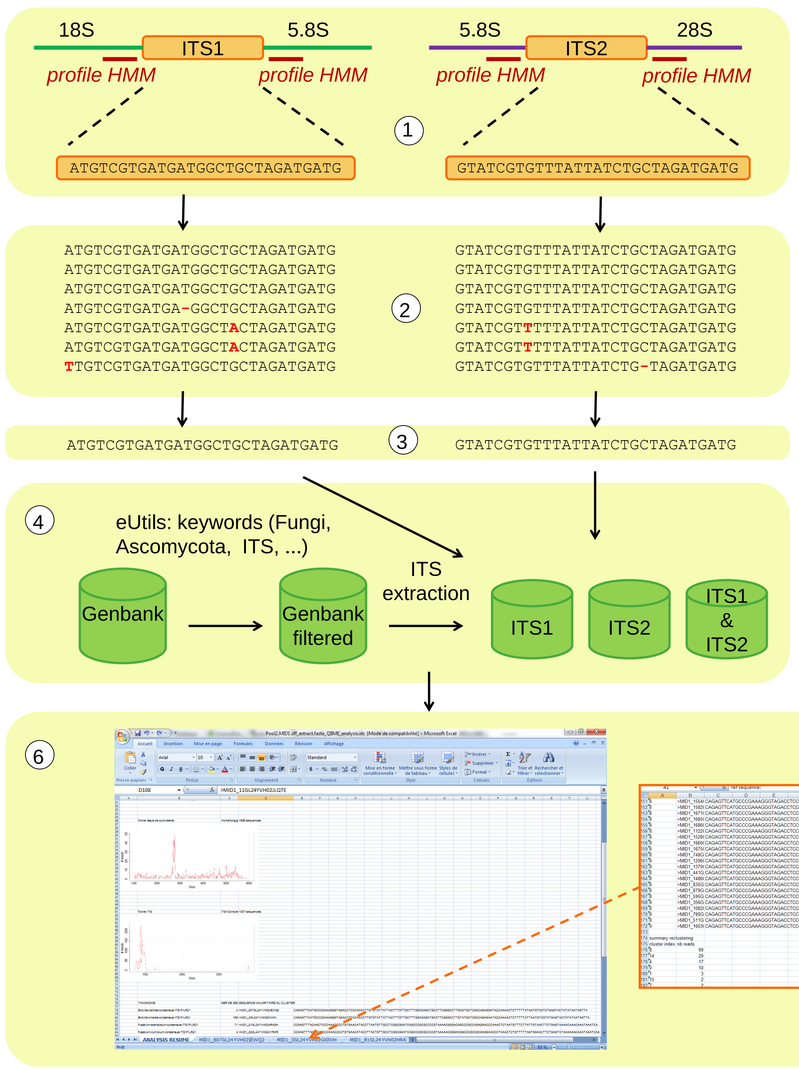


Figure 3: Bioinformatic workflow
1: ITS extraction with the FungalITSExtractor tool
2: Clustering with Uclust (sequence similarity 97%)
3: selection of the most abundant sequence
4: taxonomy assignation with Blast and customized databanks
5: Reclustering with Uclust (sequence similarity 100%)
6: Export in Excel spreadsheet

After quality filtering, data were processed with a bioinformatic workflow launched on a computing cluster (Fig 3). The first step is the ITS sequence extraction with the FungalITSExtractor[1]. Sequence extraction prevents clustering distortion, due to highly conserved ribosomal sequences. Then tools of the QIIME package[2] are launched for read clustering, reference sequence extraction and taxonomic assignation. Assignment is performed with Blast and a filtered database. An optional step with a higher clustering stringency could be launch to separate taxa with sequence similarity above the default threshold (97%). Finally, results are exported in spreadsheet for result exploration.

Results

Controls

19 cultured fungal species were used as controls for bias detection throughout the protocol (PCR, pyrosequencing). Several species, as *Pucinia*, were not recovered (Fig 4), mainly because of PCR bias. This result highlights the risk to use generic primers, which badly work with certain genera. Moreover, tests show that increasing the number of PCR cycles reduces the species diversity.

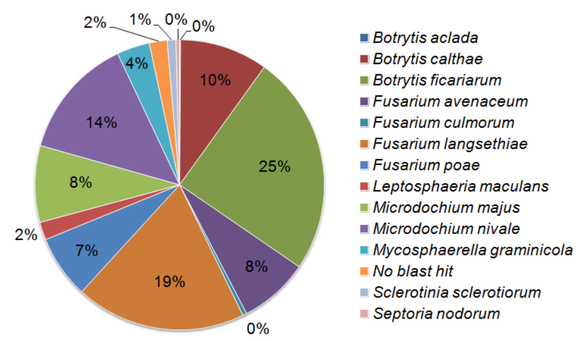


Figure 4: Pie chart of the species recovered from controls.
Percentages are the number of reads in respect to the total.

Samplings

A first analysis of Necrotic tissues vs Non-Necrotic tissues (Fig 5) reveals a higher diversity in safe tissues and overrepresented taxa in each condition, *e.g. Lecania* and *Bacidia* (Int-As-NN) and *Phialophorra*, *Eutypa* and *Phaeoacremonium*(Int-As-N). All samples were analyzed and condition comparisons are in progress.

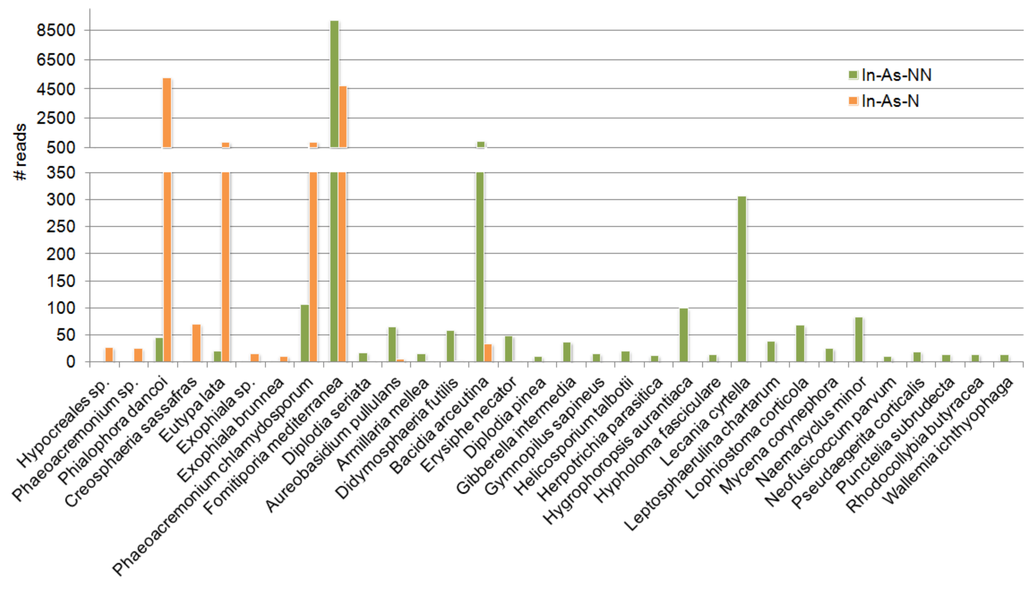


Figure 5: Barplots of read distribution per species, Internal Asymptomatic Non-Necrosis tissue (Int-As-NN) and Necrosis tissue (Int-As-N). Only species and genera with more than 10 reads are represented.

Conclusion and Perspectives

We developed a bioinformatic workflow allowing taxonomy assignation from ITS regions, the fungal kingdom barcodes. This tool was used to identify fungal communities involving in GTDs. Controls revealed PCR bias, showing that this approach must be complete with method like qPCR to quantify taxa abundance. Ongoing research on bacterial communities indicates that they are also able to colonize the various wood tissues sampled. Their association with the development of esca is currently investigated.

[1] Nilsson RH *et al.* 2010. An open source software package for rapid, automated extraction of *ITS1* and *ITS2* from fungal *ITS* sequences for use in high-throughput community assays and molecular ecology. *Fungal Ecology*
[2] Caporaso JG *et al*. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*
[3] E.Bruez, Etude comparative des communautés fongiques et bactériennes colonisant le bois de ceps de vigne ayant exprimé ou non des symptômes d'esca, Ph.D. Thesis, University of Bordeaux, France