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# NGS IDENTIFICATION OF FUNGI POTENTIALLY IMPLICATED IN THE PRODUCTION OF AGARWOOD FROM *AQUILARIA* SPP. TREES

***Clara ZAREMSKI****
AGAP, Univ Montpellier, CIRAD, INRA, Montpellier SupAgro, Montpellier, France
Address: CIRAD, UMR AGAP, F-34398 Montpellier, France
E-mail: clara.zaremski@cirad.fr

***Cédric MALANDAIN***
ENOVEO, 7 Place Antonin Poncet 69002 Lyon, France

***Olivier SIBOURG***
ENOVEO, 7 Place Antonin Poncet 69002 Lyon, France

***Claude ANDARY***
Laboratoire AMAP (UMR 5119 CNRS-CIRAD), Boulevard de la Lironde, 34398 Montpellier Cedex 5, France

***Georges MICHALOUD***
Laboratoire AMAP (UMR 5119 CNRS-CIRAD), Boulevard de la Lironde, 34398 Montpellier Cedex 5, France

***Marc DUCOUSSO***
CIRAD - UMR 113, Laboratoire des Symbioses Tropicales et Méditerranéennes, Cirad/IRD/Inra/Agro-M/UM2
Campus international de Baillarguet - TA A - 82 / J, 34398 Montpellier Cedex 5, France

***Nadine AMUSANT***
CIRAD - UMR ECOFOG "Ecologie des Forêts de Guyane", Campus Agronomique de Kourou
BP 701, 97387 Kourou Cedex, French Guyana

***Alba ZAREMSKI***
CIRAD, UMR AGAP, F-34398 Montpellier, France. AGAP, Univ Montpellier, CIRAD, INRA, Montpellier SupAgro, Montpellier, France

***Abstract:***

*Aquilaria is a tree species belonging to the Thymeleaceae family. When Aquilaria sp. is injured, it can produce agarwood. Agarwood is characterized by a darker wood colour than the healthy one and by a strong perfume that is much esteemed by perfumers and some oriental religious communities.*

*The production of agarwood is presumed to depend on environmental factors, among them fungi.*

*The aim of this work is to obtain an overview of fungi present in Aquilaria sp. from different countries. Aquilaria sp. is endemic to South East Asia including notably Cambodia, Laos and Thailand, where it is cultivated to produce agarwood. In French Guiana, farmers would like to locally produce agarwood in their field. That's why we wonder if fungal communities naturally present in French Guiana present characteristics making it compatible with the induction of agarwood.*

*In this study, NGS was used to characterize fungal communities associated with agarwood: 693,961 sequences that cover ITS2 estimated about 250bp have been obtained. These sequences have been grouped into 535 OTUs, displaying 100% identity. In this study, 87% were Ascomycetes and 10.5% were Basidiomycetes.*

*These results show also differences in fungal communities between aboveground and belowground parts of the tree. Likewise, differences between countries within fungal communities were also observed.*

***Key words:*** *Aquilaria; agarwood; fungal diversity; molecular analysis; ITS.*

## INTRODUCTION

The genus *Aquilaria* contains 17 species of trees (Andary *et al.* 2018) and belongs to the Thymaelaeaceae family. This genus is endemic to Southeast Asia and is one of major cultural and commercial value thanks to the production of oleoresin when the wood is physically wounded or infected with fungi. This modified wood is called agarwood.

When *Aquilaria* wood is contaminated by a pathogen, notably Ascomycetes belonging to genera *Chaetomium*, *Cunninghamella, Curvularia, Fusarium*, *Trichoderma*, or *Lasiodiplodia* (Kaiser 2006; Naef

* Corresponding author

2011), its defence strategy is to secrete an oleoresin. This wood produces an oleoresin rich in volatile and non-volatile molecules (Mei *et al.* 2008), which delay the spread of the fungus and also initiate the healing process. When *Aquilaria* wood is healthy, it is whitish and shiny. When it is loaded with oleoresin, it turns from pale beige to black as the oleoresin oxidizes, with a greater density, and becomes increasingly fragrant as the process evolves. At this stage, the *Aquilaria* is infected.

The aim of this work is to obtain an overview of fungi present in *Aquilaria* sp. from different countries. We wonder if fungal communities inducing agarwood present similar characteristics between these countries. To answer this question, samples of *Aquilaria* wood from Cambodia, Laos, Thailand and French Guiana will be studied. The analyzes will also be carried out on the soils from the tree's immediate environment for the French Guiana *Aquilaria* samples.

To study the diversity of microorganisms in these samples, illumina MiSeq sequencing, the so-called Next Generation Sequencing (NGS), will be used as it provides considerable opportunities (information?). In this study, the general principles of NGS will be presented from the amplification strategies to the associated bioinformatics analyzes.

Fungi were identified in wood and agarwood samples of the genus *Aquilaria* and in soils from the tree's immediate environment. To that end, the ITS of the fungi were mass sequenced of, and the fungus communities compared between infested samples and non-infested samples.

The main steps involved in this study were as follows: 1) Development of molecular characterization techniques: extraction and purification of the DNA of the fungus from *Aquilaria* tissues; 2) DNA sequencing; 3) Associated bioinformatics analyzes.

## EXPERIMENTAL METHODS

### Samples description: type, country, date of sampling

**Biological material**

A total of 17 samples of *Aquilaria* spp. were collected for this study

Samples from plantation of Cacao in French Guiana:

Twelve samples collected in April 2015 and stored at -80°C.

They were divided into four categories: root (noted "roo"), of which there were 3; trunk (noted "tru"), of which there were 3; branch (noted "bra"), of which there were 3; soil (noted "soi").

The samples were taken from 3 trees aged 3 years (noted 3a) and 4 years (noted 4a). We do not know the age of one tree; it is noted N° 2.

Samples from plantation in Thailand:

One sample collected in July 2015 and stored at -80°C.

It is noted: Th-Aga; the wood is suspected of being infected.

Samples from plantation in Vientiane Province, Laos:

One sample collected in December 2011 and stored at -80°C.

It is noted: La-Aga; the wood is suspected of being infected.

Samples from plantation "K Bal Spean Siem Reap" in Cambodia:

Two samples collected in June 2011 and stored at -80°C.

It is noted: Cam-1 and Cam-2; the wood is suspected of being infected.

Control sample:

One positive control sample: Blue wood from a forest in Montceau les Mines, Saône et Loire, France (noted "BW-Fr").

Characteristics of samples are presented in Table 1.

*Table 1*

***List of samples studied and their main characteristics***

| *Reference* | *Lab. ID.* | *Short name* | *Sampling date* | *Matrix* |
|---|---|---|---|---|
| Aquilaria 3 years Cacao: root | 1 | 3a-roo | 27/04/2015 | wood |
| Aquilaria 3 years Cacao root: bottom of trunk | 2 | 3a-tru | 27/04/2015 | wood |
| Aquilaria 3 years Cacao root: base of ramification | 3 | 3a-bra | 27/04/2015 | wood |
| Aquilaria No. 2 Cacao: soil | 4 | N2-soi | 27/04/2015 | soil |
| Aquilaria 3 years Cacao: soil | 5 | 3a-soi | 27/04/2015 | soil |
| Aquilaria 4 years Cacao: soil | 6 | 4a-soi | 27/04/2015 | soil |

| | | | | |
|---|---|---|---|---|
| Aquilaria 4 years Cacao: branch core sample | 7 | 4a-bra | 27/04/2015 | wood |
| Aquilaria 4 years Cacao: core sample, bottom of trunk | 8 | 4a-tru | 27/04/2015 | wood |
| Aquilaria 4 years Cacao: roots | 9 | 4a-roo | 27/04/2015 | wood |
| Aquilaria No. 2 Cacao: bottom of trunk | 10 | N2-tru | 27/04/2015 | wood |
| Aquilaria No. 2 Cacao: core sample, main branch | 11 | N2-bra | 27/04/2015 | wood |
| Aquilaria No. 2 Cacao: roots | 12 | N2-roo | 27/04/2015 | wood |
| Branch core sample of Thailand | 13 | Th-Aga | September 2015 | wood |
| Branch core sample of Laos | 14 | La-Aga | December 2011 | wood |
| K Bal Spean Siem Reap Cambodia, No1, 1.5 & 1.6, [1] | 15 | Cam-1 | June 2012 | wood |
| K Bal Spean Siem Reap Cambodia, No1, 1.5 & 1.6, [2] | 16 | Cam-2 | June 2012 | wood |
| Blue wood, infected with the fungus | 17 | BW-Fr | 21/07/2015 | wood |

**Extraction of total nucleic acids**

Nucleic acids were extracted from the wood samples and the soils using the PowerSoil DNA/RNA kit (MOBIO, USA).

Before applying this protocol, the wood samples were ground in liquid nitrogen. The kit was then used according to the manufacturer's protocol.

Quickly, the ground wood or soil samples (0.30g) were added to a bead beating for rapid and thorough homogenization. Cell lysis occurs by a combination of mechanical and chemical methods. PCR inhibitors (Humic acids, …) were removed by a proprietary precipitation process. Total genomic DNA is captured on a silica membrane in a spin column. DNA is then washed and eluted from the membrane. The eluted DNA is ready for PCR analysis.

*The quality and quantification of the extracted DNAs.*

The total DNA of the extracted samples was quantified by spectrometry on a Shimadzu photo spectrometer. The concentration and purity of the DNA extracted is measured in 2µl subsamples. The purity index was calculated from the ratio of the absorbance measurements at 260 and 280nm. The value of the 260/280 ratio must approach 1.8 for the sample to be qualified as pure. Smaller values indicated the presence of impurities or contaminant proteins at around the same wavelengths.

**PCR (polymerase chain reaction) amplification of the nuclear rDNA ITS**

An amplification protocol adapted to mycorrhizal fungi, inspired from White *et al.* (1990) and developed at the Symbiotes and Roots Laboratory (INRA in Montpellier) was used.

The amplification reactions were carried out in clean PCR plates. The reaction volume was as follows: 4µl of dNTP, 10µl 5X buffer, 2µl of each primer (20pmol/µl): ITS3-myc and ITS4-myc (Table 2), 5µl extracted total DNA (around 50ng), 26.7µl of Millipore or sterile water (to top up to 50µl), 0.30µl of Taq polymerase.

Negative controls without DNA were set to test for any contaminations in the reagents and buffers.

The 50µl of mixture was then covered with a drop of mineral oil to prevent evaporation and condensation in the tubes. Next, the plate was covered with adhesive paper and placed in a Thermocycler programmed as follows:

- An initial denaturation phase at 96°C for 5 min.
- 30 cycles comprising a denaturation phase at 96°C for 30sec., a hybridization phase at 55°C for 30sec, then an extension phase at 72°C for 1.30min.
- Elongation of the extension phase or final elongation phase at 72°C for 7min.
- Storage of the amplification product at 4°C or freezing at -20°C.

This protocol enabled us to properly amplify the region we were interested in. However, it was necessary to search for the DNA concentration that enabled an optimum amplification reaction to be obtained.

It was checked that the amplification reaction functioned well by visualizing PCR products on a control gel. Ten µl of PCR products was deposited on an agarose gel at 0.8% in a TAE 1X buffer. Migration was carried out by electrophoresis at 90V for one hour.

**F-RISA amplification**

F-RISA amplification consists in amplifying a precise region of the fungus genome. The PCR products generated of different sizes depending on the fungus species present. This therefore leads to generating a mixture of fragments of different sizes whose distribution depends on the community of the sample. In our case, this amplification was used to check for DNA integrity and the possibility of amplifying a different region of the ITS.

**Analyses by NGS sequencing**

The NGS sequencing was entrusted to the Research and Testing company (Illumina 2x300 sequencing).

To be a good candidate for sequencing, the genetic marker needs to have the following characteristics:

- The amplification primers has to hybridize on most fungi.
- The amplicon (amplified sequence) has to be long enough to enable efficient amplification and good quality sequencing.
- The interspecies sequence variations (between species) have to be important but the intraspecific sequence variations (within a single species) have to be limited.
- The sequences obtained have to enable alignments to most fungi (Lindahl *et al.* 2013).

While exhibiting a few limitations for the definition and the delimitation of the species for certain groups (Gazis *et al.* 2011), ITS (Intergenic Transcribed Spaces) are widely used in biodiversity studies. These regions are relatively easy to amplify for the entire fungus lineages. Many sequences are available in databanks. Within few exceptions phylogenetic information carried by these sequences is enough to distinguish close species (Gazis *et al.* 2011, Lindahl *et al.* 2013).

The amplicon selected covered ITS2 (amplification by primers ITS3F_MiSeq and ITS4R_MiSeq; Table 2).

*Table 2*

***Primers used for Illumina sequencing (size of the amplicon varied between 400 and 500 bp)***

| *Primer* | *Sequence (5' – 3') [adapter][**target primer**]* |
|---|---|
| ITS3F_MiSeq | [TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG] [**GCA TCG ATG AAG AAC GCA GC**] |
| ITS4R_Miseq | [GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG] [**TCC TCC GCT TAT TGA TAT GC**] |

The amplicon were then sequenced (MiSeq, Illumina) and the data underwent bioinformatic processing to obtain an affiliation of the sequences. The bioinformatic analyses were performed using the state-of-art workflow in Qiime (Caporaso 2010).

The taxonomic assignment was done down to the species level whenever possible. The statistical analyses were performed on the communities based on the OTU (operational taxonomic units).

When the sequence was not assigned, the closest phylogenetic parent(s) was considered.

**RESULTS AND DISCUSSION**

***Nucleic acid extractions***

The nucleic acids of all the samples were extracted. The spectrophotometry results are shown in Table 3. As the concentrations obtained were satisfactory, we proceeded with PCR quality tests.

*Table 3*

***Concentrations of the DNA extracts***

| Sample No. on agarose gel | Short name | Extraction date | NA conc. | Unit |
|---|---|---|---|---|
| | | | | |
| 1 | 3a-roo | 16/06/2015 | 295.1 | ng/µl |
| 2 | 3a-tru | 16/06/2015 | 188.3 | ng/µl |
| 3 | 3a-bra | 16/06/2015 | 87.5 | ng/µl |
| 4 | N2-soi | 29/07/2015 | 457.2 | ng/µl |
| 5 | 3a-soi | 16/06/2015 | 257.5 | ng/µl |

| | | | | |
|---|---|---|---|---|
| 6 | 4a-soi | 16/06/2015 | 206.3 | ng/µl |
| 7 | 4a-bra | 16/06/2015 | 96.5 | ng/µl |
| 8 | 4a-tru | 28/07/2015 | 69.5 | ng/µl |
| 9 | 4a-roo | 28/07/2015 | 129.6 | ng/µl |
| 10 | N2-tru | 28/07/2015 | 77.8 | ng/µl |
| 11 | N2-bra | 28/07/2015 | 163.8 | ng/µl |
| 12 | N2-roo | 28/07/2015 | 147.3 | ng/µl |
| 13 | Th-Aga | 21/07/2015 | 224.7 | ng/µl |
| 14 | La-Aga | 21/07/2015 | 243.8 | ng/µl |
| 15 | Cam-1 | 21/07/2015 | 37.3 | ng/µl |
| 16 | Cam-2 | 21/07/2015 | 13.2 | ng/µl |
| 17 | BW-Fr | 21/07/2015 | 108.3 | ng/µl |

**ITS and F-RISA amplifications**

Some positive PCR amplifications of the expected sizes (around 500 bp) were obtained for all the samples (Fig. 1).

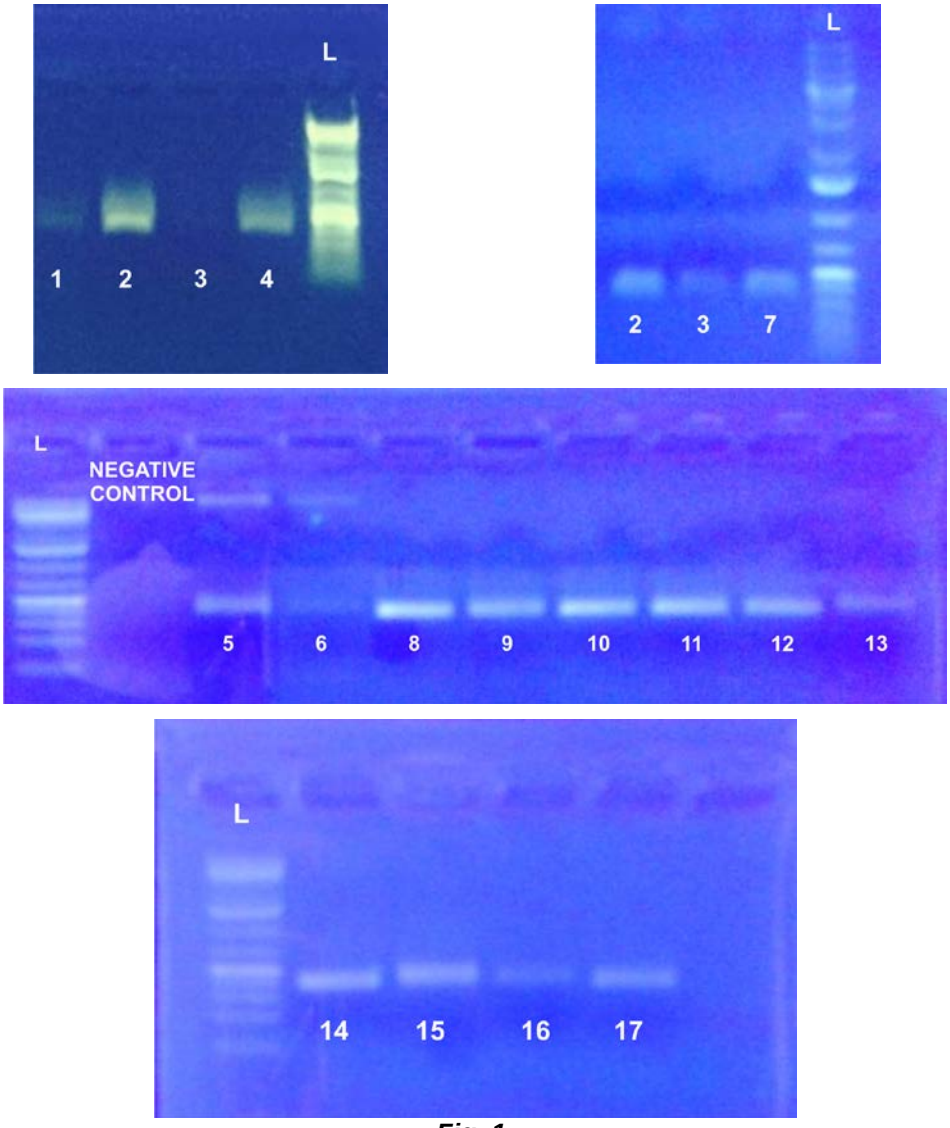


***Fig. 1.***
***Photo of the electrophoresis gels for the PCR ITS amplifications.***

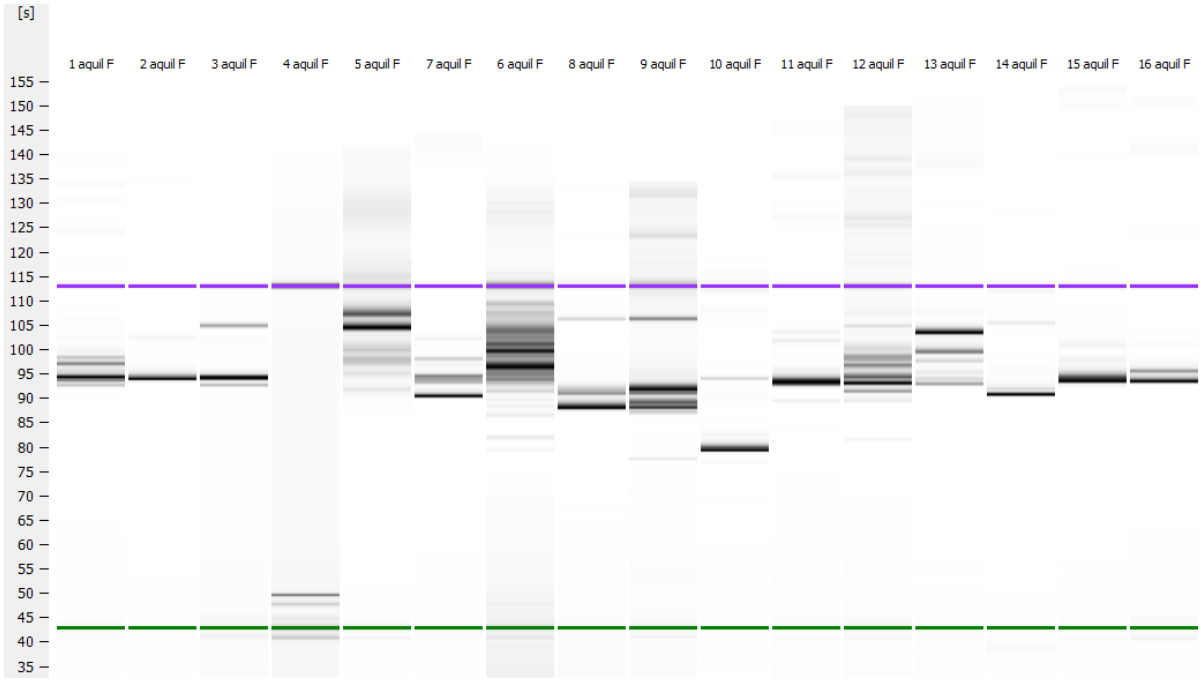


***Fig. 2.***
***Electrophoresis migration of the F-RISA amplifications. Sample 4 was validated in another experiment.***

These two steps enabled us to validate the DNA quality of each of the samples for sequencing.

**General presentation of the sequencing results**

***OTU obtained and assignment***

Sequencing led to the generation of 693, 961 sequences which were clustered to 535 OTUs. Each OTU was representative of a group of sequences displaying 98% identity.

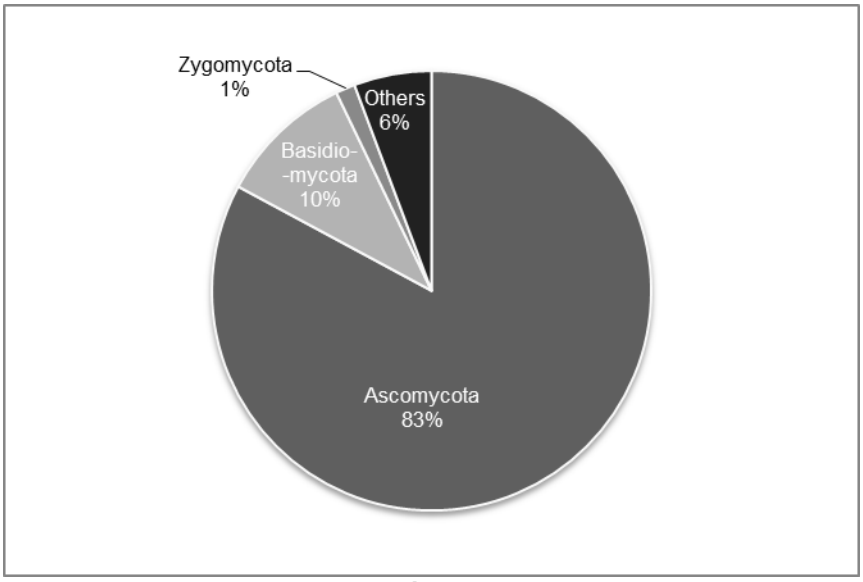


***Fig. 3.***
***693,961 sequences merged into 535 OTU, with 98% of homology.***

Of these OTUs, 19 were assigned to other kingdoms: plants (15) and animals (4, all from one of the soil samples). 183 of them were not assigned (no available correspondence in the reference database. They were labelled “No hit”).

Lastly, 333 OTUs (62%) were assigned to the fungus kingdom.

Of these 333 OTUs (Fig. 3.):

- 87% (290/333 OTUs) were assigned to Ascomycetes (Ascomycota).
- 10.5% (17/333 OTUs) were assigned to Basidiomycetes (Basidiomycota).
- 1.5% (5/333 OTUs) were assigned to Zygomycetes (Zygomycota) and more specifically the order of the Mucorales.
- 6% (17/333 OTUs) were assigned to the fungi without more detail.

***Relative abundances of the assigned genera***

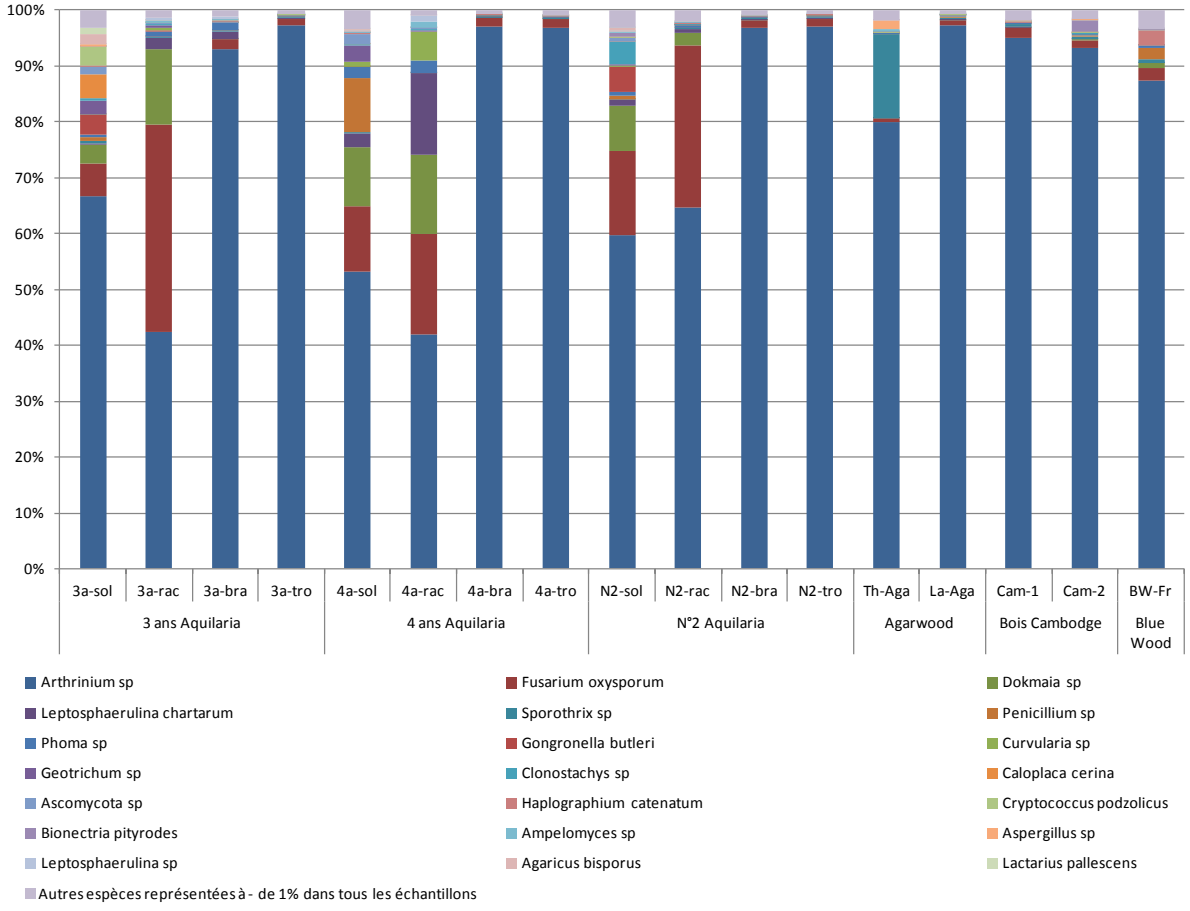


***Fig. 4.***
***Relative abundances of fungus species within the samples (Assigned sequences only).***

Fig. 4. shows the relative abundance, which is the number of sequences assigned to a taxon compared to the total number of sequences assigned.

*Arthrinium* was the most frequent genus in all the samples. Several OTUs per sample were assigned to *Arthrinium* indicating substantial diversity of *Arthrinium* sp. species (26 different OTUs were found to be affiliated to *Arthrinium* sp.). The proportion of this genus increased in the aboveground parts of the trees in French Guiana.

The genus *Arthrinium* is ubiquitous and ecologically diverse. It is commonly saprobic or saprophytic. It has been characterized as an endophyte but also as a plant pathogen (Crous and Groenewald, 2013).

One of the assigned OTUs was present in almost all samples: *Arthrinium* sp. (OTU136). Surprisingly, the *Arthrinium* sp. Sequence (OTU136) was also found in a blue wood metagenome from a decaying trunk sampled in France.

Though it cannot be ruled out that the genus may be implicated in “agarwood” production, but it is not very likely that it is a causative agent, or in any case not alone, insofar as the trees in French Guiana are not yet producing agarwood despite its consistent presence.

Among the other species of interest there were:

- The pathogenic *Fusarium oxysporum,* particularly present in the soil and root samples (non-infected trees). It was weakly present in the ”agarwood”, but it cannot be ruled out that it may be implicated in primary infection. A total of 58 OTUs were assigned to *Fusarium oxysporum*.

- The species *Dokmaia* sp. exhibited a similar pattern; like *Arthrinium, Dokmaia* is a saprobe (9 OTUs were assigned to *Dokmaia* sp.).

- *Sporothrix* sp., which was particularly represented in the Tha-Aga sample.

***Differences between aboveground and belowground fungal communities***

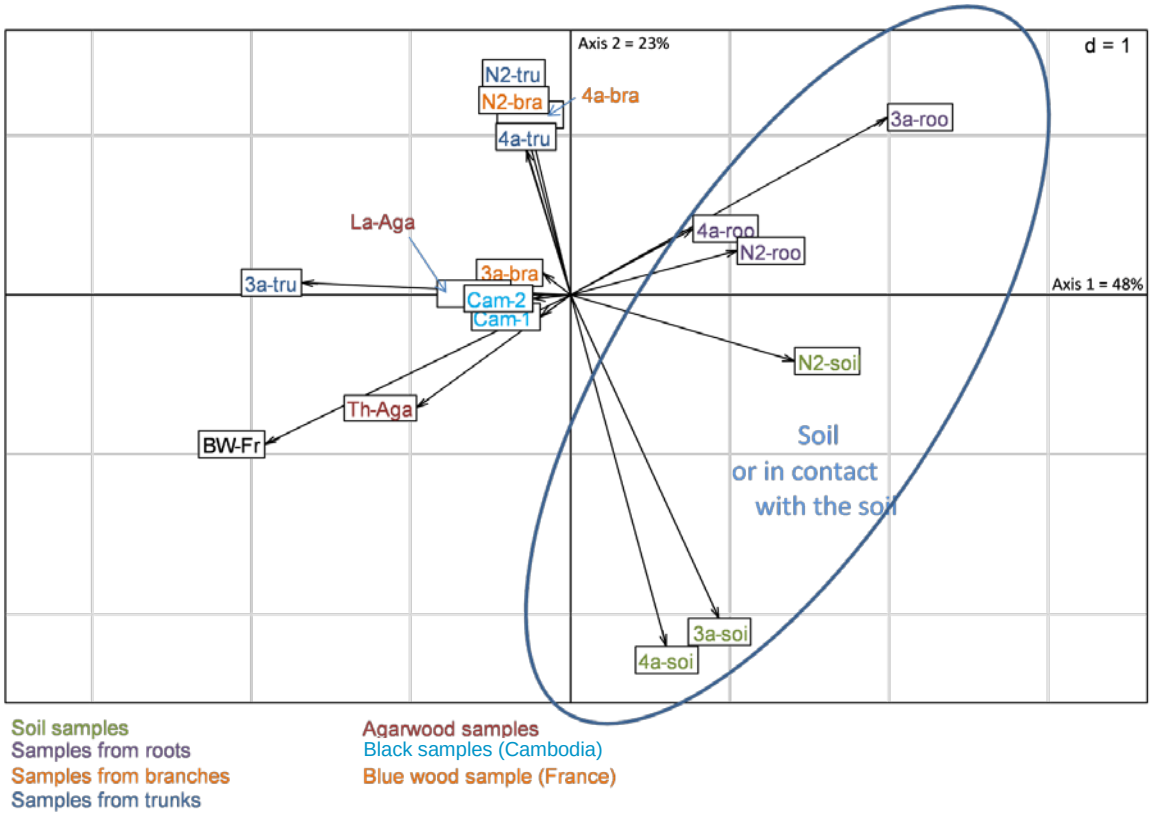


***Fig. 5.***
***PCoA of relative abundances of the OTU table (all samples) and based on the Chao distance matrix.***

Fig. 5 shows that the soil samples and the samples in contact with the soil stands out. This representation also shows that samples 3a-tru, Th-Aga and BW-Fr were relatively close. As two of these samples were infected by some fungi, one hypothesis might be that 3a-tru also displays infection or the start of infection.

***Differences between countries within fungal communities***

The differences in *Aquilaria* communities were also specifically addressed . In these analyzes the soil samples and the sample from France (blue wood) were omitted. Fig. 6 shows the PCoA of the samples specific to *Aquilaria*. The samples from the roots were again separated from the samples of the aboveground parts of the plant. The agarwood community from Thailand differs from the one from Laos.

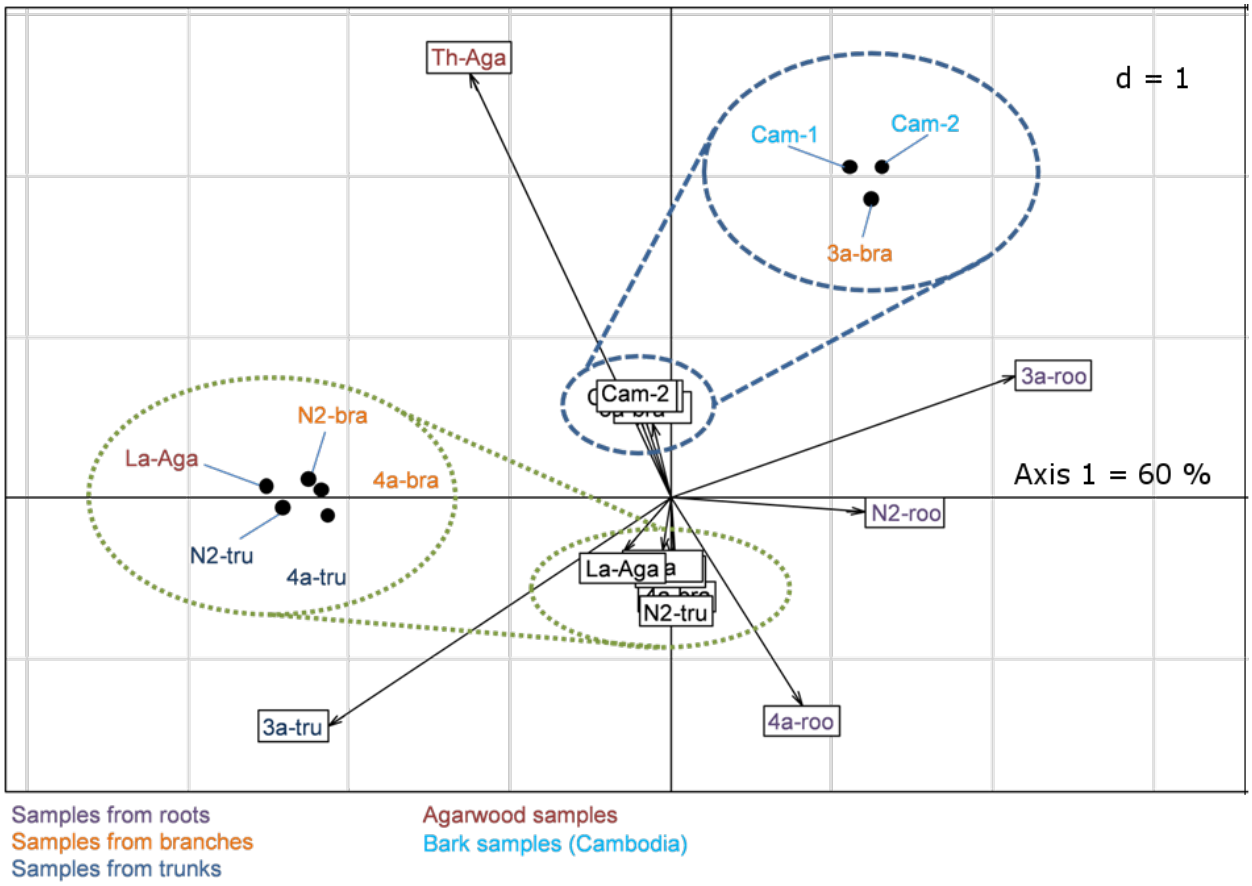


***Fig. 6.***
***PcoA of the relative abundances on the OTU of the samples specific to Aquilaria and based on the Chao distance matrix***.

## CONCLUSIONS

The soil samples and the *Aquilaria* spp. samples in direct contact with the soil (i.e. the root samples) had different communities from the samples of the aboveground parts of *Aquilaria*.

The sample from Laos displayed a community structure closer to that of the non-infected samples, which may mean: either that *Aquilaria* wood can be infected by different fungi and that, as it happens in our study, the two infections were not caused by the same fungi, or that others factors (post-infectious) influenced the communities and led them to diverge.

Overall, the genus *Aquilaria* revealed close fungi communities whatever the location of the tree.

Some candidates potentially involved in resin production by *Aquilaria,* following infection of the tissues, can be proposed. The candidate fungus genera are: *Arthrinium*, *Chloridium*, *Fusarium (*species *oxysporum*) and *Sporothrix*.

In an experiment using mechanical inoculation, Mohamed *et al.* (2014) monitored tree infection by three fungus species. The experiment revealed that the fungi communities evolved towards different populations in two neighbouring trees. It is therefore certain that several pathogens can induce the reaction and/or that a succession of the genera is to be expected.

To continue this study, fungal communities from infected trees non-infected trees needs to be compared, possibly in different locations. Also carrying out research on the fungi communities in surface soils from other sites in Southeast Asia would help to identify putative source of inoculum. Some colonization tests are ongoing to confirm the infectious nature of some of the candidate fungi.

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