Molecular characterization of fungal biodiversity and early identification of fungi associated with oil palm decay, particularly *Ganoderma boninense*

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To date, there was no information available on the diversity and molecular characterization of fungi associated with oil palm decay. This study appeared as a response to that need for essential information on the overall, global biodiversity of fungi associated with oil palm decay. In this study, 5 isolates from 30 fresh samples from the Tanah-Gambus estates (infested fresh tissues and fruiting bodies) seemed to be *Ganoderma boninense*. To date, the sequencing result for 250 samples gives a single species name and very high BLAST performance criteria (e-value, % coverage) for the best 10 results. After BLAST, we obtained 17% of the genus *Ganoderma*, 41% of Ascomycetes, yeasts and other Basidiomycetes, 17% miscellaneous (plants, etc.) and 25% of unusable sequences.

Materials and methods

- Culture medium preparation
- Isolation: culturing, obtaining pure mycelium
- Mycelium production for molecular studies
- Fungal DNA extraction using the Invitrogen "TM purelink plant total DNA purification kit"
- Quantification of the extracted DNAs.
- PCR (polymerase chain reaction) amplification of the nuclear rDNA ITS (ITS1-myc (5' TCCGTAGGTGAACCTGCGC 3') and ITS4-myc (5' TCCTCCGCTTATTGATATGC 3'; White et al. (1990)).
- BLAST (sequence alignment and comparison with databases; Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman D.J. (1997).)

Biological material

Samples from Tanah-Gambus, Indonesia:

There were two sets:

A **first set** of 31 samples collected in September 2010 and stored at -20°C:
- 17 were fruiting bodies (noted "Ind.");
- 1 fruiting body from the nursery (noted "Ind.29N";
- 13 were pieces of oil palm stems suspected of being infected (identified by numbers, e.g. "2.0" was the first fragment of stem No. 2). There were 62 stem sub-samples.

A **second set** of 28 samples collected in March 2011 was stored at 4°C:
- 16 were fruiting bodies (noted "D") (see figure 1),
- 12 were pieces of stem (noted "D.").

Samples from Benin:
- 12 samples came from Benin; they were divided into three categories: plots (noted "P"), of which there were 5;
- oleifera (noted "O"), of which there were 4;
- Benin (noted "B"), of which there were 3.

This denomination was chosen in accordance with the indications given on the slips accompanying the fungi.

Samples analysed in 2010:
- Some samples taken from the work in 2010; 23 infested and freeze-dried oil palm tissues.

The nomenclature was the same as the previous year.

Control samples:
- 4 positive control samples:
  - *Ganoderma lucidum* (noted "(+) G.l"),
  - *Ganoderma athensonii* (noted "t (+) G.a"),
  - IC 14, a fungus identified during the 2010 study,
  - 1 sample NJ3 from the March 2010 collections in Indonesia at Tanah-Gambus: this was fresh oil palm tissue (noted "t (+) NJ3").
- 1 negative control: Millipore water (noted "t –")

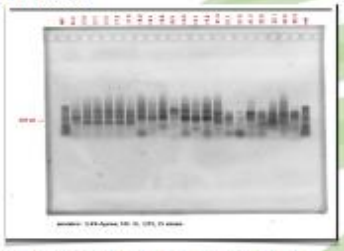
Sub-samples of oil palm stems from the September 2010 harvest:

The cross-sections of oil palm tissues were observed under a magnifier and we identified some zones of different colours using numbered discs ("0" was attributed to the palest zone) in order to take some samples from those zones for extraction. These coloured zones could have been potentially infested by the fungus (see figure 1). Here, the wood colours were

Fig.1. Oil palm tissues with some zones of different colours using numbered discs ("0" was attributed to the palest zone). Here, the wood colours were numbered 0 to 4, from palest to darkest.

Results and discussion

PCR Products



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 oil palm, fruiting bodies and pure strains.

The sequencing result was as follows:
- 17%: genus Ganoderma;
- 41 %: Ascomycetes, yeasts, other Basidiomycetes;
- 17%: miscellaneous (plants, etc.);
- 25%: unusable sequences.

Reconstruction of a phylogenetic tree for the 5.8S nuclear ribosomal sequence adding the ITS2

An initial analysis was carried out on all the sequences identified after BLAST as the genus Ganoderma.

The phylogenetic tree of the species of the genus Ganoderma based on the comparison of rDNA sequences, 5.8S and ITS 2, is presented in Figure 2. This tree shows the kinship relations between our closest strains of the genus Ganoderma extracted from NCBI. It presents five main and distinct groups:
- 3 groups of "2010 indeterminates": 2 groups for infested fresh oil palm tissues and 1 group for freeze-dried pure Ganoderma mycelia;
- 1 group of strains from Benin;
- 1 group of strains from Indonesia, set 1.

This first analysis of the interspecific phylo-genetic relations of Ganoderma, based on a comparison of rDNA sequences, 5.8S and ITS 2, reveals very distinct clades where the species tend to group according to the nature of the sample and the geographical origin, particularly the species from Benin and Indonesia, and the freeze-dried Indonesian samples.

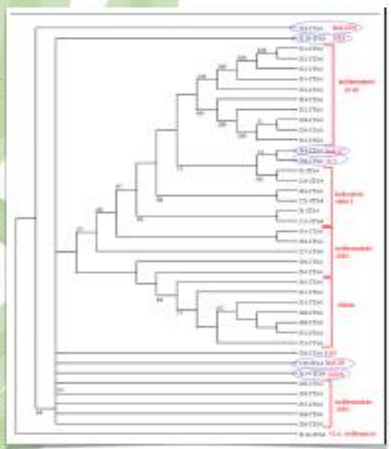


Fig 2. Phylogenetic position of species of the genus *Ganoderma* based on a comparison of rDNA sequences, ITS1, 5.8S and ITS 2. The tree was constructed by Neighbour Joining, with bootstrap resampling of 1000 replications (the bootstrap values are indicated at the main nodes of the tree).

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