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Studies on molecular diagnosis for detection, identification and differentiation of *Ganoderma*, the causal agent of basal stem rot disease in oil palm

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Contents

Summary

The objectives of this study were to develop molecular tools for detection, identification and differentiation of the oil palm pathogen *Ganoderma*. One approach for detecting the oil palm *Ganoderma* in the naturally infected plants is the use of the internal transcribed spacer (ITS) region as a target sequence for generating specific primers. To develop identification procedures for the oil palm *Ganoderma* and a differentiation against other *Ganoderma* species, that are non-pathogenic or pathogenic to other plants, four genes of the oil palm *Ganoderma*, i.e. ITS region, intergenic spacer 1 (IGS1) region, laccase and manganese superoxide dismutase (Mn-SOD) genes were chosen. Molecular approaches including the use of species-specific primers derived from variable DNA sequences, PCR/RFLP analyses with specific restriction enzymes, and DNA sequence alignment to infer a phylogenetic tree were used for identification and characterisation.

Three DNA extraction procedures were tested to evaluate detection limits in combination with the primer pair Gan1-Gan2, generating a PCR product of 167 bp, when oil palm *Ganoderma* DNA was used. Three DNA extraction methods used showed different detection limit, however, for practical reasons the NaOH extraction was chosen, still amplifying DNA from 1.5 ng freeze-dried mycelia. The other primer pairs IT1-IT2 and IT1-IT3 generated a PCR product of 450 bp and 334 bp, respectively, with the detection limit of 1.5 ng of freeze-dried mycelia using the NaOH extraction method. The specificity of three primer pairs in the PCR-based detection procedure was confirmed by verifying the absence of cross-reaction with DNA extracted from healthy palm tissue and 18 saprophytic fungi isolated from the palm. In sampling studies, oil palm *Ganoderma* was detected by PCR from naturally infected oil palm roots, using the NaOH-DNA extraction method, before any visible symptoms of oil palm infection were visible. Combined with the rapid DNA extraction of the NaOH method, PCR assays would provide a practical tool for the detection oil palm *Ganoderma* within oil palm roots.

Three primer pairs designed from conserved amino acid sequences of known laccase genes of basidiomycete fungi were used in the PCR assay to identify and differentiate between oil palm *Ganoderma* and other *Ganoderma* species studied. Primer pair Lac 2a-Lac 2b produced a unique PCR product of 327 bp, when the laccase gene was used as template of

the oil palm *Ganoderma* DNA. However, most of the other *Ganoderma* species tested with these primers produced a PCR product of the size of 1500-1600 bp within the laccase gene, starting from the copper-binding region I to the copper-binding region IV, according to the previously published laccase gene sequences of basidiomycete fungi. Primer pair Lac2a-Lac2r always generated a single PCR product of 1617 bp when genomic DNA of oil palm *Ganoderma* was used. Otherwise, two different PCR products were observed when genomic DNA of other studied *Ganoderma* species was used, i. e. a single PCR product of 1617 bp (identical size to oil palm *Ganoderma*) and a second amplificate of 1500 bp was visible. Subsequently, other *Ganoderma* species which, by using the primer pair Lac2a-Lac2r, produce a PCR product of an identical size to the oil palm *Ganoderma* can now be discriminated by using the primer pair Lac 2f-Lac 2b, generating an additional PCR product of 1265 bp in size, only when using DNA from oil palm *Ganoderma*. In contrast, genomic DNA of other *Ganoderma* species (identical PCR product in size of all tested oil palm *Ganoderma*, using the primer pair Lac 2a-Lac 2r) produced a PCR product of 1400 bp. Therefore, with the use of both primer pairs (Lac 2a-Lac 2r and Lac 2f-Lac 2b), oil palm *Ganoderma* can now be clearly distinguished from all other *Ganoderma* species studied.

Variable sequences of the oil palm *Ganoderma* within the ITS or IGS1 regions were sequenced and were compared with other published *Ganoderma* isolates and were analysed for designing species-specific primers for the identification of oil palm *Ganoderma*. Within the ITS and IGS1 regions, two specific primer pairs were designed to provide a specific DNA amplification for the oil palm *Ganoderma*. Each primer pair produced a single PCR product when oil palm *Ganoderma* DNA was used in PCR amplification, 450 bp (for primer pair IT1-IT2), 334 bp (for primer pair IT1-IT3), 630 bp (for primer pair Q-IGSa) and 1,000 bp (for primer pair Q-IGSc). No PCR amplification product was observed when other *Ganoderma* species DNA were used. Except for *Ganoderma tornatum* isolated from hardwood, both primer pairs Q-IGSa and Q-IGSc showed slight cross-reaction in the PCR amplification. Based on the results of the PCR amplification using the specific primer pairs, identification and differentiation between oil palm *Ganoderma* and other *Ganoderma* species studied are easy to interpret because it is based on the presence or absence of a single DNA fragment.

Another molecular approach for identification and differentiation of oil palm *Ganoderma* against other *Ganoderma* species studied was the use of specific restriction enzymes in the ITS/IGS1-RLFP analyses. The restriction enzymes *SacI, MluI* and *HinfI* were used to digest the ITS-PCR product and *HincII, ScaI* and *TfiI* were used to digest the IGS1-PCR product. Of the three restriction enzymes used in each rDNA region, *MluI* was specific for the digestion of the ITS regions and *TfiI* was specific for the digestion of the IGS1 region of oil palm *Ganoderma*. A restriction site of *MluI* was also not found in the ITS nucleotide sequence of 31 published *Ganoderma* species used in this study. The use of the combination of the PCR amplification and restriction analysis can be applied as a standard protocol to identify whether *Ganoderma* isolates from fields are related to the pathogenic species.

Phylogenetic trees showed that oil palm *Ganoderma* cluster together with *Ganoderma boninense* LKM but were separately from *Ganoderma boninense* RSH RS in ITS and Mn-SOD gene phylogenies. Sequences analyses of the ITS and Mn-SOD genes revealed a high sequence similarity to *Ganoderma boninense* LKM (for the ITS1 ranged from 94.2 to 98.6 %, ITS2 ranged from 90.7 to 99.5 % and Mn-SOD gene ranged from 96.5 to 98.2 %). On the other hand, oil palm *Ganoderma* have a lower sequence identity to *Ganoderma boninense* RSH RS (for the ITS1 ranged from 80.1 to 82.1 %, ITS2 ranged from 61.6 to 73.2 % and Mn-SOD gene ranged from 86.3 to 87.0 %). Apparently, *Ganoderma boninense* RSH RS was misnamed and was not associated with palms (Moncalvo, personal communication). In this case, a molecular approach has proven to be consistent to define *Ganoderma* species which are pathogenic to oil palms.

In this study, the use of species-specific primers designed from variable DNA sequences, PCR-RFLP analyses of the rDNA with specific restriction enzymes, and DNA sequence alignment to infer phylogenetic trees showed consistency in identification and differentiation of oil palm *Ganoderma* and other *Ganoderma* species studied. This finding conclude that oil palm *Ganoderma* causing the BSR disease on oil palms belongs to a single species.

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Abbreviations

AFLP	Amplified fragment length polymorphisms
BSA	Bovine serum albumin
BSR	Basal stem rot
bp	Base pair
CTAB	Cetyltrimethylammoniumbromide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
ELISA	Enzyme-linked immunosorbent assay
IGS	Intergenic spacers
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITS	Internal transcribed spacers
LB	Luria-Bertani
LB_{amp}	Luria-Bertani ampicillin
Mn-SOD	Manganese superoxide dismutase
mM	Millimolar
µM	Micromolar
µl	Microlitre
mRNA	Messeger ribonuleic acid
NCBI	National Centre for Biotechnology Information
ng	Nanogram
OD	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pg	Picogram
pmol	Picomole

PVPP	Polyvinilpolypyrrolidone
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphisms
SDS	Sodium dodecyl sulfate
Taq	*Termus aquaticus*
TBE	Tris/borate/EDTA (buffer)
TE	Tris/EDTA (buffer)
TSS	Transformation and storage solution
U	Units
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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