ONLINE ISSN 2069-7430
ISSN-L 1841-4737
PRO LIGNO
www.proligno.ro
Vol. 15 N° 3 2019
pp. 3-16

TAXONOMIC STUDY OF FRENCH GUIANA FUNGI TO IDENTIFY AND ISOLATE PURE CULTURED FUNGI FOR OLEORESIN PRODUCTION IN *AQUILARIA*: USE OF SEQUENCES FROM THE SMALL RIBOSOMAL DNA (R) SUBUNIT (SSU) AND THE TWO PRIMER PAIRS SR6/SR10R AND SR7/SR1R

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Abstract:

Fungi from tropical regions are currently under-represented in the classification system. Indeed, difficult access to tropical forests makes it complicated to study fungus species in such environments, unlike in European zones where fungal diversity and taxonomy are much better known. The purposes of this work were: a) to enhance classification by integrating new data that would bring out the importance of certain traits of these fungi, and provide a clearer understanding of how the biodiversity of fungi from the forest ecosystems of French Guiana is organized, particularly those causing wood decay through white rot, brown rot or soft rot; b) to establish a collection of fungal isolates from fruiting bodies collected in French Guiana.

This taxonomic study based on sequences of the small subunit (SSU) of ribosomal (r) DNA using the two primer pairs, SR6/SR10R and SR7/SR1R, was carried out on 39 fungus fruiting bodies from Cacao and Régina, in French Guiana. Our protocol enabled extraction, with good repeatability, of fungal DNA from a few mg of pure mycelium. With most of the strains studied we were able to obtain PCR products ranging in size from 376 to 625 base pairs.

This study enabled to specify the taxa involved wood decay: 39 species were identified by BlastN. Most of them, in the class of the Basidiomycetes, were the main genera were found, such as Antrodia, Coriolopsis, Fomitopsis, Ganoderma, Poria, Lentinus, Pycnoporus, Auricularia, Gloeophyllum, Trametes, Fomitopsis, Rigidoporus.

In addition to the above objectives, the production of pure mycelium from fruiting bodies identified in this study will be used to produce inoculum to test the ability of these fungal species in stimulating oleoresin production in Aquilaria trees from plantation established in French Guyana.

Key words*: Aquilaria; Basidiomycete; French Guiana; BlastN; primer pairs SR6/SR10R and SR7/SR1R; SSU rDNA; Wood-decaying fungi.*

INTRODUCTION

French Guiana lies in the Amazon region. With forest covering 80% of its territory, it is the largest forest mass and the largest tropical forest in the European Union.

The forest of French Guinea is characterized by a very wide diversity of trees species, with 1,200 species inventoried to date, as opposed to fewer than 200 species inventoried in mainland France. Of those species, some stand out through their great resistance to extreme climatic conditions (high moisture rate) and decomposition processes. Such processes induce the production of specific and unique enzyme systems in lignocellulolytic fungi.

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Although some recent work has led to the identification of new species of fungi in French Guiana (Horak and Cheype 2007, Cheype and Campo 2012, Cheype 2015), knowledge of fungal diversity there remains very limited. Indeed, Courtecuisse *et al*. (2012) estimated there to be 25,000 fungus species, of which only 3% are described; our knowledge of the diversity of lignocellulolytic fungi in French Guiana remains scant.

This work will focus particularly on those causing wood decay. Depending on their phenotype, they can be classed as white rots (primarily Basidiomycetes with predominant ligninolytic activity), brown rots (primarily Basidiomycetes that are both ligninolytic and hemicellulolytic), and soft rots (hemicellulolytic Deuteromycetes).

So far, conventional methods based on a description of the morphological and anatomical traits of fungi have remained unavoidable. However, they have their limitations for characterizing and identifying these fungi on an inter- and intraspecific level. They remain laborious and cannot usually be used to identify mycelium cultures.

It seems essential to propose fresh alternatives for identifying wood-decaying fungi.

Thanks to the progress made in high-throughput sequencing technologies and the development of bioinformatics tools dedicated to fungal analysis, it is possible to conceive the fungal component of microbiotas, its structuring, its dynamics, its interaction with the host, or environmental factors.

In this study, molecular techniques combining amplification and sequencing seem to be particularly suitable (Zaremski et *al*. 2005). Molecular tools such as DNA polymerization chain reaction (PCR) and sequencing could provide sufficient characterization to recognize a fungal taxon. Identification was carried out by comparing the sequence of the fungus strain to be identified with sequences from GENBANK (www.ncbi.org). In this type of application, sequence libraries are an essential element in taxonomic characterization.

All the studies carried out during this work had multiple objectives:

- Enhance classification by integrating new data that would bring out the importance of certain traits of these fungi, and provide a clearer understanding of how the biodiversity of fungi from the forest ecosystems of French Guiana is organized, particularly those causing wood decay through white rot, brown rot or soft rot;
- Establish a collection of fungal isolates from fruiting bodies collected in French Guiana.
- Establish and develop a collection of pure fungal isolates from fungal fruiting bodies for future industrial applications, including the production of agarwood in *Aquilaria* trees from plantations established in French Guiana.

The species biodiversity concept

Virtually all diversity studies are carried out using PCR to amplify rDNA existing in natural samples from which DNA has been extracted. The fidelity of microflora representativeness can therefore be questioned, as this approach has its own limitations and biases. Depending on the extraction procedure followed, lysis can be more or less selective, and the quality of the nucleic acids may vary (Theron and Cloete 2000). Consequently, microbial diversity can be more or less correctly estimated. Artefacts linked to PCR can also bias diversity evaluations. Indeed, amplifications of different DNA matrices (Reysenbach *et al*. 1992, Suzuki and Giovannoni 1996), the influence of the number of copies of rRNA genes and their microheterogeneity (Farrelly *et al*. 1995, Amann *et al*. 2000, Crosby and Criddle 2003), the effect of DNA-matrix concentration, the specificity of the primers (Weisburg *et al*. 1991) and the formation of chimerical sequences (Kopczynski *et al*. 1994) are well-known phenomena in PCR. Apart from their inherent biases, the most widely used molecular diversity analysis methods are unable to attribute a physiology and a metabolism from detected sequences. Culturing and molecular approaches, enabling each to answer different and very precise questions, therefore prove to be complementary. The emergence of culture techniques and the development of techniques making it possible to infer both a function and a phylogenetic position should considerably improve our knowledge of microbial diversity.

Taxonomic characterization methods applicable to fungi

Today, no single pair of primers or technique used to study the profiles of communities is optimum for estimating microbial diversity; they have to be chosen to suit the purpose of the research, the targeted group of microorganisms, and databank content. For example, analysing fungal diversity according to richness and relative abundance, along with community dynamics, is complicated by the mycelial nature of fungi and by the potential existence of spores in a given sample (Anderson and Cairney 2004).

Fungi, which are eukaryotic organisms, do not possess the same ribosomal RNA as bacteria, which are prokaryotic organisms. They therefore have to be analysed separately. However, studying diversity through molecular biology is less common in eukaryotes, particularly in fungi. The choice of primers, hence of the target gene to be amplified to identify the fungi of an environmental sample, is therefore still under discussion. However, studying the DNA region encoding ribosomal subunits is now established. Figure 1 shows this gene.

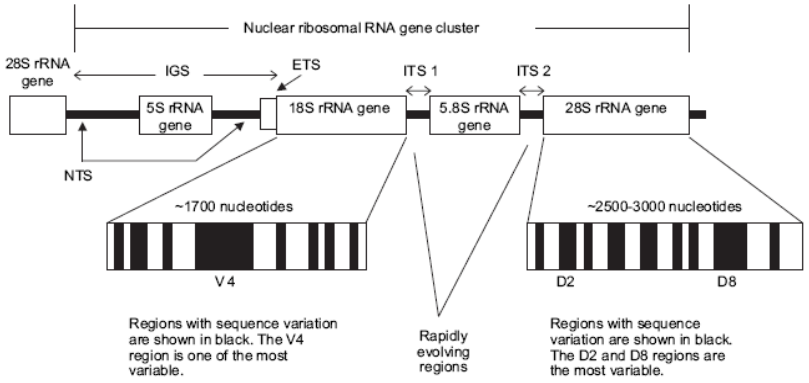

Fig. 1.
Operon of the ribosomal rRNA gene of eukaryotes. The operon comprises three main genes (molecules of 5.8S, 18S and 28S rRNA) and intermingled "interspace" regions (IGS – intergenic spacer, NTS – non-transcribed spacer, ETS – externally transcribed spacer, ITS - internally transcribed spacer; According to Mitchell and Zuccaro 2006).

Unlike for bacteria, taxonomic identification of fungi from sequences of the ribosomal subunit of eukaryotes, 18S rRNA, is more problematic. Indeed, identification is limited to the genus or family level. This is firstly due to the relative lack of variation in the 18S rRNA gene between relatively close fungal species, resulting from a relatively short period of evolution for the fungus kingdom compared to that of bacteria (Zhou *et al.* 2000; Anderson and Cairney 2004).

In our study, we chose to work on the SSU, the most widely used region for establishing phylogenetic trees and identifying strains. Two primer pairs SR6/SR10R and SR7/SR1R were chosen for this taxonomic analysis of our study species. These primers were designed to amplify the two ends of the specific regions of the gene of the 18S ribosomal small subunit (SSU) in fungi.

With these molecular tools it will be possible to study the diversity and taxonomy of 39 fungus fruiting bodies from forest stands in Cacao and Régina, in French Guiana, particularly wood-decaying fungi. The study will not be limited to morphological, histological and anatomical observations of the fruiting bodies, which remain unavoidable but laborious. Thus, the study of the specific regions of the gene of the 5.8 S ribosomal small subunit (SSU) using the two primer pairs SR6/SR10R and SR7/SR1R should enable the identification and discrimination of most of these fungus species from a pure mycelium culture.

The main stages involved in this study were as follows:

1) Isolation in pure cultures and production of pure mycelium from fruiting bodies.
2) Development of molecular characterization techniques: extraction and purification of the DNA of the fungus from pure mycelium; development of DNA amplification.
3) Sequence analyses: comparison of sequences and identification using BlastN and GENBANK.

Experimental Methods

You will find below a global diagram representing the necessary steps for the identification of the fungi studied, from sampling to identification (Fig. 2. Global diagram representing the necessary steps for the identification of the fungi studied).

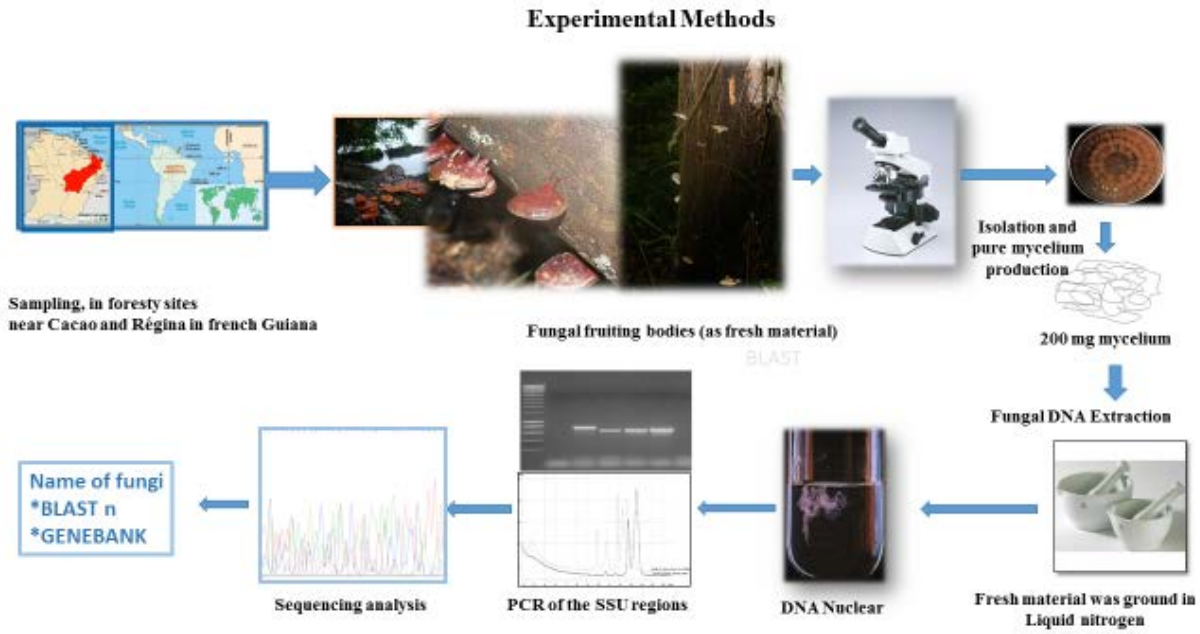

Fig. 2.
Global diagram representing the necessary steps for the identification of the fungi studied.

Sampling

For our work, collections were made in forest sites near Cacao and Régina. Régina and Cacao are two community of eastern French Guiana (on the map in red). Régina: 4° 18′ 42″N, 52° 08′ 13″W; Cacao : 4° 33′ 12.58″ N, 52° 29′ 31.78″ W.

Fungi were chosen fresh and in good condition on dead wood (25 fungi; Table1), and on standing trees (14 fungi; Table 1).

They were gently removed with a knife at the extreme base of the stem, to avoid damaging fragile elements.

Each sample was wrapped in aluminium foil with the corresponding label, before being placed in a box or case. Each sample was thus classed by numerical collection order (from C-01 to C-18; C for Cacao); from R-01 to R-21; R for Régina).

At least two specimens of each fungus were collected, as some were to be used to describe macroscopic traits and others for microscopic examination; a specimen can be kept in the refrigerator for 1 week or in the freezer at -80°C for an indefinite period.

At the same time, each fruiting body was to be photographed (general view) on site, in the forest, or after collection in the laboratory. The photos were to be used to compile technical data sheets for each fungus. An example of a file sheet in Appendix 1.

Table 1

Fungi were chosen fresh and in good condition on dead wood (25 fungi), and on standing trees (14 fungi)

Strain N°	on dead wood (W) on standing trees (T)
Fungi from Cacao	
C01	W
C-02	W
C-03	W
C-04	W
C-05	T

C-06	T
C-07	W
C-08	W
C-09	W
C-10	T
C-11	T
C-12	T
C-13	T
C-14	W
C-15	W
C-16	T
C-17	T
C-18	W
Fungi from Régina	
R-01	T
R-02	T
R-03	W
R-04	W
R-05	W
R-06	W
R-07	W
R-08	T
R-09	T
R-10	T
R-11	W
R-12	T
R-13	W
R-14	W
R-15	W
R-16	W
R-17	W
R-18	W
R-19	W
R-20	W
R-21	W

Isolation and mycelium production

All the fruiting bodies collected were cleaned (with distilled water and diluted alcohol) and stored in the freezer.

Pieces of the pileus trama of all fruiting bodies (39) were grown in Petri dishes for a week.

It was proved the necessity to search for a culture medium conducive to mycelium growth, notably for most of the samples which revealed difficulties in growing on the M.A (Malt Agar) medium conventionally used in the laboratory. It was the PDA (Potato Dextrose Agar) medium containing the antibiotic streptomycin that was chosen.

The 90 mm diameter Petri dishes contained around 25 ml of an PDA medium (Potato Dextrose Agar) prepared in the following concentrations: potatoes (39 g/l), agar (15 g/l), glucose (20 g/l), in 1 litre of distilled water. Before pouring into the Petri dishes, 1 ml (5.10^{-3} g/l) of streptomycin was added to the culture medium to reduce bacterial contaminants.

Three Petri dishes were used for each sample, to have a better chance of successful subculturing.

Cubes of 2-3 mm in size were taken from this growing culture and subcultured. The subcultures were left to incubate in the dark at 20°C and a relative humidity 75%, only bringing them out into the light to examine them. They were examined every 2 weeks for 6 weeks.

Once the culture was found to be pure, it was transferred to 3 dishes, then definitively maintained in a Petri dish to build up a fungus library.

Molecular studies

Fungal DNA extraction

200 mg of the collected fungal fruiting bodies (as fresh material) were ground in liquid nitrogen.

An insoluble tip of a PVPP (Polyvinyl-polypyrrolidine; C_6H_9NO) spatula was added to the mortar to trap the polyphenols present in the cells.

The ground mixture was then exposed to 1.4 ml of hot extraction buffer added to the mortar.

The extraction buffer (Tris 100 mM pH 9; 1.4 M NaCl; 20 mM EDTA pH 8; 2% CTAB; 0.2% β-mercaptoethanol; qs H_20 15 ml) caused the lipid membranes in the cells to burst, releasing the DNA inside them.

The solutions were placed in 2-ml Eppendorf tubes and incubated at 65°C for 30 minutes.

The proteins and polysaccharides still present in the tubes were eliminated by adding 600 µl of dichloromethane solution (CH_2Cl_2): chloroform ($CHCl_3$), isoamyl alcohol ($C_5H_{12}O$) (24:24:1). The aqueous phase was then recovered after centrifuging (13,500 rpm at 20°C, for 15 minutes).

DNA precipitation was achieved by adding 1 ml of isopropanol (C_3H_8O) along with 0.1 ml of 3M sodium acetate (CH_3CO_2Na). The tubes were placed for one hour at -20°C to facilitate DNA precipitation, then centrifuged at 10,000 rpm at 4°C for 10 minutes.

The DNA of the extracted strains was washed with 1 ml of 70% ethanol to eliminate traces of isopropanol, then centrifuged at 13,000 rpm at 0°C for 10 minutes.

The residue was dried in a Speed Vac then suspended in 100 µl of miliQ water.

DNA from the extracted strains was quantified on a Nano Drop ND-100 spectrophotometer. It measured the concentration and purity of the DNA extracted with 2 µl of sample. The purity index was calculated using the absorbance measurement ratio at 260 and 280 nm. This 260:280 ratio needed to approach 1.8 to qualify it as a pure sample. Some smaller values would seem to indicate the presence of impurities or proteins absorbing in the vicinity of the same wavelengths.

PCR of the SSU regions

Two pairs of primers, SR6/SR10R and SR7/SR1R, were chosen for the species studied. These primers were designed to amplify specific regions of the gene of the 18S ribosomal small subunit (SSU) in fungi (Bruns *et al.* 1992). These two primer pairs amplified the two ends of the 18S gene so as to have a maximum amount of genetic information for this gene (Fig. 3. Scheme of the 18S ribosomal small subunit (SSU) and the primers defined for fungi).

SR6: $^{5'}$ TGTTACGACTTTTACTT $^{3'}$; SR10R: $^{5'}$ TTTGACTCAACACGGG $^{3'}$
SR7: $^{5'}$ GTTCAACTACGAGCTTTTTAA $^{3'}$; SR1R $^{5'}$ TACCTGGTTGATQCTGCCAGT $^{3'}$

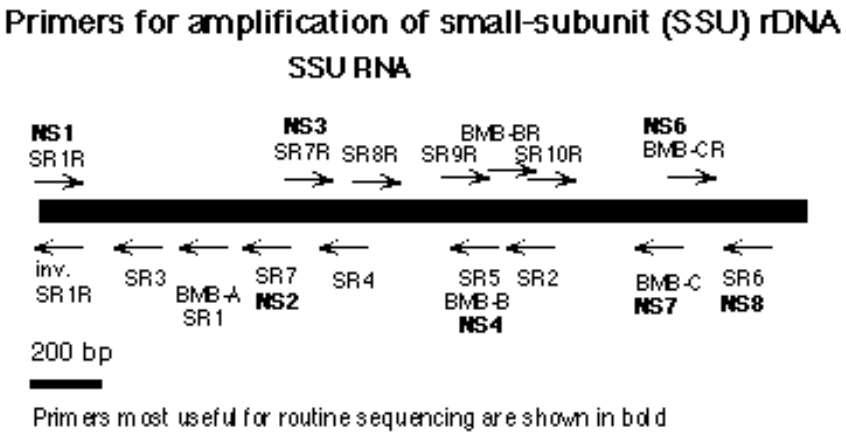

Fig. 3.
Scheme of the 18S ribosomal small subunit (SSU) and the primers defined for fungi.

PCR reactions were carried out in 96-well plates. The plates were prepared by adding the following to each well: 1.4 µl of miliQ water, 2 µl of 10X buffer, 0.4 µl of $MgCl_2$ (50 mM), 1µl of dNTP (2.5 mM), 8 µl of soluble PVP (5%), 1 µl for each forward primer (SR6 or SR7; 10 µM) and 1 µl for

each reverse primer (SR10R or SR1R; 10 µM), 0.2 µl of Taq Polymerase (5 U/µl), 5µl of matrix DNA diluted to 10^{-2}.

The PCR programme used for the samples tested was as follows:

- Initial denaturing: this was done by heating above the melting temperature (Tm) of the DNA, at 94°C for 5 min, leading to breakage of the hydrogen bonds that maintained the double-stranded DNA, established between the bases (3 bonds between G-C and 2 between A-T). This gave a single-stranded DNA molecule ready for hybridization with the primers.
- This initial denaturing was followed by 35 cycles in *3 steps*:
    1) *denaturing* at 92°C for 45 s;
    2) *hybridization*: the temperature was gradually lowered towards a temperature depending on the hybridization temperatures (Th) of the two primers used, which therefore had to be similar. This Th was around 5 to 10°C lower than the melting temperature. The latter depended on the base composition of the primers and was calculated by the following formula: Tm = 2x (A+T) + 4x (G+C). This thus gave Tm (SR6/SR10R) = 44°C and Tm (SR7/SR1R) = 58°C; hybridization took place for 1 min. Under these conditions, the primers bound themselves to each strand, either side of the region to be amplified;
    3) *elongation:* the temperature was raised to reach the optimum temperature (72°C) for Taq polymerase which then synthesized two new strands from the primers by taking up complementary dNTPs from the matrix strand in the medium. Elongation took place for 2 min.
- The last phase consisted of *final elongation* at 72°C for 5 min.

The PCR products were stored at 4°C.

The presence of PCR products was revealed under UV light after migration in 0.8% agarose gel with an ethidium bromide bath. The size marker used was the 1Kb Plus DNA Ladder.

PCR reaction residues (primers and free dNTPs) can harm sequencing quality. They were eliminated and purified by an enzyme, l'ExoSAP-IT® (Exonuclease I-Shrimp Alakaline Phosphatase). ExoSAP-IT® breaks down primers or single-stranded nucleotide sequences, alkaline phosphatase hydrolyses surplus dNTPs in PCR reactions.

For each sample, 2 µl of ExoSAP-IT® was added to 10 µl of PCR products.

In an initial stage, these two enzymes were activated for 15 min at 37°C during which excess primers and dNTPs were eliminated. ExoSAP-IT® was inactivated after incubation at 80°C for 15 min.

Sequencing of PCR products

The amplified samples were sent for sequencing to the Macrogen Company in South Korea, which uses the 3730XL DNA SEQUENCER.

In this study, after obtaining and correcting the sequences based on the interpretation of the electrophoretograms with CHROMAS PLUS software, we proceeded with BlastN on the Internet (http://www.ncbi.nlm.nih.gov/BLAST) to compare the sequences obtained with those existing in the libraries.

Sequence analysis by comparison with BLAST databases

The identity of a fungal sequence is obtained after a homology search in databases such as "Genbank" at the National Center for Biotechnology Information (NCBI, http://ncbi.nlm.nih.gov/BLAST/), supported by the European Bioinformatics Institute (EMBL-Bank, http://www.ebi.ac.uk/embl/), the "Ribosomal Database Project" (http://rdp.cme.msu.edu/http://www.psb.ugent.be/rRNA/blastrrna.html), and the AFTO project (http://aftol.biology.duke.edu/pub/blastUpload) (Mitchell and Zuccaro 2006). Unknown sequences are aligned with those that are known and present in the databases. Searches using the BlastN at NCBI supply a list of probable results corresponding to the unknown sequence, with the sequence similarity values. BLAST is a specially developed method to compare an unknown nucleic or proteic sequence to all those found in nucleic databases *(http://www.ncbi.nlm.nih.gov/BLAST).*

The purpose of sequence comparison programmes is to identify the places where identical or very similar regions are found between two sequences, and to deduce those that are significant and correspond to a biological sense of those observed by chance. BlastN detects short segments (eleven identical nucleotides or two similar tripeptides), which are locally homologous to the unknown sequence (Altschul *et al*. 1997).

Sequence comparison results are presented in the form of a homology ratio between the sequences obtained and the nearest reference sequences.

Compilation of a reference sequences file

When BlastN is performed, all the reference sequences close to our strains are extracted from the NCBI databases. They thus constitute a source of reference sequences, an essential tool for our taxonomic studies and for future phylogenetic studies.

RESULTS AND DISCUSSION

Collection of fungus fruiting bodies

It took around five days to collect 39 fungus fruiting bodies from dead wood in Cacao and Régina forest stands (Annex 2).

Isolate culture, obtaining pure mycelium

Numerous contaminations were encountered in the Petri dishes during isolation. These were mainly caused by the presence of bacteria, moulds, insects and mites from the samples. The contaminants generally developed in colonies or as mycelium radiating out into the medium. Often, the contaminants followed the evolution of the mycelium and effectively have resisted to the antibiotics. Insects and mites often brought moulds with them, being difficult to achieve isolation in those cases.

In this context, due to the large number of contaminations during isolations, also the successive subcultures were carried out before a pure strain to be obtained.

It appeared that it was essential to carry out these isolations within 24 hours after harvesting, otherwise the samples, rotting very quickly.

Other isolation techniques will be explored for this type of isolation (protocols for fresh tissue drying after harvesting, preparation of other media, addition of other antibiotics, acaricides in the culture media etc.)

DNA extraction

Extraction was optimized by adapting the protocol used in the laboratory. The DNA of the 39 fungi studied was thus extracted and amplified. The concentration and purity of the total DNAs extracted were assessed. The quantities of DNA obtained varied from 9 to 118 ng/ml depending on the samples. These results can be explained by the fact that coloured residues were obtained, probably due to the presence of pigments, tannins or impurities, "distorting" the Nano Drop measurements made. In the last case, an additional step can be envisaged in which the residue is washed during DNA extraction.

The concentrations of the extracted DNAs can be found in Annex 2.

Amplification of the rDNA SSU fragment

After testing several experimental conditions, the amplification protocol was well suited to the specific region of the gene of the rDNA SSU for 100 % of the fungi, for which we obtained a band with a molecular weight of between 223 and 635 pb. The sizes of the PCR product obtained were estimated using the 1KbPlus DNA Ladder marker. Overall, the size of the rDNA SSUs of the studied strains varied considerably.

Sequence analysis by comparison with BlastN databases

Only one band was always visualized on electrophoresis gel. This confirmed the specificity of the SR6/SR10R and SR7/SR1R primers.

All sequences were corrected after interpretation of the electrophoretograms using CHROMAS and BIOEDIT software.

The similarity search was therefore conducted on all sequences using BlastN on the Internet *(http://www.ncbi.nlm.nih.gov/*BLASl) to compare our sequences with those existing in databases.

The results of the sequence comparisons are given in the form of a homology ratio between the sequences obtained and the nearest reference sequences.

Annex 3 shows the similarity searches for our sequences with database sequences. In this annex, indicate the number of the strain studied, the name of the fungus identified by BlastN, its reference number or accession number for the primer pairs (SR6/SR10R) and (SR7/SR1R), the number of base pairs for each of the sequences (npb) and the length of the sequence that was compared using BlastN with its identity percentage in brackets.

After BlastN, we found the following results: 77% of these sequences had over 90% similarity over more than 80% of their length.

Out of 39 fungi, all can be assigned at least to the genus level.

In the order Polyporales, we found the main genera such as *Antrodia, Antrodiella, Coriolopsis, Fomitopsis, Ganoderma, Gloeophyllum, Lentinus, Pycnoporus, Poria, Rigidoporus.* These fungi all

have the particularity of causing wood decay.

CONCLUSIONS

In order to characterize the 39 fungi from forest sites, near Cacao and Régina, in French Guiana, developing in a particular ecosystem (an air temperature of 35°C with 90% humidity), we developed a rapid, discriminant method based on analysing the diversity of sequences of the rDNA SSU using two primer pairs, SR6/SR10R and SR7/SR1R. This characterization was carried out independently of macro- and microscopic traits of fruiting bodies required for the identification of genera and species.

Our protocol enabled extraction, with good repeatability, of fungal DNA from a few mg of pure mycelium. With most of the strains studied we were able to obtain a PCR product ranging in size from 223 to 635 base pairs.

BlastN analyses of the sequences obtained with 39 strains of fungi from French Guiana also enabled us to set the resolvent limits of the fragments studied to characterize these strains. The results of the BlastN analyses undertaken with our sequences in the NCBI database enabled us to consider using this tool for minimal identification of the genus of a strain for which we did not have information on the morpho-anatomical characteristics of the fruiting bodies, but for which we knew that numerous sequences of the genus were available.

In order to fine-tune this molecular taxonomic characterization, it would seem advisable to use the others molecular tools. Today, the recent contribution of molecular tools to high-throughput sequencing (meta-omic approach) sheds fresh light on the study of taxonomic diversity, ecology and the role of microbial communities. In particular, these tools have led to changes in paradigms and to new concepts (Rosenberg and Zilber-Rosenberg 2011, Vayssier-Taussat *et al.* 2014, Barret *et al*. 2016).

Lastly, this taxonomic study of fungi, which has made it possible to identify and isolate French Guiana fungi in pure culture, may be used for further research on the production of oleoresin in *Aquilaria* from the pure mycelium of these identified fungi.

ACKNOWLEDGEMENT

This study was undertaken as part of the *Aquilaria* project (Scientific and technical basis for the creation of a supply chain for top-of-the-range *Aquilaria* (Agarwood) essential oils and by-products in French Guiana), funded by the European Commission. Regional Development Fund (ERDF) for French Guiana (contract No. FEDER / 2017 / N ° 31).

REFERENCES

Altschul SF, Madden TL, Schaffer AA, Zhang J, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25: 3389-3402.

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiological Reviews* 59(1): 143-69.

Anderson IC, Cairney JWG (2004) Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. *Environmental Microbiology* 6(8): 769-779.

Barret M, Guimbaud JF, Darasse A, Jacques MA (2016) Plant microbiota affect seed transmission of phytopathogenic microorganismes. *Molecular Plant Pathology* 17(6): 91-795.

Cheype JL (2015) Quelques champignons étonnant de la Guyane française. *Bulletin de la société Mycologique et Botanique du Dauphiné-Savoie* 218: 53-63.

Cheype JL, Campo E (2012) *Russula rubropunctatissima* Cheype & E. Campo une russule découverte en Guyane française. *Bulletin de la Société Mycologique de France* 128(1-2): 127-135.

Courtecuisse R, Decock C, Fournier J, Gruhn G, Henkel T, Lechat C, Welti S, Roy M, Schimann H, Sagne A (2012) Un inventaire taxonomique et moléculaire des champignons de Guyane. Nouragues, Paracou, *Rapport de mission FunGUY*, pp. 1-35.

Crosby LD, Criddle CS (2003) Understanding systematic error in microbial community analysis techniques as a result of ribosomal RNA (rrn) operon copy number. *BioTechniques* 34(4) : 790-803.

Horak E, Cheype JL (2007) *Entoloma dragonosporum* (Singer) E. Horak et *E. luteosplendidum* sp. nov. deux espèces remarquables de la Guyane française. *Bulletin de la Société Mycologique de France* 123 (3-4): 251-262.

Farrelly V, Rainey FA, Stackebrandt E (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Applied and Environmental Microbiology* 61(7): 2798-2801.

Kopczynski ED, Bateson MM, Ward DM (1994) Recognition of chimeric small-subunit ribosomal RNA sequences composed of genes from uncultivated microorganisms. *Applied and Environmental Microbiology* 60: 746-748.

Mitchell JI, Zuccaro A (2006): Sequences, the environment and fungi. *Mycologist* 20(2): 62-74.

Reysenbach AL, Giver LG, Wickham GS, Pace NR (1992) Differential amplification of rRNA genes by polymerase chain reaction. *Applied and Environmental Microbiology* 58(10): 3417-3418.

Rosenberg E, Zilber-Rosenberg I (2011) Symbiosis and development: the hologenome concept. *Birth defects Research* 9: 56-66.

Suzuki M, Giovannoni SJ (1996) Bias caused by template annealing in the amplification of 16S rRNA genes by PCR. *Applied and Environmental Microbiology* 62: 625-630.

Theron J, Cloete TE (2000) Molecular Techniques for Determining Microbial Diversity and Community Structure in Natural Environments. *Critical Reviews in Microbiology* 26(1): 37-57.

Vayssier-Taussat M, Albina E, Citti C, Cosson JF, Jacques MA, Lebrun MH, Le Loir Y, Ogliastro M, Petit MA, Roumagnac P, Candresse T (2014) Shifting the paradigm from pathogens to pathobiome: new concepts in the light of meta-omics. *Frontiers in Cellular and Infection Microbiology* 4: 29-41.

Weisburg WG, Barns SM, Pelletier DA, Lane DL (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* 173(2): 697-703.

Zaremski A, Ducousso M, Domergue O, Fardoux J, Rangin C, Fouquet D, Joly H, Sales C, Dreyfus B, Prin Y (2005) *In situ* molecular detection of some white-rot and brown-rot basidiomycetes infecting temperate and tropical woods. *Canadian journal of forest research* 35: 1256-1260.

Zaremski A, Gastonguay L, Zaremski C, Beauchêne J (2014) Molecular characterization and biodiversity of wood-decaying fungi in French Guiana. In: IRG, *45th IRG Annual Meeting, 11-15 May 2014*, *Saint George, United States of America,* 16p.

Zaremski C, Malandain C, Sibourg O, Andary C, Michaloud G, Ducousso M, Amusant N, Zaremski A (2017) NGS analysis of fungal OTUs in *Aquilaria* sp. from French Guiana, Cambodia, Laos and Thailand. In: IRG, *48th Annual Meeting, 4-8 June 2017, Gand, Belgium, 12p.*

Zhou G, Whong WZ, Ong T, Chen B (2000) Development of a fungus-specific PCR assay for detecting low-level fungi in an indoor environment*. Molecular and Cellular Probes* 14 : 339-348.

ONLINE ISSN 2069-7430
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Annex 1: Mycological data sheet

Pycnoporus sp.

☞**Reference C.I.R.A.D. :** R-16

☞**Origin : Régina, Guyane.**

☞ **Genetic characteristics**

Les deux couples d'amorces SR6/SR10R et SR7/SR1R ont été choisis pour amplifier les régions spécifiques du gène de la petite sous unité ribosomique 18S (SSU). (SR6 : 5' TGTTACGACTTTTACTT 3' ; SR10R : 5' TTTGACTCAACACGGG 3' ; SR7 : 5' GTTCAACTACGAGCTTTTTAA 3' ; SR1R : 5' TACCTGGTTGATQCTGCCAGT 3')

- **Sequence CIRAD R-16 (SR6/SR10R) :** npb: 554

GGGGAAACTCACC-AGGTCC-AGACATGA-CTAGGATTG-ACAGATTGATAG---
CTCTTTCATGATTTTATGGGTGG
TGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGATAACGAACG-
AGACCTTAACCTGCT TAATAGCCAGGCCGGCT-TTTGCTGGTCGCCGG-
CTTCTTAGAGGGACTGTC-TGCGTCTAGCAGACGGAAGTTTG AGGCAATAA-
CAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGAGCCAGCGAGT
T ---TA--TAACCTTGGCCGGAAGGT-
CTGGGTAATCTTGTGAAACTCTGTCGTGCTGGGGATAGAGCATTGCAATTAT
TGCTCTTCAACGAGGAATTCCTAGTAAGCGTGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCC
TTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGTCTTGGGATTGGCT

- **Identity BLAST (SR6/SR10R)** (www.ncbi.org) (longueur séquence et pourcentage identité) **:** 544/547 (99%)
- **Identification BLAST-GENBANK** (www.ncbi.org) **(SR6/SR10R) :** *Pycnoporus sp.*
- **Number of accession GENBANK** (www.ncbi.org) **(SR6/SR10R) :** EU888830
- **Identification BLAST-GENBANK** (www.ncbi.org) **(SR7/SR1R) :** Ø
- **Number of accession GENBANK** (www.ncbi.org) **(SR7SR1R) :** Ø
- **Taxonomic rank** (www.mycobank.org)**:** Fungi, Basidiomycota, Agaricomycotina, Agaricomycetes, Polyporales, Polyporaceae
- **Rot** (www.mycobank.org) : fibrous

☞ **Morphological characteristics :**

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Annex 2: Quantities of the total DNA extracted - ng/ml

Fungi were chosen fresh and in good condition on dead wood (25 fungi), and on standing trees (14 fungi).

Strain N°	Quantity of total DNA extracted ng/ml	on dead wood (W) on standing trees (T)
	Fungi from Cacao	
C01	30,83	W
C-02	83,4	W
C-03	53,47	W
C-04	113,68	W
C-05	103,12	T
C-06	22,22	T
C-07	10,34	W
C-08	82,56	W
C-09	90,57	W
C-10	17,12	T
C-11	27,52	T
C-12	32,52	T
C-13	62,35	T
C-14	70,23	W
C-15	56,32	W
C-16	45,12	T
C-17	47,25	T
C-18	65,28	W
	Fungi from Régina	
R-01	102,23	T
R-02	110,15	T
R-03	45,89	W
R-04	46,52	W
R-05	42,23	W
R-06	65,25	W
R-07	25,89	W
R-08	14,52	T
R-09	17,89	T
R-10	15,64	T
R-11	13,25	W
R-12	12,25	T
R-13	78,95	W
R-14	102,32	W
R-15	18,95	W
R-16	118,23	W
R-17	23,26	W
R-18	25,63	W
R-19	25,98	W
R-20	35,89	W
R-21	58,96	W

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Annex 3: Sequence analysis by comparison with BlastN databases

In this annex, indicate the number of the strain studied, the name of the fungus named using BlastN, its reference number or accession number for the primer pairs (SR6/SR10R) and (SR7/SR1R), the number of base pairs for each of the sequences (npb) and the length of the sequence that was compared by BlastN with its identity percentage.

Fungi from Cacao

SR6/SR10R						SR7/SR1R					
N° cirad	N° accession	Identification BLAST	npb	Identity	(%)	N° cirad	N° accession	Identification BLAST	npb	Identity	(%)
C01	AB615232	*Auricularia auricula*	571	379/513	74	C01	AF291268	*Auricularia auricula*	517	364/377	97
C02	AF291269	*Auricularia delicata*	492	429/530	81	C02	AF291269	*Auricularia delicata*	501	451/493	91
C03	HM536092	*Gloeophyllum striatum*	541	329/478	69	C03	HM536092	*Gloeophyllum striatum*	547	529/545	97
C04	HM536092	*Gloeophyllum striatum*	461	450/460	98	C04	HM536092	*Gloeophyllum striatum*	560	548/561	98
C06	HM536092	*Gloeophyllum striatum*	538	523/536	98	C05	HM536092	*Gloeophyllum striatum*	531	518/531	98
C06	GU207295	*Lentinus crinitus*	603	589/589	100	C06	GU207295	*Lentinus crinitus*	623	620/620	100
C07						C07	FJ426395	*Schizophyllum commune*	590	587/590	99
C08	AB158316	*Coriolopsis caperata*	591	589/590	99	C08	JF283779	*Coriolopsis caperata*	591	247/315	78
C09	EU784225	*Geastrum coronatum*	551	480/553	87	C09	EU784225	*Geastrum coronatum*	551	472/554	85
C10	AB495008	*Xylaria sp.*	543	484/543	89	C10	AB495008	*Xylaria sp.*	515	472/530	89
C11	GU207276	*Lentinus swartzii*	463	442/460	96	C11	GU207278	*Lentinus swartzii*	612	116/157	74
C12	HM536092	*Gloeophyllum striatum*	397	267/362	74	C12	HM136871	*Fomes sp.*	376	290/326	89
	GQ354271	*Gloeophyllum abietinum*	397	214/293	73		GU731565	*Lenzites elegans*	376	257/292	83
	AJ420946	*Gloeophyllum sepiarium*	397	184/242	76						
C13	HM136871	*Fomes sp.*	580	563/565	99	C13	HM136871	*Fomes sp.*	595	586/588	99
	EF060006	*Ganoderma lipsiense*	580	533/596	89		AJ537410	*Rigidoporus sp.*	595	532/594	90
C14	AY916484	*Helicoma sp.*	529	484/529	91	C14	AY916484	*Helicoma sp.*	566	495/542	91
C15	AF363759	*Pycnoporus sanguineus*	587	512/514	99	C15					
	GQ849478	*Lentinus sp.*	621	554/624	89		EF694648	*Polyporales sp.*	617	606/611	87
	FN907915	*Cerrena unicolor*	621	508/626	81		HQ728307	*Cerrena unicolor*	617	496/623	86
C16	HQ248221	*Fomitopsis meliae*	616	605/610	99	C16	HQ248221	*Fomitopsis meliae*	605	600/606	99
C17	AY089735	*Poria placenta*	635	578/589	98	C17					
C18	AY089735	*Antrodia vaillanti*	616	600/602	99	C18					

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Fungi from Régina

SR6/SR10R						SR7/SR1R					
N° cirad	N° accession	Identification BLAST	npb	Identité	(%)	N° cirad	N° accession	Identification BLAST	npb	Identité	(%)
R01	AB540581	*Fomitopsis cf.*	616	595/609	98	R01	EU015881	*Fomitopsis sp.*	625	588/638	92
	HM126455	*Fomitopsis sp.*	616	572/621	92		GQ373177	*Basidiomycota sp.*	625	577/629	92
	AB604156	*Fomitopsis palustris*	616	563/610	92		FJ617303	*Antrodia wangii*	625	568/622	91
	FR717226	*Fomitopsis pinicola*	616	571/625	91						
R02	HQ248221	*Fomitopsis meliae*	601	599/604	99	R02	GQ982889	*Fomitopsis cf.*	605	593/608	98
	HM126460	*Fomitopsis sp.*	601	557/595	94		HM126460	*Fomitopsis sp.*	605	569/618	92
	FJ372680	*Fomitopsis cf.*	601	554/593	93		GQ373177	*Basidiomycota sp.*	605	568/620	92
	AB604156	*Fomitopsis palustris*	601	546/584	93		FJ617303	*Antrodia wangii*	605	564/618	91
R03	AB566306	*Ascomycota sp.*	325	163/176	93	R03	AY838940	*Geotrichum gigas*	352	292/331	88
R04	FM872470	*Antrodia sp.*	600	528/602	88	R04	FJ617303	*Antrodia wangii*	605	564/618	91
R05	AB512335	*Mycena sp.*	223	184/223	83	R05	AB512335	*Mycena sp.*	519	479/521	92
R06	AB470242	*Polyporales sp.*	590	468/544	86	R06	AB512335	*Mycena sp.*	583	556/584	85
R07	AM269778	*Ganoderma resinaceum*	586	546/570	96	R07	GU451247	*Ganoderma resinaceum*	586	569/591	96
R08	AY884176	*Ganoderma lucidum*	591	588/596	99	R08	EU498090	*Ganoderma lucidum*	586	585/591	99
R09	AY884176	*Ganoderma lucidum*	582	580/582	99	R09	EU498090	*Ganoderma lucidum*	589	588/589	99
R10	GU731545	*Antrodia malicola*	599	597/599	99	R10	GU731545	*Antrodia malicola*	607	566/569	99
R11	GU731545	*Antrodia malicola*	604	603/604	99	R11	GU319996	*Antrodia malicola*	607	581/610	95
R12	GU731545	*Antrodia malicola*	607	599/602	99	R12	GU319996	*Antrodia malicola*	621	582/611	95
R13	AY968082	*Trametes sp.*	575	544/582	95	R13	AY968082	*Trametes sp.*	595	571/601	95
R14						R14	HM536094	*Gloeophyllum trabeum*	571	570/571	99
R15						R15	AY336772	*Coriolopsis gallica*	610	580/583	99
R16						R16	EU888830	*Pycnoporus sp.*	554	492/492	100
R17						R17	EU888830	*Pycnoporus sp.*	551	492/492	100
R18						R18	AY497555	*Gloeophyllum sepiarium*	566	502/566	89
							HM536094	*Gloeophyllum trabeum*	566	502/568	88
R19	AY336752	*Rigidoporus vinctus*	568	566/566	100	R19	AY336752	*Rigidoporus vinctus*	578	570/600	95
R20	AY336771	*Coriolopsis polyzona*	575	564/569	99	R20	AY336771	*Coriolopsis polyzona*	595	589/592	99
R21	HM245784	*Panus sp.*	621	584/588	99	R21	HM245784	*Panus sp.*	617	610/616	90