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Molecular characterization and biodiversity of wood-decaying fungi in French Guiana

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ABSTRACT

Fungi from tropical regions are currently under-represented in the classification system. Indeed, difficult access to tropical forests and irregular occurrence carpophores make it complicated to study fungus species in such environments, unlike in European zones where fungal diversity and taxonomy are better known. The purpose of this work was 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.

In our study, we chose to work in the zone comprising the internal transcribed spacers ITS1 and ITS2, which are relatively variable, and the 5.8 S small ribosomal subunit, which is not highly variable. The primers ITS 1(5'-TCCGTAGGTGAACCTGCGC-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'), specific to fungi, were chosen for this taxonomic analysis of the studied species.

This study was carried out on 101 fungus fruiting bodies at the Paracou forest site in French Guiana. Of those 101 fungi, 72 were identified by BLASTn. Four species were Ascomycetes of the genus *Muscodor* and *Xylaria*. The other 68 species, all in the class of the Basidiomycetes, were divided into the following orders: 31 Agaricales, 1 Atheliales, 2 Boletales, 1 Gomphales, 12 Polyporales, 1 Trechisporales and 1 Tremellales. There was also an indeterminate taxon very similar to the lichens. Within the order Polyporales, the main genera were found, such as *Antrodiella, Coriolopsis, Fomitopsis, Ganoderma, Lentinus, Pycnoporus, Steccherinum, Trametes, Fomitoporia.* All these fungi have the particularity of causing wood decay.

Keywords: fungus biodiversity, tropical forest, ribosomal DNA, sequences, molecular taxonomy, BLASTn, primer ITS1, primer ITS4

1. INTRODUCTION

The forest of French Guinea is characterized by a very wide diversity of trees species, with 11600 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.

For now, very little is yet known about the diversity of tropical lignocellulolytic fungi, or about the range of enzymes capable of decaying the lignocelluloses existing in tree species with highly resistant wood. According to the ratio of diversity between filamentous fungi and higher plants in the Tropics (at least 5 to 1 according to Roberts and Spooner, 2000) and the number of plants inventoried in French Guiana, at around 5,000, fungal biodiversity is estimated at 25,000 species, of which only 3% have been described. 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 activity), and soft rots (hemicellulolytic Deuteromycetes and particularly *Trichoderma* and *Aspergillus*).

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. Molecular techniques combining amplification and sequencing seem to be particularly suitable (Zaremski et *al.*, 2005). In our study, molecular tools such as DNA polymerization chain reaction and sequencing provided 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:
- ascertain the diversity of fungal fruiting bodies at the Paracou forest site of French Guiana, where little or nothing is known about the microflora, and characterize it taxonomically.
- establish a collection of fungal isolates from those fruiting bodies
- characterize taxonomically original isolates.

In addition to the above objectives, this taxonomic approach was intended to culminate in better knowledge of fungal diversity, particularly for wood-decaying fungi.

Taxonomic characterization methods applicable to fungi
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 well established. Figure 1 shows a diagrammatic representation of the ribosomal operon with the sites of the different primers used or usable for studying nuclear ribosomal DNA.

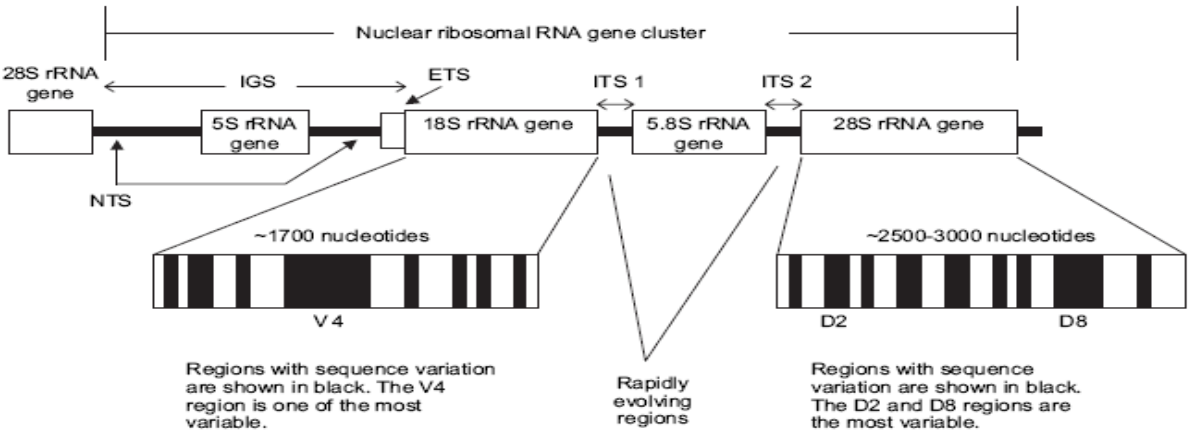

Figure 1: Operon of the ribosomal rRNA gene of eukaryotes. The operon comprises three main genes (molecules of 5.8S, 18S and 25S or 28S rRNA) and intermingled "interspace" regions (IGS – intergenic spacer, NTS – non-transcribed spacer, ETS – externally transcribed spacer, ITS - internally transcribed spacer) (According to Mitchell and Zuccaro, 2006).

The ITS regions of rDNA benefit from a rapid evolution rate, and this results in greater sequence variation between relatively close species, compared to more conserved encoding regions of the gene encoding 18S rDNA (Anderson and Cairney, 2004). This is why, in order to obtain better resolution, studies were be analysed on non-encoding spacers (ITS). However, many sequenced eukaryotic ITS regions remain limited and are therefore clearly less well represented in databanks, compared to prokaryotic 16S rDNA sequences, but the problem is lessening as databanks are increasing (Prosser, 2002).

In our study, we chose to work in the region most frequently used to identify strains and for phylogenetic trees. The primers ITS 1(5'-TCCGTAGGTGAACCTGCGC-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'), specific to fungi, were chosen for this taxonomic analysis of the studied species. These primers have been designed to amplify the zone comprising the internal transcribed spacers ITS1 and ITS2, which are relatively variable, and the 5.8 S small ribosomal subunit, which is not highly variable (Gardes and Bruns, 1993; Martin *et al*., 2002; Guerin-Laguette *et al.*, 2003; Mitchell and Zuccaro, 2006). This study was expected to result in the identification and discrimination of most of fungus species using a pure mycelium culture and contaminated the wood.

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 by BLAST and GENBANK.

With these molecular tools it was possible to study the diversity and taxonomy of 101 fungus fruiting bodies from the Paracou forest site 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, this study of the specific regions of the gene using the primer pairs ITS1/ITS4 should enable the identification and discrimination of most of these fungus species from a pure mycelium culture.

2. EXPERIMENTAL METHODS

2.1 Sampling

The network of small experimental plots in French Guiana

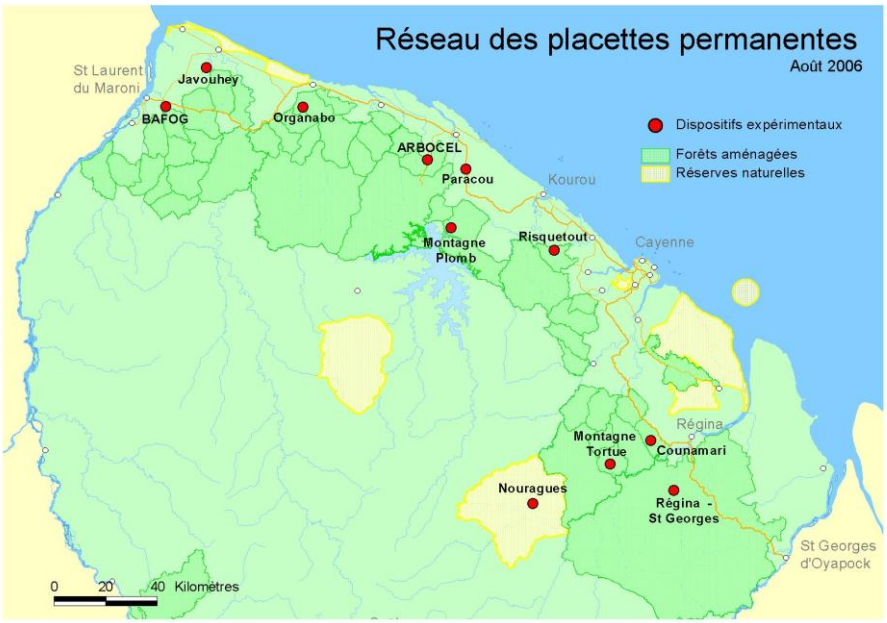

Figure 2: Network of permanent experimental plots in French Guiana

For our work, collections were made at the Paracou forest site (5°18'N, 52°53'W) which is an area of 40,000 ha defined in 1982, under an operation initiated by ECOFOG for forestry research on natural forest stands in French Guiana.

2.2 Fungal fruiting body collections

Fungi were chosen fresh and in good condition on dead wood (around 43), on standing trees (around 15) or on the leaves of trees (around 15).

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. Then individual sample was thus classed by numerical collection order (from PP-01 to PP-101).

At least two specimens of each fungus were collected, as some were to be used to describe macroscopic traits, others for a spore count, 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.

The fruiting bodies were stored in sealed plastic bags with a slip indicating the collection order number, the date, the site and the tree species if possible.

Figure 3: Fruiting bodies on Basralocus (*Dicorynia guianensis*)

Figure 4: Fruiting bodies on a standing tree

Figure 5: Fruiting bodies on a leaf

2.3 Isolation and mycelium production

2.3.1 Isolation: culturing, obtaining pure mycelium

Four pieces of fresh tissue and/or fruiting body were taken from the sample under a sterile hood using a scalpel sterilized in a bead sterilizer. Two of the pieces were sterilized in 70% ethanol. By sterilizing the surface of the pieces, the bacteria present were partly eliminated. However, as a precaution, the other two pieces were not sterilized so as not to kill all the fungal flora. Four Petri dishes containing the WA-CS medium were therefore used per sample. In all, 120 dishes were used in this first isolation step.

The cultures were left to incubate in the dark at a temperature of 20°C, only bringing them out into the light to examine them.

These isolations were examined each day for four weeks (mycelium active phase). They were inspected and identified under a magnifier and under a light microscope, either directly in the Petri dishes or with special preparations between slides and covers (with Lugol, with Cotton Blue).

Once mycelium covered a part of the surface of the WA-CS medium, and morphologically sure that it was pure mycelium of Basidiomycete, isolation could be carried out on the M-A and PDA-C media.

The cultures were once again left to incubate in the dark at 20°C, only bringing them out into the light to examine them. They were examined every two days for six weeks.

Four Petri dishes were used for each sample to have a better chance of success for subcultures. Then, using a growing culture, subcultures were carried out in the form of cubes measuring 2-3 mm per side. The cultures were left to incubate in the dark at 20°C. They were

observed daily. Each time there was mycelium development, it was subcultured, in order to isolate it from other fungi likely to develop afterwards.

When mycelium was found to be pure, it was transferred to 4 Petri dishes with Malt-Agar, and then finally maintained in two tubes in the refrigerator in the dark.

2.3.2 Mycelium production for molecular studies

For each pure strain, the mycelium was grown in a Petri dish on malt-agar culture medium (4%-2%) covered with a sheet of cellophane (Lecellier and Silar, 1994). After culturing for 10 days at 20°C in the dark, the mycelium was harvested taking care to remove all traces of the culture medium, notably the agar of the subculture implant. A method exists for obtaining mycelium free of any trace of culture medium, notably agar. Before moving on to the nuclear DNA purification stage, harvested mycelia is placed in an Eppendorf tube, frozen in liquid nitrogen, then stored in the freezer at -80°C. Under these conditions, mycelium can be stored for a few years.

2.4 Molecular methods

2.4.1 Fungal DNA extraction using the Invitrogen "TM purelink plant total DNA purification kit" and quantification of the extracted DNAs

- **From fruiting bodies**: all the samples were reduced to powder with liquid nitrogen; they were then subjected to thermal shock and mechanically ground; the protocol was followed for InvitroGen extraction with the "purelinkTM plant total DNA purification kit" (Annex1).
- **From pure mycelium:** the mycelium was separated from the agar under a sterile hood using a sterile scalpel and tweezers; it was placed in a sterile 1.5 ml Eppendorf tube; the tube was placed in liquid nitrogen for 2 min; 500 µL of R2 suspension buffer from the InvitroGen kit was added so that the mycelium was well immersed in the buffer; the mycelium was ground by hand using a sterile pestle in the Eppendorf tube; the extraction protocol established by InvitroGen was followed with the "purelinkTM plant total DNA purification kit" (Annex1).

The DNA of the extracted strains was quantified by spectrometry on a Shimadzu photospectrometer. It measured the concentration and purity of the DNA extracted with 2µl of sample. The purity index was calculated from the ratios of the absorbance measurements at 260 and 280 nm. This 260/280 ratio needed to approach 1.8 for the sample to be qualified as pure. Smaller values indicated the presence of impurities or absorbent proteins at around the same wavelengths.

2.4.2 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 Symbionts and Roots Laboratory (INRA in Montpellier) was used.
Fungi, which are eukaryotic organisms, do not have the same ribosomal RNAs as bacteria, which are prokaryotic organisms. They therefore have to be analysed separately. However, studying diversity by molecular biology is less widespread in eukaryotes, especially in fungi. The choice of primers, hence of the target gene to be amplified to identify the fungi of an environmental sample, is still under discussion. However, studying the region of the DNA encoding the ribosomal sub-units is established today. Figure 1 shows a diagrammatic representation of the ribosomal operon with the sites of the different primers used or usable for studying nuclear ribosomal DNA.

The amplification reactions were carried out in clean PCR plates. The reaction volume was as follows: 4 µl of dNTP, 10 µl 5X buffer, 2 µl of each primer (20 pmol/µl): ITS1-myc and ITS4-myc, 5 µl extracted total DNA (around 50 ng), 26.7 µl of Millipore or sterile water (to top up to 50µl), 0.30 µl of Taq polymerase.
Some controls without DNA were done to test for the existence of any contaminations in the reagents and buffers.

The 50 µl 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 30 sec, a hybridization phase at 55°C for 30 sec, then an extension phase at 72°C for 1.30 min
- elongation of the extension phase or final elongation phase at 72°C for 7 min
- 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

It was checked that the quality of amplification reaction by visualizing amplificates on a control gel. Ten µl of amplificates was deposited on an agarose gel at 0.8% in a TAE 1X buffer. Migration was carried out by electrophoresis at 120 V for one hour.

2.4.3 Sequencing

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

After obtaining and correcting the sequences based on the interpretation of the electrophoretograms with CHROMAS PLUS software, we proceeded with BlASTn (Nucleotide) on the Internet (http://www.ncbi.nlm.nih.gov/BLASl) to compare our sequences with those existing in the libraries.

2.4.4 Resemblance criteria: sequence analyses by comparison with databases – BLAST

BLAST, Basic Local Alignment and Search Tool, (Altschul *et al*., 1997) is a method that has been especially developed to compare an unknown nucleic or proteic sequence with all those that are found in nucleic databases *(http://www.ncbi.nlm.nih.gov/BLAST).*
The purpose of sequence comparison programs is to identify the places in which identical or very similar regions are found between two sequences and to deduce those of them that are significant and correspond in a biological sense to those observed by chance. BLAST detects short segments (eleven identical nucleotides or two similar tripeptides) that are locally homologous to the unknown sequence (Altschul *et al*., 1997).

A score is always associated with a given alignment. An alignment program, whichever it may be, always seeks to maximize that score. It can be imagined that the higher the score is, the more the alignment is significant. Such reasoning is valid if a uniform family is being considered where all the sequences are roughly of the same length: in this case the higher the score is, the greater the resemblance is, though this does not tell us whether it is significant (Risler *et al*., 2003).

In our study, rather than using scores, we used percentages of identity or similarity between two sequences and our likelihood criteria were as follows: if the nearest sequences extracted from the databases had over 90% similarity, and if those sequences gave the same genus and species name, the result obtained complied with our initial identification based on the morphological and anatomical criteria for the genus and for the species.

2.4.5 Constitution of a reference sequence file

When the BLAST searches were carried out, all the reference sequences close to our strains were extracted from the NCBI databases. They thus formed a source of reference sequences, an essential tool for the taxonomic and phylogenetic studies.

3. RESULTS AND DISCUSSION

3.1 Collection of fungus fruiting bodies

Fruiting bodies were collected from the Paracou forest site during the wet season characterized by the frequent and heavy showers that sweep through French Guiana twice a year (November to February and April to July), and which correspond to 20 to 30 days' rainfall per month. The average relative humidity was over 90% and the air temperature around 28°C.

It took around two months to collect 101 fungus fruiting bodies from PP-001 to PP-101 taken from dead wood, forest tree species and tree leaves.

3.2 Isolate culture

It proved necessary to find a suitable growing medium to grow mycelium, notably for most of the fungi from French Guiana, which showed difficulties in growing on MA medium (Malt, Agar), conventionally used in the laboratory. We chose MEA medium (Malt Dextrose Agar) containing the antibiotic streptomycin.

However, numerous cases of contamination occurred in the Petri dishes during isolation. They were mostly caused by the presence of bacteria, moulds, insects and mites from the fungus fruiting bodies. The contaminants developed in humid radiating colonies. They followed the evolution of the mycelium and resisted antibiotics well. Insects and mites often brought moulds and isolation was difficult to achieve in those cases.

In this tropical context, a large number of contaminations were encountered during isolation, and successive subcultures of the strains were carried out to obtain a pure strain: out of 101 fruiting bodies subcultured, 25 were contaminated.

It appeared that isolations needed to be carried out before 24 hours of collection, otherwise the fruiting body rotted quickly.

It seemed that this type of isolation under tropical conditions (very high temperature and relative humidity in the laboratory) was not suited to these fungi. Other techniques will be explored for this type of isolation (added antibiotics, miticides in the culture media, isolation from dried fruiting bodies, mycelium thallus culture on cellophane film, etc.).

As a basis for numerous studies, exploration of the diversity of microbial communities existing in an environment has long involved indirect methods comprising a stage of bacterium culture in the laboratory. Such methods are based on the notion of pure culture, whereby a clonal population derives from the cellular division of a single cell. However, despite lying at the very root of microbiology, they display numerous limitations. Indeed, it has been acknowledged for several years that "growability", or even the ability of a cell to

form colonies on a culture medium, is extremely variable depending on the microorganisms involved. It proves difficult to get microorganisms to develop under laboratory conditions. The reasons for this "non-growability" sometimes remain obscure, but it is not difficult to imagine that artificial culture conditions in the laboratory are probably too restrictive. Despite the care taken to reproduce environmental conditions as closely as possible, numerous factors potentially essential for the growth of certain microorganisms are still not mastered in the laboratory. , Interactions between microorganisms existing in the natural environment may also prove to be highly influential when growing microorganisms in the laboratory; and some will require the presence of other types of microorganisms in order to develop in an optimum manner (Herrera, 2006). Consequently, selective culturing methods have only made it possible to take into account a small share of the diversity of microoganisms that actually exists. For example, in the case of soil, the number of bacteria giving colonies on culture medium is estimated at between only 0.1 and 10%.
Since the pioneering work by Torsvik *et al*. (1990) revealing non-cultured bacteria by directly studying RNA extracted from an environmental sample, new molecular methods, called environmental genomics, have been developed for a more precise determination of the diversity of bacterial communities by circumventing the culturing step (Herrera, 2006).
The greatest benefit of molecular techniques is therefore the possibility of characterizing diversity without the need for laboratory culturing methods. Moreover, molecular identification is faster than methods based on microorganism cultures and is becoming the most popular method for identifying numerous environmental groups (Prosser, 2002).

3.3. DNA extraction and purification

Adaptation of the Invitrogen kit programme to the laboratory enabled optimum extraction. It was thus possible to extract and amplify the DNA of all the samples studied.
The concentration and purity of the extracted total DNAs was assessed. The quantities of DNA obtained varied depending on the samples, from 0.120 to 110.44 µg/ml. These results can be explained by the presence of impurities which “biased” the measurements taken with the spectrophotometer. In the latter case, an additional stage of residue washing during DNA extraction will have to be considered.

3.4 Analyses of the amplification products

The molecular weight marker was properly visualized, along with a clear band with a molecular weight of approximately 700 base pairs.
Nevertheless, some other bands, as much under 700 base pairs as over, were also visible. That would seem to mean that there were several fungus species present in the samples or that the primers chosen were not specific enough to the fungus species and amplified wood DNA and that of yeasts, fungi and bacteria.

The amplification protocol was well suited to the specific region of the gene for 83% of the fungi, for which we obtained a band with a molecular weight of between 540 and 660 pb.

3.5 Sequence analyses by comparison with databases – *BLAST*

Based on alignment quality criteria (E-value, maximum identity, coverage, etc.), the choice of most relevant sequence was made on the first ten BLAST results making it possible to identify the sequence and thereby the genus and species of the sample of infested wood, fruiting bodies and pure strains.

- 84 sequences were corrected after interpretation of the electrophoretograms by CHROMAS and BIOEDIT software: 72 were corrected and the other 12 revealed

contaminations (several curves).

- The similarity search was therefore conducted on 72 sequences: 86% of the 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.

Table 1 shows the similarity searches for our sequences with database sequences. This table indicates the number of the strain studied, the name of the fungus identified by BLASTn, the number of base pairs for each of the sequences (npb) and the length of the sequence that was compared by BLAST with its identity percentage in brackets.

Table 1: Similarity searches for our sequences with database sequences

Strain Number	**Blast Identification**	**Number of Base Pairs**	**Blast Identity, Sequence Length And [%]**
P-001	*Nolanea sericea*	660	483/490 (98%)
P-002A	*Antrodiella americana*	540	487/494 (98%)
PP_002B	*Steccherinum fimbriatum*	615	571/576 (99%)
PP_006	*Resinicium saccharicola*	638	476/494 (96%)
PP_007A	*Xylaria sp1.*	546	479/489 (97%)
PP_007B	*Muscodor albus*	610	573/581 (98%)
PP_008	*Hygrocybe conica*	553	490/495 (98%)
PP_009	*Omphalotus olivascens*	553	485/494 (98%)
PP_010	*Dentipellis separans*	610	568/579 (98%)
PP_011A	*Athelia bombacina*	611	586/597 (98%)
PP_011B	*Chaetocalathus sp1*	657	489/491 (99%)
PP_012	*Fomitiporia mediterranea*	547	487/493 (98%)
PP_013	*Marchandiomyces sp1.*	552	475/492 (96%)
PP_018	*Omphalotus olivascens*	552	489/491 (99%)
PP_019A	*Coriolopsis gallica*	610	580/583 (99%)
PP_019B	*Earliella scabrosa*	555	491/492 (99%)
PP_021	*Paxillus filamentosus*	555	492/495 (99%)
PP_025	*Coriolopsis gallica*	605	573/577 (99%)
PP_026A	*Trametes versicolor*	604	565/575 (98%)
PP_026B	*Pycnoporus sp.1*	554	492/492 (100%)
PP_028	*Campanella subdendrophora.*	552	491/492 (99%)
PP_032	*Entoloma sinuatum*	556	492/495 (98%)
PP_033	*Asterostroma andinium*	611	487/498 (97%)
PP_036A	*Coriolopsis gallica*	608	576/579 (99%)
PP_036B	*Earliella scabrosa*	552	491/492 (99%)
PP_041	*Paxillus filamentosus*	562	540/547 (98%)
PP_044	*Miladina lecithina*	555	491/492 (99%)
PP_045A	*Steccherinum fimbriatum*	612	571/579 (98%)
PP_045B	*Antrodiella americana*	548	487/494 (98%)
PP_047A	*Chrysompholiana grossulata*	612	491/495 (99%)
PP_047B	*Hohenbuehelia tristis*	555	541/552 (98%)
PP_048	*Xylaria sp2.*	551	489/489 (100%)
PP_051	*Fomitopsis rosea*	552	486/492 (98%)
PP_052A	*Marasmius oreades*	612	579/582 (99%)

Table 1 Cont'd

Strain Number	Blast Identification	Number of Base Pairs	Blast Identity, Sequence Length And [%] of similarity
PP_052B	*Omphalotus olivascens*	552	535/550 (97%)
PP_054	*Pleuroflammula flammea*	551	489/492 (99%)
PP_055A	*Rhodocollybia maculata*	556	492/493 (90%)
PP_055B	*Uncultured agaricomycotina*	612	578/583 (99%)
PP_056A	*Athelia bombacina*	610	578/583 (99%)
PP_056B	*Marasmius sp.2.*	564	470/499 (94%)
PP_059A	*Crinipellis zonata*	553	490/491 (99%)
PP_059B	*Moniliophthora roreri*	608	577/578 (99%)
PP_061	*Cryptococcus peneaus*	662	452/549 (82%)
PP_062	*Coriolopsis byrsina*	613	492/492 (100%)
PP_064	*Lentinus tigrinus*	553	528/540 (97%)
PP_066	*Muscodor albus*	605	570/589 (96%)
PP_067	*Hygrocybe miniata*	555	488/494 (98%)
PP_068	*Marasmius alliaceus*	551	488/491 (99%)
PP_069A	*Melanoleuca verrucipes*	554	543/549 (98%)
PP_069B	*Arthromyces matolae*	612	580/582 (99%)
PP_073A	*Lepiota procera*	602	583/591 (98%)
PP_073B	*Lyophyllum boudieri*	554	489/492 (98%)
PP_075	*Hohenbuehelia tremula*	592	489/492 (98%)
PP_076	*Uncultured agaricomycotina*	609	569/569 (100%)
PP_078	*Trechispora sp.1*	553	492/492 (100%)
PP_079	*Pycnoporus sp2.*	551	492/492 (100%)
PP_082	*Hygrocybe conica*	613	489/493 (99%)
PP_083	*Fomitopsis pinicola*	552	491/493 (99%)
PP_085	*Marasmius sp.3*	612	491/491 (99%)
PP_086	*Lepolichen coccophorus*	554	473/490 (96%)
PP_088	*Pycnoporus sp3*	552	492/492 (100%)
PP_090A	*Pycnoporus sp4*	549	481/551 (87%)
PP_090B	*Cymatoderma caperatum*	615	482/496 (97%)
PP_091	*Agaricus bisporus*	609	490/491 (99%)
PP_092	*Psilocybe stunzii*	553	492/492 (100%)
PP_093A	*Uncultured boletaceae*	611	578/584 (98%)
PP_093B	*Pleurotus salmoneostramineus*	553	493/493 (100%)
PP_094A	*Steccherinum fimbriatum*	611	570/575 (99%)
PP_094B	*Antrodiella americana*	551	487/494 (98%)
PP_098A	*Marasmius rotula*	611	568/575 (98%)
PP_098B	*Nolanea sericea*	617	483/490 (98%)
PP_099	*Omphalotus olearius*	608	491/491 (100%)
PP_101	*Ramaria rubella*	551	480/492 (97%)

After BLAST, we found the following results: 97% of these sequences had over 90% similarity over more than 80% of their length.

Out of 101 fungi, 72 were identified by BLASTn. Four species were Ascomycetes of the genus *Muscodor* and *Xylaria*. The other 68 species, all Basidiomycetes, were divided into the following orders: 31 Aricales, 1 Atheliales, 2 Boletales, 1 Gomphales, 12 Polyporales, 1 Trechiscoporales and 1 Tremellales. There was also one undetermined taxon very close to the lichens.

In the order Polyporales, we found the main genera such as *Antrodiella, Coriolopsis, Fomitopsis, Ganoderma, Lentinus, Pycnoporus, Steccherinum, Trametes, Fomitoporia.* These fungi all have the particularity of causing wood decay.

In our results, the undetermined taxon very close to the lichens raises a problem. In fact, genetic databases are very under-represented for many groups of fungi. NCBI's "Genbank" is the largest database and houses around 16,400 sequences representing fungal species. They are rRNA sequences, mostly of Ascomycetes, representing approximately 63% of all the sequences deposited, 34% for the Basidiomycetes and 1% of Zygomycetes. This lack of deposits may make identification of fungal sequences from Paracou difficult without access to other sources of information, such as that derived from a collection of local cultures (Mitchell and Zuccaro, 2006). Consequently, the taxonomic precision of many sequences in databases can be questioned. A study of three major taxonomic groups reported a 20% identification error rate (Mitchell and Zuccaro, 2006).

4. CONCLUSION AND PROSPECTS

In order to characterize the 101 fungi from the Paracou forest site in French Guiana, developing in a particular ecosystem (an air temperature of 28°C with 90% humidity), we developed a rapid, discriminant method based on an analysis of the diversity of partial sequences of ribosomal DNA independently from the description of macro- and microscopic morphological traits of fruiting bodies that are necessary for identifying genera and species. Adaptation of the protocol developed at CIRAD enabled us to extract DNA from all our samples using just a few mg of mycelium, of fruiting bodies and of infested wood.

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 an amplificate ranging in size from 540 to 660 base pairs.

Based on alignment quality criteria (E-value, maximum identity, coverage, etc.), the most relevant sequence was chosen from the first ten results of the BLAST search, enabling the identification of the sequences, hence of the genus and species of the fungus in the sample. To date, the sequencing result for 72 strains of fungi from French Guiana gives a single species name and very high BLAST performance criteria (e-value, % coverage) for the best 10 results. After BLAST, we obtained 4 species of Ascomycetes of the genus *Muscodor* and *Xylaria*. The other 68 species, all in the class of the Basidiomycetes, were divided into the following orders: 31 Agaricales, 1 Atheliales, 2 Boletales, 1 Gomphales, 12 Polyporales, 1 Trechisporales and 1 Tremellales. There was also an indeterminate taxon very similar to the lichens. Within the order Polyporales, the main genera were found, such as *Antrodiella, Coriolopsis, Fomitopsis, Ganoderma, Lentinus, Pycnoporus, Steccherinum, Trametes, Fomitoporia.* All these fungi have the particularity of causing wood decay.

Lastly, this taxonomic study of fungi from the Paracou forest site in French Guiana also enabled us to characterize the diversity of wood-decaying fungi belonging to the order of the olyporales and start research on phylogenetic affiliations.

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http://ncbi.nlm.nih.gov/BLAST/; http://www.invitrogen.com/; http://www.ncbi.org/; http://www.macrogen.com/.

Annex 1

Fungal DNA extraction with the Invitrogen "TM purelink plant total DNA purification kit"

The stages for plant lysate preparation are as follows:

- Add 250 µl of suspension buffer (R2) at room temperature.
- Homogenize the solution in a Vortex.
- Add 15 µl of 20% SDS and 15 µl of RNase A (20mg/ml) to obtain the lysate.
- Incubate at 55°C for 15 minutes to complete lysis.
- Centrifuge the lysate at 10,000 rpm for 5 minutes to eliminate insoluble material.
- Transfer the supernatant to a 1.5 ml sterile tube without disturbing the residue.
- Add 100 µl of precipitation buffer (N2) to the lysate. Mix in the Vortex and incubate in ice for 5 minutes. This stage precipitates proteins and polysaccharides, and all the photosynthetic pigments linked to the proteins are also precipitated. Pigments can stain the PurelinkTM cartridges and give a coloured eluate.
- Centrifuge at 15,000 rpm for 5 minutes at room temperature to obtain a clear lysate. Note: the supernatant must be clear and not viscous after this stage of precipitation.
- Transfer 250 µl of the lysate to a 1.5 ml sterile tube and add to the lysate 375 µl of binding buffer (B4) containing ethanol. Mix well in the Vortex.

Proceed with DNA fixation:

- Pour the solution into an Invitrogen kit column placed in a 2 ml tube supplied with the kit and centrifuge at 10,000 rpm for 30 seconds at room temperature.
- Discard the 2 ml tube with the filtrate and place the column in a clean 2 ml tube supplied with the kit.

DNA washing:

- Add 500 µl of washing buffer to the column (W4)
- Centrifuge at 10,000 rpm for 30 seconds at room temperature. Remove the filtrate and replace the column in the 2 ml tube.
- Add to the column 500 µl of washing buffer (W5) containing ethanol.
- Centrifuge at 10,000 rpm for 30 seconds at room temperature. Remove the filtrate and replace the column in the 2 ml tube.
- Repeat stages 3 and 4 one more time.
- Centrifuge at 15,000 rpm for 2 minutes to eliminate any washing buffer (W5) residue at room temperature. Discard the 2 ml tube.

Proceed with DNA elution:

- Place the column in a 1.5 ml sterile tube.
- Add 100 µl of elution buffer (E1) or distilled water (pH>7).
- Incubate at room temperature for 1 minute then centrifuge at 15,000 rpm for 1 minute. The tube then contains purified DNA.
- *Option: to recover more DNA, carry out a second elution stage using 100 µl of elution buffer (E1) or distilled water. The second elution can be carried out using the same elution tube or in a different tube.*
- Centrifuge the column at 15,000 rpm for 1 minute at room temperature.

The tube then contains purified DNA. Remove and discard the column.
The tubes are then stored in the freezer at -20°C to avoid any deterioration of the DNA.