Condro Utomo

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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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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1 Introduction

Oil palm (*Elaeis guineensis* Jacq.) is one of the most important estate crops in Indonesia. In 1968, the total area under oil palm cultivation was only about 120,000 hectares but by 1997 the area had extended to 2.5 million hectares, an increase of almost 21 times in 30 years (Directorate General of Estate Crops, 1997). Onwards from the second and subsequent planting cycles, oil palm is threatened by fungal pathogens, especially by *Ganoderma* spp. (Aphyllophorales, Basidiomycota), the causal agent of basal stem rot (BSR) disease that attacks the root system of oil palm.

In Southeast Asia, oil palm is planted as a monoculture on areas that previously supported other plantation crops such as rubber, cocoa or coconut, or may be planted on areas cleared from primary forest. Although oil palms are planted in areas that supported perennial crops, the influence of these different cropping systems on BSR incidence in oil palm is unclear. The BSR disease was first reported in Malaysia in 1930 and the causal agent was identified as *G. lucidum* (W.Curt.:Fr.) Karst. (Thompson, 1931). Steyaert (1967) identified six additional species associated with BSR in oil palms in Malaysia and Indonesia (Sumatra) and named them *G. boninense* Pat., *G. miniatocinctum* Steyaert sp.nov., *G. chalceum* (Cooke) Steyaert, *G. tornatum* (Pers.) Bers., *G. zonatum* Murill and *G. xylonoides* Steyaert. Even though 15 species of *Ganoderma* were later found to be associated with BSR in oil palms (Turner, 1981), recent studies in Indonesia and Malaysia indicate that BSR is caused by only one single species, *Ganoderma boninense* Pat. (Ho and Nawawi, 1985). In another area where oil palms are grown (West Africa), four species of *Ganoderma* have been identified from diseased oil palms namely, *G. zonatum* Murill, *G. colossus*, *G. encidum* and *G. applanatum* Pers.ex S.F Gray (NIFOR, 1978).

Previously it was thought that BSR does only occur on old oil palms during the first cycle of field planting. However, *Ganoderma* was recently found to attack also young palms during the second planting cycle (Singh, 1991). Following the increased planting of oil palm, infection of young oil palms was also reported in Papua New Guinea (Sanderson et al., 2000) and Thailand (Tummakate and Likhitekaraj, 1998). This fact leads to the assumption that the BSR infection takes place also in younger palms and is a result of the contact of healthy roots of young oil palm with infected tissue of previously grown oil

palms. The incubation period of the BSR disease has been determined to be several years (Turner, 1981). Unfortunately, the visible disease symptoms appear only at a very late stage of infection. By the time symptoms are observed, more than half of the bole tissue has already decayed, leaving no chance for the grower to cure the infected oil palms. An unpublished survey by the author on some oil palm plantations in North Sumatra (Indonesia) indicated that the *Ganoderma* infection rate reached 70 % in certain areas of a second planting cycle after 15 years of growing. Similar infection rates were also reported in Malaysia (Arifin et al., 1996; Hashim, 1995; Singh, 1991; Turner, 1981). In Indonesia, high incidence of BSR disease was recorded in replants in podsol and coastal clay soil (Hasan and Turner, 1998). In peat soils, which were once thought to be non-conducive for the BSR disease (Turner, 1981), serious incidences of the disease have been reported (Arifin et al., 1989; Rao, 1990). BSR is now recognised as a significant constraint to sustainable oil palm production in Southeast Asia and the development of techniques for disease management has been highlighted as a key research priority.

A limiting factor in controlling the BSR disease is the lack of reliable diagnostic methods that allow its early detection. So far only two methods have been developed for an early diagnosis of BSR. For both methods, diseased material of oil palms is sampled with a wood drill and the first method involved the use of semi-selective media for cultivating *Ganoderma* on agar plates (Arifin et al., 1993). The second method is based on the use of polyclonal antibodies which are applied in an indirect enzyme-linked immunosorbent assay (ELISA) (Utomo, 1997).

However, all conventional methods are time-consuming and their accuracy is not very high. Therefore, the availability of a rapid and accurate diagnosis, which is readily adapted to large-scale testing for detecting the presence or absence of *Ganoderma* in oil palm at an early stage of infection would benefit the detection and also facilitate decisions for taking appropriate control measures. A new approach for the early detection of BSR could be the use of molecular methods such as polymerase chain reaction (PCR) which would fulfil most of the above-mentioned requirements.

Recently, internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) have been chosen as attractive targets for PCR detection due to the generally conserved regions

within one species, but considerably high sequence variation between different species (White et al., 1990). The ITS sequences have been proven to be useful in generating primers for a species-specific PCR detection of pathogenic fungi in naturally infected plant tissue (Mazzola et al., 1996; Bunting et al., 1996; Lovic et al., 1995; Tisserat et al., 1994; Tooley et al., 1997).

Ganoderma is probably the morphologically most complex genus of the polyporus fungi. An indication of this problem is the 290 taxonomic names that have been published belonging to this genus (Moncalvo and Ryvarden, 1997). The situation can be exemplified by *G. lucidum* which alone has been described 13 times as a new occurrence in Europe (Donk, 1974). Conventional taxonomic differences are based on basidiome characteristics, which include the size of basidiospores and morphology of cutis hyphal elements of the basidiome (Adaskaveg and Gilbertson, 1986 and 1989; Bazallo and Wright, 1982; Pegler and Young, 1973; Steyaert, 1972 and 1980). However, such characters can vary under different growing conditions. For instance, Chen (1993) demonstrated in several *Ganoderma* species that the shape of basidiocarp was greatly influenced by environmental factors. Furthermore, Adaskaveg and Gilbertson (1986) revealed that basidiocarps of *Ganoderma* species have a very similar appearance that has caused confusion in identification among species. Pegler and Young (1973) subsequently stated that species identification has proved difficult in *Ganoderma*, partly due to polymorphism of the basidiocarp and partly to the need for microscopic analysis of the pileal crust and basidiospore structures. Also, Steyaert (1975) demonstrated that the size of the basidiospore of *G. tornatum* (Pers.) Bres. varies with latitude and altitude, and observations in *G. lucidum* (W.Curt.:Fr.) Karst. showed that context color was darker from more southern latitudes on the European continent (Steyaert, 1972). As a consequence, there are overlapping characters that are represented by many synonyms, species complexes, and misidentification of *Ganoderma*. Ryvarden (1991) noted that the genus *Ganoderma* represents presently a taxonomic chaos. Species identification and species circumscription are often unclear, and the taxonomic segregation is controversial (Steyaert, 1972 and 1980; Corner, 1983; Zhao, 1989). This situation is further complicated by the dubious nomenclature and inconsistencies in application of the numerous criteria by which the genus has been subdivided (Bazzalo and Wright, 1982; Gilbertson and Ryvarden, 1986; Steyaert, 1980).

Besides morphological traits of fruiting bodies, additional taxonomic characters have been investigated for the differentiation and identification of *Ganoderma*. Cultural studies to identify *Ganoderma* were conducted by Nobles (1958), Bazzalo and Wright (1982), and Adaskaveg and Gilbertson (1986 and 1989), but these attempts caused more confusion because they were often quite different from classical identifications based on morphological features. For example, Nobles (1958) described the differences in the cultural characteristics of *G. lucidum*, *G. tsugae* and *G. oregonense*. Later, the isolates previously listed as *G. lucidum* were changed to *G. sessile* (Nobles, 1965). Biochemical and molecular parameters like isozymes (Miller et al., 1995; Gottlieb et al., 1998; Smith and Sivasithamparam, 2000b) and random amplification of polymorphic DNA (RAPD) (Abu-Seman et al., 1996; Pilotti et al., 2000) were applied. Results of isozymes and RAPDs are difficult to interpret for differentiation among *Ganoderma* species because they produced variable electrophoretic patterns.

An alternative approach to the taxonomic problems in *Ganoderma* has arisen through analysis of various ribosomal DNA (rDNA) genes. Ribosomal DNA (rDNA) regions have often been chosen for taxonomic and phylogenetic studies because sequence data are available and they form a mosaic pattern of conserved and variable regions which make them attractive to be used to discriminate fungal taxa at many levels starting from family (Hibbett and Donoghue, 1995) to genera (Berbee and Taylor, 1992) and species (Cooke and Duncan, 1997; Anderson et al., 1998; Chillali et al., 1998). In fungi as well as other organisms, the noncoding regions of rDNA are known to be variable regions. These are the internal transcribed spacers (ITS) and the intergenic spacers (IGS). While genes encoding the ribosomal subunits such as the 18S, 5.8S and 28S are highly conserved, the IGS and the ITS between these subunits are less conserved and are often the source of length heterogeneity (Klassen and Buchko, 1990). The ITS regions (ITS1 and ITS2) that lie between the 18S and 28S genes lack a functional role (Nues et al., 1994), which is considered to explain the high levels of sequence variation within them. Molecular analysis in the ITS regions has proven useful in differentiating a wide variety of fungi including *Phytophthora* (Lee and Taylor, 1992), *Pythium* (Chen, 1992), *Peronosclerospora* (Yao et al., 1992), *Verticillium* (Nazar et al., 1991), *Fusarium* (O'Donnell, 1992), *Colletotrichum* (Sherriff et al., 1994; Brown et al., 1996) and *Magnaporthe* (Bunting et al., 1996). Moncalvo et al. (1995a, b) and Smith and Sivasithamparam (2000a) used rDNA-ITS

sequence to distinguish between isolates of Ganodermataceae. In some fungal genera, because the ITS regions were relatively conserved, IGS1 region was used as an alternative to discriminate among genera. For example, the IGS1 region was useful for discrimination of closely related fungi within the genus *Armillaria* (Anderson and Stasovski, 1992) and *Laccaria* (Henrion et al., 1992), and for race identification in *Puccinia* (Kim et al., 1992).

In *Ganoderma* spp., the ITS regions were suitable to distinguish between species and to infer their phylogenetic relationships, while variation in the divergent D2 domain of the 25S rDNA was too low (Moncalvo et al., 1995a). Although ITS regions have been sequenced from many *Ganoderma* species, very few DNA sequences have been obtained from isolates associated with palms, and none is available through the public access databases. In order to support the validity of the differentiation and identification of oil palm pathogenic *Ganoderma* by comparison of the ITS data sets, another additional data set of manganese superoxide dismutase (Mn-SOD) gene was applied for comparable study in the differentiation and identification of oil palm pathogenic *Ganoderma*. Sequence data of the Mn-SOD gene are available through public access databases such as GenBank and European Molecular Biology Laboratory (EMBL).

Another molecular approach was to develop a molecular diagnostic tool by exploiting the available sequence data of laccase gene from various basidiomycete fungi. In basidiomycete fungi, the gene encoding the laccase enzyme (benzenediol : O_2 oxidoreductase; E C 1.10.3.2) has been widely studied, apart from its role in delignification of plant material (Hatakka, 1994; Thurston, 1994; Eggert et al., 1997). Laccase enzyme appears to be involved also in different cellular processes such as sporulation (Leatham and Stahman, 1981), pigment production (Aramayo and Timberlake, 1990; Sannia et al., 1986), fruiting body formation (De Vries et al., 1986), rhizomorph induction (Worrall et al., 1986) and also in plant pathogenesis (Geiger et al., 1986; Choi et al., 1992). Previous studies indicated that intron sequences of laccase genes in basidomycete fungi show a low homology within the genus (D'Souza et al., 1996). Laccase gene is one of the attractive targets for the construction of species-specific primers for molecular diagnosis of oil palm *Ganoderma*. The development of these primers is based on the following observations: (i) a large number of genes encoding laccase from basidiomycete fungi has been sequenced and deposited in public databases, (ii) the genes contain relatively large number of introns,

for example, the laccase genes from *Phlebia radiata* (9 introns) (Saloheimo et al., 1991), *Coriolus hirsutus* (10 introns) (Kojima et al., 1990), *Agaricus bisporus* (14 introns) (Perry et al., 1993), *Trametes villosa* (10 introns) (Yaver et al., 1996), *Pycnoporus cinnabarinus* (10 introns) (Eggert et al., 1998), *Coprinus cinereus* (10 introns) (Yaver et al., 1999) and *Lentinula edodes* (13 introns) (Zhao and Kwan, 1999) and (iii) the protein-coding regions of the genes are generally more highly conserved in related species than intron sequences. A low homology and large number of introns among basidiomycete fungi make laccase gene sequences a good target for the development of molecular diagnostics in order to characterise the oil palm *Ganoderma* isolates that are associated with BSR disease.

The objectives of this research were to develop molecular diagnostics for detection, identification and differentiation of the oil palm pathogen *Ganoderma* (namely, oil palm *Ganoderma*). An approach for detecting the oil palm pathogen *Ganoderma* in naturally infected plants is the use of the ITS regions as a target for generating specific primers. For identification of the oil palm pathogen *Ganoderma*, two nucleotide sequence data generated from the ITS and Mn-SOD genes were used. To achieve identification, sequence data of the ITS and Mn-SOD regions of the oil palm *Ganoderma* isolates were compared with the corresponding sequences of other *Ganoderma* species that are available from GenBank. To develop identification and differentiation procedures for the oil palm *Ganoderma* against other *Ganoderma* that are non-pathogenic or pathogenic to other plants, the ITS, IGS1 sequences and the DNA sequence of the laccase gene were exploited. In the ITS and IGS1 regions, the presence of sequence variation between oil palm pathogen *Ganoderma* and other *Ganoderma* species was used to assess the potential for discriminating the oil palm pathogen *Ganoderma* against other *Ganoderma* species. The methods employed in this approach were restriction fragment length polymorphisms (RFLP) analyses of the ITS and IGS1 and generation of species-specific PCR primers for the oil palm pathogen *Ganoderma* isolates. To generate species-specific PCR primers from laccase genes for the oil palm pathogen *Ganoderma*, a sequence in the conserved copper-binding region and/or other amino acid conserved regions were used. Another approach of laccase primer design is from the conserved copper-binding region and the introns of the laccase gene.

2 Materials and methods

2.1 Media, reagents and chemicals

The culture media and other chemicals used, if not stated, were obtained from Merck, Darmstadt (Germany). Oligonucleotides (PCR primers) were synthesised by IBA-GmbH, Göttingen (Germany) and deoxynucleotide (dNTPs) mix was purchased from Promega, Madison (USA). *Taq* DNA polymerase was obtained from Mira Diagnostica GmbH, Leverkusen (Germany). The pH of the media, when necessary, was adjusted using NaOH or HCl before autoclaving. When antibiotics were used, they were added to the medium after autoclaving and cooling to about 50 °C.

2.1.1 Media for fungi

Potato dextrose agar (PDA) medium

PDA powder (Fluka)	39.0 g
Double distilled water	1,000.0 ml

Dissolve 39 g of PDA powder in 1,000 ml of double distilled water. Sterilise by autoclaving at 121 °C for 15 min.

Czapek-yeast (CY) medium for saprophytic fungi

Czapek Dox liquid (Oxoid)	34.4 g
Yeast extract (Oxoid)	10.0 g
Double distilled water	1,000.0 ml

The mixture was autoclaved at 121 °C for 15 min.

Malt-yeast (MY) medium for *Ganoderma* (Burger et al.,1994)

Malt extract	15.0 g
Yeast extract (Oxoid)	5.0 g
Double distilled water	1,000.0 ml

The mixture was autoclaved at 121 °C for 15 min.

2.1.2 Reagents and buffers for DNA extraction

DNA extraction buffer (Raeder and Broda, 1985)

Tris-HCl (pH 8.5)	200.0 mM
NaCl	250.0 mM
EDTA	25.0 mM
SDS	0.5 %

Dissolve 1.21 g of Tris in 45 ml of double distilled water, add 0.73 g of NaCl, 0.37 g of EDTA and 0.25 g of SDS. The solution was adjusted to pH 8.5 with 37 % HCl, filled up to 50 ml and autoclaved at 121 °C for 15 min.

DNA extraction buffer (50 ml) (Möller et al.,1992)

Tris-HCl (pH 8.0)	100.0 mM
EDTA	10.0 mM
SDS	2.0 %

Dissolve 0.61 g of Tris in 45 ml of double distilled water, add 0.15 g of EDTA and 1.0 g of SDS. The solution was adjusted to pH 8.0 with 37 % HCl, filled up to 50 ml and autoclaved at 121 °C for 15 min.

TE buffer (50 ml)

Tris-HCl (pH 8.0)	10.0 mM
EDTA	1.0 mM

Dissolve 0.061 g of Tris in 45 ml of double distilled water and add 0.015 g of EDTA. The solution was adjusted to pH 8.0 with 37 % HCl, filled up to 50 ml and autoclaved at 121 °C for 15 min.

Tris-HCl/BSA pH 7.8 buffer (Niepold, 1999)

Tris	0.1 M
BSA (Boehringer Mannheim)	3.0 %
Double distilled water	100.0 ml

Dissolve 1.21 g of Tris in 95 ml of double distilled water. The solution was adjusted to pH 7.8 with 37 % HCl, filled up to 100 ml and autoclaved at 121 °C for 15 min. Cool to about 50 °C and add 3 g of BSA.

Ringer's solution (500 ml)

Dissolve 1 tablet of Ringer's solution (Oxoid) in 500 ml of double distilled water. Sterilise by autoclaving at 121 °C for 15 min.

Proteinase K solution (1 ml)

Proteinase K (Fluka) 20 mg

Dissolve 20 mg of Proteinase K in 1 ml of Ringer`s solution. Solution was stored at – 20 °C.

Ribonuclease solution (20 mg/ml TE buffer)

Ribonuclease A (Sigma) 20 mg

Dissolve 20 mg of Ribonuclease A in 1 ml of TE buffer and boil for 5 min. Solution was stored at - 20 °C.

2.1.3 Reagents and buffers for PCR

10 x PCR buffer **(stock solution)**

Tris-HCl (pH 8.8)	670.0 mM
$(NH_4)_2SO_4$	160 0 mM
Tween 20	0.1 %

Dissolve 8.11 g of Tris in 95 ml of double distilled water, added with 2.11 g of $(NH_4)_2SO_4$ and 100 µl of Tween 20. The solution was adjusted to pH 8.8 with 37 % HCl, filled up to 100 ml and autoclaved at 121 °C for 15 min. The buffer was stored at – 20 °C.

$MgCl_2$ solution (stock solution)

$MgCl_2$ 100 mM

Dissolve 2.03 g of $MgCl_2$ X $6H_2O$ in 100 ml of double distilled water and autoclave at 121 °C for 15 min. The reagent was stored at – 20 °C

Nucleotide mix (dNTPs)

dATP	2.5 mM
dCTP	2.5 mM
dGTP	2.5 mM
dTTP	2.5 mM

10 µl from each nucleotide of the original 100 mM of nucleotide solution was diluted in 390 µl of millipore H_2O. The mixtures were stored at – 20 °C.

Reaction mix (20 µl) of PCR reagents for oil palm *Ganoderma* detection

Composition	Quantity (µl)	End concentration
10 x PCR buffer	2.5	1x
$MgCl_2$ (100 mM)	0.3	1.5 mM
Nucleotide mix (10 mM)	0.4	50.0 µM of each dNTP
Forward primer (25 pmol)	0.4	0.5 pmol
Reverse primer (25 pmol)	0.4	0.5 pmol
Taq polymerase (5U/µl)	0.4	2.0 U/20 µl
Double distilled water (sterile)	15.6	
Final volume	20.0	

Reaction mix (20 µl) of PCR reagents for the laccase gene amplification

Composition	Quantity (µl)	End concentration
10 x PCR buffer	2.5	1x
$MgCl_2$ (100 mM)	0.2	1.0 mM
Nucleotide mix (10 mM)	0.4	50.0µM of each dNTP
Forward primer (25 pmol)	0.4	0.5 pmol
Reverse primer (25 pmol)	0.4	0.5 pmol
Taq polymerase (5U/µl)	0.2	1.0 U/20 µl
Double distilled water (sterile)	15.9	
Final volume	20.0	

Reaction mix (20 µl) of PCR reagents for PCR amplification of the Mn-SOD gene, ITS and IGS 1 regions

Composition	Quantity (µl)	End concentration
10 x PCR buffer	2.5	1x
$MgCl_2$ (100 mM)	0.2	1.5 mM
Nucleotide mix (10 mM)	0.4	50.0 µM of each dNTP
Primer forward (25 pmol)	0.4	0.5 pmol
Primer reverse (25 pmol)	0.4	0.5 pmol
Taq polymerase (5U/µl)	0.2	1.0 U/20 µl
Double distilled water (sterile)	15.8	
Final volume	20.0	

Reaction mix (20 µl) of PCR reagents for amplification of positive clones

Composition	Quantity (µl)	End concentration
10 x PCR buffer	2.5	1x
$MgCl_2$ (100 mM)	0.3	1.5 mM
Nucleotide mix (10 mM)	0.4	50.0 µM of each dNTP
M13 forward (25 pmol)	0.4	0.5 pmol
M13 reverse (25 pmol)	0.4	0.5 pmol
Taq polymerase (5U/µl)	0.2	1.0 U/20 µl
Double distilled water (sterile)	15.8	
Final volume	20.0	

Primers for analysis of positive transformants (clones)

Positive clones were selected by PCR amplification using universal primers of M13 forward (5` GTAAAACGACGGCCAGT `3) and M13 reverse (5` CAGGAAACAGCT ATGAC `3) that flanking the inserted plasmid.

2.1.4 Media and reagents for cloning and sequencing

2.1.4.1 Media for propagation of *Escherichia coli*

Liquid Luria-Bertani (LB) medium (Maniatis et al., 1982)

Tryptone (Roth)	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g

Dissolve 10 g of Trypton, 5 g of yeast and 10 g of NaCl in 950 ml of double distilled water. The mixture was adjusted to pH 7,0 with 37 % HCl, filled up to 1000 ml and autoclaved at 121 °C for 15 min.

Solid LB medium

Liquid LB medium	1000.0 ml
Agar	1.5 %

Dissolve 15 g of agar in 1 liter of liquid LB medium, adjust to pH 7,0 with 37 % HCl and sterilise by autoclaving at 121 °C for 15 min.

Liquid or solid LB medium containing 100 mg ampicillin (LB_{amp})

Liquid or solid LB medium	1000.0 ml
Ampicillin	0.1 g

Autoclave liquid or solid LB medium at 121 °C for 15 min. Cool to about 45-50 °C and add filter sterilised solution of ampicillin (0.1 g of ampicillin dilute in 1 ml of sterile double distilled water).

Transformation and storage solution (TSS) (Chung and Miller, 1993)

Liquid LB media	10.0 ml
Polyethylene glycol/ PEG (Mr.4,000)	1.0 g
$MgCl_2$	1.9 g
DMSO	0.5 ml

Dissolve 1 g of PEG and 1.9 g of $MgCl_2$ in 10 ml of LB media, adjust to pH 6.8 with 2 N HCl and then add 0.5 ml of DMSO. The solution was sterilised by filtration (Nalge, Rochester, USA).

2.1.4.2 Reagents for cloning and sequencing

Klenow reaction

Composition	Quantity (µl)	End concentration
Klenow enzyme (2 U/µl) (Boehringer Mannheim)	1.5	3.0 U/20 µl
$MgCl_2$ (25 µM)	8.0	10.0 mM
Nucleotide mix (8 mM)	0.25	25.0 µM of each dNTP
10 x Klenow buffer	2.0	1 x
PCR product	x µl	250.0-500.0 ng
Double distilled water (sterile)	x µl	
Final volume	20.0	

Ligation reaction

Composition	Quantity (µl)	End concentration
T4 ligase enzyme (5 U/µl) (Boehringer Mannheim)	0.2	1.0 U/20 µl
Sma I digested pTZ19R	1.0	250.0 ng
10 x ligation buffer	2.0	1 x
PCR product (klenow)	x µl	125.0-250.0 ng
Double distilled water (sterile)	x µl	
Final volume	20.0	

X-Gal stock solution (20 ml)

X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside/Boehringer Mannheim) 400 mg

(Dissolve 400 mg of X-Gal in 20 ml of Dimethylformamide and store at – 20 °C).

IPTG stock solution (20 ml)

IPTG (isopropyl-β-D- thiogalactopyranoside/Boehringer Mannheim) 0.48 g

(Dissolve 0.48 g of IPTG in 20 ml of sterile water and store at – 20 °C).

Reagents for sequencing

Composition	Quantity (μl)	End concentration
Premix (Perkin Elmer, USA)	4 μl	1x
Double stranded DNA (plasmid)	x μl (250 ng)	250.0 ng/10 μl
Primer (10 pmol)	1 μl	1 pmol
H_2O (sterile) fill up to	10 μl	

2.1.5 Solution and buffers for agarose gel electrophoresis

10 x Electrophoresis buffer (TBE buffer: stock solution)

Tris	890.0 mM
Boric acid	890.0 mM
EDTA	20.0 mM
Double distilled water	1,000.0 ml

Dissolve 107.8 g of Tris, 55.0 g of boric acid and 5.85 g of EDTA in 1,000 ml of double distilled water (stock solution). Buffer was stored at room temperature. For electrophoresis, stock solution was diluted 1:10 in double distilled water.

5 x Loading buffer (10 ml)

Glycerol	50 %
TBE	5 x (half strength of stock solution)
Bromphenol blue (BPB)	0.3 %

Dissolve 30 mg of BPB in 4.25 ml of 10xTBE (stock solution) and add 5.75 ml of 87 % Glycerol. Buffer was stored at room temperature.

DNA marker

100 bp DNA ladder (100 μl)

Dilute 25 μl of 100 bp DNA ladder (GibcoBRL Life Technologies Inc., Gaithersburg, USA) in 25 μl of 5x loading buffer and 50 μl of double distilled water.

1 Kb plus DNA ladder

Dilute 25 μl of 1 Kb plus DNA ladder (GibcoBRL Life Technologies Inc., Gaithersburg, USA) in 25 μl of 5x loading buffer and 50 μl of double distilled water.

Lambda DNA/Eco47I (AvaII) Marker solution (100 µl)

Dilute 25 µl of Lambda DNA/Eco47I (AvaII) Marker (MBI Fermentas, Lithuania) in 25 µl of 5x loading buffer and 50 µl of sterilized bidest.

Eco47I digest of λ DNA yields the following 36 discrete fragments (in base pairs): 8126, 6555, 6442, 3676, 2605, 2555, 2134, 2005, 1951, 1612, 1420, 1284, 985, 974, 894, 597, 590, 513, 511, 433, 398, 345, 310, 272, 242, 215, 151, 88, 73, 67, 45, 42, 32, 28, 23.

Ethidium bromide solution (stock solution)

Ethidium bromide 1.0 %

Dissolve 1.0 g of ethidium bromide (Sigma) in 100 ml of double distilled water (stock solution). Stock solution was stored at 4 °C in brown bottle. Pipette 1 ml into Eppendorf tube for daily use. Caution: Ethidium bromide is a highly mutagenic substance. When handling ethidium bromide latex gloves should be worn.

2.1.6 Other chemicals and kits

Restriction enzyme

Restriction enzymes used in this study are, as follows: *EcoRI* (MBI Fermentas, Vilnius, Lithuania), *Sac I*, *Hinc II*, *Hinf I*, *Sca I*, *Tfi I* (New England Biolabs GmbH, Schwalbach, Germany) and *Mlu I* (GIBCO BRL Life Technologies, Eggenstein, Germany).

Kits

Various kits used in this study are, as follows:

- Plasmid (pTZ19R) DNA cat. no. SD0141(MBI Fermentas, Vilnius, Lithuania)
- QIAquick PCR purification kit cat. no. 28104 (Qiagen, Hilden, Germany)
- QIA-prep spin miniprep kit cat. no. 27106 (Qiagen, Hilden, Germany)
- TOPO TA cloning kit cat. no. K4500-01 (Invitrogen BV, Groningen, The Netherlands)
- Ready Reaction BigDye Terminator Cycle Sequencing kit /Premix cat. no. 4303149 (Perkin Elmer Corp., Carlifornia, USA).

2.1.7 Fungal isolates

The fungal isolates used in this study are shown in Table 1.

Table 1: Origin, hosts and source of fungi used in this study

Fungi	Isolate	Source	Host	Country
Ganoderma sp.	BS	IOPRI	*Elaeis guineensis*	Indonesia
Ganoderma sp.	AP	,,	,,	,,
Ganoderma sp.	SP	,,	,,	,,
Ganoderma sp.	PM	,,	,,	,,
Ganoderma sp.	Ad	,,	,,	,,
Ganoderma sp.	Mt	,,	,,	,,
Ganoderma sp.	SB	,,	,,	,,
Ganoderma sp.	BJ7	,,	,,	,,
Ganoderma sp.	BJ8	,,	,,	,,
Ganoderma sp.	GB	,,	,,	,,
Ganoderma sp.	BB	,,	,,	,,
Ganoderma sp.	BL	,,	,,	,,
Ganoderma sp.	SM	,,	,,	,,
Ganoderma sp.	Lo	,,	,,	,,
Ganoderma sp.	BM	,,	,,	,,
Ganoderma sp.	Ti	,,	,,	,,
Ganoderma sp.	Ma	,,	,,	,,
Ganoderma sp.	DS	,,	,,	,,
Ganoderma sp.	Pa	,,	,,	,,
Ganoderma sp.	DH	,,	,,	,,
G. boninense	PNG-611	PNG OPRA	,,	PNG
G. boninense	PNG-598	,,	,,	,,
G. boninense	PNG-579	,,	,,	,,
G. boninense	PNG-576	,,	,,	,,
G. boninense	PNG-600	,,	,,	,,
G. boninense	PNG-605	,,	,,	,,
G. boninense	PNG-603	,,	,,	,,
G. boninense	PNG-597	,,	,,	,,
G. boninense	PNG-753	,,	,,	,,
G. boninense	PNG-742	,,	,,	,,
G. boninense	PNG-403	,,	Coconut stump	,,
G. boninense	PNG-407	,,	Coconut stump	,,
G. boninense	PNG-410	,,	Coconut stump	,,
G. boninense	PNG-411	,,	Coconut stump	,,
G. boninense	PNG-219	,,	Coconut stump	,,
G. boninense	PNG-381	,,	Coconut stump	,,
G. boninense	PNG-240	,,	Coconut stump	,,
G. tornatum	PNG-372	,,	Hardwood stump	,,
G. tornatum	PNG-343	,,	,,	,,
G. tornatum	PNG-264	,,	,,	,,
G. tornatum	PNG-283	,,	,,	,,
G. tornatum	PNG-369	,,	,,	,,
G. tornatum	PNG-371	,,	,,	,,
G. tornatum	PNG-344	,,	,,	,,
G. tornatum	PNG-306	,,	,,	,,
G. tornatum	PNG-309	,,	,,	,,
G. tornatum	PNG-308	,,	,,	,,

Continued

Table 1. Continued

Fungi	Isolate	Source	Host	Country
G. tornatum	PNG-310	PNG OPRA	*Hardwood stump*	PNG
G. oerstedii	BAFC.178	U.B.A	Unknown	Argentina
G. oerstedii	BAFC.218	,,	*Pinus* sp.	,,
G. resinaceum	BAFC.384	,,	*Platanus acerifolia*	,,
G. tornatum (applanatum?)	BAFC.671	,,	Unknown	,,
G. resinaceum	BAFC.2288	,,	*Quercus suber*	,,
G. applanatum	BAFC2353	,,	*Eucalyptus* sp	,,
G. lucidum complex	BAFC.2374	,,	Angiosperm trunk	,,
G. tornatum	BAFC.2390	,,	Unknown	,,
G. tornatum?	BAFC.2395	,,	*Cydonia oblonga*	,,
G. applanatum	BAFC.2408	,,	*Salix* sp	,,
G. tornatum	BAFC.2424	,,	*Podocarpus* sp	,,
G. tornatum?	BAFC.2430	,,	Fallen trunk	,,
G. resinaceum	BAFC.2488	,,	Dead stump	,,
G. lucidum complex	BAFC.2495	,,	Dead root	,,
G. applanatum var. tornatum	BAFC.2501	,,	Living tree	,,
Ganoderma sp.	BAFC.2529	,,	Angiosperm trunk	,,
G. applanatum	BAFC.2552	,,	*Nothofagus alpina*	,,
G. tropicum	BAFC.2580	,,	*Cassia multijuga*	,,
G. resinaceum	BAFC.2775	,,	*Platanus acerifolia*	,,
G. lucidum	DSM 9612	DSMZ	Decayed wood	Germany
G. lucidum	DSM 103	DSMZ	Dead wood	,,
G. applanatum	DSM 3800	DSMZ	*Salix humboldtiana*	,,
G. tsugae	--	FAL	Unknown	,,
Ganoderma sp.	No.136	FAL	Dead wood	,,
G. applanatum	No.134	FAL	Dead wood	,,
G. applanatum	G 211	BBA	Dead wood	,,
G. adspersum	G 224	BBA	*Tilia* sp	,,
G. pfeifferi	G 225	BBA	*Quercus robur*	,,
G. cupreum	QFRI 8678.1	CSIRO	Dead wood	Australia
G. cupreum	DFP 4486	CSIRO	Unknown	,,
G. incrassatum	DAR 73783	CSIRO	Stump rainforest	,,
G. australe	DAR73781	CSIRO	*Banksia seminuda*	,,
*Ganoderma sp.*Group 6.3	DAR 73779	CSIRO	*Albizia lebbeck*	,,
G. weberianum	DFP 4483	CSIRO	*Casuarina* sp	,,
Trichoderma koningii	MRS 1	IOPRI	root contaminant	Indonesia
Trichoderma harzianum	MRS 2	,,	,,	,,
Trichoderma viride	MRS 3	,,	,,	,,
Aspergillus flavus	MRS 4	,,	,,	,,
Aspergillus sp.	--	,,	,,	,,
Penicillium sp.	--	,,	,,	,,
Gliocladium sp.	--	,,	,,	,,
Trichoderma sp1.	--	,,	,,	,,
Trichoderma sp2.	--	,,	,,	,,
Trichoderma sp3.	--	,,	,,	,,
Rhizopus sp.	--	,,	,,	,,
Bispora sp.	--	,,	,,	,,
Geotrichum sp.	--	,,	,,	,,
Cylindrocarpon sp.	--	,,	,,	,,
Mucor sp.	--	,,	,,	,,
Monilia sp.	--	,,	,,	,,
Fusarium sp.	--	,,	,,	,,
Botryodiplodia sp.	--	,,	,,	,,

The legend to next page

Source	
IOPRI	Indonesian Oil Palm Research Institute, Medan, Indonesia
U.B.A	Universidad De Buenos Aires, Argentina (gifts of Dr. A. M.Gottlieb)
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany
FAL	Bundesforschungsanstalt für Landwirtschaft, Braunschweig, Germany
BBA	Biologische Bundesanstalt für Land und Forstwirtschaft, Braunschweig, Germany
CSIRO	CSIRO Plant Industry, Canberra, Australia
PNG OPRA	Papua New Guinea (PNG) Oil Palm Research Association, Alotau, PNG

2.2 Detection of *Ganoderma* in oil palm based on PCR

2.2.1 Preparation of oil palm root and fungal samples

Healthy looking oil palms (without visible *Ganoderma* disease symptoms, i.e. absence of decayed tissues at the base and no fruiting bodies of *Ganoderma*) surrounding the diseased plantation area were chosen as samples. Root samples were collected from the plantations by cutting oil palm roots with a hoe or axe at a depth of 15 - 20 cm from the soil surface near the basal trunk. Root samples were taken from 15 oil palm estates in North Sumatra, Indonesia, located at Gunung bayu, Pabatu, Marihat, Bangun, Adolina, Matapao, Bukit Sentang, Dolok Hilir, Aek Pancur, Sei Pancur, Pagar merbau, Rambutan, Bah Jambi, Lonsum and Sei Bamban. Healthy and diseased root samples were separated and washed with distilled water. For each sample, three 15 cm-long pieces of diseased root were used, they were cut into pieces, mixed and weighed (as much as 1.5 g).

For *Ganoderma* isolation, fruiting bodies of pathogenic *Ganoderma* were collected from various oil palm estates in North Sumatra, Indonesia: Aek Pancur (AP), Sei Pancur (SP), Adolina (Ad), Pagar Merbau (PM), Matapao (Mt), Sei Bamban (SB), Bah Jambi 7 (BJ 7), Bah Jambi 8 (BJ 8), Gunung Banyu (GB), Bukit Sentang (BS), Bangun Bandar (BB), Batu Lokong (BB), Sei Mangkei (SM), Lonsum (Lo), Bukit Maraja (BM), Tinjauan (Ti), Dolok Sinumba (DS), Pabatu (Pb), Dolok Hilir (DH) and Marihat (Ma) estates. Basal fruiting bodies were cut into pieces, soaked in 0.5 % of sodium hypochloride for two min, dried and transferred to potato dextrose agar (PDA) plates. For isolation of saprophytic fungi, the diseased roots of oil palm were soaked in 0.5 % of sodium hypochloride for 1 min, cut longitudinally and transferred to PDA plates. After incubation at 30 °C for 4 days, pure cultures were transferred to PDA slants and stored at 10 °C.

Oil palm *Ganoderma* isolates were cultured in MY media as described in 2.1.1 and saprophytic fungi were cultured in CY media as described in 2.1.1. For inoculation, fungal isolates were grown on PDA at 30 °C for seven days. Agar plugs (disks of 1 cm diameter) were taken with a steril cork borer from the growing edge of mycelia of 7-day-old solid PDA plate cultures. Each 250-ml flask containing 100 ml liquid media was inoculated with two agar plugs. The flasks were incubated at 30 °C for all *Ganoderma* for 30 days and at 30 °C for all saprophytic fungi for 10 days, the mycelia were harvested from liquid cultures

by filtration onto Whatman No.1 filter paper and rinsed two times with double distilled water. Mycelia were freeze-dried for two days, ground to a fine powder in a pestle and mortar and then stored at -20 °C until use.

2.2.2 DNA extraction methods

Three DNA extraction methods were used to extract DNA from pure culture *Ganoderma* isolated from oil palm and diseased oil palm roots. The first method was according to Raeder and Broda (1985), the second method was according to Möller et al. (1992) and the third method was the NaOH method described by Wang et al. (1993) modified by Niepold (1999). The three procedures for DNA extraction were as follows:

DNA extraction according to Raeder and Broda (1985)

(1) Suspend 50 mg of the ground mycelia in 500 µl of extraction buffer of Raeder and Broda (1985) as described in 2.1.2.

(2) Add 350 µl of phenol and 150 µl of chloroform, mix gently and centrifuge at 15.000 rpm for 20 min at 4 °C.

(3) Remove upper aqueous layer with a cut off pipette tip and transfer into a new 1.5 ml tube, add 25 µl of ribonuclease solution and incubate at 37 °C for 10 min.

(4) Repeat extraction with phenol and chloroform one more time and collect upper aqueos layer with a cut off pipette tip.

(5) Precipitate DNA with equal volume of pre-chilled isopropanol, store at - 20 °C for 30 min and centrifuge at 14.000 rpm for 10 min.

(6) Drain all liquid, wash the pellet in 70 % ethanol, dry at 50 °C for 5 min and redissolve the pellet in 50 µl of TE buffer and store at -20 °C until use.

DNA extraction according to Möller et al. (1992)

(1) Suspend 50 mg of the ground mycelia in 500 µl of extraction buffer of Möller et al (1992) as described in 2.1.2

(2) Add 5 µl of proteinase K solution and incubate for 1 hour at 65 °C with occasional gentle mixing.

(3) Adjust salt concentration to 1.4 M with 5 M NaCl (=140 µl), add 1/10 volume (=65 µl) of 10 % Cetyltrimethylammoniumbromide (CTAB) and incubate at 65 °C for 10 min.

(4) Add 1 volume of chloroform : isoamylalcohol = 24: 1 v/v, mix gently, incubate at 0 °C for 30 min and centrifuge at 14,000 rpm for 10 min at room temperature.

(5) Transfer supernatant into a fresh tube, add 225 µl of 5 M ammonium acetate, mix gently, place on ice for approximately 30 min and centrifuge at 14,000 rpm for 5 min at room temperature.

(6) Transfer supernatant into a fresh tube, add 25 µl of ribonuclease solution and incubate for 10 min at 37 °C.

(7) Precipitate DNA with 0.55 volume of pre-chilled isopropanol, store at - 20 °C for 30 min and centrifuge at 14.000 rpm for 10 min at room temperature.

(8) Decant and drain all liquid, wash pellet with 70 % ethanol, dry at 50 °C for 5 min and dissolve the pellet in 50 µl of TE buffer and store at - 20 °C until use.

NaOH DNA extraction method based on Wang et al. (1993) modified by Niepold (1999)

(1) Suspend 30 mg of the ground mycelia in 300 µl of extraction buffer of Raeder and Broda (1985) as described in 2.1.2 and add 6 µl of proteinase K. For infected plant tissues, suspend 1.5 g of the ground tissues in 1.5 ml of extraction buffer of Raeder and Broda (1985) and add 10 µl of proteinase K

(2) Incubate at 65 °C for 60 min and centrifuge at 13,000 rpm for 2 min.

(3) Transfer 10 µl of supernatant into 20 µl of 0.5 N NaOH, incubate at 95 °C for 10 min and then incubate on ice for 10-30 min.

(4) Dilute 10 µl of supernatant in 90 µl of Tris-HCl/BSA pH 7.8 buffer or more. This suspension can be used directly for PCR.

2.2.3 Determination of DNA concentration by optical density measures

To measure the amount of the extracted DNA sample, 5 µl of DNA sample was diluted in 45 µl of TE buffer and pipetted into a cuvette. The OD-reading was taken at a wave length of 220 to 320 nm to measure DNA in the sample. The reading at OD_{260} of 1 corresponds to approximately 50 µg/ml for double-stranded DNA (Sambrook et al.,1989). The ratio of OD_{260}/OD_{280} shows an estimate for the purity of DNA. Pure preparations of DNA have OD_{260}/OD_{280} between 1.8 and 2.0 (Sambrook et al., 1989).

2.2.4 Primer design and evaluation

2.2.4.1 Primer design and PCR conditions

Primers were generated using the computer program OLIGO 4.0-2019, Primer Analysis Software, 1989-1991 by Rychlik and Rhoads (1989) from sequence of the internal transcribed spacer (ITS) region 1 of ribosomal DNA of *G. boninense* RSH RS. This sequence is derived from The European Molecular Biology Laboratory (EMBL) with accession number X78749. Other primers are also designed from the ITS regions of oil palm *Ganoderma* for detection of oil palm *Ganoderma* in the oil palm root samples (see section of the ITS regions for oil palm *Ganoderma*).

PCR procedure was carried out according to the method described by Niepold and Schöber-Butin (1997). Composition of buffer, nucleotide mix, primers and *Taq* polymerase was described in 2.1.3. For PCR amplification, 5 µl of DNA extraction was adjusted to 20 µl reaction mix. The thermocycler (MJ-Research Programmable Thermo Cycler, type PTC-100, MJ Research, Inc., Massachusetts, USA) was programmed as followed: after 5 min heating at 95 °C, the DNA amplification was carried out in 40 cycles of 35 sec denaturation at 94 °C, 35 sec annealing at 52 °C for primer pair designed from *G. boninense* RSH RS and 61 °C for primer pairs designed from oil palm *Ganoderma* and 45 sec extension at 72 °C. The 40 cycles were ended by 10 min incubation at 72 °C and cooling to 4 °C. All detection experiments were repeated three times. The PCR products were either analysed immediately or stored at -20 °C.

Visualisation of PCR products

For visualization PCR product, the plastic tray was sealed with selotape and the comb was inserted into the proper position. The agarose gel was prepared by adding 1.5 g of agarose to 100 ml of 1x TBE buffer (in a 300 ml Erlenmeyer flask). Agarose was dissolved by heating in a microwave. After cooling, 5 µl of ethidium bromide solution was added and mixed by shaking gently. The comb and tape were removed after the gel had polymerised and then transferred to electrophoresis chamber filled with 1x TBE buffer covering the gel. About 3 µl of the loading buffer was pipetted onto a parafilm strip and 10 µl of each PCR product was added. A standard size marker (2 µl) was applied in the left end-slot.

The electrophoresis was run for 1 h at 120 V. The gel was visualized on UV-transilluminator and photographed.

2.2.4.2 Determination of detection limit of oil palm *Ganoderma* DNA and specificity of primers

To determine detection limit of oil palm *Ganoderma* DNA in PCR amplification, three DNA extraction methods were used to extract DNA from pure culture of oil palm *Ganoderma* (isolate BS). The first method was based on Raeder and Broda (1985) (see 2.2.2). The extracted DNA was set up in 10-fold dilution starting with 50 ng in TE buffer. The second method used was described by Möller et al. (1992) (see 2.2.2). The extracted DNA was prepared in 10-fold dilution starting with 50 ng in TE buffer. The last method was NaOH method. The extracted DNA was prepared in 10-fold dilution of Tris-HCl/BSA pH 7.8 buffer (see 2.2.2). Each treatment was replicated three times. PCR program was described in 2.2.4.1.

To evaluate primer specificity, experiments were performed with 18 saprophytic fungi (see Table 1) that were occasionally found as saprophytes on the diseased oil palm roots. The NaOH method (see 2.2.2) was used for extracting DNA from oil palm *Ganoderma* and saprophytic fungi. The amount of the ground mycelia used for extraction for both oil palm *Ganoderma* and saprophytic fungi was 50 mg dissolved in 500 μl buffer. For control in the PCR amplification, saprophytic fungi were amplified by using primer pair ITS1-ITS4. Healthy oil palm roots were used as a negative control. At the final step of this method, the extracted DNA samples were diluted in 10-fold of Tris-HCl/BSA pH 7.8 buffer. Each treatment was repeated three times. PCR program was described in 2.2.4.1.

Another treatment to evaluate the primer specificity was to mix the ground mycelia of oil palm *Ganoderma* (isolate BS) or saprophytic fungi with healthy oil palm roots. The NaOH method (see 2.2.2) was used to extract DNA. Healthy oil palm root of 1,5 g was mixed with 50 mg of the powdered mycelia from oil palm *Ganoderma* or saprophytic fungi, extracted in 1,5 ml of buffer and 10-fold dilution series in Tris-HCl/BSA pH 7.8 buffer was made. Particularly, for primer pairs designed from oil palm *Ganoderma*, mixing of healthy oil palm root (as much as 1 g) and oil palm *Ganoderma* was performed at ratios 1:20, $1:10^2$, $1:10^3$, $1:10^4$ and $1:10^5$. DNA was extracted in 1 ml of buffer and 10-fold dilution series in Tris-HCl/BSA pH 7.8 buffer were made. Each treatment was repeated three times. PCR program was described in 2.2.4.1

To detect *Ganoderma* from the naturally infected plants, the diseased root tissues (1.5 g) from 15 oil palm estates were extracted with the NaOH method as described in 2.2.2. The extracted root samples were suspended in Tris-HCl/BSA pH 7.8 buffer. Dilution series of 1:10, $1:10^2$, $1:10^3$ were prepared for PCR amplification. PCR program was described in 2.2.3.1

2.3 Development of molecular diagnosis for identification and differentiation of oil palm *Ganoderma* against other *Ganoderma* species derived from the laccase, ITS region, IGS1 region and Mn-SOD genes

2.3.1 DNA extraction

Oil palm *Ganoderma* isolates and other *Ganoderma* species were cultured in MY media as described in 2.1.1. For DNA extraction, the amount of the powdered mycelia used was 50 mg for each *Ganoderma* and extracted by using the Möller et al. (1992) method as described in 2.2.2. At the final step of this method, DNA pellets were diluted in 250 µl TE buffer. To further purify the extracted *Ganoderma* DNA, the solution was mixed with phenol (1 vol.), shortly vortexed and centrifuged at 13,000 rpm for 5 min. The supernatants were transferred into new tubes and mixed with 1 vol. of phenol/chloroform (1:1), shortly vortexed and centrifuged at 13,000 rpm for 5 min. Subsequently, the supernatants were treated with 1 vol. of chloroform and centrifuged as before. The supernatants were transferred into new tubes and then processed for DNA precipitation.

To precipitate DNA, 6 µl of 5 M ammonium acetate (pH 4.6) was added to 100 µl of supernatant and then 250 µl of 96 % cold ethanol was added and mixed gently. The mixtures were centrifuged at 14,000 rpm for 10 min, and the supernatant was decanted. The pellet was washed with 70 % ethanol, dried in incubator at 50 °C for 5-10 min and resuspended in 50 µl of TE buffer. DNA concentration was measured using Beckman DU-50 Spectrophotometer according to 2.2.3.

2.3.2 Primer design and PCR conditions

2.3.2.1 Laccase gene

To study laccase genes in *Ganoderma*, primers were designed based on the conserved amino acid sequence in copper-binding region I, near copper-binding region II and copper-binding region IV of six laccase genes of published basidiomycete fungi. Six amino acid sequences of the laccase genes were derived from National Centre for Biotechnology Information/NCBI. The following basidiomycete fungi were used: *Trametes villosa* Lac 1 (accession number AAC41686), *T. villosa* Lac 2 (L49377), *T. villosa* Lac 4 (L78077), Basidiomycete CECT 20197 Lac 1 (AAB63443), Basidiomycete PM 1 (CAA78144), *Trametes versicolor* Lac 1 (CAA59161). Another approach to design primesr is based on the sequence from the conserved copper-binding region and introns of the laccase gene of oil palm *Ganoderma*.

In order to obtain optimum DNA concentration in the PCR amplification of the laccase gene, four different DNA concentrations of three oil palm *Ganoderma* isolates (BS, SB and PM) were tested at 200, 100, 10 and 1 ng, respectively. Three primer pairs designed from the laccase genes were used to amplify genomic DNA of oil palm *Ganoderma* isolates.

PCR conditions were carried out as described by Niepold and Schöber-Butin (1997). Composition of buffer, nucleotide mix, primers and *Taq* polymerase was described in 2.1.3. For PCR amplification, 5 µl of the extracted DNA (100 ng) was added to 20 µl reaction mix. The thermocycler was programmed, as followed: after 5 min heating at 95 °C, the DNA amplification was carried out in 40 cycles of 35 sec denaturation at 94 °C, 45 sec annealing at 65 °C and 60 sec extention at 72 °C. The 40 cycles were ended after 10 min extension at 72 °C and cooled to 4 °C. The PCR products were either analysed immediately or stored at -20 °C.

To evaluate specificity of the primer pair designed from the laccase gene, total genomic DNA of three oil palm *Ganoderma* (isolates BS, PM and SB) and purified plasmid inserted fragment of the laccase gene of oil palm *Ganoderma* (namely pU1700) was amplified with different concentrations of $MgCl_2$ and *Taq* polymerase. $MgCl_2$ concentrations used were 1mM, 2 mM and 3 mM meanwhile for *Taq* polymerase were 1U, 2U and 3U. In addition,

for PCR amplification of pU1700 (plasmid inserted fragment of the laccase gene), four different annealing temperatures 59, 61, 63 and 65 °C were used with concentration of pU 1700 was 1 ng.

2.3.2.2 ITS, IGS1 regions, Mn-SOD gene and restriction fragment length polymorphisms (RFLP) analyses

PCR primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') as forward primer and ITS 4 (5' TCCTCCGCTTATTGATATGC 3') as reverse primer were used to amplify the ITS-DNA as described by White et al. (1990). To amplify the IGS1-DNA, primer Q (5'ACGCCTCTAAGTCAGAAT 3') as forward primer (Gutell and Fox, 1988) and primer 5 SA (5'CAGAGTCCTATGGCCGTG 3') as reverse primer (designed by Duchesne and Anderson, 1990) were used. Primer pair to amplify the Mn-SOD gene was designed from nucleotide sequences of the amino acid sequences of LHHKKHH and DIWEHAF in the Mn-SOD gene of *G. boninense* RSH RS that is deposited in NCBI/GenBank with accession number U56128 for nucleotide sequence and AAB16771 for amino acid sequence. To design species-specific primers, nucleotide sequences of the ITS or IGS1 regions of oil palm *Ganoderma* were aligned with the corresponding regions of other *Ganoderma* species by using Clustal V program (MegAlign; DNAstar, Madison, USA) to search variable nucleotide sequences among oil palm *Ganoderma* and other *Ganoderma* species.

PCR procedure was carried out according to the method described by White et al. (1990). Composition of buffer, nucleotide mix, primers and *Taq* polymerase was described in 2.1.3. For PCR amplification, 5 µl (10 ng) of extracted DNA was adjusted to 20 µl reaction mix. The thermocycler was programmed as follows: after 5 min heating at 95 °C, the DNA amplification was carried out in 35 cycles of 30 sec denaturation at 94 °C, 45 sec annealing at 55 °C and 60 sec extension at 72 °C. The 35 cycles were ended after 10 min extension at 72 °C and cooled to 4 °C. To amplify the Mn-SOD genes of oil palm *Ganoderma*, annealing temperature was changed from 55 to 61 °C. For PCR amplification of species-specific primers derived from the ITS region, the annealing temperature was set to 65 °C and for the IGS1 region to 61 °C. The PCR products were either analysed immediately or stored at -20 °C.

To search specific restriction enzymes in the ITS or IGS1 regions of oil palm *Ganoderma*, the restriction sites in the nucleotide sequences of the ITS or IGS1 regions (oil palm *Ganoderma* and other *Ganoderma* species) were determined by using MapDraw program (DNAstar, Madison, USA). *Ganoderma*-ITS sequences deposited in databases were also examined by the specific restriction enzymes using MapDraw program (DNAstar, Madison, USA). For RFLP analyses, the reactions consisted of 10 μl of PCR product, 1.5 μl (10x concentration) restriction buffer, 3.2 μl double distilled water and 0.3 μl (3U) restriction enzyme were used. Incubation temperatures were according to the manufacturer`s recommendations.

2.3.3 Cloning of PCR products

Before cloning, PCR-amplified products were purified by using QIAquick PCR purification kit (Qiagen, Germany) according to the manufacturer`s instructions. For each purification, 50 μl of PCR product was prepared and at the final step, the PCR product was eluted in 50 μl of 10 mM Tris-HCl, pH 8.5. The concentration of PCR product was measured by Beckman DU-50 Spectrophotometer and checked by agarose gel electrophoresis.

2.3.3.1 Cloning of laccase genes into plasmid pTZ19R

Oil palm *Ganoderma* (isolate BS) DNA was amplified by primer pairs designed from the laccase gene and PCR products were cloned into plasmid pTZ19R, as follows:

Klenow and ligation reaction

For Klenow reaction, composition of buffer, nucleotide mix, the purified PCR product and Klenow enzyme was as described in 2.1.4.2. The thermocycle program was 30 min at 30 °C (incubation) and 10 min at 75 °C (enzyme inactivation). For ligation reaction, composition of buffer, plasmid (*Sma I* digested pTZ19R), T4 DNA ligase and PCR product after treating with Klenow enzyme as described in 2.1.4.2. The thermocycle program was 6 hr at 15 °C (incubation) and at 4 °C overnight.

Transformation of plasmids into *Escherichia coli*

Two transformation protocols were used to transform plasmids into competent bacterial cells. The first method was described by Mandel and Higa (1970) and Hanahan (1983).

This method is based on the treatment of bacteria with $CaCl_2$ and a heat shock. The second method described by Chung and Miller (1993) is based on treating bacteria on ice with transformation and storage solution (TSS) containing polyethylene glycol (PEG), dimethyl sulfoxide (DMSO) and magnesium.

Transformation of plasmids into *E. coli* based on Mandel and Higa (1970) and Hanahan (1983)

(1) Inoculate 20 ml of LB medium with 0.15 ml of an overnight culture (*E. coli* DH 10ß) and incubate with shaking (200 rpm) at 37 °C until the $OD\lambda_{600}$ reaches about 0.4 (usually after approximately 2.5 h.).

(2) Harvest cell by centrifugation at 6,000 rpm for 10 min at 4 °C. Suspend the pellet in one-half volume (10 ml) of cold, sterile $CaCl_2$ (50 mM). Place on ice for 15 min and centrifuge at 6,000 rpm for 10 min at 4 °C

(3) Dissolve the pellets in 1 ml of cold $CaCl_2$ (50 mM), place 0.15 ml in pre-chilled glass tubes and maintain on ice for approximately 15 min.

(4) Add pre-chilled DNA (10 µl of DNA ligation) and incubate on ice for an additional 30 min.

(5) Heat-shock by placing the tubes in a water bath at 42 – 45 °C for 3 min. Place the tubes at room temperature for 10 min.

(6) Add to 1 ml of LB medium to the tube and incubate without shaking at 37 °C for 60 min. Plate onto solid LB_{amp} plates with different concentrations of *E. coli* (20 µl, 100 µl and 300 µl), add 70 µl of X-Gal and 5 µl of IPTG, and then homogenize. Incubate the plates at 37 °C overnight.

(7) Select white colonies by picking with sterile toothpick and suspend in PCR reaction mix as described in 2.1.3 to analyse positive transformants. The rest of *E. coli* on toothpick was inoculated on solid LB_{amp} and incubated at 37 °C overnight.

Transformation of plasmid into *E. coli* based on Chung and Miller method (1993)

(1) LB broth (50 ml) was inoculated directly with *E. coli* DH 10ß from the plate and grown at 37 °C with shaking (200 rpm) to the early exponential phase ($OD\lambda_{600}$ = 0.4, usually after approximately 2.5 h.).

(2) Bacteria were pelleted by centrifugation at 6,000 rpm for 10 min at 4 °C and the cell pellets were resuspended with 5 ml of ice-cold TSS.

(3) TSS-treated bacteria were incubated on ice for 15 min and then pipetted into Eppendorf tubes (100 µl per tube).

(4) For transformation, 10 µl of DNA ligation was added to the tubes, followed by gentle mixing and incubated on ice for 30 min.

(5) After the incubation period on ice, 0.9 ml of LB medium was added and the tubes were incubated at 37 °C for 1 h.

(6) Plate on solid LB_{amp} plates with different concentration of *E. coli* (20 µl, 100 µl and 300 µl), add 70 µl of X-Gal and 5 µl of IPTG and then homogenise. Incubate the plates at 37 °C overnight.

(7) Select white colonies by picking with sterile toothpick and suspend in PCR reaction mix as described in 2.1.3 to analyse positive transformants. The rest of *E. coli* on toothpick was inoculated on solid LB_{amp} and incubated at 37 °C overnight.

2.3.3.2 Cloning of the ITS, IGS1 and Mn-SOD gene using the TOPO cloning kit

For cloning of the ITS DNA, eight oil palm *Ganoderma* isolates of Ad, Ap, BJ 7, BJ 8, BS, GB, Mt and SB) were used. For cloning of the IGS1 DNA, four oil palm *Ganoderma* isolates of BJ8, Lo, PM and SB plus *G. adspersum* G224, *G. applanatum* DSM3800, *G. applanatum* G211, *G. australe* DAR73781, *G. cupreum* DFP4336, *G. cupreum* QFRI8678, *G. pfeifferi* G225 and *Ganoderma* sp DAR73779 were used. Oil palm *Ganoderma* isolates of AP, BJ8 and BS were used for cloning of the Mn-SOD gene fragments.

After purification of PCR products, 1 µl (20 ng/µl) of PCR product was directly ligated in plasmid vector of pCR^R 2.1-TOPO from TOPO TA cloning kit (Invitrogen, cat. no. K4500-01) according to the manufacturer`s instructions. Basic principle of the direct ligation for PCR product was based on characteristics of PCR fragments produced by *Taq* polymerase that has single 3`A-overhangs. On the other hand, the linearised plasmid vector of pCR^R 2.1-TOPO has single 3` T-overhangs. After ligation, plasmid vectors were transformed into *E. coli* (TOP 10) and plated onto solid $LB_{amp}$ containing X-gal (50 µl or 1mg/ml) according to the manufacturer`s instructions. White colonies were selected, picked with toothpick and suspend in PCR reaction mix as described in 2.1.3 to analyse positive transformants.

Analysis of positive transformants.

PCR procedure was carried out according to the method described in instruction manual of TOPO TA Cloning kit version J. Composition of buffer, nucleotide mix, primers and *Taq* polymerase used was described in 2.1.3. For PCR amplification, white colonies were picked with sterile toothpicks and transferred into 20 µl of PCR reaction mix. The thermocycler was programmed as follows: after 5 min heating at 95 °C, DNA amplification was carried out in 35 cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 55 °C and 90 sec extention at 72 °C. The 35 cycles were ended after 10 min extension at 72 °C and cooled to 4 °C. The PCR products were either analysed immediately or stored at -20 °C.

Propagation of positive transformants and plasmid purification

Positive transformants were picked with sterile toothpicks and inoculated into vials containing 10 ml of liquid LB_{amp}. Bacteria were grown overnight at 37 °C on a shaker at 200 rpm, harvested and transferred into 1.5-ml Eppendorf tubes. Bacteria were centrifuged at 6,000 rpm for three minutes. To isolate and purify plasmids from bacteria, the pellets were processed with QIA-prep spin miniprep kit (Qiagen, Germany) according to the manufacturer's instructions. Plasmid concentrations were measured by spectrophotometry and checked by agarose gel electrophoresis. The purified plasmids could be directly sequenced or stored at - 20 °C.

2.3.4 Sequencing of plasmid inserted with PCR product

Cycle Sequencing protocol and thermocycler program

Sequencing of plasmid inserts was carried out on both strands using forward and reverse universal primers M13. To continue sequencing, internal sequencing primers were designed every 400 bp along either strand. Sequencing method is based on *Taq* polymerase cycle sequencing (Murray, 1989) with fluorescent labeled dideoxy nucleotides as described by Zimmermann et al. (1994) according to the modified standard procedure described by Sanger et al., (1977). Composition of plasmid, primer and premix used was described in 2.1.4.2. The mixtures were denatured in thermocycler at 96 °C for 5 min, cooled down and then 4 µl of premix was added. PCR conditions for DNA sequencing were, as follows: 10 sec denaturation at 96 °C, continued with ramp temperature at -1 °C /sec to 60 °C, 5 sec annealing at 60 °C, 4 min of extension at 60 °C and then continued with ramp temperature at 1 °C/sec to 96 °C. The cycle was repeated 30 times.

Processing and sequence determination of thermocycler products

The mixture reactions were pipetted into sterile Eppendorf tubes and added with 90 µl of distilled water, 10 µl of 3 M Na-acetate pH 4.6 and 250 µl of 100 % ethanol while gently mixing at room temperature. The tubes were centrifuged at 14,000 rpm for 15 min at room temperature and the ethanol was decanted. To the pellets, 250 µl of 70 % ethanol was added, gently mixed and centrifuged at 14,000 rpm for 5 min at room temperature. Pellets were dried in Speed-Vacuum for 5 min, dissolved in 20 µl of template suppression reagent/TSR (Perkin-Elmer Corp., California, USA) and denatured at 90 °C for 2 min. 15 µl of the solution was pipetted into tubes and the reactions were read in an automated DNA sequencer (model ABI 310, Perkin Elmer Corp., California, USA).

2.3.5 Nucleotide sequence data analysis

Determination of IGS1 region of *Ganoderma* isolates

To determine the 3` end of the large subunit (LS) rDNA of *Ganoderma* isolates, nucleotide sequences of the LS rDNA of 13 *Ganoderma* isolates from primer Q to the 3`end of the gene were aligned with the published genes of *Saccharomyces. cerevisiae* CEN.PK2 (GenBank accession number AJ302029), *Metharhizium. anisopliae* 5530 (AF218207), *Puccinia. graminis* 5297 (Z11586), *Tricholoma. matsutake* 40145 (U62964) and *Schizophyllum. commune* FL06.1 (AF249487). To determine the 5`end of the 5S rDNA of 13 *Ganoderma* isolates, six nucleotide sequences of the first 21 bases at the 5`end of 5S rDNA from other fungi such as *S. commune* 5334 (X06853), *Lentinula. edodes* 5353 (AB030581), *Laccaria. bicolor* S238N (AF116534), *T. matsutake* 40145 (Z49275), *Pleurotus. ostreatus* 5322 (K03168), *Coprinus. cinereus* 5346 (K02344) and *G. applanatum* FP-125024-T (X73589) were aligned with 13 *Ganoderma* isolates.

Data analysis

For the laccase genes, computer-assisted comparisons of the nucleotide sequences were made to find the similarities of nucleotide sequences in NCBI/GenBank, using BLASTN program (Altschul et al., 1997). Nucleotide sequences that encoded amino acid (exons) for the laccase and Mn-SOD genes were translated to the (deduced) amino acid sequences by using EditSeq (DNAstar, Madison, USA). To compare with ingroup sequences, additional sequences from GenBank were used as described in Table 3 for the ITS region and in Table 4 for the Mn-SOD gene. To compare the laccase gene of oil palm *Ganoderma* with

other laccase genes, several laccase genes of basidiomycete fungi were used as described in Table 2. *Fomitopsis rosea* T92-10 (GenBank accession numbers X78754 and X78763) was used as outgroup for sequence analysis and reconstruction of a phylogenetic tree based on the ITS, while *Amauroderma rude* JMM ASP.1 (U56109) was used as outgroup for phylogeny reconstruction based on the Mn-SOD genes. *Armillaria singula* (D89926). was used as outgroup for phylogeny reconstruction based on the IGS1 region. Alignments of sequences were done using Clustal V algorithm method (MegAlign; DNAstar, Madison, USA). Percent identities, alignment reports and phylogenetic trees of the laccase gene, Mn-SOD gene, ITS and IGS1 regions were calculated and constructed using MegAlign program (DNAstar, Madison, USA).

Table 2: Laccase amino acid sequences of various basidiomycete fungi used for comparison with the laccase genes from oil palm *Ganoderma* BS

Fungi and gene	Laccase amino acid sequence GenBank accession numbers
1. *Agaricus bisporus* Lac 1	AAC18877
2. Basidiomycete CECT 20197 Lac pox 1	AAB63443
3. Basidiomycete PM 1 Lac	CAA78144
4. *Coprinus cinereus* Lac 1	AAD30964
5. *Ceriporiopsis subvermispora* Lac	AAC97074
6. *Lentinula edodes* Lac 1	AAF13037
7. *Marasmius quercophilus* Lac 1	AAF06967
8. *Pycnoporus cinnabarinus* Lac 1	AAF13052
9. *Pleurotus ostreatus* Lac	CAA06291
10. *Phlebia radiata* Lac	CAA36379
11. *Schizophylum commune* Lac (mRNA)	BAA31217
12. *Trametes versicolor* Lac 1	CAA59161
13. *Trametes villosa* Lac 1	AAC41686

Table 3: Published nucleotide sequences of the ITS1 and ITS2 regions from the different *Ganoderma* species obtained from GenBank

Ganoderma	ITS 1	ITS 2	Host
	GenBank acc. numbers	GenBank acc. numbers	
G. adspersum CBS 351.74	X78742	X78763	*Salix* sp.
G. ahmadii FWP 14329	Z37047	Z37098	*Dalbergia sissoo*
G. australe 07505	X78750	X78771	Unknown
G. boninense RSH RS	X78749	X78770	Unknown
G. colossum CBS 216.36	Z37071	Z37091	Unknown
G. cupreum DFP 3896	-	-	*Casuarina* sp.
G. cupreum DFP 4336	-	-	Softwood
G. cupreum QFRI 8678	-	-	Dead wood
G. formicatum RSH 0814	Z37067	Z37087	Hardwood
G. formosanum 0109	X78752	X78773	Unknown
G. gibbosum ACCC 5.151	X78741	X78762	Unknown
G. lipsiense BAFC 2424	AF169977	AF169978	*Podocarpus* sp.
G. lobatum BAFC 2411	AF169989	AF169990	*Salix* sp
G. lucidum ATCC 324.71	X78744	X78765	*Acrocarpus* sp.
G. lucidum BAFC 2419	AF170007	AF170008	From soil/roots
G. microsporum RSH 0821	X78751	X78772	*Salix babylonica*
G. oerstedii ATCC 52410	X78739	X78760	Unknown
G. oregonense CBS 177.30	Z37060	Z37100	Conifer
G. pfeifferi CBS 747.84	X78738	X78759	*Quercus* sp.
G. platense BAFC 384	AF170001	AF170002	*Platanus acerifolia*
G. resinaceum CBS 194.76	X78737	X78758	*Fagus sylvatica*
G. sessile BAFC 2373	AF170005	AF170006	Angiosperm
G. sessiliforme BAFC 2580	AF169999	AF170000	*Cassia multijuga*
G. sinense ZHANG 1734	Z37066	Z37103	Unknown
G. subamboinense ATCC 52419	X78736	X78757	*Platanus* sp.
G. tornatum BAFC 1172	AF169975	AF169976	Rotted trunk
G. tornatum BAFC 1139	AF169979	AF169980	Unknown
G. tropicum HK 93-8	Z37069	Z37089	Hardwood
G. tsugae CBS 428.84	X78735	X78756	*Abies concolor*
G. tuberculosum BAFC 2414	AF169997	AF169998	*Salix* sp
G. valesiacum CBS 282.33	Z37056	Z37081	*Larix* sp.?
G. weberianum CBS 219.36	X78734	X78755	*Mangifera* sp
G. zonatum BAFC 2374	AF170003	AF170004	Angiosperm
Gano PNG	-	-	*Elaeis guineensis*
G. boninense LKM	-	-	Palm

Nucleotide sequences of the ITS regions of three *G. cupreum* were quoted from Smith and Sivasithamparam (2000a). The ITS region of Gano PNG (*Ganoderma* isolated from basal stem of diseased oil palm) was quoted from Bridge et al. (2000). The ITS region of *G. boninense* LKM was provided by Dr. Moncalvo.

Table 4: Nucleotide sequences of the Mn-SOD genes from *Ganoderma* species deposited in GenBank

Ganoderma	Mn-SOD nucleotide		Host
	GenBank Acc. No.	Sizes (bp)	
G. adspersum CBS 351.74	U56111	738	*Salix* sp.
G. ahmadii FWP 14329	U56137	704	*Dalbergia sissoo*
G. australe RSH 07505	U56112	776	Unknown
G. boninense RSH RS	U56128	683	Unknown
G. capense ACCC 5.71	U56125	708	Unknown
G. formicatum RSH 0184	U56126	708	Hardwood
G. formosanum RSH 0109	U56110	717	Unknown
G. lucidum ACCC 5.65	U56119	807	Unknown
G. lucidum CBS 270.81	U56133	789	Unknown
G. lucidum CBS 430.84	U56129	766	*Quercus hypoleucoides*
G. lucidum HMAS 60537	U56120	807	Hardwood
G. lucidum RSH 0626	U56121	826	Unknown
G. lucidum ATCC 324.71	U56122	737	*Acrocarpus* sp.
G. lucidum RYV 33217	U56134	705	*Betula* sp
G. microsporum RSH 0821	U56127	708	*Salix babylonica*
G. oerstedii ATCC 52410	U56131	704	Unknown
G. oregonense CBS 177.30	U56130	675	Conifer
G. resinaceum CBS 152.27	U56123	705	Unknown
G. tropicum RSH 1111	U56113	851	Unknown
G. tsugae RSH 1109	U56115	760	Unknown
G. tsugae RSH H2	U56114	717	Unknown
G. valesiacum CBS 282.33	U56136	704	*Larix* sp. ?
G. weberianum CBS 219.36	U56124	708	*Mangifera* sp
G. boninense LKM	-	375*	Palm

* Mn-SOD gene of *G. boninense* LKM (provided by Dr. Moncalvo) is available only 375 bp or only until the beginning of the second intron (other *Ganoderma* species in this table consist of two complete introns)

3 Results

3.1 Detection of oil palm *Ganoderma*

3.1.1 Primer designs from ITS region and determination of detection limits of the generated primers

Primer Gan1 (5' TTGACTGGGTTGTAGCTG 3') as forward primer and Gan2 (5' GCGT TACATCGCAATACA 3') as reverse primer were designed from the nucleotide sequence of ITS 1 of *G. boninense* RSH RS as shown in Fig.1. Species-specific primer pairs IT1 (5`AGCTCGTTCGTTTGACGA´3)-IT2 (5`TTGTCCCAATAACGGGAC`3) and IT1-IT3 (5`CGATCAATAAAAGACCGA`3) were designed from the variable ITS sequence of oil palm *Ganoderma* and other *Ganoderma* species (see the ITS results in 3.3.2).

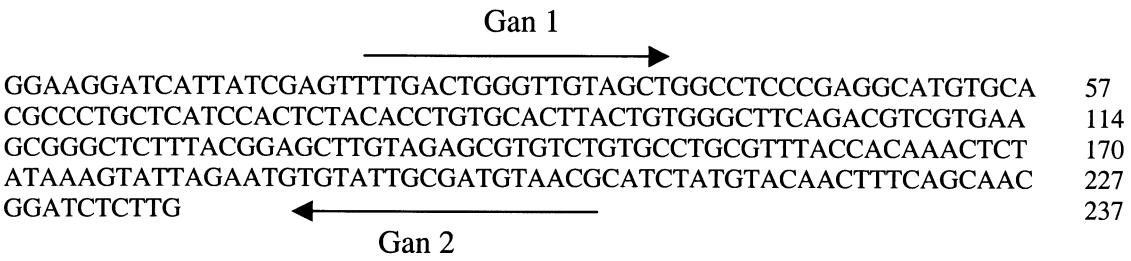


Fig.1: DNA sequence of ITS region 1 of ribosomal DNA from *G. boninense* RSH RS. The deduced size of PCR product of primer Gan1- Gan2 was 167 base pairs.

Template DNA dilution series of oil palm *Ganoderma* were performed to determine the smallest amount of *Ganoderma* DNA that would result in a PCR product. Three different DNA extraction methods of Raeder and Broda (1985), Möller et al. (1992) and Wang et.al., (1993) or the NaOH method were tested to evaluate detection limits of primer pair Gan1-Gan2. Of the three DNA extraction methods used, the DNA extracted by the NaOH method could not be measured directly by UV spectrophotometer due to possible impurity of DNA (method did not precipitate proteins). Primer pair Gan1-Gan2 generated the expected PCR product of 167 bp only when oil palm *Ganoderma* was used. With the DNA extraction method used, the detection limit of 5 pg of template DNA (for the first and second DNA extraction method) and 1.5 ng of freeze-dried mycelia (or dilution of 1: 10^5), respectively were detectable in agarose gels (Fig. 2). By using the NaOH method, other primer pairs IT1-IT2 and IT1-IT3, each generated a single PCR product of about 450 bp and 334 bp for oil palm *Ganoderma* with the detection limit of 1.5 ng of freeze-dried mycelia (corresponding to a dilution 1:10^5) (Fig. 2).

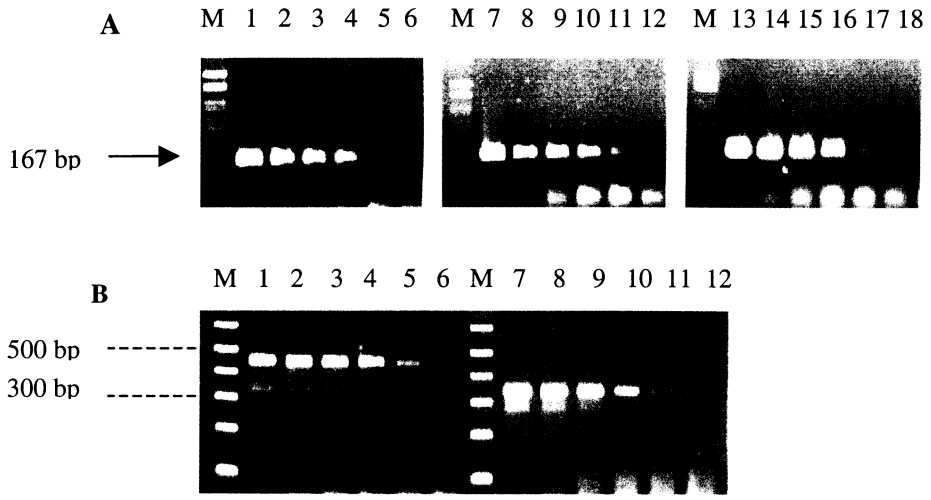


Fig. 2: Determination of the detection limit of oil palm *Ganoderma* by using different DNA concentrations, primer pairs and DNA extraction methods.

A. Results of PCR amplification of primer Gan1-Gan2. Lanes 1-6, 7-12: 50, 5, 0.5, 0.05, 0.005 and 0.0005 ng of template DNA from Raeder and Broda method and Möller method and lanes 13-18: 1: 10, 1:10^2, 1:10^3, 1:10^4, 1:10^5 and 1:10^6 of DNA dilution series from NaOH method.

B. Results of PCR amplification using the primer combinations of IT1-IT2 and IT1-IT3. Lanes 1-6, 7-12: 1: 10, 1:10^2, 1:10^3, 1:10^4, 1:10^5 and 1:10^6 of DNA dilution series from the NaOH method.

To further determine the detection limits of oil palm *Ganoderma* via the ITS-PCR products, oil palm *Ganoderma* was mixed with healthy oil palm roots. This mixture was extracted by using the NaOH method and diluted at 10-fold dilution series. The results of this experiment showed no difference in the detection limit levels in comparison with a pure culture of oil palm *Ganoderma*. No PCR product in the dilution 1:10 was observed for PCR amplification using primer pairs IT1-IT2 and IT1-IT3. No PCR amplification product was also observed using the extracted healthy oil palm roots as DNA template. The results of PCR amplifications using primer pairs Gan1-Gan2, IT1-IT2 and IT1-IT3 for this mixture are summarised in Table 5. PCR amplification using primer pairs IT1-IT2 and IT1-IT3 at different mixing ratios gave no PCR product for dilution 1:10. No PCR amplification was observed using the mixture at a concentration 1:10^5 (w/w) of oil palm *Ganoderma*/healthy root as template DNA. The results of PCR amplification following the mixing of oil palm *Ganoderma* with healthy oil palm root at different mixing and dilution series using primer pairs IT1-IT2 and IT1-IT3 are summarised in Table 6.

Table 5: The detection limit of oil palm *Ganoderma* mixed with healthy oil palm root by using the DNA extraction NaOH method

Ganoderma : healthy root	Primers		
	Gan 1 and Gan 2	IT 1 and IT2	IT1 and IT 3
1:10	+++	-	-
$1:10^2$	+++	+++	+++
$1:10^3$	+++	+++	+++
$1:10^4$	++	++	++
$1:10^5$	+	+	+
$1:10^6$	-	-	-
Healthy root	-	-	-
Negative control (water)	-	-	-

+++: a strong band, ++: a moderate band, +: a weak band, and -: no PCR product
Healthy root was diluted in 10-fold dilution series (1:10, $1:10^2$,...,$1:10^6$)

Table 6: Results of PCR amplification of mixing oil palm *Ganoderma* with healthy oil palm root using different mixing and dilution series using primer pairs IT1-IT2 and IT1-IT3

Ganoderma : Healthy root	DNA dilution series					
	1	2	3	4	5	6
1:20 (w/w)	-	+++	+++	++	+	-
$1:10^2$ (w/w)	-	+++	+++	++	+	-
$1:10^3$ (w/w)	-	+++	+++	+	-	-
$1:10^4$ (w/w)	-	++	+	-	-	-
$1:10^5$ (w/w)	-	-	-	-	-	-

The number (1 to 6) designates for 10-fold dilution series and each dilution amplified by primer pairs IT1-IT2 and IT1-IT3
+++: a strong band, ++: a moderate band, +: a weak band, -: no PCR amplification product

3.1.2 Cross reaction test of saprophytic fungi by using three primer sets

To determine the specificity of the primer pairs Gan1-Gan2, IT1-IT2 and IT1-IT3, the extracted DNA of saprophytic fungi isolated from diseased oil palm roots was amplified. In dilution series 1:10, $1:10^2$ and $1:10^3$, primer set Gan1-Gan2 cross-reacted with saprophytic fungi, however, the PCR products of the saprophytic fungi were different in DNA fragment sizes compared to oil palm *Ganoderma* (Fig. 3). In contrast, when saprophytic fungi DNA were mixed with healthy oil palm roots and diluted at 1:10, $1:10^2$ and $1:10^3$, no PCR amplification product of the saprophytic fungi could be observed. Detailed results of PCR amplification are summarised in Table 7. None of the saprophytic fungi tested using primer pairs IT1-IT2 and IT1-IT3 generated a PCR amplification

product (Fig. 3). When using primer pair ITS1-ITS4, PCR amplification of saprophytic fungi DNA produced a single PCR product ranging from about 550 to 600 bp as summarised in Table 8.

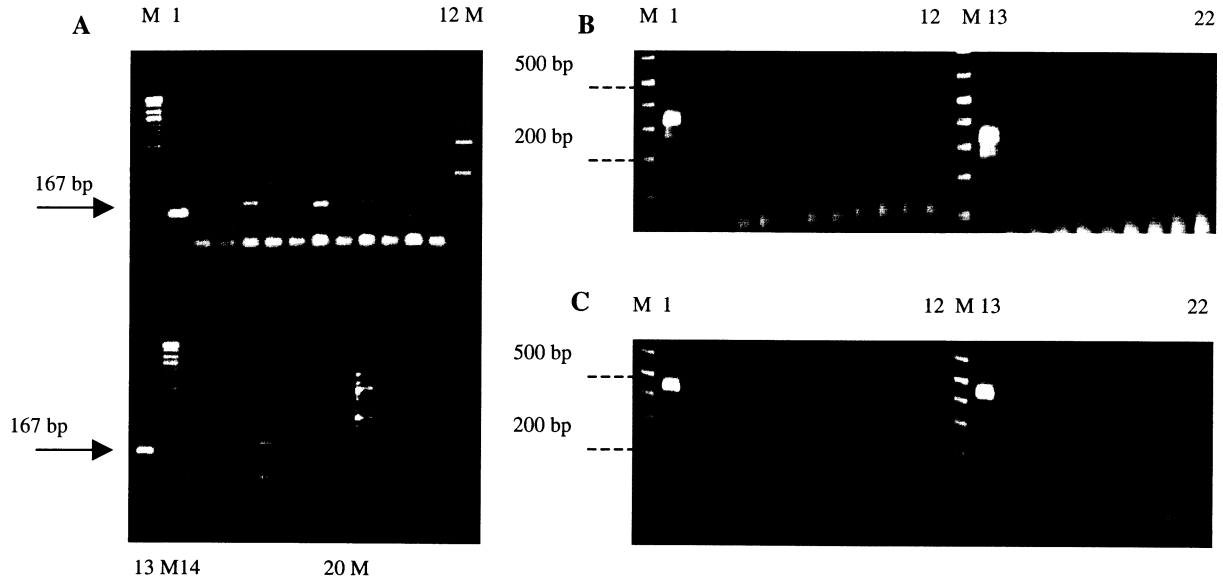


Fig. 3: PCR cross-reaction tests of three primer pairs for 18 saprophytic fungi at dilution 1:10^2.

A. PCR amplification of primer pair Gan1-Gan2 for saprophytic fungi. Lanes 1-20: oil palm *Ganoderma* (BS), *T. koningii*, *T. harzianum*, *T. viride*, *A. flavus*, *Penicillium* sp, *Trichoderma* sp.1, *Rhizopus* sp, *Bispora* sp, *Geotrichum* sp, *Tricho derma* sp.2, *Trichoderma* sp.3, oil palm *Ganoderma* (AP), *Gliocladium* sp., *Mucor* sp., *Cylindrocarpon* sp., *Monilia* sp., *Fusarium* sp., *Aspergillus* sp., and *Botryodiplodia* sp.

B. and C. Primer pairs IT1-IT3 and IT1-IT2, lanes 1-20: the fungi as the same in A and lanes 21-22: healthy oil palm roots. M: DNA marker

Table 7: Saprophytic fungi were mixed with healthy root, diluted in 10-fold dilution series and amplified using primer pair Gan1-Gan2

Saprophytic fungi + healthy root	Primer pair Gan1-Gan2 and DNA dilution series		
	1:10	$1:10^2$	$1:10^3$
Trichoderma koningii	-	-	-
T.harzianum	-	-	-
T.viride	-	-	-
Aspergillus flavus	-	-	-
Penicillium sp.	-	-	-
Trichoderma sp. 1	-	-	-
Rhizopus sp.	-	-	-
Bispora sp.	-	-	-
Geotrichum sp.	-	-	-
Trichoderma sp. 2	-	-	-
Trichoderma sp. 3	-	-	-
Gliocladium sp.	-	-	-
Mucor sp.	-	-	-
Cylindrocarpon sp.	-	-	-
Monilia sp.	-	-	-
Fusarium sp.	-	-	-
Aspergillus sp.	-	-	-
Botryodiplodia sp.	-	-	-

-: no PCR amplification product

Table 8: Cross reaction test of saprophytic fungi by using primer pairs IT1-IT2 IT1-IT3 and ITS1-ITS4 (as a control PCR) in order to amplify the ITS regions of saprophytic fungi.

Saprophytic fungi	Primers and DNA dilutions					
	IT 1 and IT2		IT 1 and IT3		ITS1 and ITS4	
	$1:10^2$	$1:10^3$	$1:10^2$	$1:10^3$	$1:10^2$	$1:10^3$
Trichoderma koningii	-	-	-	-	600	600
T.harzianum	-	-	-	-	600	600
T.viride	-	-	-	-	600	600
Aspergillus flavus	-	-	-	-	550	550
Penicillium sp.	-	-	-	-	600	600
Trichoderma sp. 1	-	-	-	-	600	600
Rhizopus sp.	-	-	-	-	600	600
Bispora sp.	-	-	-	-	550	550
Geotrichum sp.	-	-	-	-	550	550
Trichoderma sp. 2	-	-	-	-	600	600
Trichoderma sp. 3	-	-	-	-	600	600
Gliocladium sp.	-	-	-	-	580	580
Mucor sp.	-	-	-	-	600	600
Cylindrocarpon sp.	-	-	-	-	550	550
Monilia sp.	-	-	-	-	550	550
Fusarium sp.	-	-	-	-	600	600
Aspergillus sp.	-	-	-	-	550	550
Botryodiplodia sp.	-	-	-	-	550	550

-: no PCR amplification
Primer pair ITS1-ITS4 produced a single strong band ranging of about 550 to 600 bp when saprophytic fungi DNA was amplified

3.1.3 Detection of *Ganoderma* DNA from naturally infected oil palm roots

Of the 15 oil palm root samples taken from various oil palm estates, four samples (from Pabatu, Rambutan, Pagar Merbau and Sei Bamban estates) showed no PCR amplification signal or 80 % of samples showed positive detection. One sample (from Bukit Sentang estate) gave an expected weak PCR product at a dilution of $1:10^2$ for all primer pairs used (Fig. 4), otherwise, at a dilution of $1:10^3$, no PCR signal was observed. The positive samples showed an expected PCR amplification signal at a dilution of $1:10^2$ and $1:10^3$ using primer pairs Gan1-Gan2, IT1-IT2 and IT1-IT3. On the other hand, no PCR signal of the positive samples in the dilution of 1:10 was observed using primer pairs IT1-IT2 and IT1-IT3. Detailed results are summarised in Table 9. Initial screening showed that primer pairs IT1-IT2 and IT1-IT3 only produced a PCR product of about 450 and 334 bp, respectively, in oil palm *Ganoderma*, and these bands were not obtained by PCR with DNA from saprophytic fungi and healthy oil palm roots. Therefore, the presence of PCR

amplification product from root samples confirms that oil palm root samples tested contained oil palm *Ganoderma*.

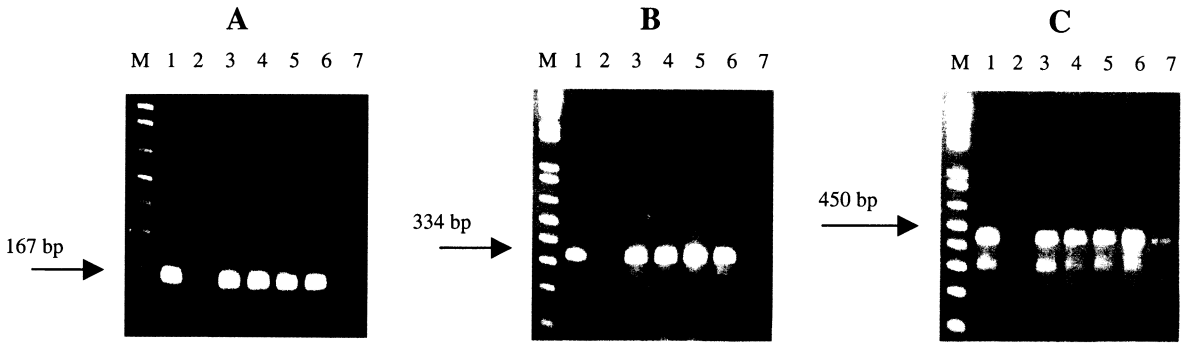


Fig. 4: PCR amplification of the naturally infected oil palm root samples using three primer pairs.
A, B and C. Samples in dilution 1:10^2 were amplified by using primer pairs of Gan1-Gan2, IT1-IT3 and IT1-IT2, respectively. Lanes 1-7: samples from Marihat, Sei Bamban, Adolina, Dolok Hilir, Sei Pancur, Gunung Bayu and Bukit Sentang estates.

Table 9: The results of PCR amplification from oil palm root samples using primer pairs Gan1-Gan2, IT1-IT2 and IT1-IT3

Oil palm root origin	Primer pairs and DNA dilution								
	Gan1 and Gan2			IT 1 and IT 2			IT 1 and IT 3		
	1:10	1:10^2	1:10^3	1:10	1:10^2	1:10^3	1:10	1:10^2	1:10^3
Gunung Bayu estate	+++	+++	+++	-	+++	+++	-	+++	+++
Pabatu estate	-	-	-	-	-	-	-	-	-
Marihat estate	+++	+++	+++	-	+++	+++	-	+++	+++
Bangun estate	+++	+++	+++	-	+++	+++	-	+++	+++
Adolina estate	+++	+++	+++	-	+++	+++	-	+++	+++
Matapao estate	+++	+++	+++	-	+++	+++	-	+++	+++
Bukit Sentang estate	+	+	-	-	+	-	-	+	-
Dolok Hilir estate	+++	+++	+++	-	+++	+++	-	+++	+++
Aek Pancur estate	++	++	++	-	++	++	-	++	++
Sei Bamban estate	-	-	-	-	-	-	-	-	-
Sei Pancur estate	+++	+++	+++	-	+++	+++	-	+++	+++
Pagar Merbau estate	-	-	-	-	-	-	-	-	-
Bah Jambi estate	++	++	++	-	++	++	-	++	++
Rambutan estate	-	-	-	-	-	-	-	-	-
Lonsum estate	+++	+++	+++	-	+++	+++	-	+++	+++
Healthy root	-	-	-	-	-	-	-	-	-

+++: a strong fragment, ++: a moderate fragment, +: a weak fragment
Primer pairs Gan1-Gan2, IT1-IT2 and IT1-IT3 generated each PCR product of 167, 450 and 334 bp, respectively.
-: no PCR amplification product

3.2 Identification and differentiation of oil palm *Ganoderma* based on the laccase genes

3.2.1 Primer design and PCR amplifications of *Ganoderma* DNA using the primer pairs designed from the laccase genes

Four 17- or 18-base pair sequences designed as primers Lac 2a, Lac 2f, Lac 2b and Lac 2r were selected from DNA sequences of the conserved amino acid sequences of six laccase genes from the published sequences of basidiomycete fungi. The DNA sequences are shown in Table 10. The primers were used in combination to amplify *Ganoderma* DNA.

Table 10: Selection of primers from nucleotide sequences of the conserved amino acid of the laccase genes of 6 known basidiomycete fungi

Basidiomycete fungi and primers	Nucleotide sequence of amino acid sequence WHGFFQ (copper-binding region I)
T. villosa Lcc2	5`TGGCACGGCTTCTTCCAG 3`
T. villosa Lcc1	5`TGGCACGGTTTCTTCCAG 3`
Basidiomycete PM1	5`TGGCACGGCTTCTTCCAA 3`
Basidiomycete CECT 20107	5`TGGCACGGCTTCTTCCAG 3`
T. villosa Lcc4	5`TGGCACGGCTTCTTCCAG 3`
T. versicolor LccI	5`TGGCACGGCTTCTTCCAG 3`
Lac 2a (forward primer)	**5`TGGCACGGCTTCTTCCAG 3`**

Basidiomycete fungi and primers	Nucleotide sequence of TQYCDG (near copper-binding region II)
T. villosa Lcc2	5`ACGCAGTACTGTGACGGG 3`
T. villosa Lcc1	5`ACGCAGTACTGTGATGGT 3`
Basidiomycete PM1	5`ACTCAATACTGTGACGGT 3`
Basidiomycete CECT 20107	5`ACGCAATACTGTGACGGC 3`
T. villosa Lcc4	5`ACGCAGTACTGCGATGGT 3`
T. versicolor LccI	5`ACGCAGTACTGCGACGGG 3`
Lac 2f (forward primer) `	**5`ACGCAGTACTGTGACGG 3**

Basidiomycete fungi and primers	Nucleotide sequence of WFLHCHI (copper-binding region IV)
T. villosa Lcc2	5`GTTCCTCCACTGCCACAT 3`
T. villosa Lcc1	5`GTTCCTCCACTGCCACAT 3`
Basidiomycete PM1	5`GTTCCTCCACTGCCACAT 3`
BasidiomyceteCECT 20107	5`GTTCCTCCACTGCCACAT 3`
T. villosa Lcc4	5`GTTCCTCCACTGCCACAT 3`
T. versicolor LccI	5`GTTCCTCCACTGCCACAT 3`
Lac 2b (reverse primer)	**5`GTTCCTCCACTGCCACAT 3**

Continued

Basidiomycete fungi and primers	Nucleotide sequence of HCHIDF (copper-binding region IV)
T. villosa Lcc2	5'CACTGCCACATCGACTTC 3'
T. villosa Lcc1	5'CACTGCCACATCGACTTC 3'
Basidiomycete PM1	5'CACTGCCACATCGACTTC 3'
Basidiomycete CECT 20107	5'CACTGCCACATCGACTTC 3'
T. villosa Lcc4	5'CACTGCCACATCGACTTC 3'
T. versicolor LccI	5'CACTGCCACATCGACTTC 3'
Lac 2r (reverse primer)	**5'CACTGCCACATCGACTTC 3'**

The DNA purity from 80 *Ganoderma* isolates using the DNA extraction method of Möller et al. (1992) was estimated by UV spectrophotometry. DNA concentrations gave a ratio of optical density (OD) 260/280 ranged from 1.62 to 2.11 (Table 1A, see Appendix). Four different DNA concentrations (200 ng, 100 ng, 10ng and 1 ng) of three oil palm *Ganoderma* (isolates BS, PM and SB) were amplified by using the primer pairs Lac 2a - Lac 2b, Lac 2a - Lac 2r and Lac 2f - Lac 2b. These primer pairs generated a single PCR product of about 327 bp, 1617 bp and 1265 bp (see the sequence result in this section). The result showed that at a DNA concentration of 100 ng, a strong DNA fragment was obtained. The intensity of this band was similar to the result of amplification of DNA using a concentration of 200 ng. For further experiments, a DNA concentration of 100 ng was used as an optimum concentration for PCR amplification of the laccase genes of *Ganoderma* species studied. The results of PCR amplification by using these primers are summarised in Table 11.

Table 11: Determination of the optimum DNA concentration of oil palm *Ganoderma* for PCR amplification

Oil palm *Ganoderma*	Amplified by primer pairs of											
	Lac 2a and Lac 2b				Lac 2a and Lac 2r				Lac 2f and Lac 2b			
	Co.1	Co.2	Co.3	Co.4	Co.1	Co.2	Co.3	Co.4	Co.1	Co.2	Co.3	Co.4
Ganoderma BS	+++	+++	++	+	+++	+++	++	+	+++	+++	++	+
Ganoderma PM	+++	+++	++	+	+++	+++	++	+	+++	+++	++	+
Ganoderma SB	+++	+++	++	+	+++	+++	++	+	+++	+++	++	+

Co.1, 2, 3 and 4 : concentration of 200, 100, 10 and 1 ng of the extracted DNA
+++ : a strong band
++ and + : a moderate to weak band

PCR amplification of the deduced laccase gene fragment from *Ganoderma* spp. using primer pair Lac 2a-Lac 2b

All oil palm *Ganoderma* isolates and *G. boninense* isolated from coconut stumps, when amplified using the primer pair Lac 2a-Lac 2b, generated a single PCR product of about 327 bp. However, when DNA of other *Ganoderma* species was used, two different banding patterns were observed: banding pattern 1 consisted of a single PCR product of about 1,610 bp and banding pattern 2 consisted of a double band of about 1,610 bp and 1,500 bp as shown in Fig. 5. *Ganoderma* species that exhibit banding pattern 1 are *G. lucidum* 9612, *G. lucidum* complex BAFC2374, *G. lucidum* complex BAFC2495, *G. resinaceum* BAFC384, *G. resinaceum* BAFC2288, *G. resinaceum* BAFC2775, *G. resinaceum* BAFC2488, *G. tropicum* BAFC2580 and *G. tsugae*, and for banding pattern 2 are *G. tornatum* BAFC671, *G. tornatum* BAFC2390, *G. tornatum* BAFC2395, *G. tornatum* BAFC2424, *G. tornatum* BAFC2430, *G. applanatum* BAFC2353, *G. applanatum* BAFC2408, *G. applanatum* BAFC2501, *G. applanatum* BAFC2552, *G. applanatum* DSM3800 and *Ganoderma* sp. BAFC2529 (Fig. 5). No PCR amplification product was observed when *G. oerstedii* DNA was used. The PCR amplification results of the *Ganoderma* species studied by using these primer pairs are summarised in Table 12.

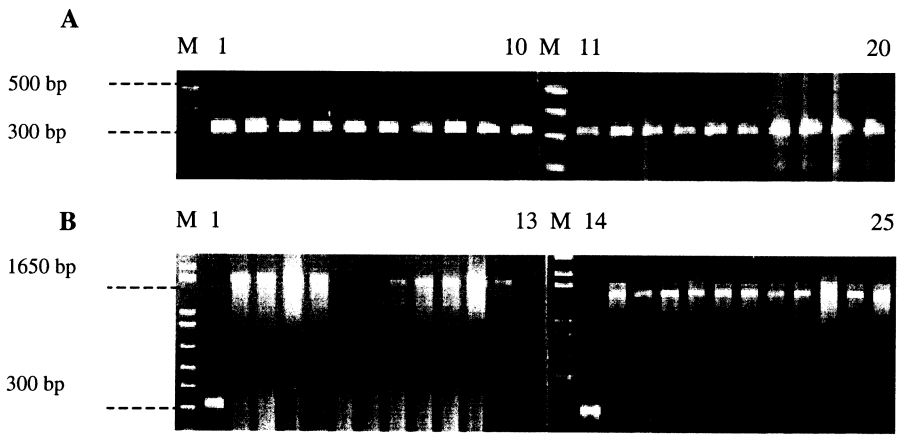


Fig. 5: Results of PCR amplification of *Ganoderma* spp. DNA using primer pair Lac 2a-Lac 2b

A. Oil palm *Ganoderma*, lanes 1-20: isolates of BS, AP, SP, PM, Ad, Mt, SB, BJ7, BJ8,GB, BB, BL, SM, Lo, BM, Ti, Ma, DS, Pa and DH.

B. Other *Ganoderma* species, lanes 1-25: oil palm *Ganoderma* (BS), *Ganoderma* of BAFC2374, BAFC2495, DSMZ9612, BAF 384, BAFC178, BAFC218, BAFC2288, BAFC2275, BAFC2488, BAFC2580, *G. tsugae*, water (negative control), oil palm *Ganoderma* (AP), BAFC671, BAFC2390, BAFC2395, BAFC2430, BAFC2529, BAFC2353, BAFC2424, BAFC2501, BAFC2408, BAFC2552 and DSMZ3800. M: DNA marker

PCR amplification of the deduced laccase gene fragment from *Ganoderma* spp. using the primer pair Lac 2a-Lac 2r

By using the primer pair Lac 2a-Lac 2r, all oil palm *Ganoderma* and *G. boninense* isolated from coconut stumps produced a single PCR product of about 1,617 bp. Two banding patterns were observed when other *Ganoderma* species were amplified by using primer pair Lac 2a-Lac 2r. The first banding pattern showed a single band of a PCR-amplification product of about 1,617 bp (identical size with oil palm *Ganoderma*) which include: *G. lucidum* 9612, *G. lucidum* complex BAFC2374, *G. lucidum* complex BAFC2495, *G. resinaceum* BAFC384, *G. resinaceum* BAFC2288, *G. resinaceum* BAFC2775, *G. resinaceum* BAFC2488, *G. tropicum* BAFC2580 and *G. tsugae*. The second banding pattern showed a double band of about 1,617 bp and 1,500 bp in size with the following isolates: *G. tornatum* BAFC671, *G. tornatum* BAFC2390, *G. tornatum* BAFC2395, *G. tornatum* BAFC2424, *G. tornatum* BAFC2430, *G. applanatum* BAFC2353, *G. applanatum* BAFC2408, *G. applanatum* BAFC2501, *G. applanatum* BAFC2552, *G. applanatum* DSM3800 and *Ganoderma* sp. BAFC2529 (Fig. 6). No PCR amplification product was observed using this primer pair when two *G. oerstedii* isolates were used. PCR amplification of DNA from other *Ganoderma* species using this primer pair was similar to the PCR result obtained with the primer pair Lac 2a and Lac 2b. The amplification results of the *Ganoderma* species studied by using these primer pairs are summarised in Table 12.

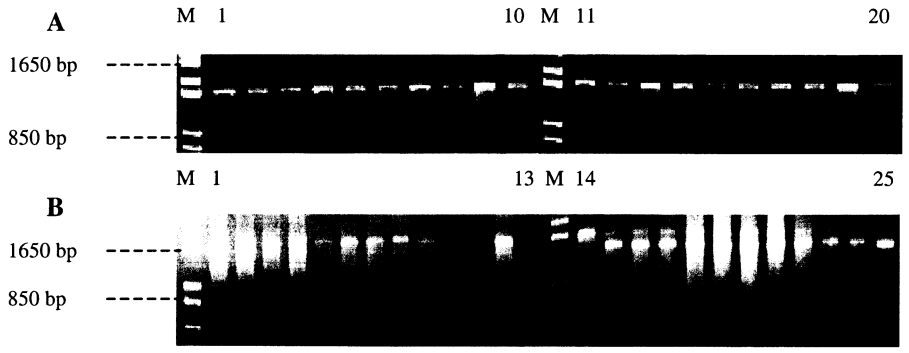


Fig. 6: Results of PCR amplification of *Ganoderma* spp. DNA using primer pair Lac 2a-Lac 2r

A. Oil palm *Ganoderma*, lanes 1-20: isolates of BS, AP, SP, PM, Ad, Mt, SB, BJ7, BJ8, GB, BB, BL, SM, Lo, BM, Ti, Ma, DS, Pa and DH

B. Other *Ganoderma* species, lanes 1-25: oil palm *Ganoderma* (BS), *Ganoderma* of DSMZ9612, BAFC2374, BAFC2495, BAFC384, BAFC2288, BAFC2275, BAFC2488, BAFC2580, BAFC178, BAFC218, *G. tsugae*, water (negative control), oil palm *Ganoderma* (AP), BAFC671, BAFC2390, BAFC2395, BAFC2430, BAFC2529, BAFC2353, BAFC2424, BAFC2501, BAFC2408, BAFC2552 and DSMZ3800. M: DNA marker.

PCR amplification of the deduced laccase gene fragment from *Ganoderma* spp. using the primer pair Lac 2f-Lac 2b

All oil palm *Ganoderma* species and *G. boninense* isolated from coconut stumps produced a single band of about 1,265 bp in size when amplified with the primer pair Lac 2f-Lac 2b. Two banding patterns were also observed when DNA from other *Ganoderma* species were amplified using primer pair Lac 2f-Lac 2b. The first banding pattern revealed a single PCR amplification product of about 1,265 bp in size (identical size with the oil palm *Ganoderma*) that was common to the following: *G. tornatum* BAFC671, *G. tornatum* BAFC2390, *G. tornatum* BAFC2395, *G. tornatum* BAFC2424, *G. tornatum* BAFC2430, *G. applanatum* BAFC2353, *G. applanatum* BAFC2408, *G. applanatum* BAFC2501, *G. applanatum* BAFC2552, *G. applanatum* DSM3800 and *Ganoderma* sp. BAFC2529. The second banding pattern revealed a single PCR product of about 1,400 bp that was common to the following: *G. oerstedii* BAFC178, *G. oerstedii* BAFC218 *G. lucidum* 9612, *G. lucidum* complex BAFC2374, *G. lucidum* complex BAFC2495, *G. resinaceum* BAFC384, *G. resinaceum* BAFC2288, *G. resinaceum* BAFC2775, *G. resinaceum* BAFC2488, *G. tropicum* BAFC2580 or double bands of about 1,265 bp and 1,400 bp unique to *G. tsugae* (Fig. 7). The amplification results of the *Ganoderma* species studied with this primer pair are summarised in Table 12. By using combinations of the primer pairs Lac 2a-Lac 2r and Lac 2f-Lac 2b, oil palm *Ganoderma* could be distinguished from other *Ganoderma* species as summarised in Table 12.

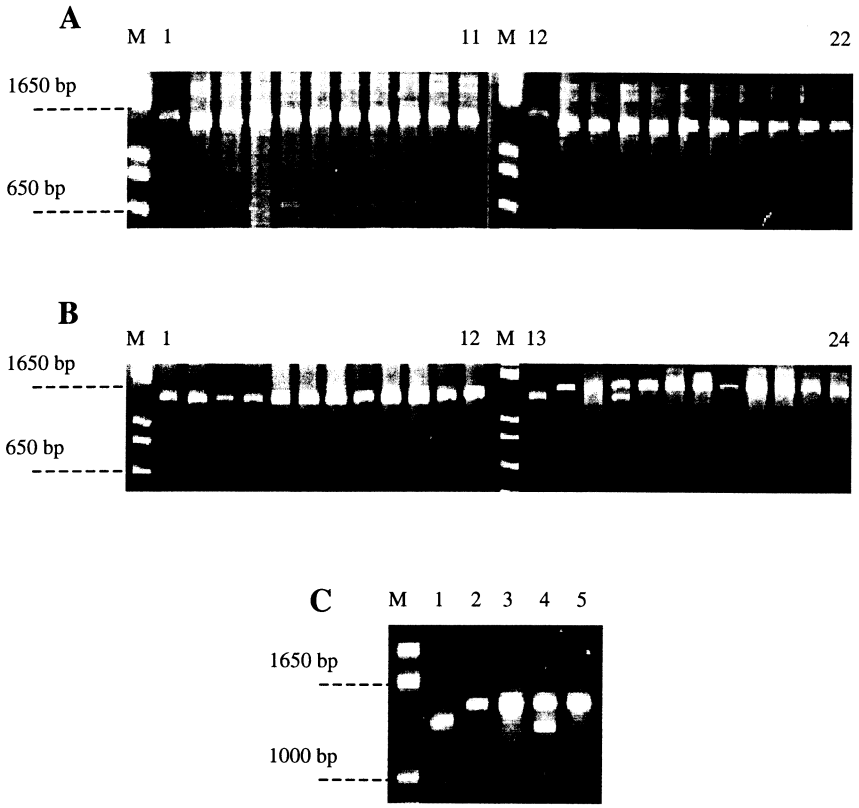


Fig.7: Results of PCR amplification of *Ganoderma* spp. DNA using primer pair Lac 2f-Lac 2b

A. Oil palm *Ganoderma*, lanes 1-22: *G. lucidum* BAFC2374, oil palm *Ganoderma* isolates of BS, AP, SP, PM, Ad, Mt, SB, BJ7, BJ8, GB, *G. lucidum* BAFC 2495, oil palm *Ganoderma* isolates of BB, BL, SM, Lo, BM, Ti, Ma, DS, Pa and DH

B. Other Ganoderma species, lanes 1-24: oil palm *Ganoderma* (BS), *Ganoderma* of BAFC671, BAFC2390, BAFC2395, BAFC2430, BAFC2529, BAFC2353, BAFC2424, BAFC2501, BAFC2408, BAFC2552, DSMZ3800, oil palm *Ganoderma* (BJ7), BAFC178, BAFC218, *G. tsugae,* DSMZ9612, BAFC2374, BAFC2495, BAFC384, BAFC2288, BAFC2275, BAFC2488 and BAFC2580

C. Other Ganoderma species running in a longer time in agarose gel, lanes 1-5: oil palm *Ganoderma* (AP), *Ganoderma* of BAFC178, BAFC218, *G. tsugae*, DSMZ9612. M: DNA marker

Table 12: Results of PCR amplification using the primer pair of Lac 2a-Lac 2b, Lac 2a-Lac 2r and Lac 2f-Lac 2b for different *Ganoderma* species.

Ganoderma species	PCR amplifications by primer pairs of		
	Lac 2a-Lac 2b	Lac 2a-Lac 2r	Lac 2f-Lac 2b
Oil palm *Ganoderma* (Indonesia), 20 isolates	327 bp	1	1
G. boninense (oil palm, PNG), 10 isolates	„	1	1
G. boninense (coconut stump, PNG), 7 isolates	„	1	1
G. tornatum (hardwood, PNG), 11 isolates	2	2	1
G. oerstedii BAFC 178	-	-	2
G. oerstedii BAFC 218	-	-	2
G. resinaceum BAFC 384	1	1	2
G. tornatum (applanatum?) BAFC 671	2	2	1
G. resinaceum BAFC 2288	1	1	2
G. applanatum BAFC 2353	2	2	1
G. lucidum complex BAFC 2374	1	1	2
G. tornatum BAFC 2390	2	2	1
G. tornatum? BAFC 2395	2	2	1
G. applanatum BAFC 2408	2	2	1
G. tornatum BAFC 2424	2	2	1
G. tornatum? BAFC 2430	2	2	1
G. resinaceum BAFC 2488	1	1	2
G. lucidum complex BAFC 2495	1	1	2
G. applanatum var. tornatum BAFC 2501	2	2	1
Ganoderma sp. BAFC 2529	2	2	1
G. applanatum BAFC 2552	2	2	1
G. tropicum BAFC 2580	1	1	2
G. resinaceum BAFC 2775	1	1	2
G. lucidum DSM 9612	1	1	2
G. applanatum DSMZ 3800	2	2	1
G. tsugae	1	1	2*
G. applanatum 134	2	2	2*
G. applanatum G 211	2	2	2*
G. adspersum G 224	1	1	2
G. pfeifferi G 225	1	1	2
G. cupreum QFRI 8678.1	2	2	2*
G. australe DAR 73781	2	2	1
G. incrassatum DAR 73783	2	2	2*
G. cupreum DFP 4336	2	2	2*
*Ganoderma sp.*Group 6.3 DAR 73779	1	1	2
G. weberianum DFP 4483	1	1	2

The number (1, 2 and 2*) designates the banding pattern for each primer pair

PCR product using primer pair Lac 2a-Lac 2b

- Banding pattern 1 shows a PCR product of about 1,610 bp
- Banding pattern 2 shows PCR products of about 1,610 bp and 1,500 bp

PCR product using primer pair Lac 2a-Lac 2r

- Banding pattern 1 shows a PCR product of about 1,617 bp
- Banding pattern 2 shows PCR products of about 1,617 bp and 1,500 bp

PCR product using primer pair Lac 2f-Lac 2b

- Banding pattern 1 shows a PCR product of about 1,265 bp
- Banding pattern 2 shows a PCR product of about 1,400 bp
- Banding pattern 2* shows PCR products of about 1,265 bp and 1,400 bp.

3.2.2 Characterization of the nucleotide sequences of PCR products of oil palm *Ganoderma* isolate BS amplified using the primer pairs Lac 2a-Lac 2b, Lac 2a-Lac 2r and Lac 2f-Lac 2b

Computer-assisted comparison with nucleotide sequences in GenBank.

To confirm the identity of nucleotide sequences of oil palm *Ganoderma* amplified by the primer pairs Lac2a-Lac 2b, Lac2a-Lac 2r and Lac 2f-Lac 2b, a computer-assisted comparison of the nucleotide sequences with the existing nucleotide sequences in gene databases was performed by using the BLASTN program (Altschul et al., 1997). By sending a partial nucleotide sequence of 300-500-bp to the gene databases, the identity of the sequenced DNA fragment could be determined. Results of the nucleotide sequence comparison from the GenBank for the nucleotide sequence obtained by amplifying with primers Lac 2a-Lac 2b, Lac 2a-Lac 2r and Lac 2f-Lac 2b are shown, as follows:

Nucleotide sequence obtained by amplifying with Lac 2a-Lac 2b

TGGCACGGCTTCTTCCAGAAGGGCACGAACTGGGCGGACGGCGTTGCCTTCGTCAACCAGTGCC
CGATCTCCAGCGGCAACTCCTTCCTGTACGACTTCCAAGTCCCTGGCCAGCCGGTAAGCATCGC
CGTCCCCTTCGGCCTGACATCAAGATGATGTTCACGGTAGTTGCGCAGGGCACCTATTGGTATC
ACAGCCATCTGTCCACTCAGTACTGCGATGGTCTCAGGGGCCCGTTCGTCGTATACGACCCTGA
AGACCCGCTGTTGTCCATGTATGACGTCGATGATGGTGAGATTCCCC

Nucleotide sequence obtained by amplifying with Lac 2a-Lac 2r

GAAGTCGATGTGGCAGTGGAGGATCCACGGCCCCGGGTTGTTCGTCTGGAACCGGATCGTCACG
TTGTCGCCCGCCACGCCCGTCGACACGACGTCGCGCCACACGGAGGTTGTCGTAGTTGTACTCG
GTCGAGCCCGCGCTGCGGATCACCGCGAACTCGTGCTGCACGCGACGGGAGGGGTGTGGTCAG
CGTCGGCATCGGACAGGGGAGGAGGGAATAAGGGGAGACTTACACCGTGCAAGTGNAACGGG
TGCGGGGTGCCGGGGGCGCTGGCGGTCGCGGGGAAGGTGAGCTCGATGGAGGAGTTCATTGGG
AGCTCGTAGACGCTGCCGGAGGGGAGGAGCTCCTGCGCGGTCTGTGCGCCGCTGAGGATCTGC
AGGAGCACGGGCACGGTGGGCGGGACGAAGGTGTCGCCGTTGATGAAGAAGCGGG

Nucleotide sequence obtained by amplifying with Lac 2f-Lac 2b

ACGCAGTACTGCGACGGTCTGCGGGGCCCGATGGTGGTGTACGACCCGGAGGACCCGCACGCG
AGCCTGTACGACGTGGACGACGAGACGACGGTGATCACGCTTGCGGACTGGACGCACACGGCG
GCGCGGCTGGGCAACCGCTTCCCGTGAGTCTTTTCCGAACATACACATGCATATACAGCGATGG
TGCTAACGATACGCTTGGGACAGCGGTGGGGCGGACTCGACGCTGATCAACGGTCTGGGGCGG
TTCGCGGGGGGCGCGTCGAGCGATCTCGCGGTGTTCAACGTGACGCAGGGCCAGCGGACCGGT
TCCGGCTGGTGTCGATGGCGTGCGACCCGAATTTCACGTTCTCGGTCGACGGGCACAA

Similarity report of the nucleotide sequence amplified by primer pair Lac 2a-Lac 2b with nucleotide sequence in GenBank (here only one similarity report is presented as an example):

```
AF176230.1 Polyporus ciliatus laccase (lcc3-1) mRNA, complete cds, Length = 1829
 Score = 105 bits (53), Expect = 1e-20
 Identities = 89/101 (88%)
 Strand = Plus / Plus
```

```
Query: 1   tggcacggcttcttccagaagggcacgaactgggcggacggcgttgccttcgtcaaccag 60
           |||||||| |||||||||||| ||||| |||||||| |||||   ||||||||| ||||||
Sbjct: 327 tggcacgggttcttccagaaaggcaccaactgggctgacggtcctgccttcgtgaaccag 386

Query: 61  tgcccgatctccagcggcaactccttcctgtacgacttcca 101
           |||||||| ||||||| |||||||||||||| ||||||||
Sbjct: 387 tgcccgattgccagcggaaactccttcctgtatgacttcca 427

 Score = 91.7 bits (46), Expect = 2e-16
 Identities = 67/74 (90%)
 Strand = Plus / Plus

Query: 177 ggcacctattggtatcacagccatctgtccactcagtactgcgatggtctcaggggcccg 236
           ||||||| ||||| |||||||||||||||||||||||||| |||||||| ||||| |||
Sbjct: 444 ggcaccttctggtaccacagccatctgtccactcagtactgtgatggtctgaggggtccg 503

Query: 237 ttcgtcgtatacga 250
           |||||||| |||||
Sbjct: 504 ttcgtcgtgtacga 517
```

Similarity report of the nucleotide sequence amplified by primer pair Lac 2a-Lac 2r with nucleotide sequence in GenBank (here only one similarity report is presented as an example):

```
L49376.1 TMTLCCA Trametes villosa (clone LCC1) laccase gene, exons 1-9,
complete cds.Length = 2417

 Score = 111 bits (56), Expect = 3e-22
 Identities = 193/239 (80%)
 Strand = Plus / Minus


Query: 236  ccgtgcaagtgnaacgggtgcggggtgccgggggcgctggcggtcgcggggaaggtgagc 295
           |||||||||||| || ||||| || |  |||||||||  |||||| |||||||||| || |
Sbjct: 1925 ccgtgcaagtggaaggggtggggcgcaccgggggcggcggcggtggcggggaaggagatc 1866

Query: 296  tcgatggaggagttcattgggagctcgtagacgctgccggaggggaggagctcctgcgcg 355
           ||||||  || ||||   || |||  ||||||||| |||||||| ||||| |||||||||
Sbjct: 1865 tcgatgtcggcgttcgagggaagcgagtagacgctaccggagggcaggaggtcctgcgcg 1806

Query: 356  gtctgtgcgccgctgaggatctgcaggagcacgggcacggtgggcgggacgaaggtgtcg 415
            |||| |||||||||| |||||| || || || |||||||| || ||   ||| |    |
Sbjct: 1805 ttctgcgcgccgctgatgatctggagcaggacaggcacggtcgggggcgtgaaagacgtg 1746

Query: 416  ccgttgatgaagaagcgggagccgttgaagttgaacgctaagttgatcgccaggtcgac 474
           ||||||||||||||  || |||||||||||||||| | |||||| ||||||||||
Sbjct: 1745 ccgttgatgaagaagttggtgccgttgaagttgaacgccatgttgatggccaggtcgac 1687

Score = 58.0 bits (29), Expect = 4e-06
 Identities = 38/41 (92%)
 Strand = Plus / Minus

Query: 1    gaagtcgatgtggcagtggaggatccacggccccgggttgt 41
           ||||||||||||||||||||||| |||||| || |||||||
Sbjct: 2094 gaagtcgatgtggcagtggaggaaccacgggccggggttgt 2054
```

Similarity report of the nucleotide sequence amplified by primer pair Lac 2f-Lac 2b with nucleotide sequence in GenBank (here only one similarity report is presented as an example):

```
AF176232.1 Polyporus ciliatus laccase (lcc3-3) mRNA, partial cds Length = 1694
 Score = 129 bits (65), Expect = 1e-27
 Identities = 143/169 (84%)
 Strand = Plus / Plus

Query: 296 acgcagggccagcggtaccggttccggctggtgtcgatggcgtgcgacccgaatttcacg 355
           ||||||||| |||| |||||||||| || || |||||  ||||||||||||| |||
Sbjct: 505 acgcagggcaagcgttaccggttccgccttgtctcgatctcgtgcgacccgaacttcgtc 564

Query: 356 ttctcggtcgacgggcacaacatgacggtgatcgaggcggacgcggtcaaccacgagcca 415
           |||||| |||||  |||||||||||||| |||||||||||||||||| ||||||||||
Sbjct: 565 ttctcgatcgacaagcacaacatgacggtcatcgaggcggacgcggtcagccacgagcca 624

Query: 416 gtcgtggtggactcgatccagatctacgcgggccagcgctactcgttcg 464
           |||   || || || |||||||||||||| ||||| || ||||| ||||
Sbjct: 625 gtcaccgtcgattccatccagatctacgccggccaacgatactccttcg 673

 Score = 58.0 bits (29), Expect = 4e-06
 Identities = 83/101 (82%)
 Strand = Plus / Plus

Query: 1   acgcagtactgcgacggtctgcggggcccgatggtggtgtacgacccggaggacccgcac 60
           |||||||||||||||||||| || || || ||||| || || |||||| | ||||||||
Sbjct: 271 acgcagtactgcgacggtcttcgtggtcccatggttgtatatgacccgaacgacccgcat 330

Query: 61  gcgagcctgtacgacgtggacgacgagacgacggtgatcac 101
           |||    |||||||||| ||||||||| | ||||| |||||
Sbjct: 331 gcgtcattgtacgacgtcgacgacgagtctacggtcatcac 371
```

Based on nucleotide comparison with the published nucleotide sequence in GenBank, it could be shown that the sequenced DNA fragment of the oil palm *Ganoderma* (isolate BS) had similarity with the laccase genes of other basidiomycete fungi. Therefore, the PCR product amplified by these primers was confirmed as DNA fragments of the laccase genes.

Deduced exons (amino acid sequence) and introns of laccase genes from oil palm *Ganoderma* amplified by the primer pairs Lac 2a-Lac 2b, Lac 2a-Lac 2r and Lac 2f-Lac 2b

Deduced intron sequences were based on the comparison with the published laccase gene sequences from other basidiomycete fungi such as *Trametes villosa* (Yaver et al., 1996), Basidiomycete CECT 20197 (Mansur et al., 1997) and *Pycnoporus cinnabarinus* (Eggert et al., 1998) and consensus sequence for 5‘ splicing GT(AG)(AT)GT and 3‘ splicing (CT)AG junctions present in filamentous fungi (Ballance, 1986; Hahn et al., 1997). For better visualisation, the deduced exon and intron sequences are presented as capital and lowercase characters, respectively. The deduced exon sequences were translated to the predicted amino acid sequences by using EditSeq program (DNAstar, Madison, USA).

Introns and exons within the nucleotide sequence of laccase gene amplified by the primer pair Lac 2a-Lac 2b.

Fig. 8 shows the total length of the nucleotide sequence of oil palm *Ganoderma* BS (DNA fragment of *Ganoderma* Lac 7) amplified by using primers Lac 2a and Lac 2b was 327 bp. According to the consensus intron, *Ganoderma* Lac 7 contains two introns. In the middle of the second intron, the binding site of primer Lac 2b is located.

Fig. 8. Partial nucleotide sequence of the laccase gene of oil palm *Ganoderma* BS (DNA fragment of *Ganoderma* Lac 7)
The deduced amino acid and intron sequences are presented as bold and lowercase characters. Copper-binding regions are indicated by roman numerals (I to II) The arrows are the binding sites of the primers Lac 2a (forward) and Lac 2b (reverse)

Introns and exons in the nucleotide sequence of the laccase gene amplified by the primer pair Lac 2a-Lac 2r

Fig. 9 shows the total length of nucleotide sequence (1617 bp) of oil palm *Ganoderma* BS (DNA fragment of *Ganoderma* Lac 1700) amplified by the primer pair Lac 2a-Lac 2r. To completely sequence this fragment, internal sequencing primer P1: 5‘ TTGGGA AAACGCAGGCTT 3‘, P2: 5‘ GGGCTTGTTATCCGAAGG 3‘ and P3: 5‘ GGGAGGG GTGTGGTCAGC 3‘ were used. Based on the consensus introns, *Ganoderma* Lac 1700 has seven putative introns ranging from 55 to 71 bp in size. Within the *Ganoderma* Lac 1700, the binding site of primer Lac 2b (5‘ATGTGGCAGTGGAGGAAC‘3) was found in two positions with one base pair mismatch. The first binding site position located within 2nd intron (shadow box) with a mismatch at the 13th position (5‘GTTCCTCCA

CTGACACAT‘3) and the second binding site position located at the nucleotide sequence of WIFHCHI (shadow box) with a mismatch at the 2nd position (5‘GATCCTCCACTGCCACT‘3). Nucleotide sequence in *Ganoderma* Lac 7 when compare to *Ganoderma* Lac 1700 (from nucleotide no.1 up to 327) had 99.0 % similarity, therefore, the DNA fragment of *Ganoderma* Lac 7 is possibly a part of *Ganoderma* Lac 1700.

Introns and exons within the nucleotide sequence of the laccase gene amplified by the primer pair Lac 2f-Lac 2b

Fig. 10 shows the total length of nucleotide sequence (1265 bp) of oil palm *Ganoderma* BS (DNA fragment of *Ganoderma* Lac 1.8) amplified by primer pair Lac 2f-Lac 2b. To completely sequence this fragment, internal sequencing primer P4: 5‘ GTTCGTCG TAAGTCCTTTGTC 3‘ and P5: 5‘ GTCCCCCGCCCTCCTACA 3‘ were used. According to the consensus introns, *Ganoderma* Lac 1.8 contains four putative introns ranging from 57 to 63 bp. By comparison to *Ganoderma* Lac 1700, *Ganoderma* Lac 1.8 showed that from the amino acid sequence TQYCDG to WFLHCHI there are four introns, whereas *Ganoderma* Lac 1700 has six introns in this corresponding position. The length of the nucleotide sequence from amino acid TQYCDG to WFLHCHI in *Ganoderma* Lac 1700 is 1,404 bp. This means that the length of nucleotide sequence of *Ganoderma* Lac 1.8 is 139 bp shorter than that of *Ganoderma* Lac 1700 at the corresponding nucleotide sequence.

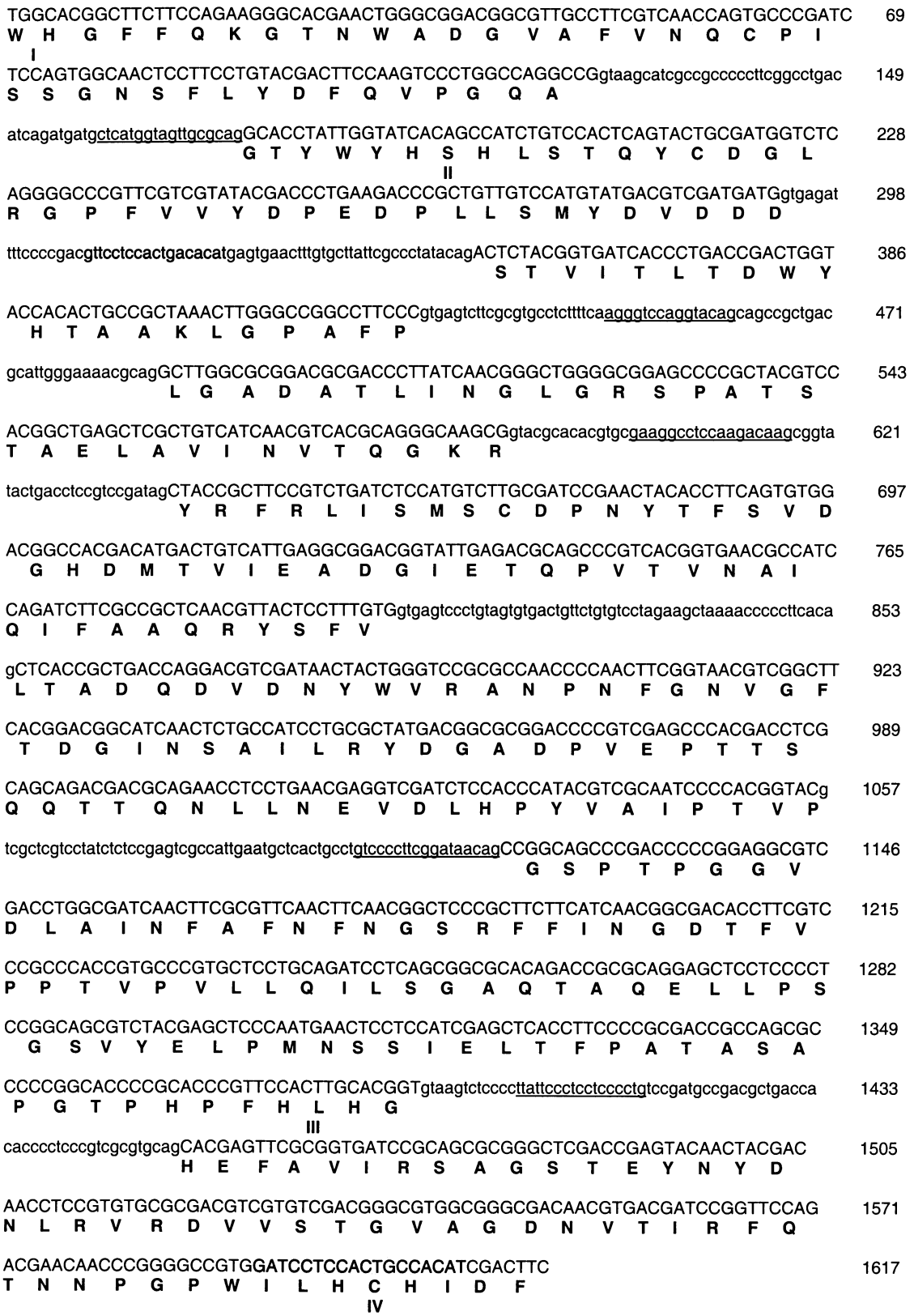


Fig. 9: Partial nucleotide sequence of the laccase gene of oil palm *Ganoderma* BS (*Ganoderma* Lac 1700). The deduced amino acid and intron sequences are presented as bold and lowercase characters. Copper-binding regions are indicated by roman numerals (I to IV). Nucleotides with a shadow box showed the annealing sites of primer Lac 2b, differing by one base. The underlined introns were used to design specific primers

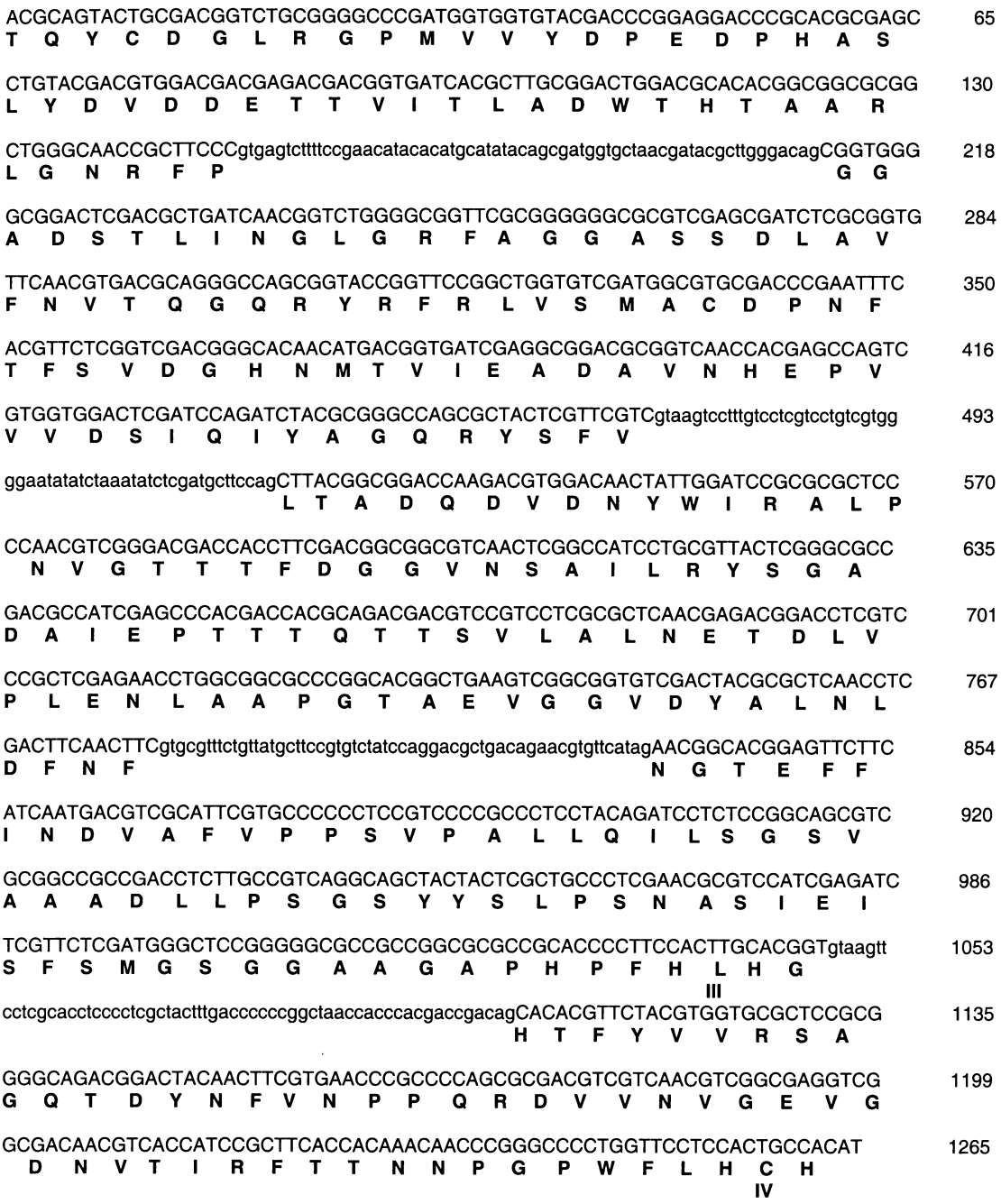


Fig. 10: Partial nucleotide sequence of the laccase gene of oil palm *Ganoderma* BS (DNA fragment of *Ganoderma* Lac 1.8).
The deduced amino acid and intron sequences are presented as bold and lowercase characters. Copper-binding amino acids regions are indicated by roman numerals (III to IV).

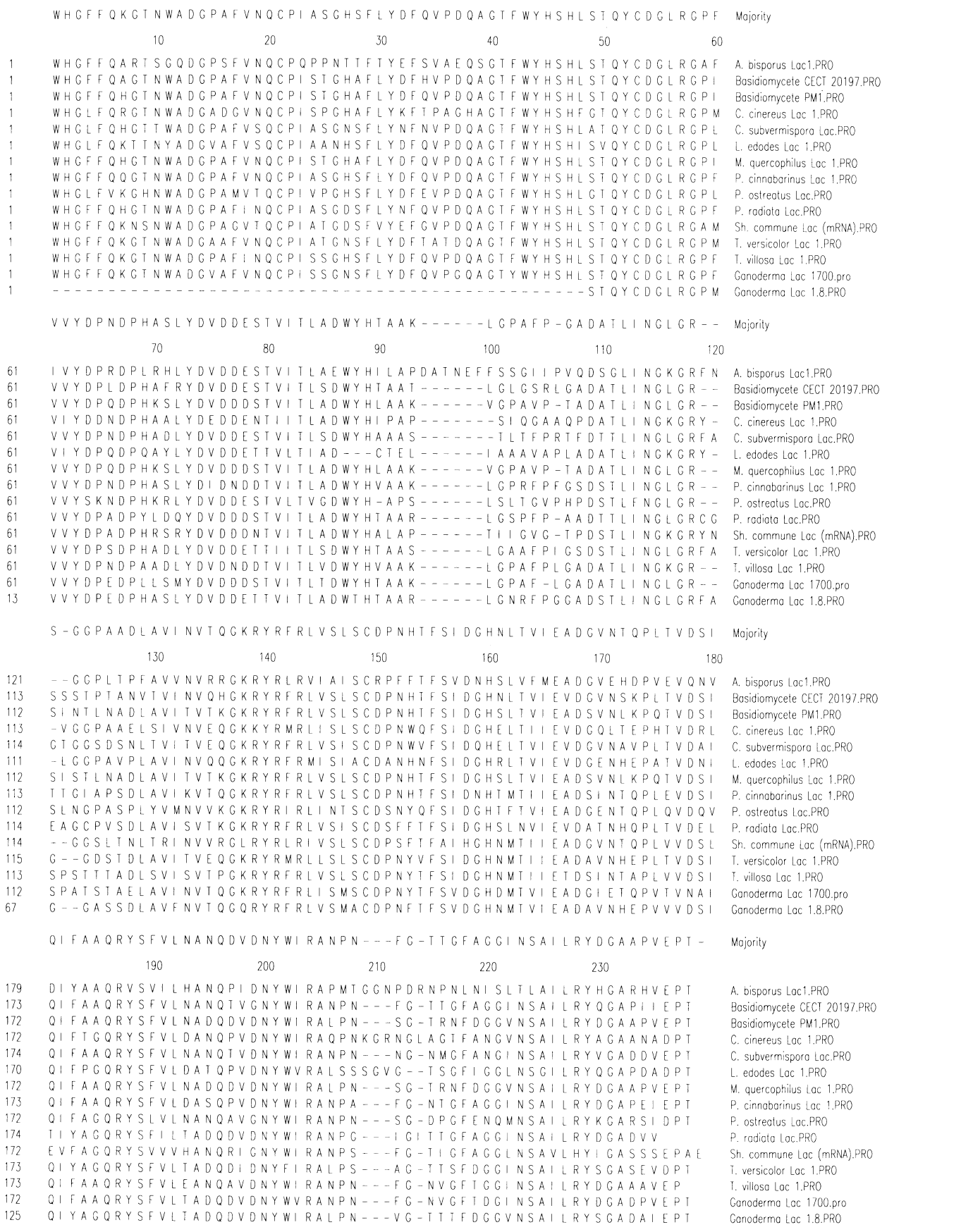
Comparison of the partial amino acid sequences of *Ganoderma* Lac 1700 and *Ganoderma* Lac 1.8 with the laccase genes of other basidiomycete fungi.

The deduced amino acid sequences of the laccase genes from *Ganoderma* Lac 1700 and *Ganoderma* Lac 1.8 fragments had identities to laccase genes of other basidiomycete fungi ranging from 44.5 to 76.7 % and 40.4 to 76.0 %, respectively (Table 13). Amino acid sequences of the laccase genes of *Ganoderma* Lac 1700 and *Ganoderma* Lac 1.8 were aligned with those of published sequences of other basidiomycete fungi (Fig. 11) to infer phylogenetic trees. The resulting phylogenetic trees are shown in Fig. 12. The laccase gene of *Ganoderma* Lac 1700 clustered to *T.villosa* Lac1 and *Ganoderma* Lac 1.8 clustered to *T.versicolor* Lac1.

Table 13: Percent amino acid identities of the partial sequenced laccase genes of *Ganoderma* Lac 1700 and *Ganoderma* Lac 1.8 compared to published laccase genes of other basidiomycete fungi

Fungi	% Identity of laccase amino acid sequence	
	Ganoderma Lac 1700	*Ganoderma* Lac 1.8
A. bisporus Lac 1	44.5	40.4
Basidiomycete CECT 20197	71.4	65.0
Basidiomycete PM1	72.8	67.2
C. cinereus Lac 1	57.8	54.2
C. subvermispora Lac	63.4	59.8
L. edodes Lac 1	54.4	54.0
M. quercophilus Lac 1	72.8	67.2
P. cinnabarinus Lac 1	73.7	69.1
P. ostreatus Lac	56.5	50.5
Ph. radiata Lac	66.2	65.9
Sh. commune Lac (mRNA)	55.8	54.8
T. versicolor Lac 1	65.0	76.0
T. villosa Lac 1	76.7	66.2
Ganoderma Lac 1.8	63.9	-

Percent identities of amino acid sequences of the laccase genes among other basidiomycete fungi are not shown.


WHGFFQKGTNWADGPAFVNQCPIASGHSFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPF Majority
10 20 30 40 50 60
1 WHGFFQARTSGQDGPSFVNQCPQPPNTTFTYEFSVAEQSGTFWYHSHLSTQYCDGLRGAF A. bisporus Lac1.PRO
1 WHGFFQAGTNWADGPAFVNQCPISTGHAFLYDFHVPDQAGTFWYHSHLSTQYCDGLRGPI Basidiomycete CECT 20197.PRO
1 WHGFFQHGTNWADGPAFVNQCPISTGHAFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPI Basidiomycete PM1.PRO
1 WHGLFQRGTNWADGADGVNQCPISPGHAFLYKFTPAGHAGTFWYHSHFGTQYCDGLRGPM C. cinereus Lac 1.PRO
1 WHGLFQHGTTWADGPAFVSQCPIASGNSFLYNFNVPDQAGTFWYHSHLATQYCDGLRGPL C. subvermispora Lac.PRO
1 WHGLFQKTTNYADGVAFVSQCPIAANHSFLYDFQVPDQAGTFWYHSHISVQYCDGLRGPL L. edodes Lac 1.PRO
1 WHGFFQHGTNWADGPAFVNQCPISTGHAFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPI M. quercophilus Lac 1.PRO
1 WHGFFQQGTNWADGPAFVNQCPIASGHSFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPF P. cinnabarinus Lac 1.PRO
1 WHGLFVKGHNWADGPAMVTQCPIVPGHSFLYDFEVPDQAGTFWYHSHLGTQYCDGLRGPL P. ostreatus Lac.PRO
1 WHGFFQHGTNWADGPAFINQCPIASGDSFLYNFQVPDQAGTFWYHSHLSTQYCDGLRGPF P. radiata Lac.PRO
1 WHGFFQKNSNWADGPACVTQCPIATGDSFVYEFGVPDQAGTFWYHSHLSTQYCDGLRGAM Sh. commune Lac (mRNA).PRO
1 WHGFFQKGTNWADGAAFVNQCPIATGNSFLYDFTATDQAGTFWYHSHLSTQYCDGLRGPM T. versicolor Lac 1.PRO
1 WHGFFQKGTNWADGPAFINQCPISSGHSFLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPF T. villosa Lac 1.PRO
1 WHGFFQKGTNWADGVAFVNQCPISSGNSFLYDFQVPGQAGTYWYHSHLSTQYCDGLRGPF Ganoderma Lac 1700.pro
1 ----------STQYCDGLRGPM Ganoderma Lac 1.8.PRO
VVYDPNDPHASLYDVDDESTVITLADWYHTAAK------LGPAFP-GADATLINGLGR-- Majority
70 80 90 100 110 120
61 IVYDPRDPLRHLYDVDDESTVITLAEWYHILAPDATNEFFSSGIIPVQDSGLINGKGRFN A. bisporus Lac1.PRO
61 VVYDPLDPHAFRYDVDDESTVITLSDWYHTAAT------LGLGSRLGADATLINGLGR-- Basidiomycete CECT 20197.PRO
61 VVYDPQDPHKSLYDVDDDSTVITLADWYHLAAK------VGPAVP-TADATLINGLGR-- Basidiomycete PM1.PRO
61 VIYDDNDPHAALYDEDDENTIITLADWYHIPAP-------SIQGAAQPDATLINGKGRY- C. cinereus Lac 1.PRO
61 VVYDPNDPHADLYDVDDESTVITLSDWYHAAAS-------TLTFPRTFDTTLINGLGRFA C. subvermispora Lac.PRO
61 VIYDPQDPQAYLYDVDDETTVLTIAD---CTEL------IAAAVAPLADATLINGKGRY- L. edodes Lac 1.PRO
61 VVYDPQDPHKSLYDVDDDSTVITLADWYHLAAK------VGPAVP-TADATLINGLGR-- M. quercophilus Lac 1.PRO
61 VVYDPNDPHASLYDIDNDDTVITLADWYHVAAK------LGPRFPFGSDSTLINGLGR-- P. cinnabarinus Lac 1.PRO
61 VVYSKNDPHKRLYDVDDESTVLTVGDWYH-APS------LSLTGVPHPDSTLFNGLGR-- P. ostreatus Lac.PRO
61 VVYDPADPYLDQYDVDDDSTVITLADWYHTAAR------LGSPFP-AADTTLINGLGRCG P. radiata Lac.PRO
61 VVYDPADPHRSRYDVDDDNTVITLADWYHALAP------TIIGVG-TPDSTLINGKGRYN Sh. commune Lac (mRNA).PRO
61 VVYDPSDPHADLYDVDDETTIITLSDWYHTAAS------LGAAFPIGSDSTLINGLGRFA T. versicolor Lac 1.PRO
61 VVYDPNDPAADLYDVDNDDTVITLVDWYHVAAK------LGPAFPLGADATLINGKGR-- T. villosa Lac 1.PRO
61 VVYDPEDPLLSMYDVDDDSTVITLTDWYHTAAK------LGPAF-LGADATLINGLGR-- Ganoderma Lac 1700.pro
13 VVYDPEDPHASLYDVDDETTVITLADWTHTAAR------LGNRFPGGADSTLINGLGRFA Ganoderma Lac 1.8.PRO
S-GGPAADLAVINVTQGKRYRFRLVSLSCDPNHTFSIDGHNLTVIEADGVNTQPLTVDSI Majority
130 140 150 160 170 180
121 --GGPLTPFAVVNVRRGKRYRLRVIAISCRPFFTFSVDNHSLVFMEADGVEHDPVEVQNV A. bisporus Lac1.PRO
113 SSSTPTANVTVINVQHGKRYRFRLVSLSCDPNHTFSIDGHNLTVIEVDGVNSKPLTVDSI Basidiomycete CECT 20197.PRO
112 SINTLNADLAVITVTKGKRYRFRLVSLSCDPNHTFSIDGHSLTVIEADSVNLKPQTVDSI Basidiomycete PM1.PRO
113 -VGGPAAELSIVNVEQGKKYRMRLISLSCDPNWQFSIDGHELTIIEVDGQLTEPHTVDRL C. cinereus Lac 1.PRO
114 GTGGSDSNLTVITVEQGKRYRFRLVSISCDPNWVFSIDQHELTVIEVDGVNAVPLTVDAI C. subvermispora Lac.PRO
111 -LGGPAVPLAVINVQQGKRYRFRMISIACDANHNFSIDGHRLTVIEVDGENHEPATVDNI L. edodes Lac 1.PRO
112 SISTLNADLAVITVTKGKRYRFRLVSLSCDPNHTFSIDGHSLTVIEADSVNLKPQTVDSI M. quercophilus Lac 1.PRO
113 TTGIAPSDLAVIKVTQGKRYRFRLVSLSCDPNHTFSIDNHTMTIIEADSINTQPLEVDSI P. cinnabarinus Lac 1.PRO
112 SLNGPASPLYVMNVVKGKRYRIRLINTSCDSNYQFSIDGHTFTVIEADGENTQPLQVDQV P. ostreatus Lac.PRO
114 EAGCPVSDLAVISVTKGKRYRFRLVSISCDSFFTFSIDGHSLNVIEVDATNHQPLTVDEL P. radiata Lac.PRO
114 --GGSLTNLTRINVVRGLRYRLRIVSLSCDPSFTFAIHGHNMTIIEADGVNTQPLVVDSL Sh. commune Lac (mRNA).PRO
115 G--GDSTDLAVITVEQGKRYRMRLLSLSCDPNYVFSIDGHNMTIIEADAVNHEPLTVDSI T. versicolor Lac 1.PRO
113 SPSTTTADLSVISVTPGKRYRFRLVSLSCDPNYTFSIDGHNMTIIETDSINTAPLVVDSI T. villosa Lac 1.PRO
112 SPATSTAELAVINVTQGKRYRFRLISMSCDPNYTFSVDGHDMTVIEADGIETQPVTVNAI Ganoderma Lac 1700.pro
67 G--GASSDLAVFNVTQGQRYRFRLVSMACDPNFTFSVDGHNMTVIEADAVNHEPVVVDSI Ganoderma Lac 1.8.PRO
QIFAAQRYSFVLNANQDVDNYWIRANPN---FG-TTGFAGGINSAILRYDGAAPVEPT- Majority
190 200 210 220 230
179 DIYAAQRVSVILHANQPIDNYWIRAPMTGGNPDRNPNLNISLTLAILRYHGARHVEPT A. bisporus Lac1.PRO
173 QIFAAQRYSFVLNANQTVGNYWIRANPN---FG-TTGFAGGINSAILRYQGAPIIEPT Basidiomycete CECT 20197.PRO
172 QIFAAQRYSFVLNADQDVDNYWIRALPN---SG-TRNFDGGVNSAILRYDGAAPVEPT Basidiomycete PM1.PRO
172 QIFTGQRYSFVLDANQPVDNYWIRAQPNKGRNGLAGTFANGVNSAILRYAGAANADPT C. cinereus Lac 1.PRO
174 QIFAAQRYSFVLNANQTVDNYWIRANPN---NG-NMGFANGINSAILRYVGADDVEPT C. subvermispora Lac.PRO
170 QIFPGQRYSFVLDATQPVDNYWVRALSSSGVG--TSGFIGGLNSGILRYQGAPDADPT L. edodes Lac 1.PRO
172 QIFAAQRYSFVLNADQDVDNYWIRALPN---SG-TRNFDGGVNSAILRYDGAAPVEPT M. quercophilus Lac 1.PRO
173 QIFAAQRYSFVLDASQPVDNYWIRANPA---FG-NTGFAGGINSAILRYDGAPEIEPT P. cinnabarinus Lac 1.PRO
172 QIFAGQRYSLVLNANQAVGNYWIRANPN---SG-DPGFENQMNSAILRYKGARSIDPT P. ostreatus Lac.PRO
174 TIYAGQRYSFILTADQDVDNYWIRANPG---IGITTGFAGGINSAILRYDGADVV P. radiata Lac.PRO
172 EVFAGQRYSVVVHANQRIGNYWIRANPS---FG-TIGFAGGLNSAVLHYIGASSSEPAE Sh. commune Lac (mRNA).PRO
173 QIYAGQRYSFVLTADQDIDNYFIRALPS---AG-TTSFDGGINSAILRYSGASEVDPT T. versicolor Lac 1.PRO
173 QIFAAQRYSFVLEANQAVDNYWIRANPN---FG-NVGFTGGINSAILRYDGAAAVEP T. villosa Lac 1.PRO
172 QIFAAQRYSFVLTADQDVDNYWVRANPN---FG-NVGFTDGINSAILRYDGADPVEPT Ganoderma Lac 1700.pro
125 QIYAGQRYSFVLTADQDVDNYWIRALPN---VG-TTTFDGGVNSAILRYSGADAIEPT Ganoderma Lac 1.8.PRO

Continued

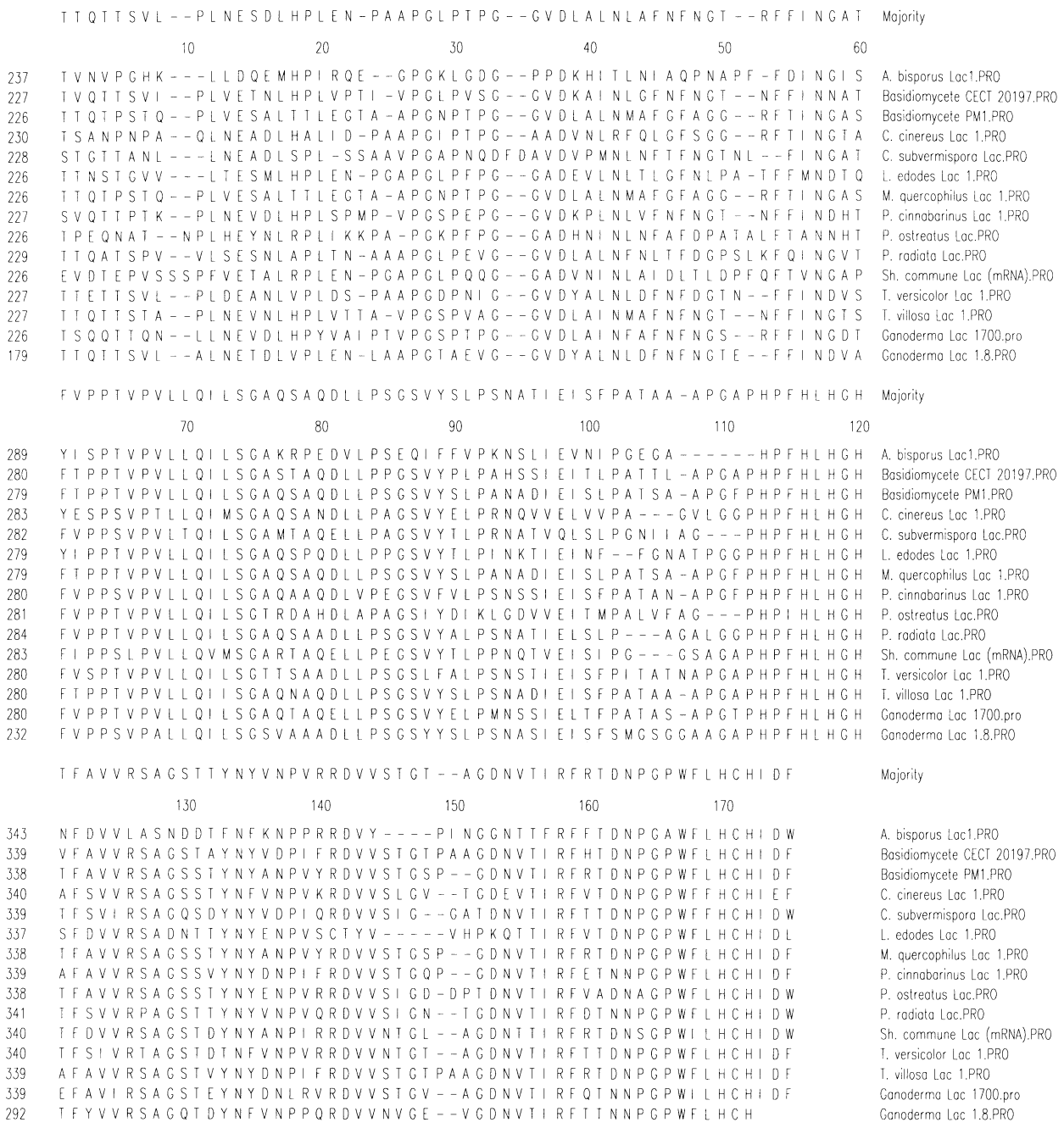


Fig. 11: Alignment of the partial deduced amino acid sequences of oil palm *Ganoderma* BS (*Ganoderma* Lac 1700 and *Ganoderma* Lac 1.8) with other basidiomyceteous laccase sequences. Amino acid residues in the conserved positions were shown on the top. Gaps were introduced for optimal alignment and indicated by dashes. The sequences were aligned with the Clustal method (MegAlign, DNAstar)

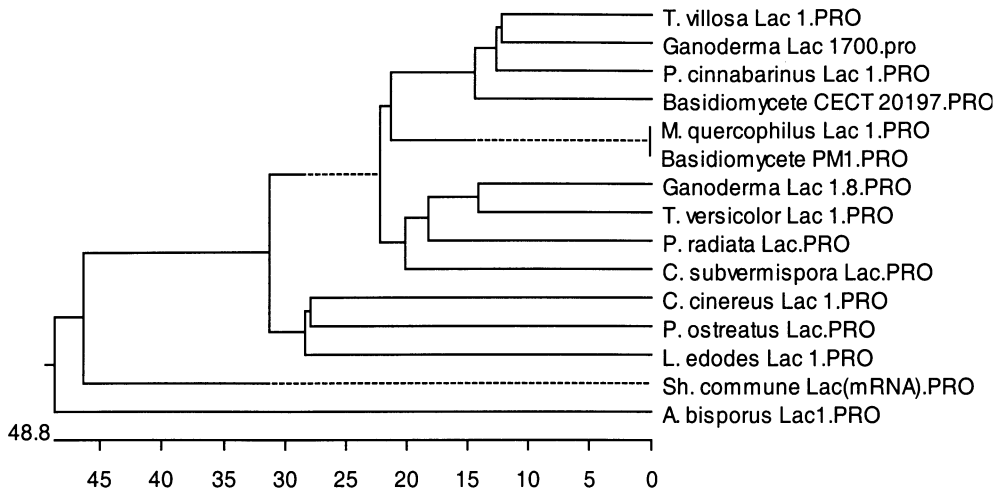


Fig. 12:A phylogenetic tree of the partial amino acid sequences of laccases was constructed using the Clustal method with the PAM 250 residue weight table. Multiple alignment parameters used were: gap penalty = 10 and gap length penalty = 10. Pairwise alignment parameters were: ktuple = 2, gap penalty = 5, window = 4 and diagonals = 4. The length of each pair of branches represents the distance between sequence pairs. The units at the bottom of the tree indicate the number of substitution events.

3.2.3 Evaluation of the primer specificity of Lac 2a-Lac 2b in the PCR amplification of oil palm *Ganoderma* DNA

3.2.3.1 PCR amplifications using primer pair Lac 2a-Lac 2b with different concentrations of *Taq* polymerase and $MgCl_2$

Standard concentrations of *Taq* polymerase and $MgCl_2$ in PCR mix reaction for the primer pair Lac 2a-Lac 2b are 1U for *Taq* polymerase and 1mM for $MgCl_2$ (see materials and methods). To study the specificity of the primer pair Lac 2a-Lac 2b, different concentrations of *Taq* polymerase and $MgCl_2$ were used. The effects of *Taq* polymerase and $MgCl_2$ concentration on the PCR amplification of the laccase gene fragments of oil palm *Ganoderma* are shown in Fig. 13. None of the oil palm *Ganoderma* isolates amplified by this primer pair produced a DNA fragment of about 1610 bp.

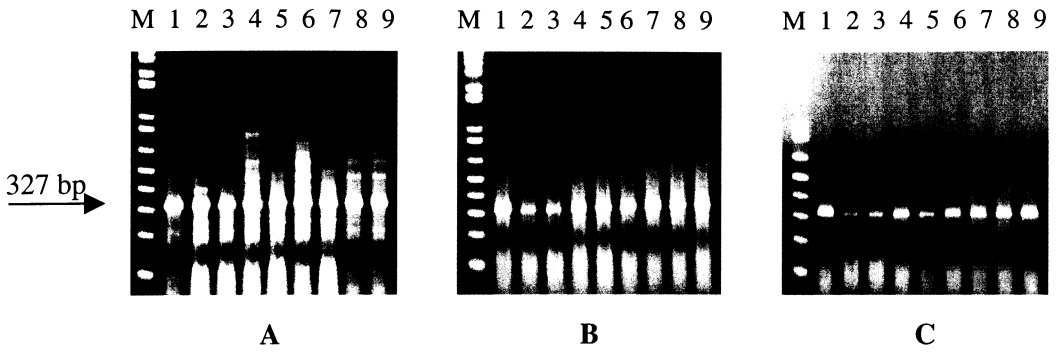


Fig.13:PCR amplification using the primer pair Lac 2a and Lac 2b in order to amplify an additional DNA fragment of 1610-bp by increasing concentration of *Taq* polymerase and $MgCl_2$
Oil palm *Ganoderma* isolates BJ7 (A), SM (B) and Ma (C) were amplified by using different concentrations of *Taq* polymerase and $MgCl_2$. Lane 1-3: *Taq* polymerase concentration (1U as normal standard) with 1mM (normal standard), 2 mM and 3 mM of $MgCl_2$. Lane 4-6: *Taq* polymerase concentration (2U) with 1mM, 2mM and 3 mM of $MgCl_2$. Lane 7-9: *Taq* polymerase concentration (3U) with 1mM, 2 mM and 3 mM of $MgCl_2$. M: DNA marker

3.2.3.2 PCR amplification of DNA fragment of *Ganoderma* Lac 1700 (designation pU1700) using the primer pair Lac 2a-Lac 2b at different temperatures, concentrations of *Taq* polymerase and $MgCl_2$.

The previous data of nucleotide sequence of *Ganoderma* Lac 1700 showed that the nucleotide sequence of the DNA fragment of *Ganoderma* Lac 7 is part of the DNA fragment of *Ganoderma* Lac 1700. By amplifying the purified pU1700, the specificity of the primer pair Lac 2a-Lac 2b could be evaluated. Fig. 14 shows the result of the amplification of pU1700 with the primer pair Lac 2a-Lac 2b at different annealing temperatures in the PCR standard mix reaction. This primer pair specifically produced a single band of 327 bp and no amplification of an additional PCR product of about 1610 bp was observed at annealing temperatures of 61, 63 and 65 °C. However, at 59 °C, a faint band of about 1610 bp was detected.

The results of the effects of different concentrations of *Taq* polymerase and $MgCl_2$ on the PCR amplification of pU1700 are shown in Fig. 13. At a *Taq* polymerase concentration of 2U and a $MgCl_2$ concentration of 3mM, an additional PCR product of about 1610 bp was detected. On the other hand, at *Taq* polymerase concentration of 3U and $MgCl_2$ concentrations of 1, 2 and 3 mM, an additional band of 1610-bp was clearly observed at three different $MgCl_2$ levels.

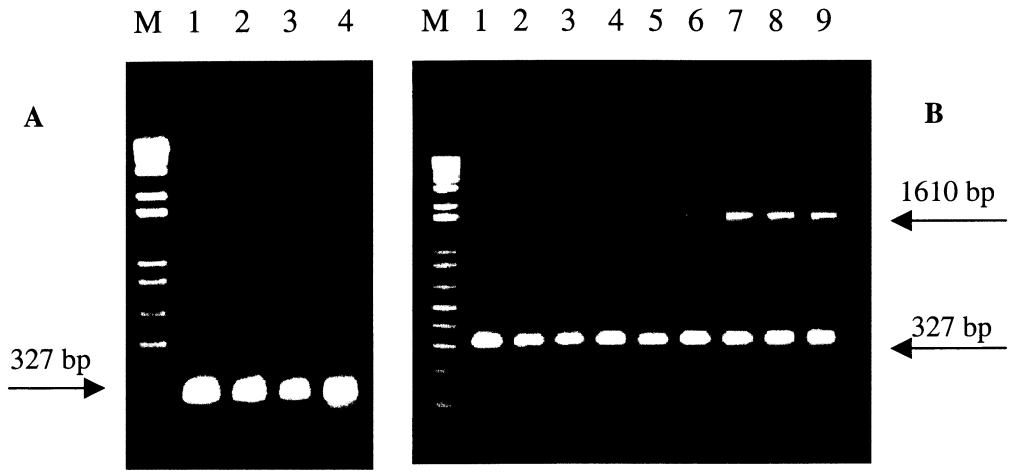


Fig. 14: PCR amplification experiments with primer Lac 2a and Lac 2b to amplify an additional DNA fragment of 1610-bp at different annealing temperatures and increasing concentrations of *Taq* polymerase and $MgCl_2$.

A. 1 ng of plasmid pU1700 was amplified at different annealing temperatures. Lane 1-4: annealing temperatures 65, 63, 61 and 59 °C with normally used concentration of *Taq* polymerase (1U) and $MgCl_2$(1 mM)

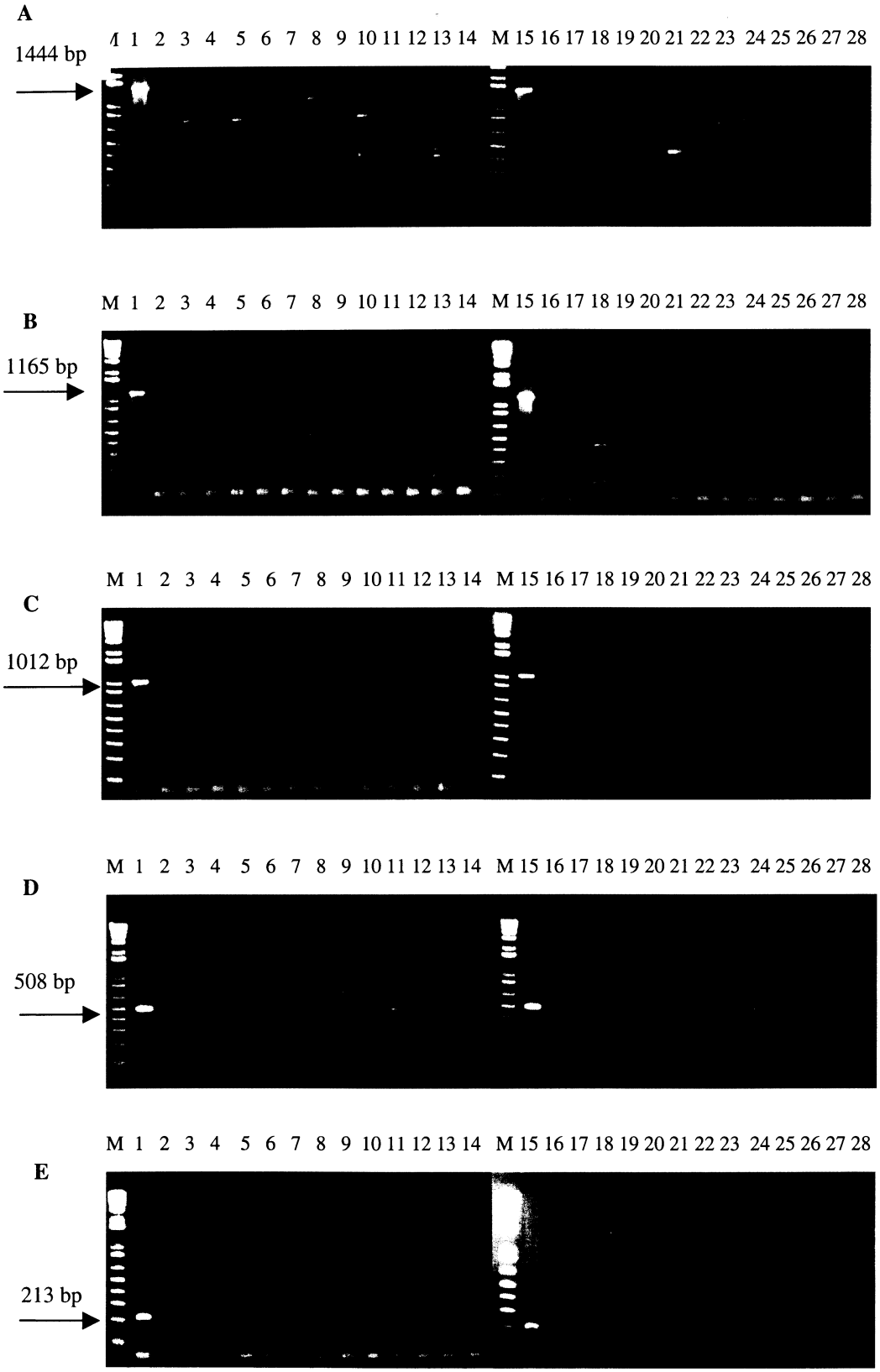
B. 1 ng of plasmid pU1700 was amplified at different concentrations of *Taq* polymerase and $MgCl_2$. Lane 1-3: *Taq* polymerase concentration (1U) with 1 mM, 2 mM and 3 mM of $MgCl_2$. Lane 4-6: *Taq* polymerase concentration (2U) with 1mM, 2 mM and 3 mM of $MgCl_2$. Lane 7-9: *Taq* polymerase concentration (3U) with 1mM, 2 mM and 3 mM of $MgCl_2$. M: DNA marker

3.2.4 Primer specificity for oil palm *Ganoderma* designed from specific nucleotide sequence corresponding to conserved amino acid sequence of ILHCHI and introns of *Ganoderma* Lac 1700 fragment

Primer Lac 2b 2 (5'GATGTGGCAGTGGAGGAT3') was designed as a reverse primer from the nucleotide sequence corresponding to the amino acid sequence ILHCHI. The following forward primers of introns were selected: Intron 9 (5'CTCATGGTA GTTGCGCAG 3'), Intron 1 (5'AGGGTCCAGGTACAGCAG 3'), Intron 2 (5'GAAGGC CTCCAAGACAAG 3'), Intron 4 (5'GTCCCCTTCGGATAACAG 3') and Intron 5 (5'TATTCCCTCCTCCCC TGT 3'), see Fig. 9. Based on the expected size, PCR amplification of genomic DNA of oil palm *Ganoderma* BS by using primer pairs Lac 2b 2-Intron 9, Lac 2b 2-Intron 1, Lac 2b 2-Intron 2, Lac 2b 2-Intron 4 and Lac 2b 2-Intron 5 generated a single band of about 1444 bp, 1165 bp, 1012 bp, 508 bp and 213 bp.

The specificity of the five primer pairs was evaluated against other *Ganoderma* species to allow the discrimination of oil palm *Ganoderma* from other *Ganoderma* species. Of the five primer pairs tested, two primer pairs (Lac 2b 2-Intron 5 and Lac 2b 2-Intron 2) showed no cross-reaction and no additional band with any other *Ganoderma* species tested, indicating that they are species-specific primers for oil palm *Ganoderma* (Fig.14). The primer pairs Lac 2b 2- Intron 1 and Lac 2b 2-Intron 9 showed no cross-reaction with any other *Ganoderma* species tested but there were additional bands observed as the results of the random amplification using the primers in several other *Ganoderma* species (Fig.15). Amplification with primer pair Lac 2b 2-Intron 4 indicated a cross-reaction by producing a faint band of about 508 bp for *G. applanatum* BAFC 2552 and *G. applanatum* BAFC 2408 (Fig. 15). The results of the PCR amplification using five primer pairs for oil palm *Ganoderma* and other *Ganoderma* species studied are summarised in Table 14.

To further evaluate primer specificity, the annealing temperature of the primers was decreased from the initial temperature 65 to 55 ^{o}C. For this evaluation, only 12 other *Ganoderma* species were used. The specificity of the primer pair Lac 2b 2-Intron 5 was not altered by decreasing the annealing temperature, as indicated by the occurrence of a single band of about 213 bp when oil palm *Ganoderma* DNA was used. No amplification of DNA was observed in any of the 12 other *Ganoderma* species using primer pair Lac 2b 2-Intron 5. The other four primer pairs produced additional bands when oil palm *Ganoderma* DNA was amplified. The randomly generated PCR products observed with DNA from 12 other *Ganoderma* species are shown in Fig. 15.


A
1444 bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M 15 16 17 18 19 20 21 22 23 24 25 26 27 28
B
1165 bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M 15 16 17 18 19 20 21 22 23 24 25 26 27 28
C
1012 bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M 15 16 17 18 19 20 21 22 23 24 25 26 27 28
D
508 bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M 15 16 17 18 19 20 21 22 23 24 25 26 27 28
E
213 bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 M 15 16 17 18 19 20 21 22 23 24 25 26 27 28

Continued

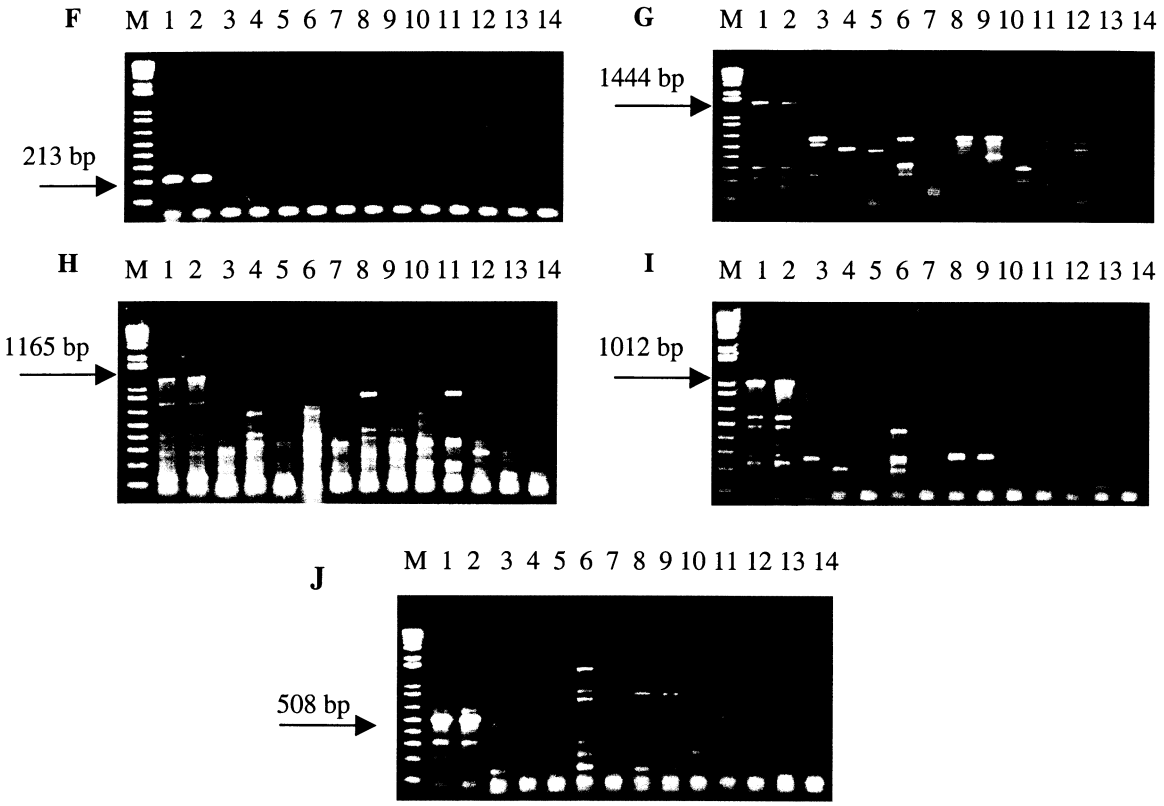


Fig.15: PCR amplification of DNA from oil palm *Ganoderma* and other *Ganoderma* species using the primer Lac 2b 2 combined with primers designed from intron sequences of the laccase gene of *Ganoderma* Lac 1700

A, B, C, D and E: Results of PCR amplification using the primer pairs Lac2b 2 with Intron 9, Intron 1, Intron 2, Intron 4 and Intron 5, respectively. Lane 1-28: oil palm *Ganoderma* BS, *Ganoderma* of BAFC2775, BAFC2390, *G. tsugae*, BAFC 2495, BAFC 2424, BAFC 2288, DSM 9612, BAFC 2374, BAFC2580, BAFC218, BAFC 2552, BAFC 2529, DSMZ 3800, PNG-*597*, BAFC2488, G211, G 224, G 225, 134, QFRI 8678.1, DAR 73781, DAR 73783, DFP 4483,BAFC 2408, DFP 4483, PNG-208 and PNG-209

F, G, H, I and J: DNA fragments of the PCR amplification (at annealing temperature 55 °C) using primer pairs Lac 2b 2 with Intron 5, Intron 9, Intron 1, Intron 2 and Intron 4, respectively. Lane 1-12: oil palm *Ganoderma* PM, *Ganoderma* of PNG-240, BAFC2775, BAFC2495, BAFC2288, DSM 9612, BAFC 2529, BAFC 2390, BAFC 2395, G 211, G 224, G 225, 134 and PNG-208. M: DNA marker.

Table 14: Results of the PCR amplification of DNA from *Ganoderma* using the primer pairs Lac 2b2 with Intron 9, Intron 1, Intron 2, Intron 4 and Intron 5

Ganoderma species	PCR amplifications by primer pairs				
	1*	2*	3	4	5
Oil palm *Ganoderma* (Indonesia), 20 isolates	+++	+++	+++	+++	+++
G. boninense (oil palm, PNG), 10 isolates	+++	+++	+++	+++	+++
G. boninense (coconut stump, PNG), 7 isolates	+++	+++	+++	+++	+++
G. tornatum (hardwood, PNG), 11 isolates	-	-	-	-	-
G. oerstedii BAFC 178	-	-	-	-	-
G. oerstedii BAFC 218	-	-	-	-	-
G. resinaceum BAFC 384	-	-	-	-	-
G. tornatum (applanatum?) BAFC 671	-	-	-	-	-
G. resinaceum BAFC 2288	-	-	-	-	-
G. applanatum BAFC 2353	-	-	-	-	-
G. lucidum complex BAFC 2374	-	-	-	-	-
G. tornatum BAFC 2390	-	-	-	-	-
G. tornatum? BAFC 2395	-	-	-	-	-
G. applanatum BAFC 2408	-	-	-	+	-
G. tornatum BAFC 2424	-	-	-	-	-
G. tornatum? BAFC 2430	-	-	-	-	-
G. resinaceum BAFC 2488	-	-	-	-	-
G. lucidum complex BAFC 2495	-	-	-	-	-
G. applanatum var. tornatum BAFC 2501	-	-	-	-	-
Ganoderma sp. BAFC 2529	-	-	-	-	-
G. applanatum BAFC 2552	-	-	-	+	-
G. tropicum BAFC 2580	-	-	-	-	-
G. resinaceum BAFC 2775	-	-	-	-	-
G. lucidum DSM 9612	-	-	-	-	-
G. applanatum DSMZ 3800	-	-	-	-	-
G. tsugae	-	-	-	-	-
G. applanatum 134	-	-	-	-	-
G. applanatum G 211	-	-	-	-	-
G. adspersum G 224	-	-	-	-	-
G. pfeifferi G 225	-	-	-	-	-
G. cupreum QFRI 8678.1	-	-	-	-	-
G. australe DAR 73781	-	-	-	-	-
G. incrassatum DAR 73783	-	-	-	-	-
G. cupreum DFP 4336	-	-	-	-	-
*Ganoderma sp.*Group 6.3 DAR 73779	-	-	-	-	-
G. weberianum DFP 4483	-	-	-	-	-

The numbers (1, 2, 3, 4 and 5) designates for the primer pairs of Lac 2b 2-Intron 9, Lac 2b 2-Intron 1, Lac 2b 2-Intron 2, Lac 2b 2-Intron 4 and Lac 2b 2-Intron 5.

+++: a strong single band of PCR product as the result of PCR amplification from Lac 2b 2-Intron 9 (1,444 bp), Lac 2b 2-Intron 1 (1,165 bp), Lac 2b 2-Intron 2 (1,012 bp), Lac 2b 2-Intron 4 (508 bp) and Lac 2b 2-Intron 5 (213 bp)

+: a weak band of PCR product, in the same column shows the same size of PCR product of oil palm *Ganoderma*

-: no PCR amplification identical to oil palm *Ganoderma* was observed

1* and **2***: no PCR product identical to oil palm *Ganoderma* was observed using the primer pairs Lac 2b 2-Intron 9 and Lac 2b 2-Intron 1 for other *Ganoderma* species, but generate randomly additional bands in several other *Ganoderma* species

3.3 Identification and differentiation of oil palm *Ganoderma* based on ITS, Mn-SOD and IGS1 genes

3.3.1 ITS region and ITS-RFLP analyses

3.3.1.1 ITS region sequences

PCR amplification of genomic DNA of eight oil palm *Ganoderma* isolates using primer pair ITS1-ITS4 produced a single PCR amplification product of about 650 bp. These primers amplify a region from the 3`-end of the 18S rDNA to the 5`-end of the 28S rDNA, including the ITS1, 5.8S rDNA and ITS2 regions. Alignment of nucleotide sequences in the ITS region and the 5.8S rDNA of eight oil palm *Ganoderma* isolates is shown in Fig. 16. Boundaries of the ITS1 and ITS2 sequences of the oil palm *Ganoderma* were determined by a comparison with the published sequences of the ITS region of *Ganoderma* spp. (Moncalvo et al., 1995a). The nucleotide size of ITS 1 was 217 bp with percent identities ranging from 93.8 to 100 % (Table 2A, see appendixes). ITS 2 ranged from 193 to 197 bp with percent identities ranged from 90.2 to 100 % within 8 oil palm *Ganoderma* isolates (Table 3A see appendixes). The length of nucleotide sequences of *Ganoderma* isolates BS and SB in the ITS2 region is 197 bp, i.e. 4 bp longer than the other oil palm *Ganoderma* sequences. Of eight oil palm *Ganoderma* isolates sequenced, *Ganoderma* isolate SB had the highest nucleotide sequence variation in the ITS region. No nucleotide variation (i.e. 100 % homolog) was apparent in the region of the 5.8S r DNA sequences within the 8 oil palm *Ganoderma* isolates.

3.3.1.2 Restriction fragment length polymorphism (RFLP) analyses for characterising the PCR products of the ITS region of oil palm *Ganoderma*

PCR amplifications of total genomic DNA from the *Ganoderma* species using primer pair ITS1-ITS4 produced a single PCR product of about 650 bp, except for *G. lucidum* DSM 103 and *Ganoderma* sp.136 which produced larger PCR products than the expected size for *Ganoderma* species. *G. lucidum* DSM 103 produced a double band of about 700 bp and 550 bp, whereas *Ganoderma* sp. 136 produced a PCR product of about 800 bp (Fig. 17). Both *Ganoderma* were excluded from further analyses because they were considered an outgroup of *Ganoderma* (non-*Ganoderma* species). In order to identify and discriminate between oil palm *Ganoderma* and other *Ganoderma* species, the PCR product was digested with several restriction enzymes that were specific to the ITS regions of oil palm

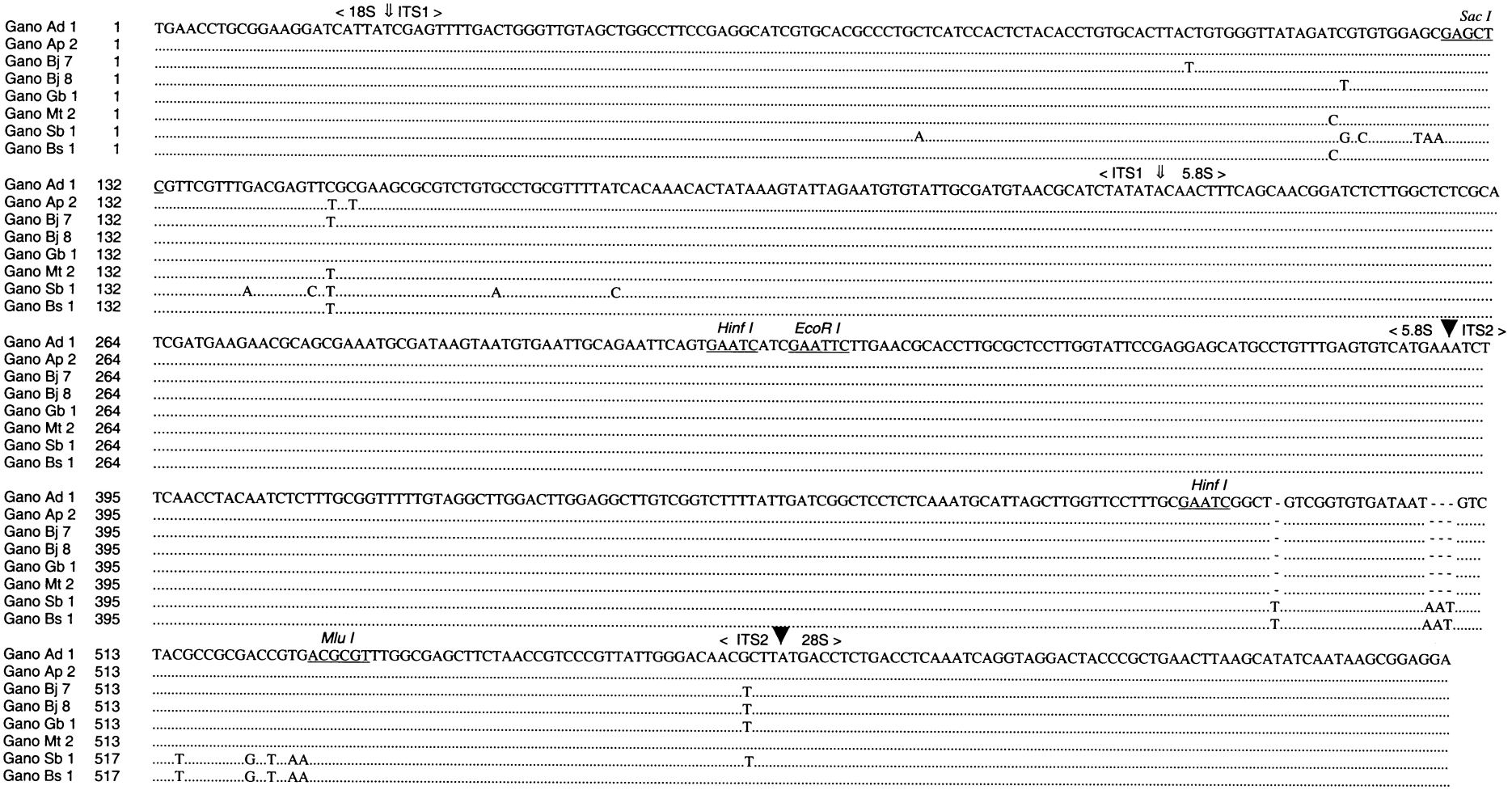


Fig. 16: Comparison of the nucleotide sequences of the internal transcribed spacer (ITS) region and 5.8S rDNA from 8 isolates of oil palm *Ganoderma*. Start and end of ITS1 region are indicated by white arrow and start and end of ITS 2 region are indicated by black arrow. Dots (..) in the sequence indicate conserved bases, and dashes (-) indicate gaps.

Ganoderma (the determination of restriction sites based on MapDraw of the DNAstar program). The selected restriction enzymes were *EcoRI, SacI*, *MluI* and *Hinf I*. Positions of restriction sites of *EcoRI* in the the 5.8 rDNA, *Sac I* in the ITS1 sequence, *Mlu I* in the ITS2 sequence and *Hinf I* in the 5.8S rDNA and the ITS2 sequence were shown in Fig. 16. PCR product digested by *Hinf I* was generated by using primer pair ITS3-ITS4. These primers produced a single PCR product about 390 bp for *Ganoderma* spp. Restriction digestion by using *EcoRI* of PCR product of primer pair ITS1-ITS4 produced the same pattern for all *Ganoderma* species tested. Restriction enzyme *Sac I* digested the ITS-PCR product amplified by primer pair ITS1-ITS4 for all oil palm *Ganoderma* and *G. boninense* isolated from coconut stumps including other *Ganoderma* species such as *G. tornatum* (*applanatum*?) BAFC671, *G. tornatum* BAFC2390, *G. tornatum* BAFC2395, *G. tornatum* BAFC2430, *Ganoderma* sp. BAFC2529 and *G. applanatum* DSMZ3800. The restriction patterns of *G. applanatum* DSMZ3800 and *G. tornatum* BAFC2430 are rather different compared to four other digested *Ganoderma* with an intense band of about 650 bp as shown in Fig. 17. *MluI* digested the ITS-PCR product amplified by primer pair ITS1-ITS4 for all oil palm *Ganoderma* and all *G. boninense* isolated from coconut stump but did not digest any other *Ganoderma* species tested. Following digestion with *MluI,* oil palm *Ganoderma* could be differentiated from other *Ganoderma* species studied.

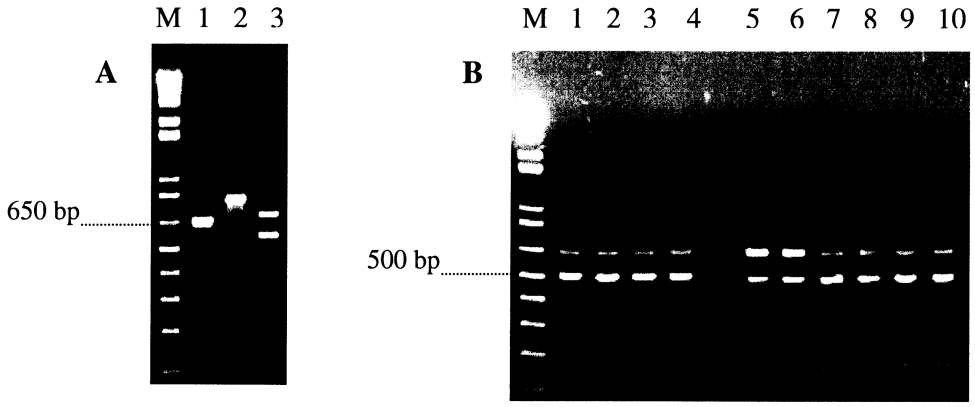


Fig. 17: PCR amplification products of non-*Ganoderma* amplified by primer pair ITS1-ITS 4 and RFLP analyses of *Ganoderma* spp. using *Sac I*

A. Non *Ganoderma* Lane 1-3: oil palm *Ganoderma* isolate BS, *Ganoderma* sp. 136 and *G. lucidum* DSM 103

B. *Ganoderma* digested with *Sac I*, Lane 1-10: oil palm *Ganoderma* isolates BS, Ad, SB, Mt, *Ganoderma* of DSMZ3800 BAFC2430, BAFC2529, BAFC671, BAFC2390 and BAFC2395. M: DNA marker.

Table 15: PCR amplification products of *Ganoderma* species amplified by primer pairs ITS1-ITS4 and ITS3-ITS4 primers were digested by several restriction enzymes in order to discriminate oil palm *Ganoderma* from other *Ganoderma* spp.

Ganoderma	Restriction enzymes used			
	EcoRI	*SacI*	*MluI*	*Hinf I*
Oil palm *Ganoderma* (Indonesia), 20 isolates	308, 342	650, 523, 127	537, 113	60, 170, 160
G. boninense (oil palm, PNG), 10 isolates	+	+	+	+
G. boninense (coconut stump, PNG), 7 isolates	+	+	+	+
G. tornatum (hardwood, PNG), 11 isolates	+	-	-	330, 60
G. oerstedii BAFC 178	+	-	-	+
G. oerstedii BAFC 218	+	-	-	+
G. resinaceum BAFC 384	+	-	-	+
G. tornatum (applanatum?) BAFC 671	+	+	-	330, 60
G. resinaceum BAFC 2288	+	-	-	+
G. applanatum BAFC 2353	+	-	-	330, 60
G. lucidum complex BAFC 2374	+	-	-	330, 60
G. tornatum BAFC 2390	+	+	-	330, 60
G. tornatum? BAFC 2395	+	+	-	330, 60
G. applanatum BAFC 2408	+	-	-	330, 60
G. tornatum BAFC 2424	+	-	-	330, 60
G. tornatum? BAFC 2430	+	+	-	330, 60
G. resinaceum BAFC 2488	+	-	-	330, 60
G. lucidum complex BAFC 2495	+	-	-	330, 60
G. applanatum var. tornatum BAFC 2501	+	-	-	330, 60
Ganoderma sp. BAFC 2529	+	+	-	330, 60
G. applanatum BAFC 2552	+	-	-	330, 60
G. tropicum BAFC 2580	+	-	-	+
G. resinaceum BAFC 2775	+	-	-	+
G. lucidum DSM 9612	+	-	-	330, 60
G. applanatum DSM 3800	+	+	-	330, 60
G. tsugae	+	-	-	330, 60
G. applanatum 134	+	-	-	330, 60
G. applanatum G 211	+	-	-	+
G. adspersum G 224	+	-	-	330, 60
G. pfeifferi G 225	+	-	-	+
G. cupreum QFRI 8678.1	+	-	-	330, 60
G. australe DAR 73781	+	-	-	330, 60
G. incrassatum DAR 73783	+	-	-	330, 60
G. cupreum DFP 4336	+	-	-	330, 60
*Ganoderma sp.*Group 6.3 DAR 73779	+	-	-	330, 60
G. weberianum DFP 4483	+	-	-	+

+: in the same column showed identical PCR (amplified by ITS1-ITS4) digested product sizes with oil palm *Ganoderma*

- : in the same column showed PCR amplification product(amplified by ITS1-ITS4) about 650 bp or not digested by restriction enzyme

***Hinf I*:** this restriction enzyme was used to digest PCR amplification product (ITS2 region) amplified by primer pair ITS3 (5' GCATCGATGAAGAACGCAGC 3')-ITS4 primers

Table 16: Presumed RFLP of the published ITS1 and ITS2 region from other *Ganoderma* species digested by the three restriction enzymes *SacI, MluI* and *HinfI*

Ganoderma species	Restriction enzymes used		
	SacI	*MluI*	*HinfI*
Oil palm *Ganoderma*	+	+	+
G. adspersum CBS 351.74	-	-	-
G. ahmadii FWP 14329	-	-	+
G. australe 07505	-	-	-
G. boninense RSH RS	-	-	+
G. colossum CBS 216.36	-	-	+
G. cupreum DFP 3896	-	-	-
G. formicatum RSH 0814	-	-	+
G. formosanum 0109	+	-	+
G. gibbosum ACCC 5.151	-	-	-
G. lipsiense BAFC 2424	-	-	-
G. lobatum BAFC 2411	+	-	-
G. lucidum ATCC 324.71	-	-	-
G. lucidum BAFC 2419	-	-	-
G. microsporum RSH 0821	-	-	+
G. oerstedii ATCC 52410	-	-	+
G. oregonense CBS 177.30	-	-	+
G. pfeifferi CBS 747.84	-	-	+
G. platense BAFC 384	-	-	+
G. resinaceum CBS 194.76	-	-	+
G. sessile BAFC 2373	-	-	+
G. sessiliforme BAFC 2580	-	-	+
G. sinense ZHANG 1734	-	-	+
G. subamboinense ATCC 52419	-	-	+
G. tornatum BAFC 1172	-	-	-
G. tornatum BAFC 1139	-	-	-
G. tropicum HK 93-8	-	-	-
G. tsugae CBS 428.84	-	-	+
G. tuberculosum BAFC 2414	-	-	+
G. valesiacum CBS 282.33	-	-	+
G. weberianum CBS 219.36	-	-	+
G. zonatum BAFC 2374	-	-	+

Hinf I: searching for restriction site was done only in the ITS2 region
Sac I, Mlu I: searching for restriction site was done in both ITS1 and ITS2 region
+: showed that the deduced digestion pattern was identical to oil palm *Ganoderma*
-: showed that no restriction site was found

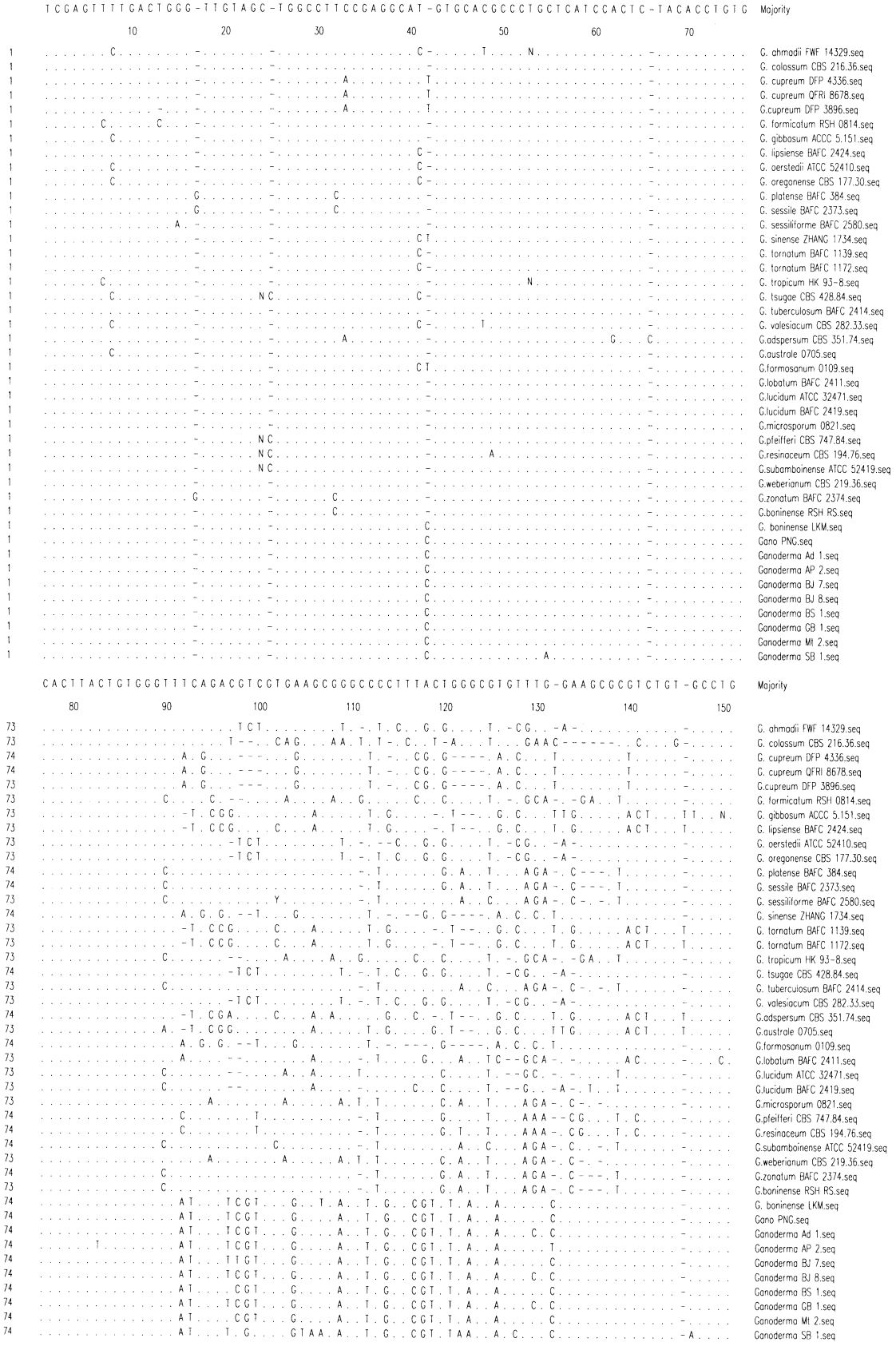
Based on RFLP analysis, *MluI* allowed a specific recognition of *Ganoderma* isolates that are associated with BSR disease in oil palm. PCR-amplified products of the ITS2 region from the oil palm *Ganoderma* isolates and all *G. boninense* isolates obtained from coconut stumps including several other *Ganoderma* species, when digested by *Hinf I*, produced

DNA fragment of about 60 bp, 160 bp and 170 bp in size whereas the rest of the *Ganoderma* species tested produced digestion products of about 60 bp and 330 bp. The results of digestion of the ITS-PCR products with *EcoRI, SacI, MluI* and *Hinf I* are summarised in Table 15.

A restriction site of *MluI* was not found in the ITS nucleotide sequence of 31 published *Ganoderma* species. However, a restriction site of *SacI* was found in the published ITS nucleotide sequence of *G. formosanum* 0109 and *G. lobatum* BAFC 2411. On the other hand, a restriction site of *HinfI* was found in many of 31 published *Ganoderma* species. Putative restriction sites of the three restriction enzymes *SacI, MluI* and *HinfI* for 31 published ITS sequences of *Ganoderma* species are summarised in Table 16. The location of the restriction sites of the three restriction enzymes *SacI, MluI* and *HinfI* within the ITS region of oil palm *Ganoderma* and other published *Ganoderma* sequences are shown in Fig. 18 and Fig. 19.

3.3.2 Species-specific primer design for the oil palm *Ganoderma* PCR amplification

Alignment of nucleotide sequences of the ITS1 and ITS2 regions of eight oil palm *Ganoderma* isolates with the corresponding regions of published sequence of other *Ganoderma* species showed that sequence variations are located in the central region within the ITS1 and close to the termini within the ITS2 (Fig. 18 and Fig. 19). In comparison with published ITS sequences of other *Ganoderma* species, there were variable sequences of 18-base-pair in the ITS1 region and two 18 base pairs in the ITS2 in the oil palm *Ganoderma.* These regions could be used to design species-specific primers for discriminating oil palm *Ganoderma* from other *Ganoderma* species by PCR amplifications. Species-specific primers for oil palm *Ganoderma* designed from the variable sequence within the ITS region are shown in Fig. 20. As forward primer, a variable sequence of ITS1 region was designed as IT1 (5'AGCTCGTTCGTTTGA CGA'3). For reverse primers, two ITS2 variable sequences were constructed as IT2 (5`TTGTCCCAATAACGGGAC`3) and IT3 (5`CGATCAATAAAAG ACCGA`3).



Decoration "Decoration #1": Hide (as '.') residues that match the Consensus exactly.

SacI

Continued

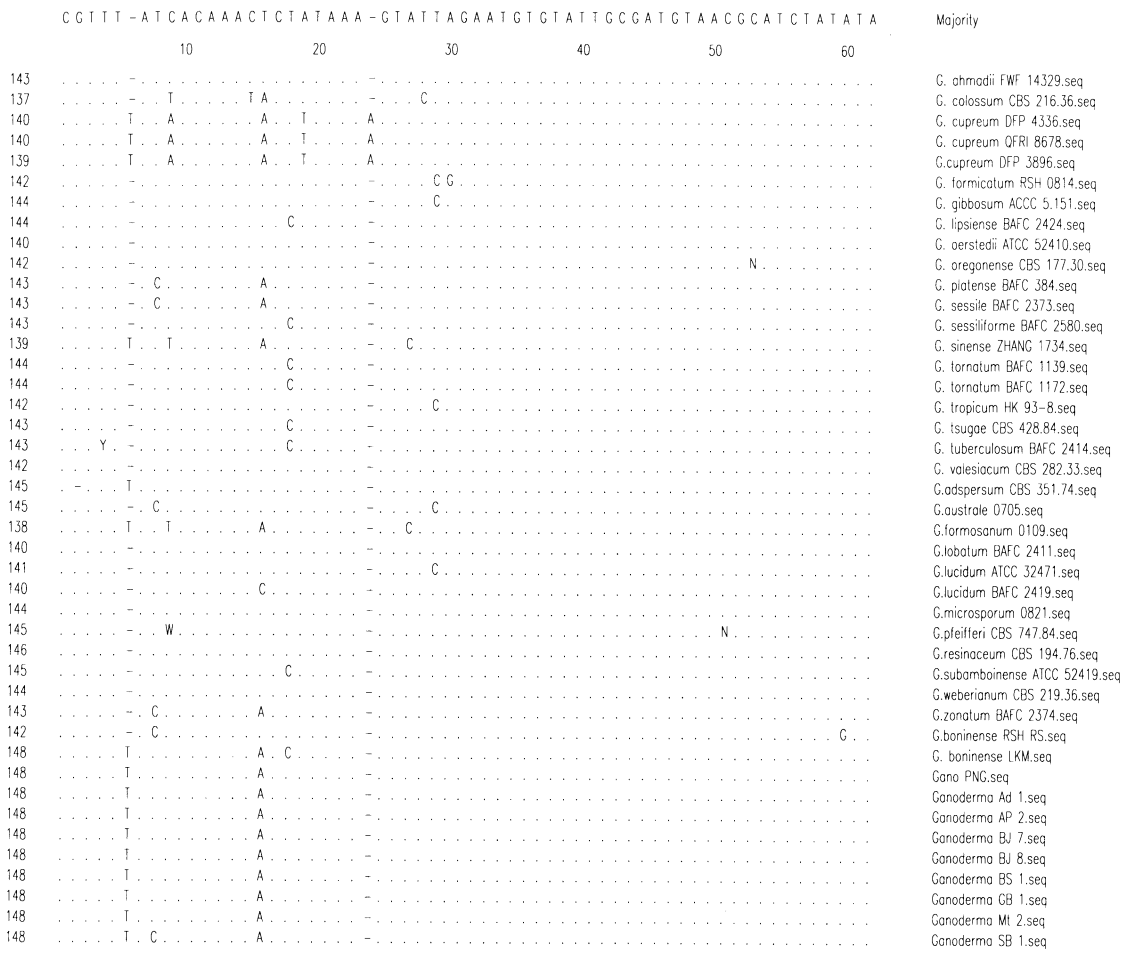
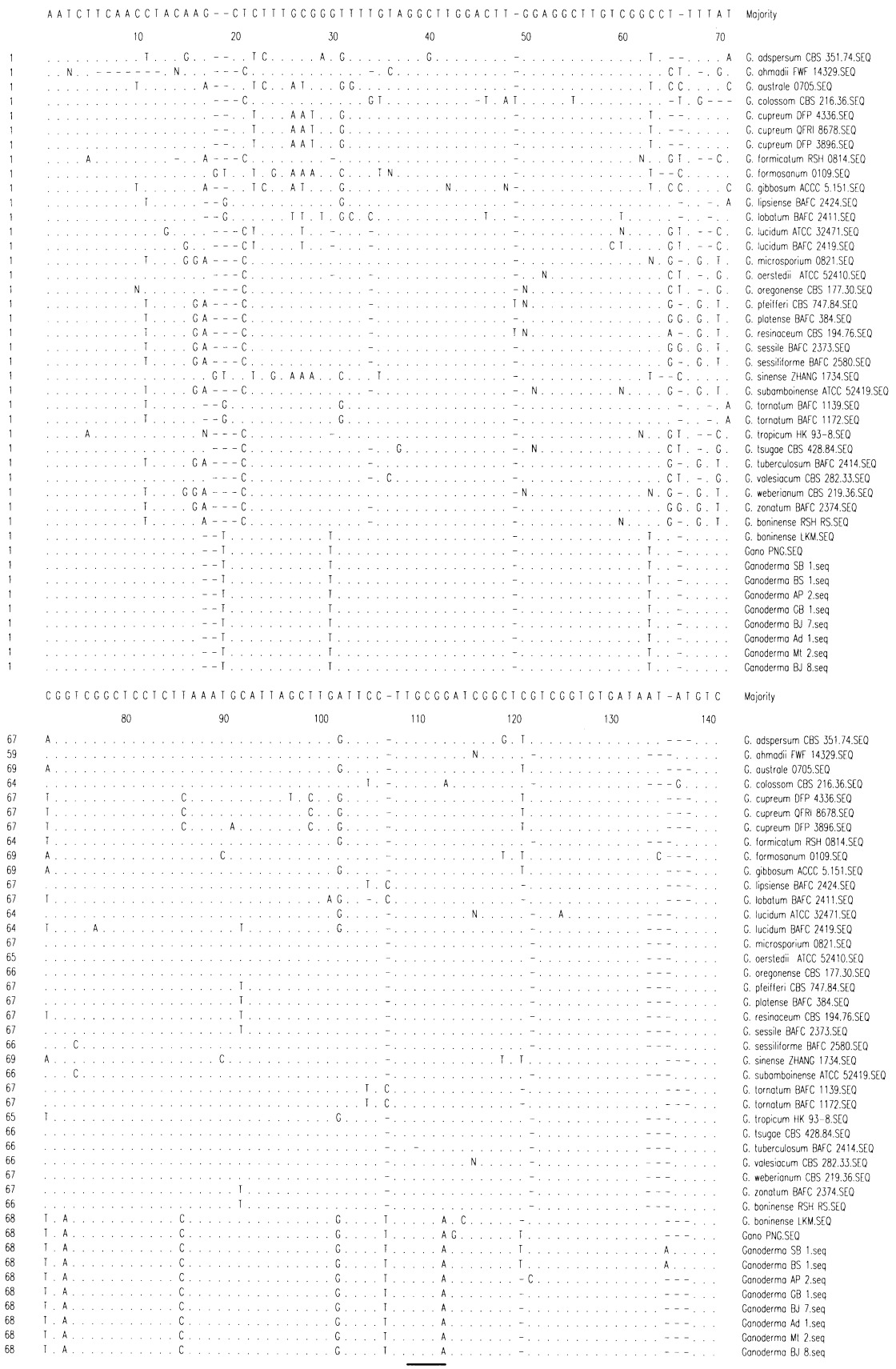


Fig. 18: Aligned sequences of the ITS1 region of rDNA from oil palm *Ganoderma* with other *Ganoderma* species. Ambiguities of bases within *Ganoderma* species are as follows: N = {A, C, T or G}, Y = {C, T} and W = {A, T}. Alignment gaps are indicated by dashes. The DNA sequence from left to right reads from 5‘ to 3‘ ends. The position of the restriction site of *SacI* within the ITS1 region of oil palm *Ganoderma* is underlined



Decoration "Decoration #1": Hide (as ".") residues that match the Consensus exactly.

Continued

HinfI

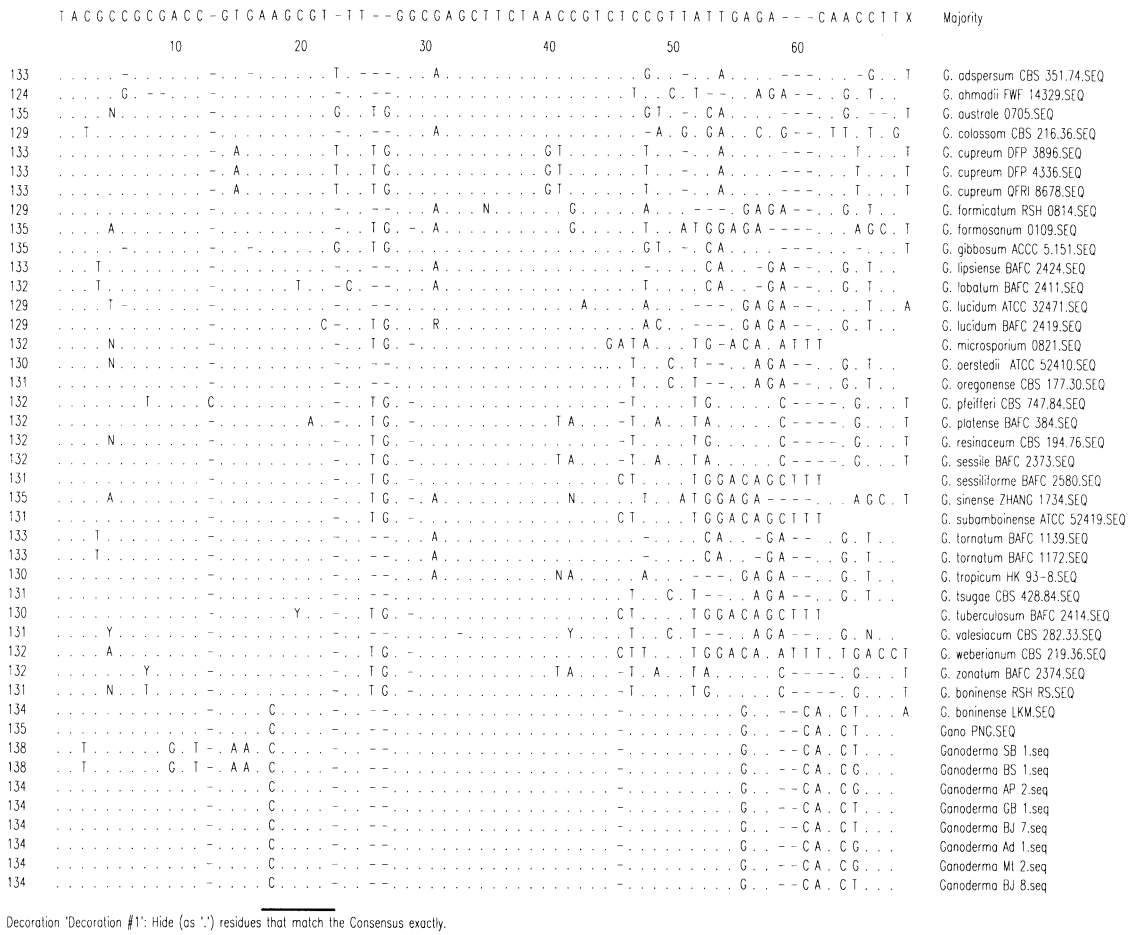


Fig. 19: Aligned sequences of the ITS2 region of rDNA from oil palm *Ganoderma* with other *Ganoderma* species. Ambiguities within *Ganoderma* species are as follows: N = {A, C, T or G} and Y = {C, T}. Alignment gaps are indicated by dashes. The DNA sequence from left to right reads from 5‘ to 3‘ ends. The positions of restriction sites of *Hinf I* and *Mlu I* within the ITS2 region of oil palm *Ganoderma* are underlined

```
      G G C T C G T T T A - T T C G G G C        Majority

                      10

1     A G C T C G T T C G T T T G A C G A        Ganoderma BS.SEQ
1     G . . C . . . . . A - . . C G G . C        G. adspersum CBS 351.74.SEQ
1     G . . . . . . . T A G . . C G G . C        G. australe 0705.SEQ
1     G . . . . . . . T A - . . C G G . C        G. gibbosum ACCC 5.151.SEQ
1     G . T C . C . . T A - - C C G G . C        G. lucidum ATCC 32471.SEQ
1     G . - C . C . . . A - - C C G G . C        G. lucidum BAFC 2419.SEQ

Decoration 'Decoration #1': Hide (as '.') residues that match Ganoderma BS.SEQ exactly..
```

Within ITS1

```
      G T C T C G T T A T A G A G A C A G        Majority

                      10

1     G T C C C G T T A T T G G G A C A A        Ganoderma BS.SEQ
1     . . . T . . . . . C A . A . . . G          G. australe 0705.SEQ
1     . . . T . . G . . . A . A . . . .          G. adspersum CBS 351.74.SEQ
1     . . . T . . . . . C A . A . . . G          G. gibbosum ACCC 5.151.SEQ
1     A . . T . A G . . - G . A . . . .          G. lucidum ATCC 32471.SEQ
1     . . . T . A C . T - G . A . . . G          G. lucidum BAFC 2419.SEQ

Decoration 'Decoration #1': Hide (as '.') residues that match Ganoderma BS.SEQ exactly..
```

Within ITS2

```
      T C G G T C G T T T T A C A G G T C G      Majority

                      10

1     T C G G T C T - T T T A T T G A T C G      Ganoderma BS.SEQ
1     . . . . . . - - . . . . A A . G . . .      G. adspersum CBS 351.74.SEQ
1     . . . . . . C C . . . . C A . G . . .      G. australe 0705.SEQ
1     . . . . . . C C . . . . C A . G . . .      G. gibbosum ACCC 5.151.SEQ
1     . N . . C . G T . C . - - C . G . . .      G. lucidum ATCC 32471.SEQ
1     C T . . C . G T . C . - - . . . . . .      G. lucidum BAFC 2419.SEQ

Decoration 'Decoration #1': Hide (as '.') residues that match Ganoderma BS.SEQ exactly..
```

Within ITS2

Fig.20: Variable regions in the ITS1 and ITS2 sequences of oil palm *Ganoderma* (isolate BS) aligned with other *Ganoderma* species to design specific primers for oil palm *Ganoderma*. Ambiguities of bases indicated as N = {A, C, T or G}

3.3.3 Specificity tests of the primer sets IT1-IT2 and IT1-IT3 for *Ganoderma*

The primer pairs IT1-IT2 and IT1-IT3 were used in the PCR to amplify ITS fragments from total genomic DNA extracted from a range of oil palm *Ganoderma* and other *Ganoderma* species tested. Each primer pair IT1-IT2 or IT1-IT3 generated a single PCR product of about 450 or 334 bp when genomic DNA of oil palm *Ganoderma* isolates and all *G. boninense* isolated from coconut stumps was used. No amplification of PCR products of both sizes using the primer pairs IT1-IT2 and IT1-IT3 was observed when genomic DNA from any other *Ganoderma* species studied was used (Fig. 21). With the primer pair ITS1-ITS4 as control PCR, all genomic DNA of *Ganoderma* species studied was amplified and produced a single PCR product of about 650 bp. The PCR amplification results of *Ganoderma* species using the primer pairs IT1-IT2, IT1-IT3 and ITS1-ITS4 are summarised in Table 17.

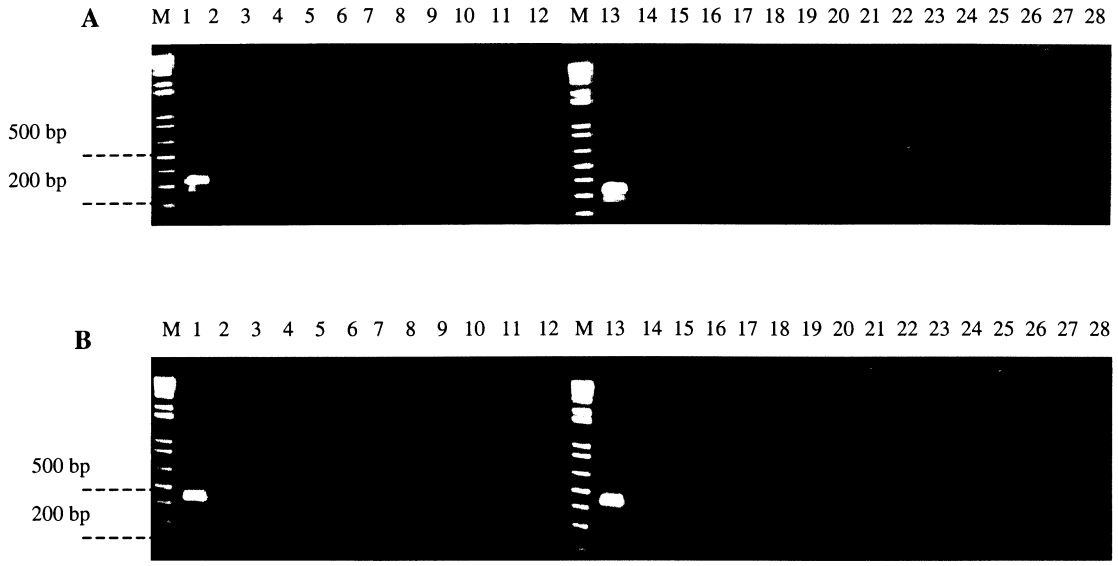


Fig. 21: Results of PCR amplification of the ITS region of *Ganoderma* species using the primer pairs IT1-IT2 and IT1-IT3

A, B: *Ganoderma* amplified by using primer pairs IT1-IT3 and IT1-IT2, Lanes 1-28: oil palm *Ganoderma* (SB), *Ganoderma* of BAFC2775, BAFC2501, *G. tsugae*, BAFC2495, BAFC2424, BAFC2288, DSMZ9612, BAFC2374, BAFC2430, BAFC2552, BAFC2580, oil palm *Ganoderma* (AP), BAFC218, BAFC671, BAFC2395, BAFC2529, BAFC178, DSMZ3800, BAFC2390, BAFC2353, BAFC2488, BAFC384, BAFC2408, G211, G224, G225 and 134. M: DNA marker

Table 17: PCR results obtained from the genomic DNA of *Ganoderma* species amplified by using the primer pairs IT1-IT2, IT1-IT3 and ITS1-ITS4 (as a control PCR)

Ganoderma species	Amplification of the ITS region by primer sets		
	IT1-IT2	IT1-IT3	ITS1-ITS4
Oil palm *Ganoderma* (Indonesia), 20 isolates	+++	+++	+++
G. boninense (oil palm, PNG), 10 isolates	+++	+++	+++
G. boninense (coconut stump, PNG), 7 isolates	+++	+++	+++
G. tornatum (hardwood, PNG), 11 isolates	-	-	+++
G. oerstedii BAFC 178	-	-	+++
G. oerstedii BAFC 218	-	-	+++
G. resinaceum BAFC 384	-	-	+++
G. tornatum (applanatum?) BAFC 671	-	-	+++
G. resinaceum BAFC 2288	-	-	+++
G. applanatum BAFC 2353	-	-	+++
G. lucidum complex BAFC 2374	-	-	+++
G. tornatum BAFC 2390	-	-	+++
G. tornatum? BAFC 2395	-	-	+++
G. applanatum BAFC 2408	-	-	+++
G. tornatum BAFC 2424	-	-	+++
G. tornatum? BAFC 2430	-	-	+++
G. resinaceum BAFC 2488	-	-	+++
G. lucidum complex BAFC 2495	-	-	+++
G. applanatum var. tornatum BAFC 2501	-	-	+++
Ganoderma sp. BAFC 2529	-	-	+++
G. applanatum BAFC 2552	-	-	+++
G. tropicum BAFC 2580	-	-	+++
G. resinaceum BAFC 2775	-	-	+++
G. lucidum DSM 9612	-	-	+++
G. applanatum DSMZ 3800	-	-	+++
G. tsugae	-	-	+++
G. applanatum 134	-	-	+++
G. applanatum G 211	-	-	+++
G. adspersum G 224	-	-	+++
G. pfeifferi G 225	-	-	+++
G. cupreum QFRI 8678.1	-	-	+++
G. australe DAR 73781	-	-	+++
G. incrassatum DAR 73783	-	-	+++
G. cupreum DFP 4336	-	-	+++
*Ganoderma sp.*Group 6.3 DAR 73779	-	-	+++
G. weberianum DFP 4483	-	-	+++

Primer pairs IT1-IT2 and IT1-IT3 produced a single PCR product of 450 and 344 bp for oil palm *Ganoderma* and *G. boninense* isolated from coconut stump. Primer pair ITS1-ITS4 as a control produced a single PCR amplification product about 650 bp for all *Ganoderma* species.

+++: Produced a strong band at the same size of PCR amplification product of the same column.

-: No PCR amplification product was observed.

3.3.4 Phylogenetic analysis of oil palm *Ganoderma* based on ITS1 and ITS2 regions

Nucleotide sequences of ITS1 and ITS2 of eight oil palm *Ganoderma* isolates were aligned with those of published *Ganoderma* species to infer phylogenetic trees. The resulting phylogenetic trees are shown in Fig. 22 and Fig. 23. Within the ITS1 and ITS2 sequences, eight oil palm *Ganoderma* isolates from Indonesia and one isolate from Papua New Guinea, designated as Gano PNG clustered within G. *boninense* LKM. *Fomitopsis rosea* T92-10 was used as an outgroup (non-*Ganoderma* species but basidiomycete fungus) for ITS sequence analysis to infer phylogenetic tree. The sequences of the ITS1 and ITS2 regions of eight oil palm *Ganoderma* had percent identities that ranged from 94.2 to 98.6 % and 90.7 to 99.5 % when compared with *G. boninense* LKM (Table 2A and Table 3A, see appendixes). All other *Ganoderma* species analysed within the ITS1 and ITS2 regions, when compared with eight oil palm *Ganoderma* isolates, showed identities ranging from 70.6 to 87.1 % and 57.2 to 83.0 %. The percent identities of the ITS1 and ITS2 sequences of *G. boninense* RSH RS, when compared to eight oil palm *Ganoderma*, ranged from 80.1 to 82.1 % and 61.6 to 73.2 %.

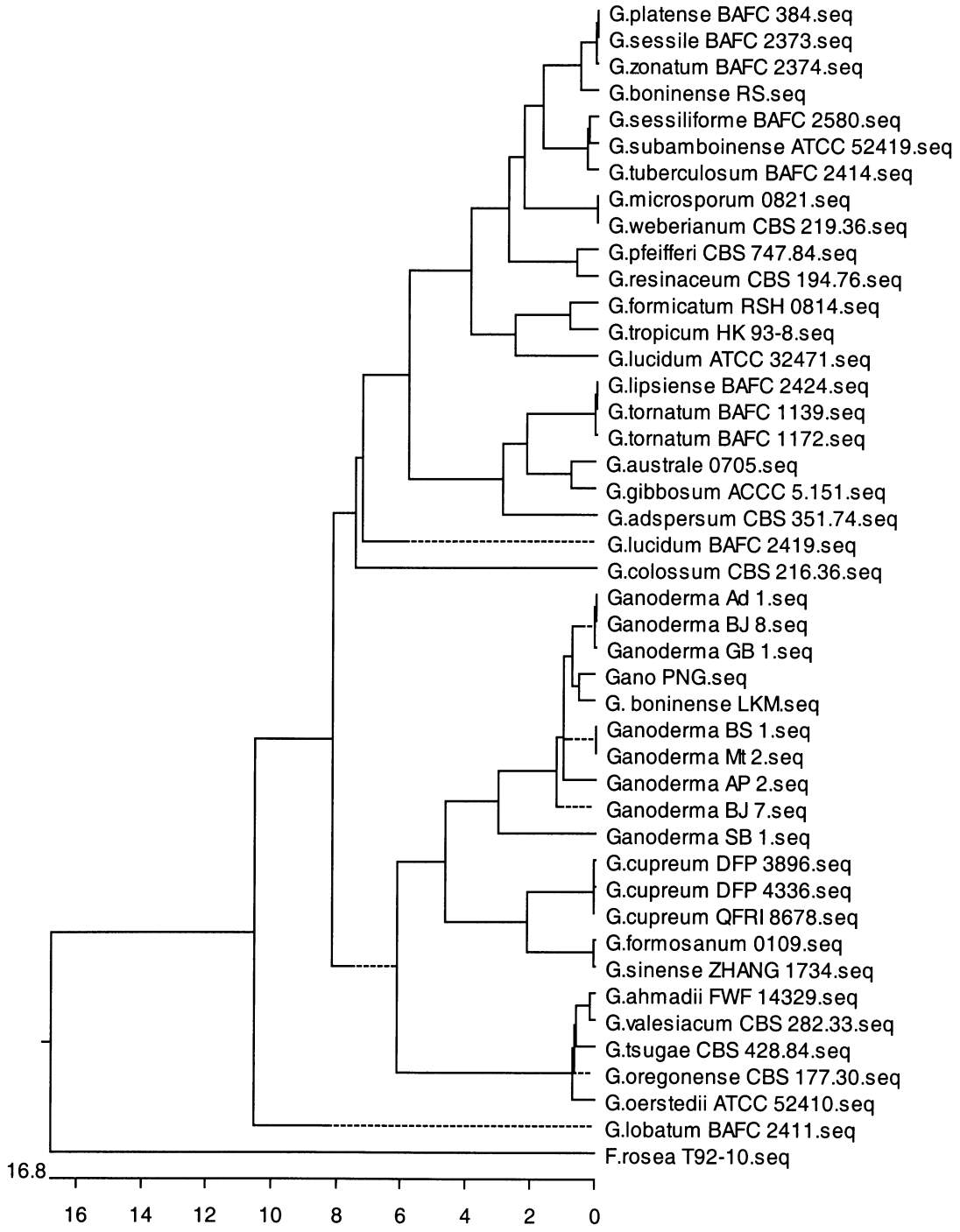


Fig. 22: Phylogenetic relationships of *Ganoderma* species inferred from the nucleotide sequences of the ITS 1

***Fomitopsis rosea* T92-10 was used as outgroup for phylogenetic analysis. Phylogenetic tree was generated using the Clustal method with weighted residue weight table. Multiple alignment parameters were: gap penalty = 10 and gap length penalty = 10. Pairwise alignment parameters were: ktuple = 2, gap penalty = 5, window = 4 and diagonals = 4. The length of each pair of branches represents the distance between sequence pairs. The units at the bottom of the tree indicate the number of substitution events.**

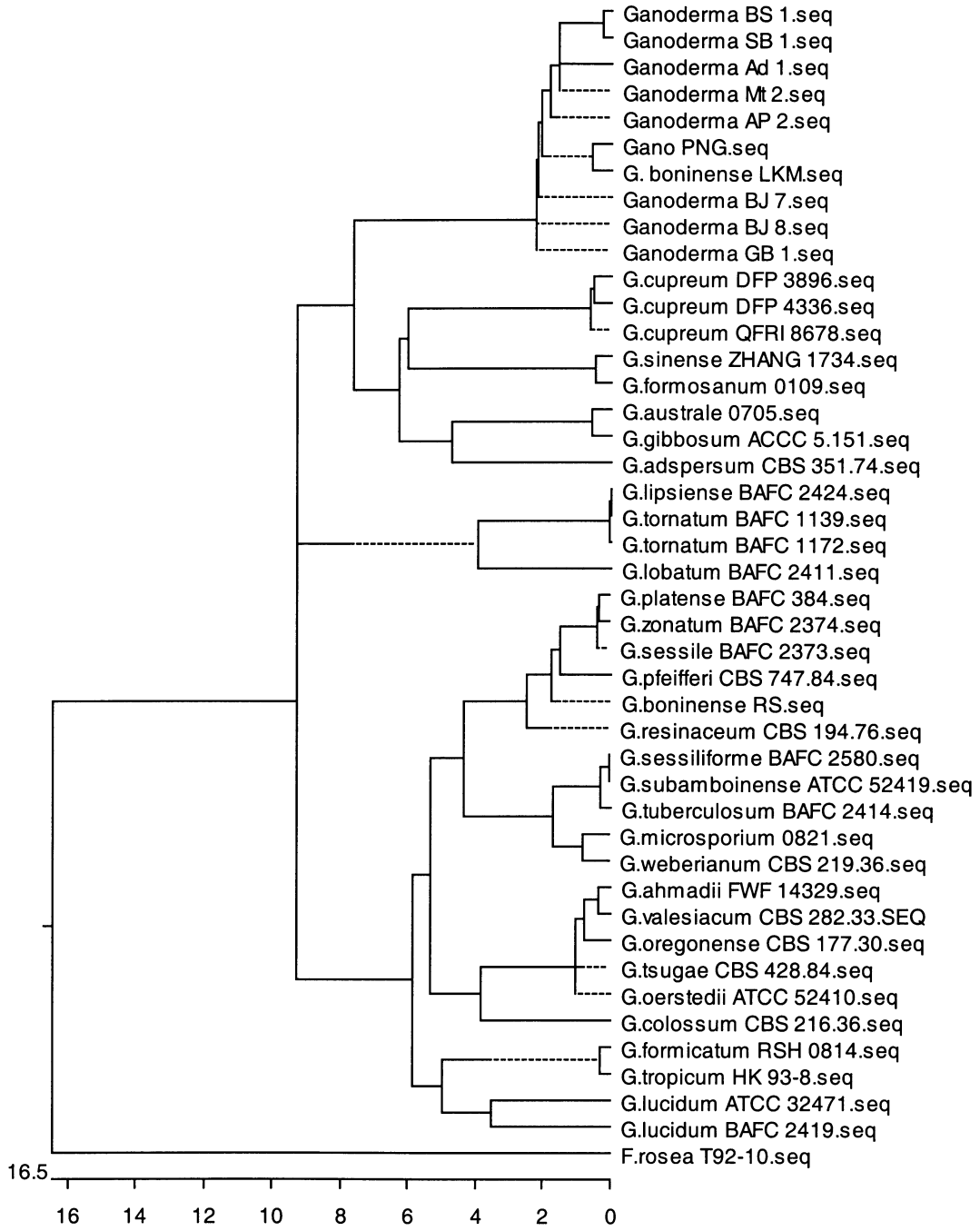


Fig. 23: Phylogenetic relationships of *Ganoderma* species inferred from the nucleotide sequences of the ITS 2

***Fomitopsis rosea* T92-10 was used as outgroup for phylogenetic analysis. Phylogenetic tree was generated using the Clustal method with weighted residue weight table. Multiple alignment parameters were: gap penalty = 10 and gap length penalty = 10. Pairwise alignment parameters were: ktuple = 2, gap penalty = 5, window = 4 and diagonals = 4. The length of each pair of branches represents the distance between sequence pairs. The units at the bottom of the tree indicate the number of substitution events.**

3.4 Identification of oil palm *Ganoderma* based on the Mn-SOD gene sequence

3.4.1 Primer design and PCR amplification

Primer Mn-SOD 1 (5‘ CTCCACCACAAGAAGCACCAC‘3) and Mn-SOD 2 (5‘GAA GGCGTGCTCCCAGATGTC‘3) were designed from nucleotide sequences of amino acid sequence regions LHHKKHH and DIWEHAF in the Mn-SOD gene of *G. boninense* RSH RS. Partial amino acid sequences of the Mn-SOD gene of *G. boninense* RSH RS and primers design are shown in Fig. 24.

A. Amino acid sequence of the Mn-SOD gene within *G. boninense* RSH RS

1. L H H K K H H (Designed for forward primer)
2. D I W E H A F (Designed for reverse primer)

B. Nucleotide sequence in *G. boninense* RSH RS

1. 5‘CTCCACCACAAGAAGCACCAC 3‘
2. 5‘GACATCTGGGAGCACGCCTTC 3‘

C. PCR primers

1. Primer Mn-SOD 1: 5‘CTCCACCACA AGAAGCACCAC 3‘
2. Primer Mn-SOD 2: 5‘GAAGGCGTGCTCCCAGATGTC 3‘

Fig. 24: Design of PCR primers to isolate the Mn-SOD gene of oil palm *Ganoderma*

A. Partial amino acid sequence of Mn-SOD gene of *G. boninense* RSH RS

B. Nucleotide equences of *G. boninense* RSH RS corresponding to amino acid sequences of LHHKKHH and DIWEHAF

C. PCR primers were designed from the nucleotide sequence in panel B

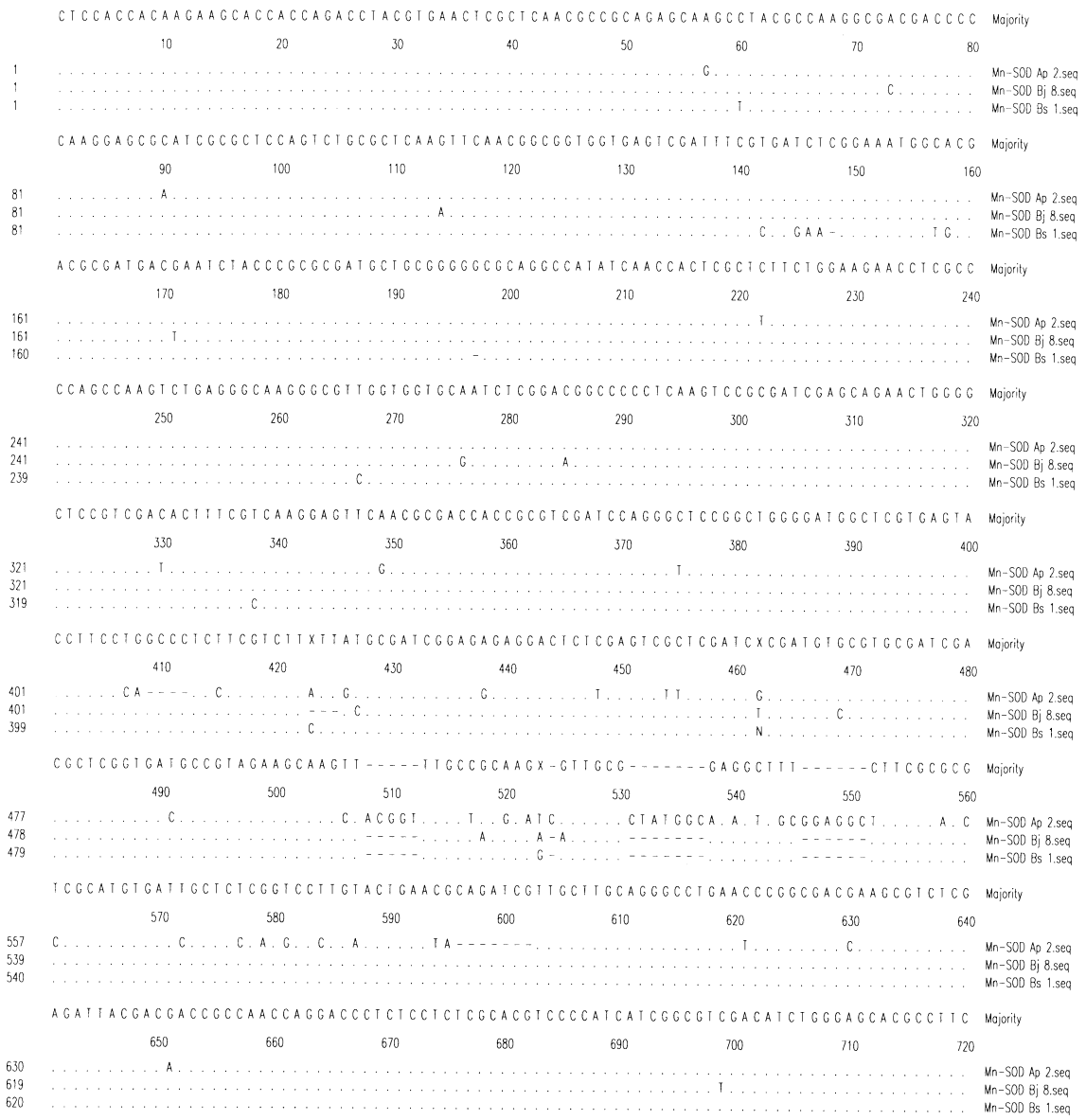


Fig. 25: Nucleotide sequence alignment of the partial Mn-SOD gene from 3 oil palm *Ganoderma* (isolates AP, BJ 8 and BS). Dashes (-) indicate gaps and X indicates a base that could not be determined

PCR amplification of the genomic DNA of three oil palm *Ganoderma* isolates using the primer pair Mn-SOD1 and Mn-SOD2 produced a single PCR product of about 700 bp. The sequence shows that the size of the nucleotide sequences of the Mn-SOD gene from three oil palm *Ganoderma* isolates varied from 698 to 709 bp. The nucleotide sequence alignment of the Mn-SOD genes of the three oil palm *Ganoderma* isolates indicated sequence variations in two locations, due to the presence of introns. This partial Mn-SOD gene contains two introns, the first deduced intron started from the alignment number 128

to 200 and the second deduced intron started from the alignment number 394 to 598 as shown in Fig. 25. The first intron showed more conserved and shorter nucleotide sequences compared to the second intron.

Deduced exons (amino acid sequence) and introns and of partial Mn-SOD gene of oil palm *Ganoderma* (isolate BS)

The deduced intron sequences were based on the comparison of amino acid sequences of the Mn-SOD gene of oil palm *Ganoderma* (isolate BS) with published Mn-SOD amino acid sequences from other *Ganoderma* species (see Table 4 in materials and methods) and the consensus sequences for 5' splicing GT(AG) (AT)GT and 3' splicing (CT)AG junctions of filamentous fungi (Ballance, 1986; Hahn et al., 1997). For a better visualisation, the deduced exon and intron sequences are presented as capital and lowercase characters, respectively, as shown in Fig. 26. The deduced exon sequences were translated to the predicted amino acid sequences by using EditSeq program (DNAstar, Madison, USA).

```
CTCCACCACAAGAAGCACCACCAGACCTACGTGAACTCGCTCAACGCCGCAGAGCAAGCTTACGCCAAG   69
L  H  H  K  K  H  H  Q  T  Y  V  N  S  L  N  A  A  E  Q  A  Y  A  K

GCGACGACCCCCAAGGAGCGCATCGCGCTCCAGTCTGCGCTCAAGTTCAACGGCGGTGgtgagtcgatttcgcg  144
A  T  T  P  K  E  R  I  A  L  Q  S  A  L  K  F  N  G  G  G

AgaaggaaatggtgcgacgcgatgacgaatctacccgcgcgatgctgcggggcgcagGCCATATCAACCACTCGCTCTTCTGGAA  231
                                                          H  I  N  H  S  L  F  W  K

GAACCTCGCCCCAGCCAAGTCTGAGGGCAAGGGCGTCGGTGGTGCAATCTCGGACGGCCCCCTCAAGT  299
 N  L  A  P  A  K  S  E  G  K  G  V  G  G  A  I  S  D  G  P  L  K  S

CCGCGATCGAGCAGAACTGGGGCTCCGTCGACACTTTCGCCAAGGAGTTCAACGCGACCACCGCGTCG  367
  A  I  E  Q  N  W  G  S  V  D  T  F  A  K  E  F  N  A  T  T  A  S

ATCCAGGGCTCCGGCTGGGGATGGCTCgtgagtaccttcctggccctcttcgtcttcttatgcgatcggagagaggactctcgagtcgctc  458
I  Q  G  S  G  W  G  W  L

gatcncgatgtgcgtgcgatcgacgctcggtgatgccgtagaagcaagttttgccgcaagggttgcggaggctttcttcgcgcgtcgcatgtgattgctctcgg  562

tccttgtactgaacgcagatcgttgcttgcagGGCCTGAACCCGGCGACGAAGCGTCTCGAGATTACGACGACCGCCAAC  642
                              G  L  N  P  A  T  K  R  L  E  I  T  T  T  A  N

CAGGACCCTCTCCTCTCGCACGTCCCCATCATCGGCGTCGACATCTGGGAGCACGCCTTC                699
Q  D  P  L  L  S  H  V  P  I  I  G  V  D  I  W  E  H  A  F
```

Fig. 26: Partial nucleotide sequence of the Mn-SOD gene of oil palm *Ganoderma* (isolate BS). The deduced amino acid sequences are presented as bold characters. The deduced intron sequences are presented as lowercase characters

3.4.2 Phylogenetic analysis of oil palm *Ganoderma*

Nucleotide sequences of the partial Mn-SOD gene of the oil palm *Ganoderma* isolates AP, BJ 8 and BS were compared with those from other published *Ganoderma* species. For phylogenetic analysis, *G. boninense* LKM was used as a reference and *Amauroderma rude* JMM ASP.1 (accession number U56109) was used as an outgroup (non-*Ganoderma* species but basidiomycete fungus). The size of the nucleotide sequence of the partial Mn-SOD gene of *G. boninense* LKM was only 283 bp after eliminating the first and the second intron. These introns were excluded from the analysis because nucleotide sequences could not be unambiguously aligned across all *Ganoderma* species tested. The variations in the sequences ranged from a single base pair change to multiple changes representing transition and transversion. However, no deletion and insertion were observed (Fig. 28). Multiple sequence alignment was used to infer a phylogenetic tree and the generated tree showed that all oil palm *Ganoderma* isolates clustered with G. *boninense* LKM (Fig. 27). Identities of the partial Mn-SOD gene within three isolates of oil palm *Ganoderma* ranging from 95.8 to 96.8 %. When compared with *G. boninense* LKM, identities of three oil palm *Ganoderma* isolates ranged from 96.5 to 98.2 %. All other *Ganoderma* species studied showed identities ranged from 84.2 to 90.2 % when compared with the three oil palm *Ganoderma* isolates. Identities of the partial sequence of Mn-SOD gene of *G. boninense* RSH RS, when compared with the three oil palm *Ganoderma* species, ranged from 86.3 to 87.0 % (Table 4A, see Appendixes).

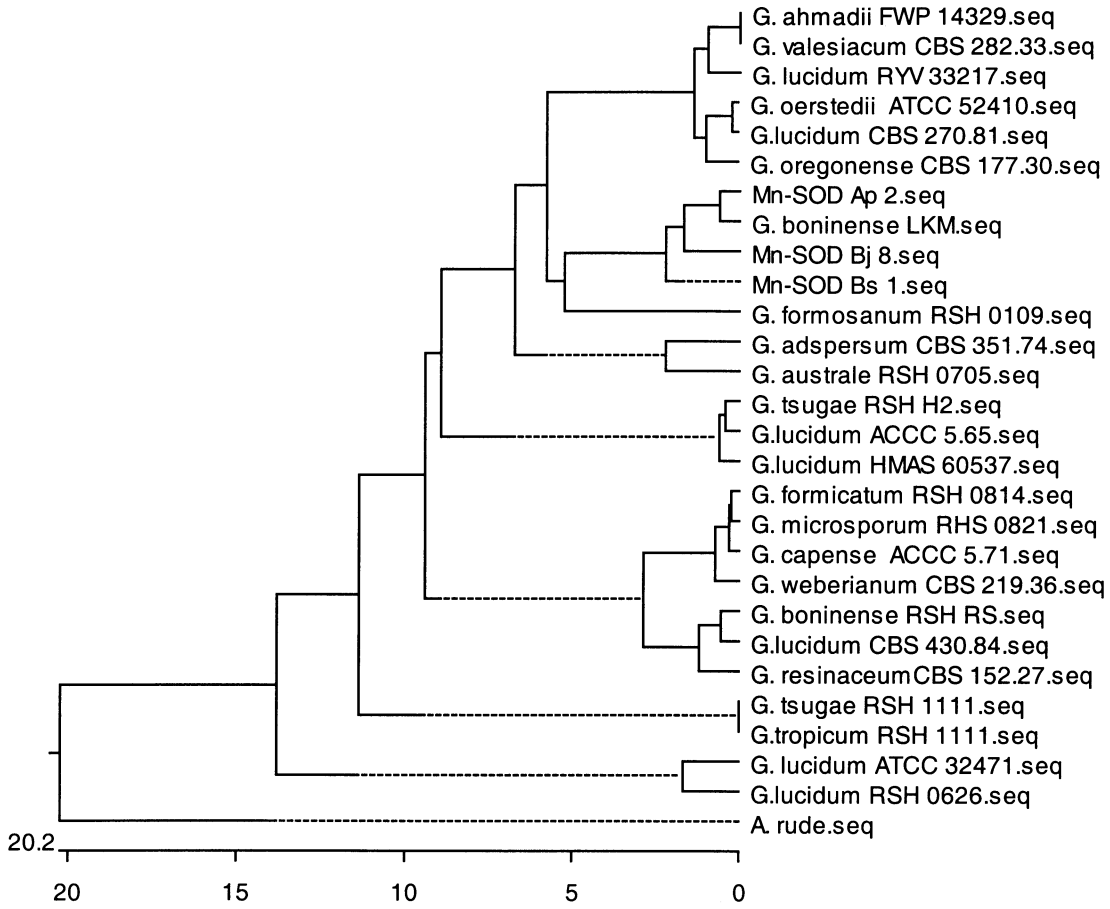
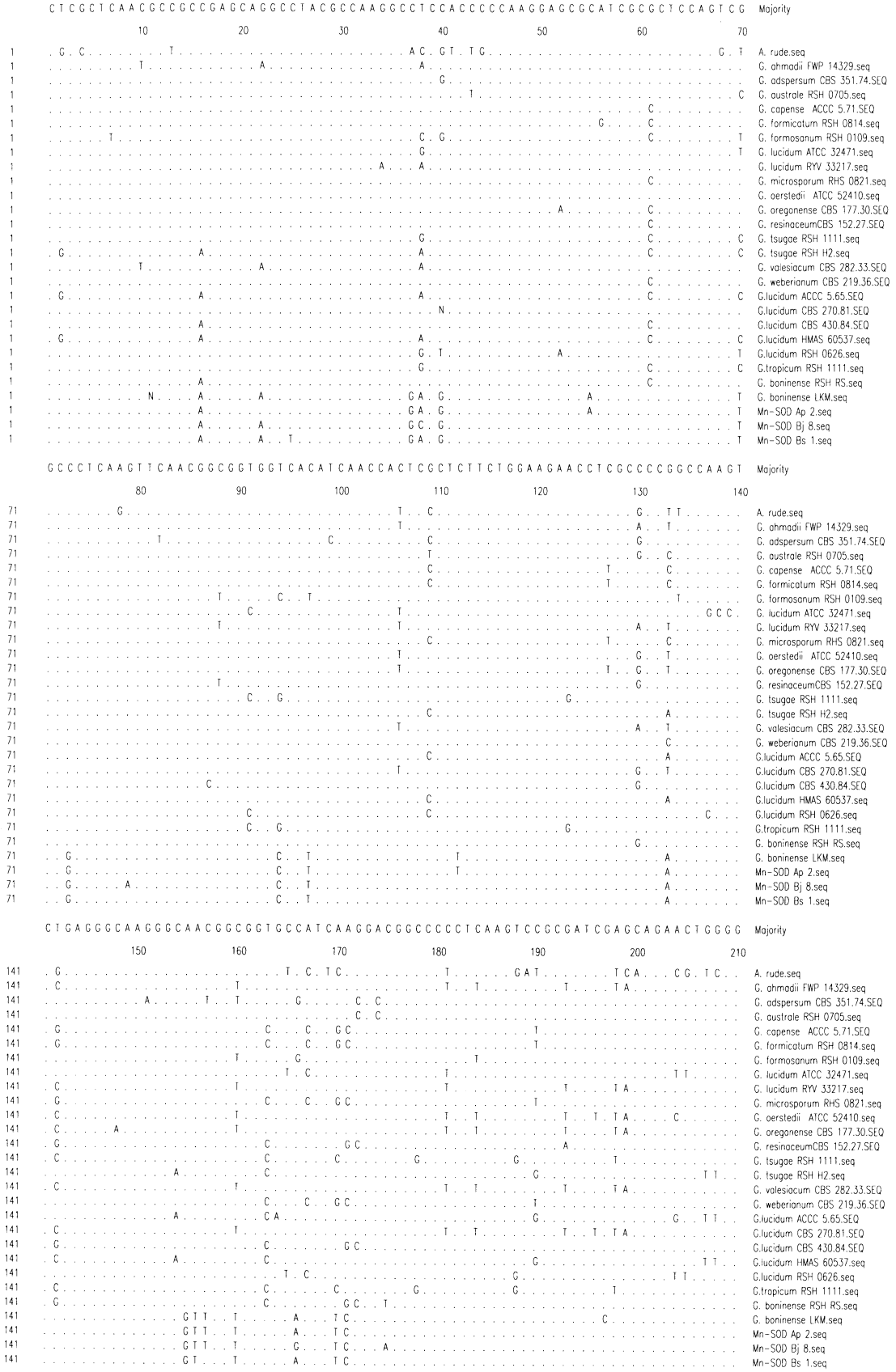


Fig. 27: Phylogenetic relationships of *Ganoderma* species inferred from the nucleotide sequences of the Mn-SOD genes.

Amauroderma rude **was used as an outgroup for phylogenetic analysis. A phylogenetic tree was generated using the Clustal method with weighted residue weight table. Multiple alignment parameters were: gap penalty = 10 and gap length penalty = 10. Pairwise alignment parameters were: ktuple = 2, gap penalty = 5, window = 4 and diagonals = 4. The length of each pair of branches represents the distance between sequence pairs. The units at the bottom of the tree indicate the number of substitution events.**



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

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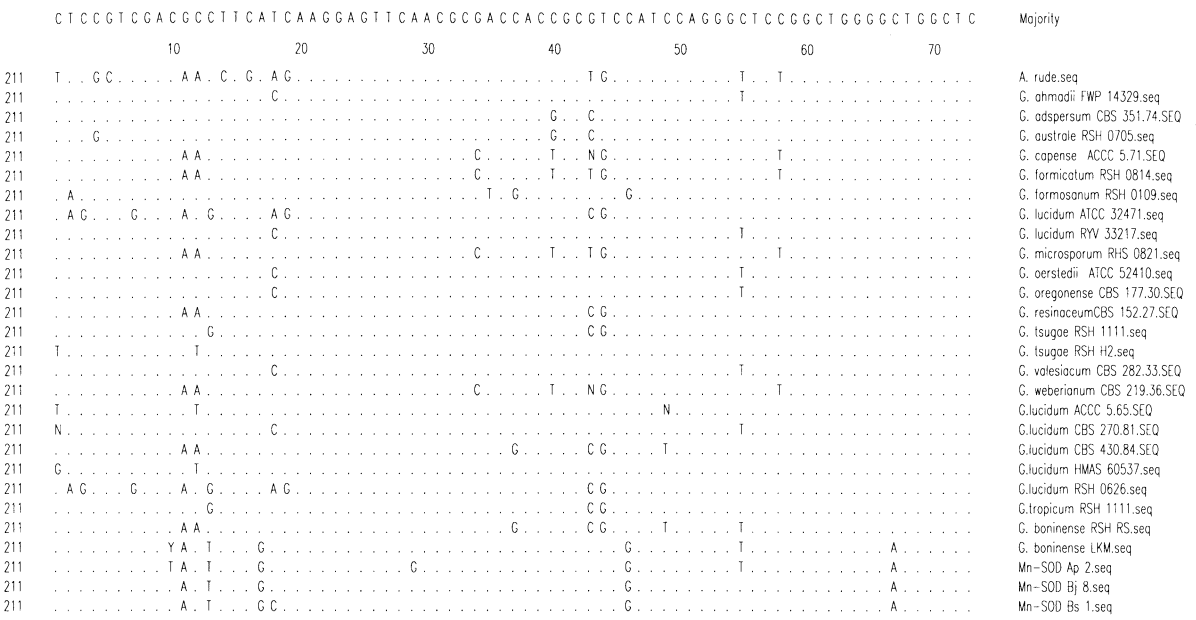


Fig. 28: Nucleotide sequence alignment of the partial Mn-SOD gene of *Ganoderma* species. Introns in all these *Ganoderma* species were characterised and excluded prior to alignment. Ambiguities within *Ganoderma* species are as follow: N = {A, C, T or G} and Y = {C, T}. The DNA sequence from left to right reads from 5'to 3' ends.

3.5 Identification and differentiation of oil palm *Ganoderma* based on the IGS1 region

3.5.1 IGS1 sequence and the IGS1-RFLP analysis

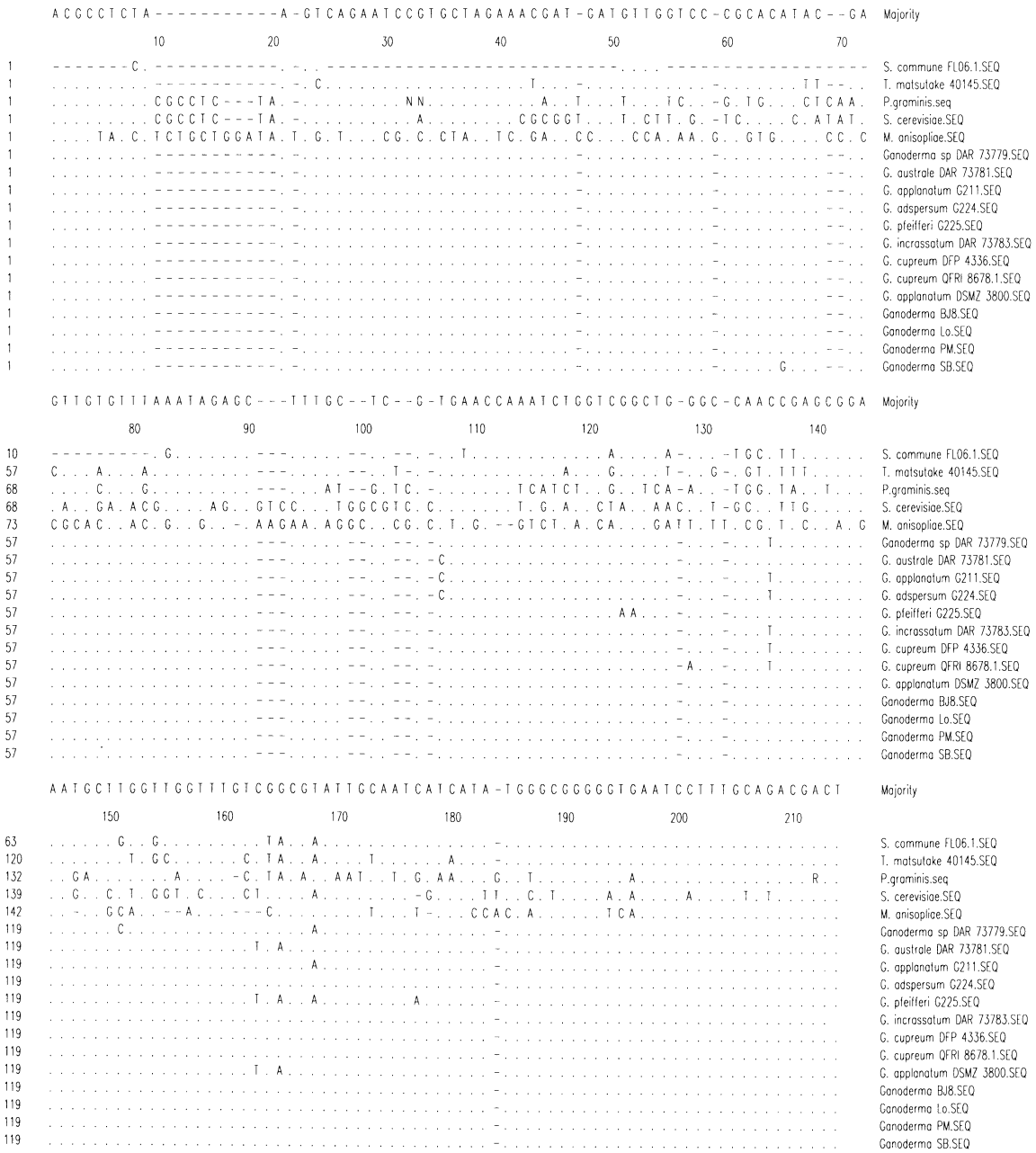
3.5.1.1 Determination of the IGS1 region of 13 *Ganoderma* isolates

Amplifications of IGS1 from the genomic DNA of 13 *Ganoderma* isolates using the primer Q and 5SA produced a single PCR product of about 1,100 bp. This PCR amplified product corresponded to the large subunit (LS) rDNA, IGS1 and 5S rDNA regions. The sequences of the LS rDNA of 13 *Ganoderma* isolates were determined from the primer Q to the 3'end of this gene by alignment with published genes from *S. cerevisiae* CEN.PK2, *M. anisopliae* 5530, *P. graminis* 5297, *T. matsutake* 40145 and *S. commune* FL06.1. The last 7 bases at the 3'end of the aligned gene were found to be GATTTGT, except for *M. anisopliae* that was GATTTCC. The size of the partial LS rDNA of 13 *Ganoderma* isolates was identical (271 bp) and thus highly conversed. However, sequence variability within oil palm *Ganoderma* was only a single base pair. Between the oil palm *Ganoderma* isolates and *Ganoderma* isolated from other hosts, sequence variability ranging from 1 to 6 base pairs was recorded (Fig. 29). To determine the 5S rDNA of 13 *Ganoderma* isolates, six sequences of the first 21 bases at the 5'end of 5S rDNA from other fungi such as *S. commune* 5334, *L. edodes* 5353, *L. bicolor* S238N, *T. matsutake* 40145, *P. ostreatus* 5322, *C. cinereus* 5346 and *G. applanatum* FP-125024-T were aligned with 13 *Ganoderma* isolates. The first 21 bases at the 5'end of 5S rDNA of these sequences were highly conserved among these basidiomycetes, except for *G. applanatum* FP-125024-T, which was different in one base pair near at the end of the first 21 bases as shown in Fig. 30. The primer 5SA anneals at the fourth position of the beginning of the 5S rDNA of 13 *Ganoderma* isolates.

3.5.1.2 Nucleotide sequences of the IGS1 region from 13 *Ganoderma* isolates.

After obtaining the partial sequence data of all surveyed 13 *Ganoderma* isolates, an alignment was made to find a conserved sequence among all 13 *Ganoderma* isolates. A conserved sequence was found and an internal sequencing primer was designed as primer Ut (5'CATTCTCTTCCCCGTCTT 3'). This primer worked well to sequence the complete IGS1 region of 13 *Ganoderma* isolates. The size of the IGS1 region of 13 *Ganoderma* isolates ranged from 791 to 835 bp (Table 18). However, within the oil palm *Ganoderma*, this region had a size of 791 to 802 bp. All sequences obtained are aligned in 885 positions

(Fig. 31). Sequence variations were scattered within the IGS1 region and were preferentially located in the centre or near to the termini region of the IGS1 sequences. Mostly there were sequence changes (substitutions) in all 13 *Ganoderma* species. Single base pair changes were unique to particular *Ganoderma* species. Several sites were ambiguous for aligning (represented as gaps) in some *Ganoderma* species, due to short nucleotide insertions or deletions.



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

Continued

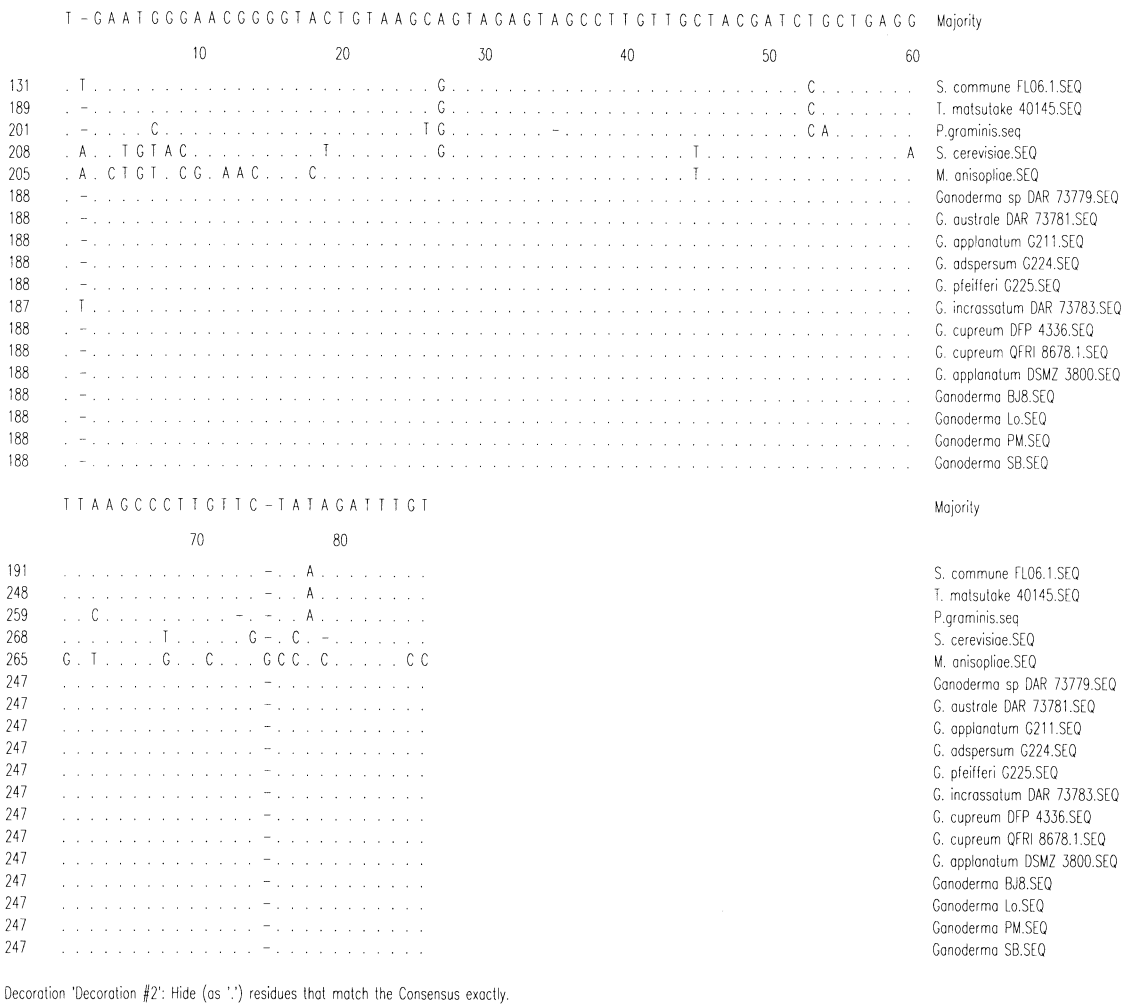


Fig. 29: Determination of the 3‘ end of the LS rDNA of 13 *Ganoderma* isolates
The sequences of the LS rDNA of 13 *Ganoderma* isolates from primer Q to the 3‘end of the LS rDNA were aligned with the published genes from *S. cerevisiae* CEN.PK2, *M. anisopliae* 5530, *P. graminis* 5297, *T. matsutake* 40145 and *S. commune* FL06.1 for determining the 3‘ end of the LS rDNA. Dashes (-) indicates gaps

Table 18: The length of intergenic spacer (IGS1) sequence of 13 *Ganoderma* isolates

Ganoderma species	Code of isolates	Length of IGS1 region (bp)
G. adspersum	G 224	817
G. applanatum	DSM 3800	796
G. applanatum	G 211	811
G. australe	DAR 73781	816
G. cupreum	DFP 4336	792
G. cupreum	QFRI 8678	793
G. incrassatum	DAR 73783	835
G. pfeifferi	G 225	799
Ganoderma sp	DAR 73779	816
Ganoderma (oil palm)	BJ 8	802
Ganoderma (oil palm)	Lo	792
Ganoderma (oil palm)	PM	791
Ganoderma (oil palm)	SB	800

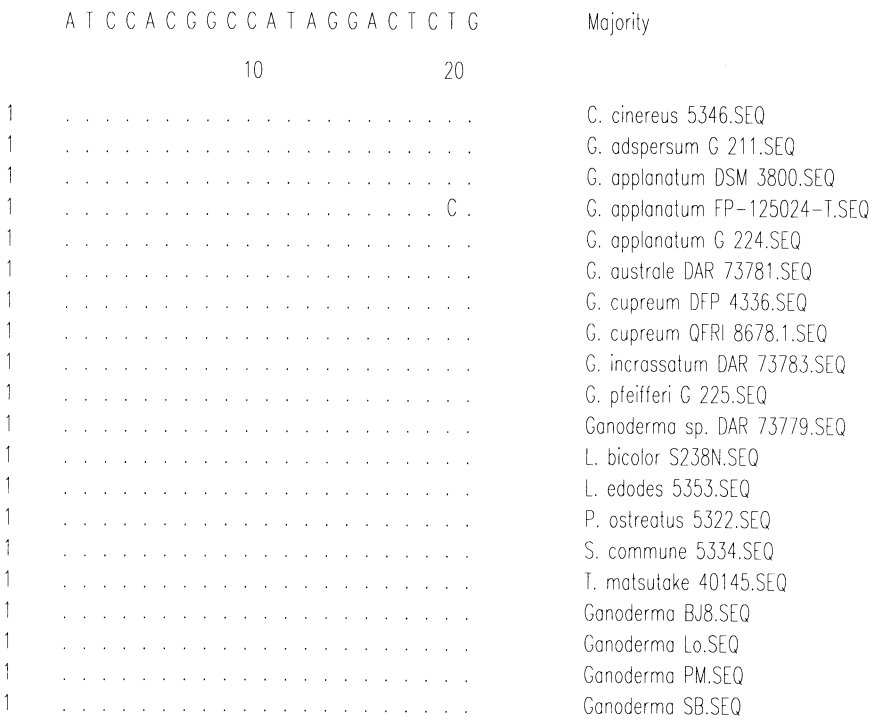
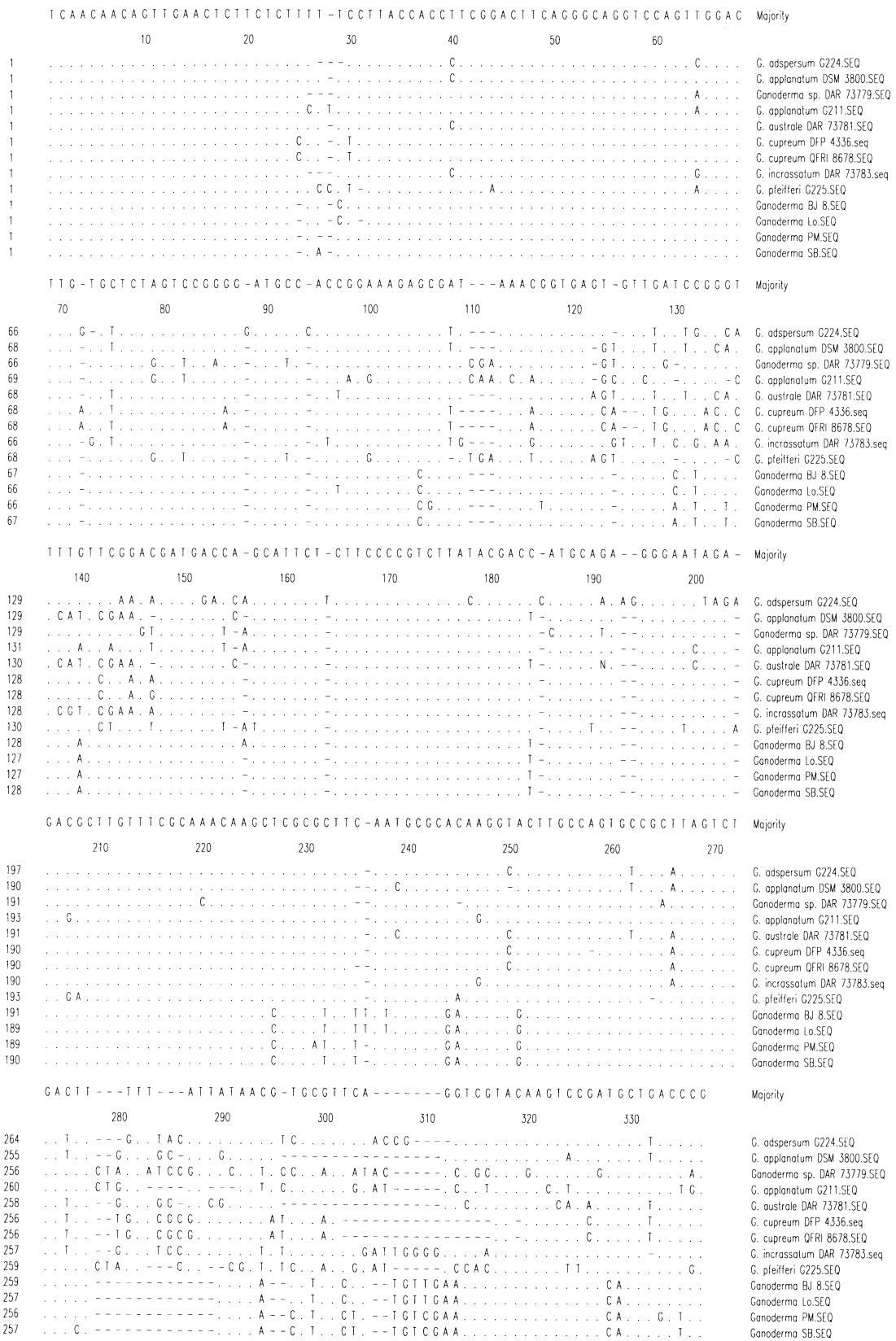


Fig. 30: Determination of the 5'end of the 5S rDNA of 13 *Ganoderma* isolates. The sequences of the first 21 bases of 5S rDNA of the published basidiomycetes were used and aligned to corresponding region with the 13 *Ganoderma* isolates studied for determining the 5'end of the 5S rDNA



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

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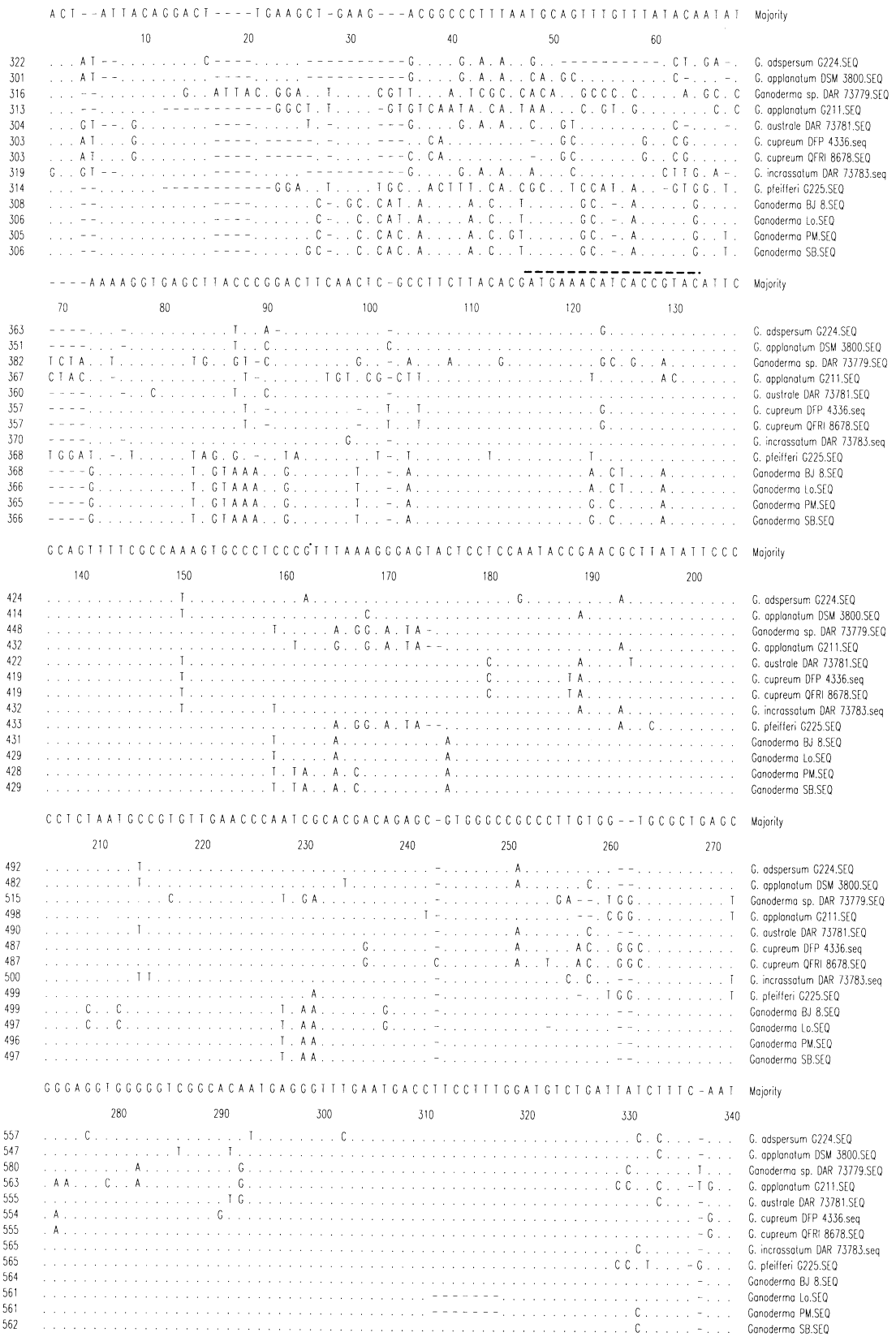

Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

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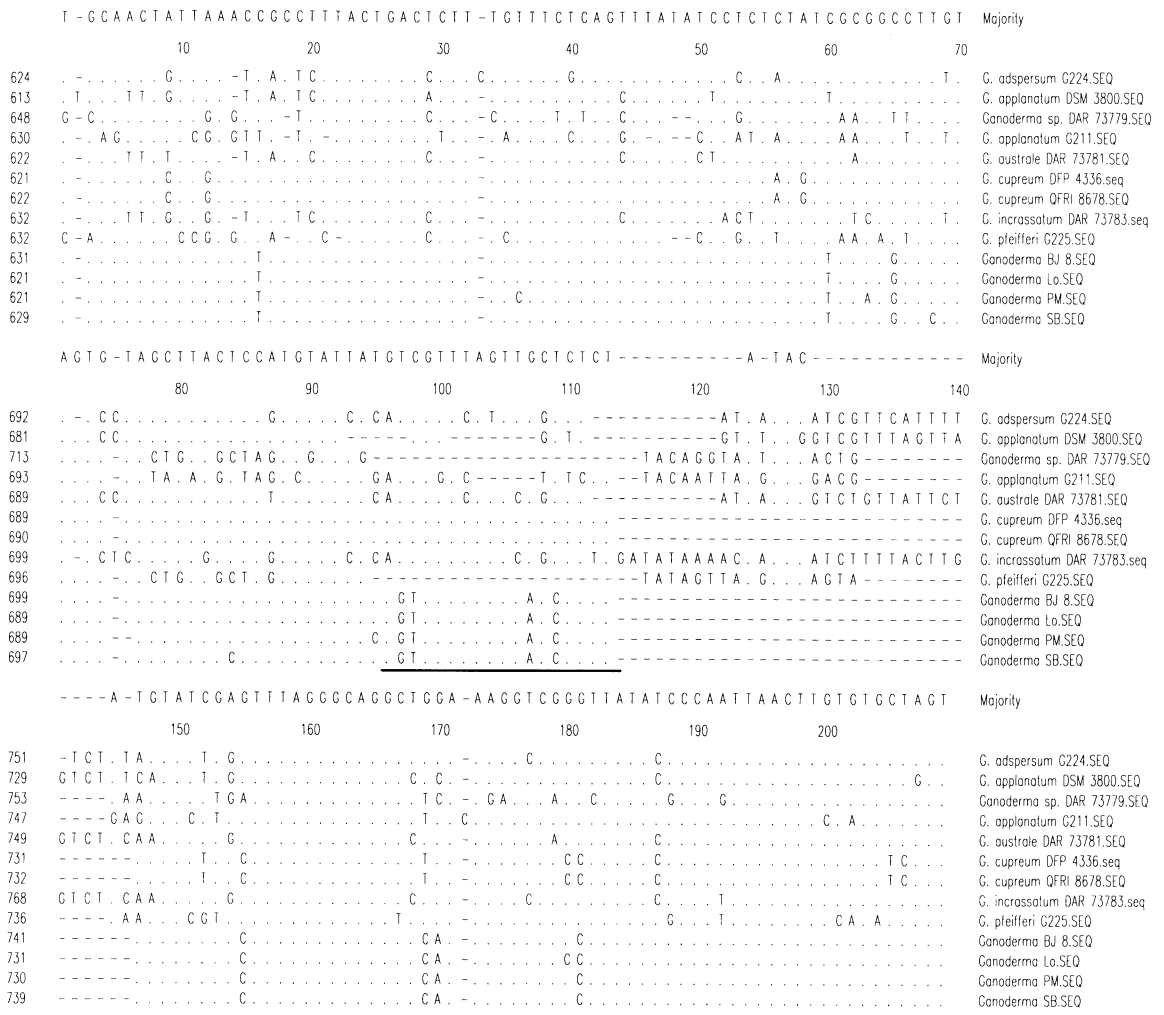

Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

Fig.31: Multiple nucleotide sequence alignments of the complete IGS1 region of 13 *Ganoderma* isolates. Dashes (-) indicates gaps and N={A, C, T or G}. The DNA sequence from left to right reads from 5'to 3'ends. Line (-----) is the annealing position of the primer IGSa and line (—) is the annealing position of the primer IGSc

3.5.2 IGS1-RFLP analyses for oil palm *Ganoderma*

The three restriction enzymes *Hinc II*, *Sca I* and *Tfi I* were selected by using MapDraw program analysis (DNASTAR, USA) to characterise oil palm *Ganoderma* isolates. Depending on each restriction enzyme, two to four distinct restriction patterns were obtained when the IGS1-*Ganoderma* species tested were digested. *Hinc II* digested the IGS1 region of *Ganoderma* species into three digestion patterns: digestion pattern type 1 with DNA fragments of about 1,100, 707 and 393 bp, represented by oil palm *Ganoderma* and *G. boninense* isolated from coconut stumps (Fig. 32A); digestion pattern type 2 with DNA fragments of about 1,100, 960 and 140 bp represented by *G. tornatum* BAFC 2390 (Fig. 32A), and digestion pattern type 3 with DNA fragments of about 1,100, 936, 700, 390, 230, 210, 137 and 98 bp, represented by *G. lucidum* complex BAFC 2374 (Fig. 32A). *Sca* I digested the IGS1 region of *Ganoderma* species into two digestion patterns: digestion pattern type 1 with DNA fragments of about 1,100, 700, 370, 350 and 230 bp, represented by *G. tornatum* PNG-371 (Fig. 32 B) and digestion pattern type 2 with DNA fragments of about 1,100, 700, 350 and 50 bp, represented by, *G. applanatum* DSM 3800 (Fig. 32B). On the other hand, *Sca I* did not digest oil palm *Ganoderma* and *G. boninense* isolated from coconut stumps. *Tfi I* digested the IGS1 region of *Ganoderma* species studied into four digestion patterns: digestion pattern type 1 with DNA fragments of about 950, 540, 400 and 150 bp, represented by oil palm *Ganoderma* and *G. boninense* isolated from coconut stumps; digestion pattern type 2 with DNA fragments of about 950 and 150 bp, represented by *G. oerstedii* BAFC 218; digestion pattern type 3 with DNA fragments of about 950, 600, 350 and 150 bp, represented by*G. tornatum* PNG-371 and digestion pattern type 4 with DNA fragments of about 950, 700, 200 and 150 bp, represented by *G. tsugae*. The digestion results of *Hinc II*, *Sca I* and *Tfi I* for all *Ganoderma* are summarised in Table 19.

Table 19: Results of the IGS1 DNA patterns of *Ganoderma* species studied digested with *Hinc II, Sca I and Tfi I*

Ganoderma species	Restriction enzymes used and DNA digestion pattern		
	Hinc II	*Sca I*	*Tfi I*
Oil palm *Ganoderma* (Indonesia), 20 isolates	1	-	1
G. boninense (oil palm, PNG), 10 isolates	1	-	1
G. boninense (coconut stump, PNG), 7 isolates	1	-	1
G. tornatum (hardwood, PNG), 11 isolates	-	1	3
G. oerstedii BAFC 178	1	2	2
G. oerstedii BAFC 218	1	2	2
G. resinaceum BAFC 384	3	2	2
G. tornatum (applanatum?) BAFC 671	2	2	2
G. resinaceum BAFC 2288	3	2	2
G. applanatum BAFC 2353	2	2	2
G. lucidum complex BAFC 2374	3	2	2
G. tornatum BAFC 2390	2	2	2
G. tornatum? BAFC 2395	2	2	2
G. applanatum BAFC 2408	2	2	2
G. tornatum BAFC 2424	2	2	2
G. tornatum? BAFC 2430	2	2	3
G. resinaceum BAFC 2488	1	-	4
G. lucidum complex BAFC 2495	2	-	2
G. applanatum var. tornatum BAFC 2501	2	2	2
Ganoderma sp. BAFC 2529	2	2	2
G. applanatum BAFC 2552	2	2	2
G. tropicum BAFC 2580	3	2	2
G. resinaceum BAFC 2775	3	2	2
G. lucidum DSM 9612	-	2	4
G. applanatum DSMZ 3800	2	2	2
G. tsugae	-	2	4
G. applanatum 134	1	2	2
G. applanatum G 211	1	2	2
G. adspersum G 224	-	2	2
G. pfeifferi G 225	3	2	4
G. cupreum QFRI 8678.1	-	2	2
G. australe DAR 73781	2	2	2
G. incrassatum DAR 73783	-	2	3
G. cupreum DFP 4336	-	2	2
*Ganoderma sp.*Group 6.3 DAR 73779	1	-	4
G. weberianum DFP 4483	2	2	2

-: not digested by enzyme (no restriction site is found)

1, 2, 3 and 4 showed digestion patterns, in the same column shows different restriction DNA fragment patterns

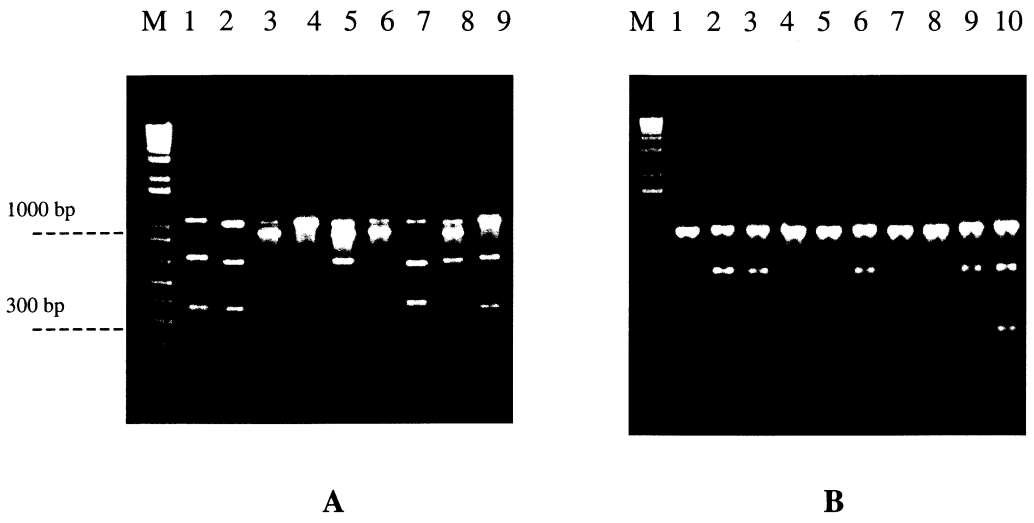

Fig. 32: Restriction patterns of the IGS1 region of *Ganoderma* species digested with the restriction enzymes *Hinc II* and *Sca I*

A. Agarose gel of the *Ganoderma* IGS1 region digested with *Hinc II*. Lanes 1-9: oil palm *Ganoderma* Ad (lane 1, digestion pattern type 1), *Ganoderma* of BAFC 218 (lane 2, typel), BAFC 2390 (lane 3, type 2), DSM 9612 (lane 4, not digested), BAFC 2374 (lane 5, type 3), BAFC 2424 (lane 6, type 2), BAFC 2488 (lane 7, type 2) BAFC 2775 (lane 8, type 3) and G 211 (lane 9, type 1)

B. Agarose gel of the *Ganoderma* IGS1 region digested with *Sca I*. Lanes: 1-10, oil palm *Ganoderma* BS (lane 1, not digested), *Ganoderma* PNG- 371 (lane 2, digestion pattern type 1), PNG-310 (lane 3, type 1) PNG-579 (lane 4, not digested), PNG-600 (lane 5, not digested), PNG-309 (lane 6, type 1), PNG-742 (lane 7, not digested), PNG-602 (lane 8, not digested), PNG-343 (lane 9, type 1) and DSM 3800 (lane 10, type 2). M= DNA markers.

3.5.3 Species-specific primer design for oil palm *Ganoderma* isolates

Based on the IGS1 sequence variability among the 13 *Ganoderma* isolates, 17-18-mer species-specific primers were designed for oil palm *Ganoderma* isolates. Primer IGSa (5'ATCGTATATAAGCCTGC'3) as reverse primer aligned to bp 349 to 365 and primer IGSc (5'AAGAGGGTAACTAAACAC'3) as reverse primer aligned to bp 724 to 741 in the IGS1 region of oil palm *Ganoderma* isolates (see Fig. 31). For PCR amplification, primers IGSa and IGSc were combined with the primer Q as a forward primer. Each primer pair Q-IGSa or Q-IGSc amplified a single PCR product of the expected size of 630 or 1,000 bp when the genomic DNA of all oil palm *Ganoderma* (including *G. boninense* isolated from coconut stumps) was used. Both primer pairs Q-IGSa and Q-IGSc showed no cross-reaction in PCR amplification with any other *Ganoderma* species tested (except for *G. tornatum* from hardwood), indicating that these primer pairs were specific for oil palm *Ganoderma*. Weak bands of the same

size were amplified with the primer pair Q-IGSa when DNA of *G. tornatum* isolates (from hardwood) was used (Fig. 33). On the other hand, a DNA fragment of about 2,000 was amplified from the genomic DNA of *G. tornatum* BAFC 2430 and *G. applanatum* BAFC 2552 using the primer pair Q and IGSa (Fig. 33).

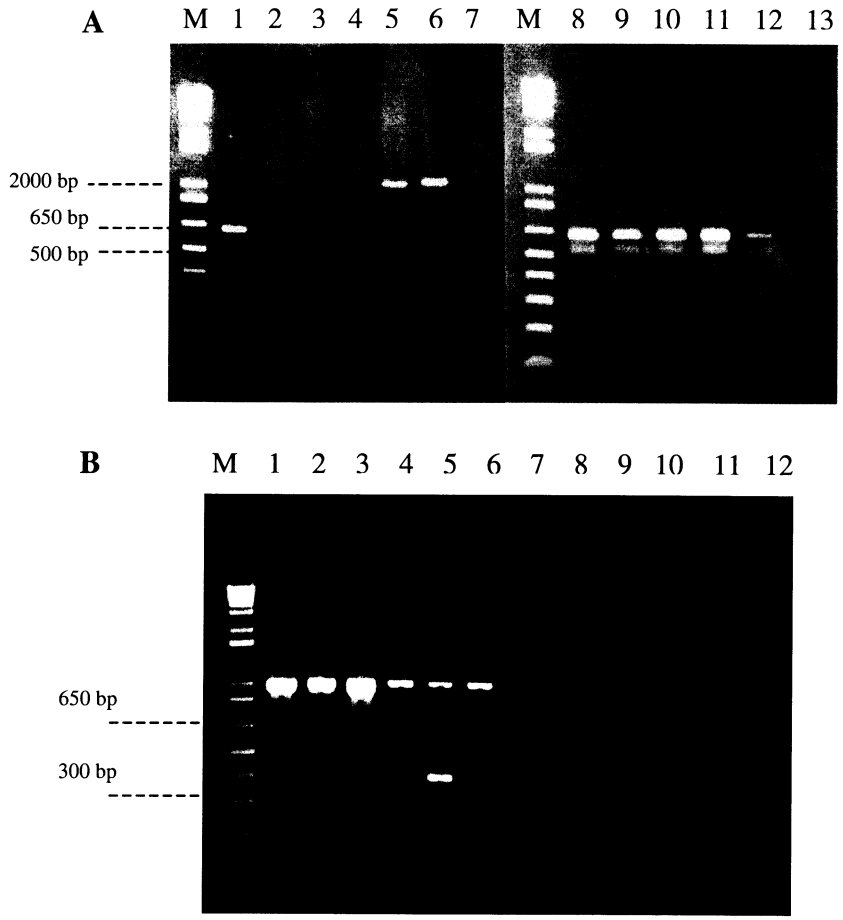

Fig. 33: Agarose gel showing PCR products obtained from *Ganoderma* DNA using the species-specific primers Q-IGSa and Q-IGSc

A. PCR products amplified with primer pairs Q and IGSa. Lanes 1-13: oil palm *Ganoderma* Mt (lane 1), *Ganoderma* of DSM 9612 (lane 2), BAFC2374 (lane 3), BAFC 2580 (lane 4), BAFC 2552 (lane 5), BAFC 2430 (lane 6), BAFC 218 (lane 7), PNG-742 (lane 8), PNG 597 (lane 9), PNG- 381 (lane 10), PNG-600 (lane 11), PNG-371 (lane 12) and BAFC 2529 (lane 13).

B. PCR products amplified with primer pairs Q and IGSc. Lanes 1-12: oil palm *Ganoderma* Ad (lane 1), *Ganoderma* of PNG-579 (lane 2), PNG- 410 (lane 3), PNG-308 (lane 4), PNG-369 (lane 5), PNG-283 (lane 6), DFP 4483 (lane 7), DAR 7377 (lane 8), DFP 4336 (lane 9), DAR 73783 (lane 10), DAR 73781 (lane 11) and QFRI 8678.1 (lane 12). M= DNA marker

With the primer pair Q-IGSc, PCR amplification of the genomic DNA of *G. tornatum* (hardwood) produced the same size band as oil palm *Ganoderma* but an additional DNA fragment of about 400 bp also appeared (Fig. 33). The specificity of

amplification products generated by both species-specific primers was confirmed by PCR amplification of the genomic DNA of all *Ganoderma* species using the primer pair Q-5SA as control PCR at the same annealing temperature. These primers (primer pair Q-5SA) amplified the expected IGS1-PCR products of about 1,100 bp for all *Ganoderma* tested (Table 20).

3.5.4 Phylogeny analysis of the IGS1 region

Multiple nucleotide sequence alignments of the IGS1 region of 13 *Ganoderma* isolates were constructed by the Clustal method to infer phylogenetic relationships using *Armillaria singula* (non-*Ganoderma* species but basidiomycete fungus, GenBank accession number D89926) as outgroup (Fig. 34). Percent identities within oil palm *Ganoderma* ranged from 93.3 to 97.1% while other different *Ganoderma* species had percent identities to oil palm *Ganoderma* ranged from 59.2 to 73.5 % (Table 5A, see appendixes). Within oil palm *Ganoderma* isolates, there were subspecific populations among the isolates. Oil palm *Ganoderma* isolates BJ 8 and Lo were separated from oil palm *Ganoderma* isolates PM and SB within phylogenetic tree (Fig. 34).

Table 20: Results of PCR amplification results of total genomic DNA from *Ganoderma* species using the primer pairs Q-IGSa, Q-IGSc and Q-5SA

Ganoderma species	PCR amplification of IGS1 region by primer pairs		
	Q-IGSa	Q-IGSc	Q-5SA
Oil palm *Ganoderma* (Indonesia), 20 isolates	+++	+++	+++
G. boninense (oil palm, PNG), 10 isolates	+++	+++	+++
G. boninense (coconut stump, PNG), 7 isolates	+++	+++	+++
G. tornatum (hardwood, PNG), 11 isolates	+	++	+++
G. oerstedii BAFC 178	-	-	+++
G. oerstedii BAFC 218	-	-	+++
G. resinaceum BAFC 384	-	-	+++
G. tornatum (applanatum?) BAFC 671	-	-	+++
G. resinaceum BAFC 2288	-	-	+++
G. applanatum BAFC 2353	-	-	+++
G. lucidum complex BAFC 2374	-	-	+++
G. tornatum BAFC 2390	-	-	+++
G. tornatum? BAFC 2395	-	-	+++
G. applanatum BAFC 2408	-	-	+++
G. tornatum BAFC 2424	-	-	+++
G. tornatum? BAFC 2430	*	-	+++
G. resinaceum BAFC 2488	-	-	+++
G. lucidum complex BAFC 2495	-	-	+++
G. applanatum var. tornatum BAFC 2501	-	-	+++
Ganoderma sp. BAFC 2529	-	-	+++
G. applanatum BAFC 2552	*	-	+++
G. tropicum BAFC 2580	-	-	+++
G. resinaceum BAFC 2775	-	-	+++
G. lucidum DSM 9612	-	-	+++
G. applanatum DSMZ 3800	-	-	+++
G. tsugae	-	-	+++
G. applanatum 134	-	-	+++
G. applanatum G 211	-	-	+++
G. adspersum G 224	-	-	+++
G. pfeifferi G 225	-	-	+++
G. cupreum QFRI 8678.1	-	-	+++
G. australe DAR 73781	-	-	+++
G. incrassatum DAR 73783	-	-	+++
G. cupreum DFP 4336	-	-	+++
*Ganoderma sp.*Group 6.3 DAR 73779	-	-	+++
G. weberianum DFP 4483	-	-	+++

Primer pairs Q-IGSa and Q-IGSc produced a single PCR product of 630 and 1,000 bp for oil palm *Ganoderma* and *G. boninense* isolated from coconut stumps. Primer pair Q-5SA as control produced a single PCR product of about 1,100 bp for all *Ganoderma* species.

+++, ++ and +: Produced a strong, moderate and weak band of the same size of PCR product indicated in the same column.

*: Produced PCR amplification product of about 2,000 bp.

-: No PCR amplification product was observed.

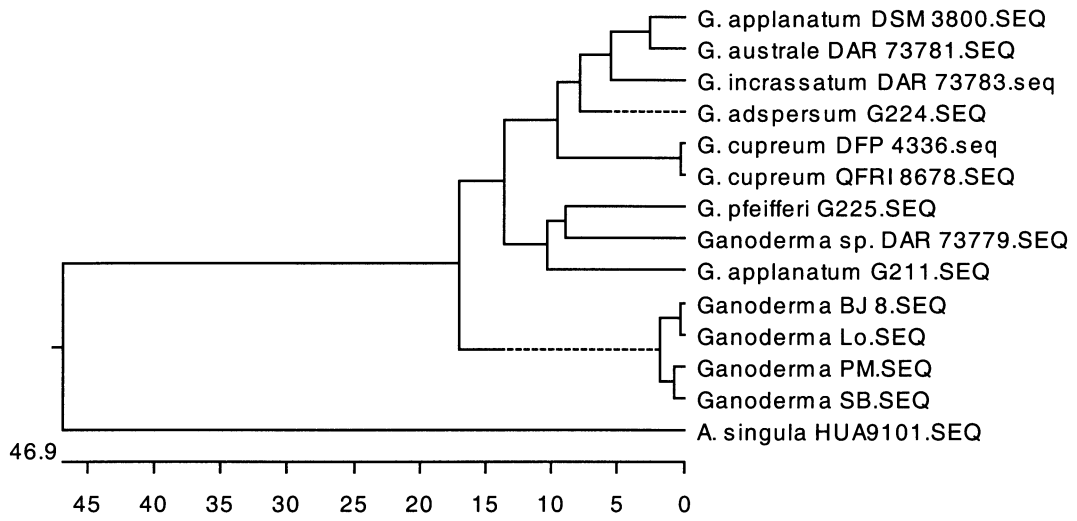

Fig. 34: Phylogenetic relationships of *Ganoderma* species inferred from the IGS1 region

***Armillaria singula* HUA9101 was used as an outgroup for phylogenetic analysis. A phylogenetic tree was generated using the Clustal method with weighted residue weight table. Multiple alignment parameters were: gap penalty = 10 and gap length penalty = 10. Pairwise alignment parameters were: ktuple = 2, gap penalty = 5, window = 4 and diagonals = 4. The length of each pair of branches represents the distance between sequence pairs. The units at the bottom of the tree indicate the number of substitution events.**

4 Discussion

4.1 Detection of oilpalm *Ganoderma*

Prior to the development of DNA-based methods, the detection of BSR disease in oil palm was based on visual observation and isolation of the pathogen from infected tissue on semi-selective medium (Arifin, 1993) or on ELISA assays (Utomo, 1997). Visual observation requires placement of infected tissue on semi-selective medium to observe the mycelia characteristics of pathogenic *Ganoderma*, which may take four days or longer. PCR testing for oil palm *Ganoderma* is relatively expensive in comparison to culturing the fungus in semi-selective medium or an ELISA test, but it may be more sensitive and rapid. The PCR assay can be performed within three to four hours, including sample processing, PCR amplification, gel electrophoresis and staining. In addition, the use of species-specific primers designed specifically for detecting pathogenic *Ganoderma* may prevent cross-reactions with DNA from non-target organisms, for example, saprophytic fungi associated with the diseased tissue. Combined with the use of a rapid DNA extraction method, PCR assays would offer a method that is faster than other methods described earlier for specifically detecting those *Ganoderma* species that infect oil palms.

As the primary factors in developing a PCR detection procedure are specificity and sensitivity, it is essential that the parameters used in the PCR are optimal. The specificity of the PCR-based detection procedure was verified by the absence of cross-reaction with DNA from the host (healthy palm tissue) and 18 saprophytic fungi (except for primers Gan1 and Gan2). The sensitivity of PCR detection was high as it successfully detected an amount of 5 pg genomic DNA of oil palm *Ganoderma* (for both DNA extraction methods, i.e. those by Raeder and Broda (1985) and Möller et al. (1992). Similar results using the DNA extraction of Möller et al. (1992) were also reported for the detection of *Fusarium graminearum* (Schilling et al., 1996). PCR amplification of oil palm *Ganoderma* DNA was not adversely affected by the presence of large quantities of host (oil palm) DNA, as demonstrated by successful amplification of template DNA at *Ganoderma* : oil palm root DNA ratio of 1: 10^4. The unsuccessful use of primer pairs IT1-IT2 and IT1-IT3 to amplify ITS-rDNA spacer elements from saprophytic fungi indicates that negative results obtained with saprophytic fungi was not associated with DNA degradation or the presence of PCR

inhibitors. These results were confirmed by PCR amplification using the primer pair ITS1-ITS4 as control PCR that amplified ITS-rDNA of saprophytic fungi.

One of the problems in performing PCR on the target DNA from infected host tissues is the presence of inhibitory substances such as polysaccharides (Demeke and Adam, 1992) or phenolic compounds (Cenis, 1992; John, 1992; Tsai and Olson; 1992; Johanson, 1994) which may drastically reduce the sensitivity of PCR tests. For example, because of the presence of inhibitors, PCR failed to amplify *Gaeumannomyces graminis* (Henson et al., 1993) and *Magnaporthe poae* DNA (Bunting et al., 1996) extracted from infected plant tissues. For this reason, further purification steps were performed to remove inhibitors, including cation exchange columns (Stein and Raoult, 1992), polyvinil polypyrrolidone (PVPP) which binds polyphenolic compounds (Parry and Nicholson, 1996) or the use of commercial DNA purification kits such as Magic DNA Clean-up Columns (Johanson, 1994). Since all these procedures are time-consuming and expensive, the NaOH method (1993) for DNA extraction from infected tissue with no additional purification steps, represents an advantage in routine PCR tests. In this study, PCR amplification using primer pairs IT1-IT2 and IT1-IT3 with artificial mixing of *Ganoderma*-oil palm root or naturally infected roots at dilution 1:10 were unsuccessful. A possibility of the failure of PCR reaction could be the presence of inhibitory substances. On the other hand, the successful PCR amplifications of the naturally infected roots at dilution of 1: 10^2 and 1: 10^3 were recorded. This requirement for higher dilution may be explained by diluting inhibitory compounds present in crude DNA extracts from infected tissues.

For routine detection purposes, a simple and fast DNA extraction method that allows quick extraction of DNA from a large number of samples is required. In the NaOH method, only a few steps were required to obtain the target DNA, by grinding root samples and then directly boiling in NaOH solution without requiring other reagents to eliminate proteins or other substances. The sample preparation procedure of the NaOH method represents a significant simplification in comparison with the two other methods by Raeder and Broda (1985) and Möller et al. (1992). With this method it was found that infected plant tissues released enough of the target DNA to obtain a positive PCR test. This method also reduced much of the handling of fungal DNA samples and, consequently, reduced the time required for DNA processing and limited the possibility of contamination by extraneous DNA

which otherwise could be amplified and would give false results. Similar results using the NaOH method have also been reported for *Phytophthora* spp. (Tooley et al., 1997) and *Ralstonia solanacearum*, as well as for *Clavibacter michiganensis* ssp *sepedonicus* (Niepold, 1999). It is hoped that the PCR assay described here will prove useful to assist BSR disease management in the quest for a rapid, sensitive and accurate method of *Ganoderma* detection in the fields. In future, research will attempt to define additional parameters that are important in field application of the PCR assay, including sampling methods and approaches for assaying tissue samples from different parts of the diseased oil palm.

The fact that oil palm *Ganoderma* was detectable by PCR before the appearance of visible symptoms indicates that the PCR assay described may prove useful in determining levels of latent infection in symptomless oil palm. Such information could assist growers in making decisions about treatment of the infected plants. Because the PCR assay is successful in detecting *Ganoderma* in infected tissue, it may also have applicability in studying pathogen dispersal within an oil palm or to trace the pathogenic *Ganoderma* in other host plants in the fields or in yet uncultivated areas.

4.2 Identification and differentiation of oil palm *Ganoderma*

Identification of *Ganoderma* spp. pathogenic on oil palm is problematic, due to the presence of morphological similarity of other *Ganoderma* occurring in oil palm (Turner, 1981). This situation is even more complicated by the fact that morphological variation in *Ganoderma* appears to be affected by environmental conditions during basidiocarp development (Seo and Kirk, 2000). Context colour of basidiocarp is highly variable, especially in dried specimens, not only in the same species but also in a single specimen (Zhao, 1989). Development of the basidiocarp is also sensitive to light and ventilation. Under dark conditions with poor ventilation, the pileus does not expand and often an abnormal pileus is produced (Shin and Seo, 1988; Stamets, 1993). For these reasons, a number of authors concluded that the use of morphological characters alone is insufficient and has very limited value for identification of *Ganoderma* species (Bazzalo and Wright, 1982; Gilbertson and Ryvarden, 1986; Ryvarden, 1995). For defining species, most taxonomic studies on species of *Ganoderma* originating from Southeast Asia are based on

the system developed by Steyaert (1967 and 1975). Among the species found on Asian oil palm, there were two pathogenic *Ganoderma* spp., i.e. *G. boninense* and *G. miniatocinctum* (Steyaert, 1967). Identification of these two species was strongly refuted by Corner (1983) who suggested synonymy with *G. chalceum* for *G. boninense* and *G. miniatocinctum*. In this case, identification of *G. boninense* based on morphological characters has proven inconsistent.

Recently, random amplified polymorphic DNA (RAPD) (Abu-Seman et al., 1996; Pilotti et al., 2000) and amplified fragment length polymorphisms (AFLP) (Rolph et al., 2000) analyses have been used to examine populations within oil palm *Ganoderma*. These techniques can also be used to differentiate between oil palm *Ganoderma* and other *Ganoderma* species, but the results are not specific so that they are more difficult to interpret than those obtained by the method described here. In addition, the use of the RAPD and AFLP techniques are extremely sensitive to contamination by other non-target organisms (Dyer and Leonard, 2000). In this study, four genes of the oil palm *Ganoderma*, i.e. ITS region, IGS1 region, laccase and Mn-SOD genes were exploited in order to provide tools for unambiguous identification and differentiation between oil palm *Ganoderma* and other *Ganoderma* species. Molecular approaches include 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 for identification.

PCR amplification of the laccase gene of oil palm *Ganoderma* is much less sensitive compared to the nuclear rDNA gene, because of lower copy number of this gene. Sensitivity threshold of the laccase gene in PCR amplification was 1 ng of genomic DNA as compared to the sensitivity threshold of rDNA gene, which was 5 pg of genomic DNA. Lower sensitivity thresholds (1 ng of genomic DNA) are also reported in amplifying the glycerinaldehyde-3-phosphate dehydrogenase gene (Kreuzinger et al., 1996) and the chitin synthase gene (Glen et al., 2001) of ectomycorrhizal fungi. Therefore, the availability of alternative genomic regions may be desirable for some studies, but for studies requiring species identification of a large number of samples, a target fragment which is easily amplified from small quantities of template DNA is preferable. In addition, the use of more

than one genomic region would provide an extra level of confidence in oil palm *Ganoderma* identification and differentiation.

The unexpected size of PCR-amplified laccase gene fragment of 327 bp in oil palm *Ganoderma* and *G. boninensis* isolated from coconut stumps was unique since primer pair Lac 2a-Lac 2b was designed from the conserved region of the amino acid sequence WHGFFQ (copper-binding region I) and WFLHCHI (copper-binding region IV) of the laccase gene with the expected DNA-size of about 1,500-1,600 bp. Amplification of this unique PCR product revealed the specificity of these primers used for amplifying the laccase gene of oil palm *Ganoderma*. However, most other *Ganoderma* species tested with these primers, produced a PCR product of the expected size of about 1500-1600 bp starting from copper-binding region I to copper-binding region IV in the laccase genes as described in the previously published laccase gene sequences of basidiomycete fungi (Saloheimo et al., 1991; Coll et al., 1993; Yaver et al., 1996; Mansur et al., 1997; Eggert et al., 1998).

Other primer pairs designed from conserved regions of the laccase gene that provide tools for identification and differentiation of oil palm *Ganoderma* are primer pairs Lac 2a-Lac 2r and Lac 2f-Lac2b. 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 *Ganoderma* species studied was used. The two different PCR products generated using primer pair Lac2a-Lac2r are derived from genomic DNA of other *Ganoderma* species that generate a PCR product of 1617 bp (identical size to oil palm *Ganoderma*) and another genomic DNA of other *Ganoderma* species that generate a double band in the PCR of 1617 bp and 1500 bp. Subsequently, other *Ganoderma* species, which produce a PCR product that identical in size to oil palm *Ganoderma* using primer pair Lac2a-Lac2r can be discriminated by using primer pairs Lac 2f-Lac 2b. By using primer pairs Lac 2f-Lac 2b, genomic DNA of oil palm *Ganoderma* always generated a PCR product of 1265 bp. In contrast, genomic DNA of other *Ganoderma* species (identical PCR product to oil palm *Ganoderma* when using primer pairs Lac 2a-Lac 2r) produced a PCR product of 1400 bp. Therefore, with the use of both primer pairs, oil palm *Ganoderma* could be clearly distinguished from all other *Ganoderma* species studied. Sequence analyses show that each primer pair generated a DNA fragment belonging to a different laccase gene family within oil palm *Ganoderma*.

The laccase gene of a PCR product amplified with the primer pair Lac2f-Lac2b is 139 bp smaller compared to the PCR product of the laccase gene generated with the primer pair Lac2a-Lac2r at the corresponding region. The failure of primer pair Lac2f-Lac2b to amplify the larger laccase gene could be due to a single base mismatch of primer Lac2b and two base mismatches of primer Lac2f to their complementary binding sites at the larger laccase gene.

Since introns have potentially high rates of sequence evolution, their analysis has become an important tool in studies of evolutionary relationships among species (Slade et al., 1993). It is known, however, that many introns are highly variable at the species level. These changes in intron structure include nucleotide substitutions, insertions/deletions, or the presence or absence of introns in a gene (Nellen et al., 1981; Tarlow et al., 1993; Palumbi and Baker, 1996). The reason for the existence and distribution of introns is debated continuously and whether these intervening sequences have a function remains unclear (Fink, 1987; Mattick, 1994).

In this study, introns in laccase gene fragments of *Ganoderma* Lac 1700 (oil palm *Ganoderma* isolate BS) were targeted to discriminate between oil palm *Ganoderma* and other *Ganoderma* species studied. From the introns of the laccase gene of *Ganoderma* Lac 1700, five primers were constructed and used as forward primers, paired with primer Lac 2b2 as reserve primer and tested for their ability to amplify fragments of a laccase gene from oil palm *Ganoderma* and other *Ganoderma* species studied. The results of PCR amplification of five primer pairs showed that the expected specific PCR products of 1,444, 1,165, 1,012, 508, 213 bp were observed when tested with oil palm *Ganoderma* (including *G. boninense* from coconut stumps). The primers did not amplify a fragment of a laccase gene from other *Ganoderma* species studied, except for *G. applanatum* BAFC2552 and BAFC2408 that generated a weak band identical in size to oil palm *Ganoderma* using one of the primer pairs. Almost all other *Ganoderma* species studied were successfully amplified by primer pairs Lac2a-Lac2b, Lac2a-Lac2r and Lac2f-Lac2b (primers designed to amplify the laccase gene as control for PCR). Therefore, the lack of PCR amplification products of other *Ganoderma* species studied by using primers designed from intron sequences of strain was not due to DNA degradation or the presence of PCR inhibitors.

Of the primers designed from intron sequences to amplify *Ganoderma* laccase genes, primer Intron 5 in combination with the primer Lac 2b2 is the most specific primer pair for oil palm *Ganoderma* laccase genes. The specificity is confirmed by changing annealing temperature from 65 to 55 °C. Decreasing the annealing temperature of the PCR did not affect the specificity of primer pair Intron 5- Lac 2b2 whereas other primers designed from introns in combination with the primer Lac 2b2 generated additional bands with genomic DNA of oil palm *Ganoderma* and other *Ganoderma* spp. used. PCR amplification of the laccase gene fragment using primers designed from introns in combination with the primer Lac 2 indicates that the primer binding sites in oil palm *Ganoderma* are highly specific and provide an alternative strategy for identification and differentiation of oil palm *Ganoderma*. This is in agreement with the result of differentiation between *Saccharomycetes carlsbergensis* and *S. cerevisiae* in which actin genes are identical except for their introns (Nellen et al., 1981).

Several studies have shown that ITS regions are more variable than small and large ribosomal subunit genes (18S and 28S) among fungal species (Sherriff et al., 1994; Jasalavich et al., 1995; Cooke and Duncan, 1997; Paulus et al., 2000; Crous et al., 2001). Small and large ribosomal subunit genes are highly conserved and thus have been generally used for comparison at higher taxonomic level (Bruns and Szaro, 1992; Berbee et al., 1995; Hibbett et al., 1997; Harrington et al., 1999; Gottlieb and Lichtwardt, 2001). In fungi, the entire ITS region usually has the size of 500 to 800 bp (Gardes and Bruns, 1993), even though longer ITS sequences were also reported in *Entomophaga aulicae*, where the complete ITS region comprises 1100 bp (GenBank U35394); *Cantharellus* spp. with the ITS1 region ranging from 250 to 1110 bp (Feibelman, et al., 1994) and *Smittium* spp. with a complete ITS region ranging from 650 to 2,000 bp (Gottlieb and Lichtwardt, 2001). The complete ITS region of *Ganoderma* species is about 400 bp and the size of both ITS1 and ITS2 regions was similar. By including the 5.8S region the size is about 650 bp when amplified by primers ITS1 and ITS4. In *Ganoderma* species, ITS sequence length is relatively constant. Therefore, the constant ITS sequence length in *Ganoderma* species could be used to test if fungal samples are from the *Ganoderma* group or not by amplifying with primers ITS1-ITS4. For example, *G. lucidum* DSM 103 (produced a double band of about 700 bp and 550 bp) and *Ganoderma* sp. 136 are considered an outgroup of *Ganoderma* and therefore they are excluded from molecular analyses. Other advantages of

using the constant *Ganoderma*-ITS sequence length are that it is suitable for RFLP analyses and phylogenetic studies. In contrast, partial sequences of the Mn-SOD gene from various *Ganoderma* derived from the GenBank showed sequence length variations. At the corresponding amino acid sequences from LHHKKHH to DIWEHAF, the length of these partial genes ranged from 675 to 851 bp. The sequence length variations were due to two introns and these sequence length variations make sequence alignments problematic. Therefore, exclusion of these introns allows unambiguous nucleotide sequence alignments across the entire data set.

In fungi, location of the 5S rDNA repeats follows two different patterns. In the first pattern, the 5S rDNA repeats are not located within the rDNA gene cluster but they are dispersed throughout the genome, as for example in *Neurospora crassa* (Selker et al., 1981) and *Aspergillus nidulans* (Lockington et al., 1982). In the second pattern, 5S rDNA repeats are located between 28S and 18S of the rDNA repeat units, for example in basidiomycete fungi *Flammulina velutipes*, *Agaricus bisporus*, *Coprinus* spp. (Cassidy et al., 1984 and Cassidy and Pukkila, 1987), *Schizophyllum commune* (Buckner et al., 1988), *Armillaria* spp. (Duchesne and Anderson, 1990; Anderson and Stasovski, 1994; Terashima et al., 1998) and *Puccinia* spp. (Kim et al., 1992). Amplification of 13 *Ganoderma* isolates with the primer pair Q-5SA and sequencing of the PCR product showed that the 5S rDNA could be discovered in *Ganoderma* isolates. The location and orientation of the *Ganoderma* 5S rDNA is similar to that reported in other basidiomycetes (Cassidy et al., 1984). This is the first report of the location of the 5S RNA gene (Fig. 35) in *Ganoderma* species.

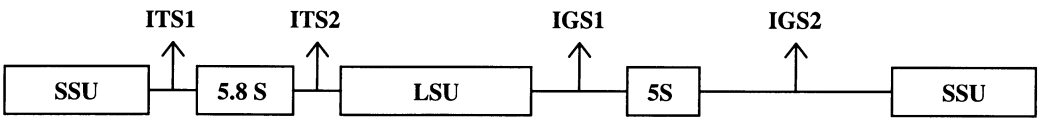

Fig. 35. Schematic ribosomal DNA repeat unit of *Ganoderma*

Amplification of total genomic DNA of all *Ganoderma* spp. studied, using the primer pair Q-5SA, produced a single PCR product about 1,100 bp, indicating that the location of the 5S rDNA in inter- and intra-species of *Ganoderma* is not variable in the rDNA repeat

units. In contrast, in some other basidiomycetes fungi it is reported that the location of 5S rDNA is variable. In *Puccinia* spp., the location of the 5S rDNA shows inter- and intra-species variability (Kim et al., 1992). These variable positions are also reported in *Laccaria* spp. (Henrion et al., 1992, 1994; Selosse et al., 1996). However, the appearance of the invariable position of the 5S rDNA repeats in the IGS region of *Ganoderma* makes it suitable for phylogenetic and RFLP studies.

Alignment of ITS sequences of eight oil palm *Ganoderma* isolates with the corresponding region of other published *Ganoderma* spp. indicates that the most variable sequence in the ITS1 region is located in the central and in the ITS2 region near the termini of the sequences. Within oil palm *Ganoderma* isolates, a small nucleotide variation was revealed in both ITS regions, as shown by identities ranged from 93.8 to 100 % in the ITS1 region and 90.2 to 100 % in the ITS2 region. On the other hand, Bridge et al. (2000) reported that the ITS region of four oil palm *Ganoderma* isolates from New Papua Guinea are identical. Moncalvo et al. (1995a,b) reported that the ITS sequence variation in *Ganoderma* is about 2-3 % within *Ganoderma* species studied. This level of sequence variation would suggest that ITS sequences could be used to define *Ganoderma* species. The source of sequence variation in these ITS regions was used to infer phylogeny but sequence variation alone can not distinguish between species until variation in a given region for a given species is established and analysed. For analysis of sequence variations in order to infer phylogenetic relationships, the Clustal V algorithm method (Higgin and Sharp, 1989) was used. In this method, sequences are grouped into clusters by examining the distances between all sequence pairs. Clusters are aligned as pairs and then collectively as sequence groups to produce the overall alignment. After the multiple alignment is completed, a neighbor-joining method (to cluster the variants from the distance data) as described by Saitou and Nei (1987) is employed to reconstruct phylogeny for the putative alignment.

The phylogenetic trees showed that oil palm *Ganoderma* species cluster together with *G. boninense* LKM (isolated from palm) but separately from *G. boninense* RSH RS (unknown host) in ITS and Mn-SOD gene phylogeny, although morphological characters of *G. boninense* RSH RS matched with *G. boninense* of Steyaert-based description (Moncalvo et al., 1995). Apparently, *G. boninense* RSH RS was misnamed and was not associated with a palm (Moncalvo, personal communication). Therefore, the placement of *G. boninense*

RSH RS is inconsistent with that of oil palm *Ganoderma* based on the ITS and Mn-SOD gene phylogenies, demonstrating the limitation of morphological identification in this species complex. Additional data of percent sequence identity also showed that oil palm *Ganoderma* has high sequence identity to *G. 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* has lower sequence identity to *G. 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 %). In this study, a molecular approach has proven to be more accurate and consistent than morphological approaches to define *Ganoderma* species pathogenic on oil palm.

Analyses of restriction fragment length polymorphisms (RFLP) of the ITS regions of *Ganoderma* spp. for molecular taxonomic studies, using a set of restriction enzymes have been reported (Gottlieb et al., 2000). In these analyses, the choice of restriction enzymes was random and based on mostly 4-base pair-cutting restriction enzymes. However, the results of 4-base pair-cutting restriction enzymes did not confer specificity. Sequence analyses of the ITS regions to identify *Ganoderma* allowed detailed information to be obtained, but sequencing is time-consuming and costly. Alternatively, to identify and characterise large numbers of samples, digesting ITS amplification products by specific restriction enzymes is more efficient in terms of simplicity and rapidity.

Digestion of the ITS regions with selected restriction enzymes (*SacI*, *MluI* and *HinfI*) and the IGS1 region with the restriction enzymes (*Hinc II*, *ScaI* and *TfiI*) allowed oil palm *Ganoderma* to be distinguished from all other *Ganoderma* species studied. Of the three restriction enzymes used in each r DNA 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*. *MluI* identically cut the amplified ITS region of all oil palm *Ganoderma* species including *G. boninense* from the coconut stumps, but did not cut all other *Ganoderma* spp. studied. *TfiI* reveals a species-specific restriction pattern for the IGS1 region of oil palm *Ganoderma*. Both restriction enzymes (*MluI* and *TfiI*) allowed the generation of data for identification and differentiation between oil palm *Ganoderma* and other *Ganoderma* spp. studied.

From the RLFP analyses of the ITS and IGS1 regions using specific restriction enzymes, it was shown that *Ganoderma* attacking oil palm survived saprophytically in the coconut stumps. In the ITS regions, the specificity of the three restriction enzymes also appears to be well-suited for presumptive analyses of various *Ganoderma*-ITS sequences deposited in the database. Simulation analysis using these restriction enzymes showed that oil palm *Ganoderma* could be discriminated from the deposited *Ganoderma* spp. used in this study. A restriction site for *MluI* was not found in the ITS sequence of the deposited *Ganoderma* spp.

Restriction fragment patterns of the ITS/IGS1 regions of the oil palm *Ganoderma* species studied were identical for each specific restriction enzyme used. These findings lead to the conclusion that restriction sites of the three restriction enzymes in oil palm *Ganoderma* are highly conserved and they provide an excellent criterion for species identification and differentiation in oil palm *Ganoderma*. ITS PCR-RFLP analyses in other basidiomycetes also exhibited intraspecific uniformity; the specific fragment pattern could be used for identification of respective isolates of species such as *Serpula himantioides* (Schmidt and Moreth, 1998), *S. lacrymans* (Schmidt and Moreth, 2000), *Tylospora fibrillosa* (Erland et al., 1994) and *Armillaria ostoyae* (Schulze et al., 1997). PCR-RFLP analyses of the ITS/IGS1 regions are considered to be useful for routine identification and differentiation of oil palm *Ganoderma*. Because of the specificity of the restriction sites found within the ITS/IGS1 regions of oil palm *Ganoderma* isolates, the use of combination of the restriction enzymes can be applied as a standard protocol to identify whether *Ganoderma* samples from the field are capable of infecting healthy oil palms.

Although PCR-RFLP analyses have proven to be a practical and accurate tool for identification of oil palm *Ganoderma*, some technical limitations particularly in the selection of the specific restriction enzyme must be taken into consideration. This method requires a large number of corresponding sequences to align with the target sequence. Another theoretical limitation is the availability of a single base variation of the restriction sites in the target sequence.

The amplification of specific rDNA sequences using species-specific primers derived from nucleotide sequence was tested for possible cross reaction with related fungi and

considered the most valuable tool for fungal diagnosis developed so far (Garbelotto et al., 1996; Schulze et al., 1997). Multiple sequence alignment of the ITS and IGS1 regions of oil palm *Ganoderma* with other *Ganoderma* species confirms the presence of a species-specific sequence variation in the ITS and IGS1 regions across a broad range of *Ganoderma* species. Based on the species-specific sequences in oil palm *Ganoderma*, primers were constructed and generated a single PCR product when genomic DNA of oil palm *Ganoderma* was used. In other fungi, differences of several base pairs among species in the ITS region was used to design species-specific primers to identify plant pathogenic fungi (Beck and Ligon, 1995; Goodwin et al., 1995; Ristaino et al., 1998; Förster and Adaskaveg, 2000; Freeman et al., 2000). These regions are an attractive target for PCR amplification because they are always present in high copy number. For example, 60 to 90 copies for *Coprinus cinereus* (Cassidy et al., 1984), 190 copies for *Neurospora crassa* (Free et al., 1979) and 120 copies for *Schizophyllum commune* (Dons and Wessels, 1980) per haploid genome have been reported.

PCR amplification with species-specific primers allows identification by a single PCR step, while PCR-RFLP analysis requires two steps: PCR amplification and digestion with restriction enzymes. By PCR amplification with specific primers, samples which may contain DNA of several fungal species or other organisms, such as DNA extracted from the diseased oil palm tissues can be analysed reliably. In PCR-RFLP analyses, it is preferable to use samples that contain DNA from a single species. The result of species-specific primers is clear because the identification is based on the presence or absence of the specific band. Therefore, for diagnostic purposes, PCR amplifications using species-specific primers are more appropriate than PCR-RFLP analyses. On the other hand, in connection with the closely related species that may have minor nucleotide differences, PCR amplification of DNA fragments may occur in closely related species. In this case, the additional use of specific restriction enzymes in PCR-RFLP to identify the target species offers advantages over species-specific primers.

The development of PCR-RFLP or species-specific PCR primers for the discrimination between pathogenic *Ganoderma* in oil palms and non-pathogenic *Ganoderma* species is not only required for practical agronomic purposes but also for detecting the source of inoculum. The discrimination has an impact on crop rotation from rubber or cocoa plants

to oil palms, because usually the stumps of both plants are left in the fields. After a certain period of time the stumps are colonised by *Ganoderma* and other basidiomycete fungi. However, it is difficult to determine whether the *Ganoderma* species colonising these stumps represent the same species that can also infect healthy oil palms. This is an important question to be solved for the planters to make their decision whether or not to remove stumps in order to minimise the source of infection. One has to keep in mind that removal of stumps is very expensive.

In future, an alternative approach by using a combination of a species-specific primer and a specific restriction enzyme could be applied in order to reduce time in identification of oil palm *Ganoderma.* For example, the PCR product amplified by primers IT1-IT2 contains the restriction site of *MluI* or a PCR product amplified by primers Q-IGSc contains the restriction sites of *TfiI* when DNA of oil palm *Ganoderma* is present. This approach could be used to amplify directly DNA from fruiting bodies of *Ganoderma* samples from the field without purifying and culturing the fungi, which usually takes at least a month.

Multigene approaches (laccase genes, ITS and IGS1 regions) show consistency in identification and differentiation of oil palm *Ganoderma* and other *Ganoderma* spp. tested. Although limited genetic variability is found in the ITS and IGS1 regions, conserved species-specific primers and specific restriction enzymes for oil palm *Ganoderma* have been established. The unambiguous results of species-specific primer amplifications (in the laccase genes, ITS and IGS1 regions) and specific restriction enzyme digestion (in the ITS and IGS1 regions) of the oil palm *Ganoderma* isolates indicate that oil palm *Ganoderma* causing BSR disease in oil palm belongs to a single species.

5 Perspective

In this study, PCR assay has proved to be a reliable and sensitive method to detect oil palm *Ganoderma* in symptomless palms. The impact of this detection method on the disease management is to inspect *Ganoderma* infection at an early stage. By treating the palms at an early stage of infection with an appropriate systemic fungicide, large numbers of palms could possibly be saved.

The main problem in the subsequent oil palm planting (after replanting) would be the increase of the *Ganoderma*-infection rate. Any methods of disposal of the previous palms involving chopping or clean-clearing technique to reduce the pathogen have been conducted, but these methods in the large-scale practices would become unmanageable. A possible alternative method that economically acceptable could be poisoning of old palms using herbicide followed by felling the palms. After certain period, different numerous basidiocarps of *Ganoderma* are produced from the felled trunks. Molecular diagnosis to confirm whether those *Ganoderma* species are associated with the BSR disease or not would be crucial to determine the further treatment of the trunks.

6 Zusammenfassung

In dieser Arbeit wurden molekulare Methoden zum Nachweis, zur Identifikation und zur Differenzierung des Ölpalmen-Pathogens *Ganoderma* verwendet. Eine Vorgehensweise zum Nachweis des Ölpalmen-Pathogens *Ganoderma* in natürlich infizierten Pflanzen war die Verwendung von intern transkribierten Spacern (ITS) zur Herstellung spezifischer Primer. Um Nachweis- und Differenzierungsmethoden für das Ölpalmen-Pathogens *Ganoderma* gegenüber anderen Ganodermen, die apathogen oder pathogen bei anderen Pflanzen sind, zu entwickeln, wurden insgesamt vier DNA-Bereiche des Ölpalmen-Pathogens *Ganoderma* verwendet, nämlich die ITS Region, der Intergenetische Spacer 1 (IGS 1), das Laccasegen, und das Gen der Mangan Superoxid Dismutase (Mn-SOD). Molekulare Methoden, wie die Herstellung von sequenz-spezifischen Primern, PCR/RFLP-Analysen mit Restiktionsenzymen und Sequenzvergleich (Alignment) zur Ermittlung der phylogenetischen Abstammung wurden zur Differenzierung des Ölpalmen-Pathogens *Ganoderma* verwendet.

Insgesamt wurden drei DNA-Extraktionsmethoden getestet, um die Nachweisgrenze zu erfassen. Das Primerpaar Gan1-Gan2 amplifizierte ein DNA-Fragment von ca. 167 bp, wenn DNA des Ölpalmen-Pathogens *Ganoderma* verwendet wurde. Mit zwei der drei getesteten Methoden ließen sich zwar eine höhere Nachweisempfindlichkeit erzielen, für praktische Erwägungen wurde aber die NaOH Extraktion vorgezogen, die noch aus 1,5 ng DNA aus gefriergetrocknetem Myzel amplifizieren konnte. Andere Primerpaare (ITS1-ITS2 und ITS1-ITS3) amplifizierten ein PCR-Produkt von 450 bp bzw. 334 bp mit einer Nachweisgrenze von ebenfalls 1,5 ng, bei Verwendung der NaOH-Extraktion und gefriergetrocknetem Pilzmyzel. Die Spezifität der drei Primerpaare konnte im PCR-Nachweis mit der Abwesenheit einer Kreuzreaktion des gesunden Palmengewebes und 18 von Palmen isolierten, saprophytischen Pilzisolaten bestätigt werden. Bei Probenahmen konnte das Ölpalmenpathogen *Ganoderma* aus Palmwurzeln mit der NaOH-Methode isoliert werden, bevor erste sichtbare Symptome entstanden. Eine Kombination der schnellen DNA-Extraktion durch die NaOH-Methode und der PCR könnte für einen praktischen Nachweis des Ölpalmenpathogen *Ganoderma* in Ölpalmwurzeln verwendet werden.

Insgesamt wurden drei Primerpaare aus dem konservierten Aminosäure-Bereich des sequenzierten Laccase Genes hergestellt, um das Ölpalmenpathogen *Ganoderma* von anderen untersuchten *Ganoderma* Species zu unterscheiden. Das Primerpaar Lac2a-Lac2b produzierte in der PCR eine spezifische Bande von 327 bp, wenn als DNA-Templat das Laccase-Gen des Ölpalmenpathogens *Ganoderma* verwendet wurde. Hingegen zeigten die meisten anderen

getesteten *Ganoderma*-Spezies mit diesen Primern ein PCR-Produkt mit der Größe von 1500 bis 1600 bp innerhalb des Laccase-Genes, beginned an der Kupferbindungsstelle I bis zur Kupferbindungsstelle IV, wie es auch bei den veröffentlichten Laccasegenen von Basidiomyzeten der Fall ist. Eine andere Primerpaar-Kombination (Lac2a-Lac2r) erzeugte immer dann ein einzelnes PCR-Produkt von 1617 bp, wenn DNA des Ölpalmenpathogens *Ganoderma* verwendet wurde. Bei den anderen getesteten *Ganoderma* Spezies wurden zwei unterschiedliche PCR-Produkte von 1617 bp, was identisch war mit dem PCR-Fragment des Ölpalmenpathogens *Ganoderma*, und ein zusätzliches zweites, kleineres Amplifikat von 1500 bp amplifiziert. Mit dem Primerpaar Lac2f und Lac2b hingegen können nun auch andere *Ganoderma* Spezies unterschieden werden, da beim Ölpalmenpathogen *Ganoderma* ein amplifiziertes DNA-Fragment von 1265 bp auftritt, während bei den anderen *Ganoderma*-Spezies mit den gleichen Primern ein größeres, 1400 bp Fragment amplifiziert wird. Daher kann bei Verwendung beider Primerpaare (Lac2a-Lac2r und Lac2f-Lac2b) das Ölpalmenpathogen *Ganoderma* von anderen *Ganoderma* Spezies eindeutig unterschieden werden.

Die variablen DNA-Sequenzen innerhalb der ITS und IGS1 Regionen des Ölpalmenpathogens *Ganoderma* wurden sequenziert und mit veröffentlichten Sequenzen von anderen *Ganoderma* Isolaten verglichen, um spezies-spezifische Primer zu konstruieren und somit das Ölpalmenpathogen *Ganoderma* nachzuweisen. Innerhalb der ITS und IGS1 Region konnten zwei Bereiche gefunden werden, bei denen eine spezifische Amplifikation des Ölpalmenpathogens *Ganoderma* möglich war. Jedes Primerpaar produzierte ein einzelnes Amplifikat von der Größe 450 bp (ITS1 und ITS2), 334 bp (ITS1 und ITS3), 630 bp (Q und IGSa) sowie 1000 bp (Q und IGSc). Kein Amplifikat war auffindbar, wenn DNA anderer *Ganoderma* Isolate verwendet wurde. Nur bei *Ganoderma tornatum*, isoliert aus Harthölzern, zeigten die beiden Primerpaare Q und IGSa, sowie Q und IGSc in der PCR eine schwache Kreuzreaktion. Somit konnte gezeigt werden, daß ein negatives PCR-Ergebnis nicht infolge einer Zerstörung der DNA oder Anwesenheit von Inhibitoren verursacht wurde. Basierend auf den PCR-Ergebnissen mit den spezifischen Primern ist nun eine Identifizierung und Differenzierung zwischen den ölpalmenpathogenen *Ganoderma* Isolaten und anderen untersuchten *Ganoderma* Isolaten möglich und leicht zu interpretieren, da eine Unterscheidung auf Grund einer Anwesenheit oder dem Fehlen eines einzigen DNA-Amplifikates möglich ist.

Ein anderer, molekularer Weg zur genaueren Identifizierung des Ölpalmenpathogens *Ganoderma* und Differenzierung von anderen, bislang untersuchten *Ganoderma* Spezies, stellt

die ITS/IGS1-RFLP Analyse durch die Verwendung von spezifischen Restriktionsenzymen dar. Basierend auf den ITS und IGS1 Regionen konnten drei Restriktionsenzyme für jede einzelne Region gefunden werden. Von den drei getesteten Restriktionsenzymen *Sac*I, *Mlu*I und *Hinf*I konnte nur das Enzym *Mlu*I das ITS-PCR Produkt verdauen. Von den drei Restriktionsenzymen *Hinc*II, *Sca*I und *Tfi*I verdaute nur das *Tfi*I das IGS1-Produkt des Ölpalmenpathogens *Ganoderma*. Im amplifizierten ITS-DNA-Bereich konnte anhand der DNA-Sequenz von insgesamt 31 veröffentlichten *Ganoderma* Isolaten keine *Mlu*I-Restriktionsschnittstelle gefunden werden. Eine Verwendung der Kombination aus PCR-Amplifikation und Restriktionsanalyse können als Standardprotokoll zum Nachweis von *Ganoderma* Feld-Isolaten dienen, die dann als Pathogene angesehen werden können und somit auch gesunde Ölpalmen infizieren.

Das phyllogenetische Dendrogramm zeigte, daß das Ölpalmenpathogen *Ganoderma* ein Cluster mit dem Isolat *Ganoderma boninense* LKM bildet, sich aber bei der ITS-DNA-Sequenz und dem Mn-SOD Gen vom Isolat *Ganoderma boninense* RSH RS unterschied. Sequenzanalysen des ITS Bereiches und des Mn-SOD Gens zeigten, daß eine hohe Übereinstimmung der DNA-Sequenz mit dem Isolat *Ganoderma boninense* LKM (zwischen 94,2 und 98,6% bei der ITS1-Region, zwischen 90,7 und 99,5 % bei der ITS2-Region und 96,5 bis 98,2% bei dem Mn-SOD Gen) besteht. Auf der anderen Seite hat das Ölpalmenpathogen *Ganoderma* eine geringere Sequenz-Übereinstimmung zum Isolat *Ganoderma boninense* RSH RS (zwischen 80,1 und 82,1% bei der ITS1-Region, zwischen 61,6 und 73,2 % bei der ITS2-Region und 86,3 bis 87,0% bei dem Mn-SOD Gen). So wurde wohl das Isolat *Ganoderma boninense* RSH RS in der Vergangenheit falsch benannt, da es nicht mit Palmen (Moncalvo, persönliche Mitteilung) assoziiert ist. Für den Fall *Ganoderma boninense* RSH RS konnte gezeigt werden, daß die durchgeführten molekularen Untersuchungen so exakt sind, daß nur noch diejenigen *Ganoderma* Spezies identifiziert werden, die auch pathogen für Ölpalmen sind.

In dieser Promotionsarbeit konnte gezeigt werden, daß molekulare Techniken, wie der Einsatz von Spezies-spezifischen Primern, gewonnen aus variablen DNA-Sequenzen, die PCR-RFLP Analyse der rDNA mit spezifischen Enzymen, sowie dem DNA-Sequenzvergleich zur Herstellung eines phyllogenetischen Dendrogramms und zur Identifizierung, sowie Differenzierung ölpalmenpathoger *Ganoderma* Isolate von anderen in dieser Arbeit untersuchten *Ganoderma* Isolaten verwendet werden konnten. So lassen sich nun ölpalmenpathogene *Ganoderma* Isolate, Verursacher der BSR Krankheit an Ölpalmen, eindeutig nachweisen und gehören somit einer einzigen Spezies an.

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8 Appendixes

Table 1A: *Ganoderma* DNA concentration and purity

Ganoderma species	DNA conc. (ng/μl)	DNA purity (OD_{260}/OD_{280})
Ganoderma BS	357.0	1.87
Ganoderma AP	294.6	1.89
Ganoderma SP	361.6	2.07
Ganoderma PM	255.8	1.67
Ganoderma Ad	559.0	1.71
Ganoderma Mt	668.1	2.06
Ganoderma SB	227.3	1.80
Ganoderma BJ 7	475.7	1.77
Ganoderma BJ 8	342.9	1.87
Ganoderma GB	560.5	1.82
Ganoderma BB	576.3	1.74
Ganoderma BL	518.0	1.84
Ganoderma SM	259.5	1.67
Ganoderma Lo	508.1	1.70
Ganoderma BM	470.4	1.92
Ganoderma Ti	460.6	1.73
Ganoderma Ma	646.5	1.81
Ganoderma DS	420.3	1.76
Ganoderma Pa	292.2	1.68
Ganoderma DH	399.2	1.78
G. boninense PNG-611	355.6	1.79
G. boninense PNG-598	276.0	1.88
G. boninense PNG-579	435.6	2.09
G. boninense PNG-576	278.9	1.92
G. boninense PNG-600	507.9	1.89
G. boninense PNG-605	211.3	1.81
G. boninense PNG-603	321.7	1.83
G. boninense PNG-597	376.9	1.67
G. boninense PNG-753	455.6	1.87
G. boninense PNG-742	199.8	1.77
G. boninense PNG-403	511.5	1.89
G. boninense PNG-407	356.8	1.79
G. boninense PNG-410	277.7	1.88
G. boninense PNG-411	332.1	1.90
G. boninense PNG-219	366.0	2.01
G. boninense PNG-381	422.6	1.87
G. boninense PNG-240	254.7	1.92
G. tornatum PNG-372	379.0	1.82
G. tornatum PNG-343	226.1	1.69
G. tornatum PNG-264	271.3	1.77
G. tornatum PNG-283	306.6	1.85
G. tornatum PNG-369	435.0	1.91
G. tornatum PNG-371	233.5	1.72
G. tornatum PNG-344	256.0	1.79
G. tornatum PNG-306	388.0	1.89

Continued

Table 1A: *Ganoderma* DNA concentration and purity

Ganoderma species	DNA conc. (ng/μl)	DNA purity (OD_{260}/OD_{280})
G. tornatum PNG-309	189.0	1.84
G. tornatum PNG-308	550.5	1.75
G. tornatum PNG-310	435.2	1.73
G. oerstedii BAFC 178	371.7	2.09
G. oerstedii BAFC 218	402.3	1.89
G. resinaceum BAFC 384	178.6	1.67
G. tornatum (applanatum?) BAFC 671	603.0	1.79
G. resinaceum BAFC 2288	518.8	1.85
G. applanatum BAFC 2353	273.0	1.99
G. lucidum complex BAFC 2374	432.7	2.11
G. tornatum BAFC 2390	241.6	1.89
G. tornatum? BAFC 2395	385.1	2.09
G. applanatum BAFC 2408	351.6	1.87
G. tornatum BAFC 2424	294.6	1.89
G. tornatum? BAFC 2430	426.0	1.94
G. resinaceum BAFC 2488	361.6	2.07
G. lucidum complex BAFC 2495	208.5	2.01
G. applanatum var. tornatum BAFC 2501	442.2	1.67
Ganoderma sp. BAFC 2529	698.4	1.62
G. applanatum BAFC 2552	252.2	1.65
G. tropicum BAFC 2580	258.0	1.94
G. resinaceum BAFC 2775	188.2	2.08
G. lucidum DSM 9612	205.5	1.78
G. lucidum DSM 103	256.4	1.84
G. applanatum DSM 3800	326.2	1.73
G. tsugae	481.6	1.76
Ganoderma sp. 136	357.5	1.82
G. applanatum 134	482.6	1.89
G. applanatum G 211	564.3	2.11
G. adspersum G 224	177.3	1.77
G. pfeifferi G 225	173.4	1.97
Ganoderma sp. DAR 73779	243.7	1.92
G. australe DAR 73781	345.8	1.83
G. incrassatum DAR 73783	411.3	1.76
G. cupreum DFP 4336	350.9	1.89
G. cupreum QFRI 8678.1	314.2	1.79
G. weberianum DFP 4483	277.9	1.89

Table 2A: Percent identities of the ITS1 region of oil palm *Ganoderma* compared with other *Ganoderma* species

Ganoderma	Percent Identity of ITS 1 (%)							
	Gano Ad	*Gano* AP	*Gano* BJ 7	*Gano* BJ 8	*Gano* BS	*Gano* GB	*Gano* Mt	*Gano* SB
*G.ad.*CBS 351.74	70.6	72.5	72.1	70.6	72.1	70.6	72.1	73.0
*G.ah.*FWP14329	81.9	81.4	80.9	81.9	81.4	81.9	81.4	76.0
*G.au.*0705	73.5	75.0	75.0	73.5	75.0	73.5	75.0	75.5
G.bo. RS	80.1	81.6	81.6	80.1	82.1	80.1	82.1	80.6
*G.co.*CBS216.36	75.3	76.3	75.3	75.3	75.8	75.3	75.8	76.3
*G.cu.*DFP3896	80.5	82.0	82.0	80.5	82.5	80.5	82.5	79.5
*G.cu.*DFP4336	82.3	83.7	83.7	82.3	84.2	82.3	84.2	81.3
*G.cu.*QFRI8678	82.3	83.7	83.7	82.3	84.2	82.3	84.2	81.3
*G.fo.*RSH0814	73.4	72.9	73.4	73.4	72.9	73.4	72.9	71.4
*G.fo.*0109	87.4	86.4	85.9	87.4	85.9	87.4	85.9	83.3
*G.gi.*ACCC5.151	74.6	76.1	76.1	74.6	76.1	74.6	76.1	75.6
*G.li.*BAFC2424	73.2	75.1	74.6	73.2	74.6	73.2	74.6	74.6
*G.lo.*BAFC2411	82.9	80.4	81.4	82.9	81.4	82.9	81.4	79.9
*G.lu.*ATCC32471	80.0	79.5	80.5	80.0	80.5	80.0	80.5	79.5
*G.lu.*BAFC2419	79.4	80.9	80.4	79.4	80.4	79.4	80.4	78.9
*G.mi.*0821	78.8	79.3	79.3	78.8	80.3	78.8	80.3	78.3
*G.oe.*ATCC52410	81.6	81.1	80.1	81.6	79.6	81.6	79.6	76.6
*G.or.*CBS177.30	81.3	80.8	79.8	81.3	79.3	81.3	79.3	76.4
*G.pf.*CBS747.84	75.0	76.0	76.0	75.0	76.5	75.0	76.5	73.5
*G.pl.*BAFC384	77.5	78.9	78.9	77.5	79.4	77.5	79.4	77.9
*G.re.*CBS194.76	77.6	76.1	77.6	77.6	78.0	77.6	78.0	73.2
*G.se.*BAFC2373	77.5	78.9	78.9	77.5	79.4	77.5	79.4	77.9
*G.se.*BAFC2580	75.0	76.0	76.0	75.0	76.5	75.0	76.5	74.5
*G.si.*Zhang1734	87.1	86.1	85.6	87.1	85.6	87.1	85.6	81.6
*G.su.*ATCC52419	72.5	73.5	73.5	72.5	74.0	72.5	74.0	72.1
*G.to.*BAFC1139	73.9	75.9	75.4	73.9	75.4	73.9	75.4	75.4
*G.to.*BAFC1172	73.2	75.1	74.6	73.2	74.6	73.2	74.6	74.6
*G.tr.*HK93-8	74.9	74.4	74.9	74.9	74.9	74.9	74.9	72.4
*G.ts.*CBS428.84	77.9	77.5	76.5	77.9	76.0	77.9	76.0	73.0
*G.tu.*BAFC2414	76.5	77.5	77.5	76.5	77.9	76.5	77.9	75.5
*G.va.*CBS282.33	81.3	80.8	79.8	81.3	79.3	81.3	79.3	76.4
*G.we.*CBS219.36	78.8	79.3	79.3	78.8	80.3	78.8	80.3	78.3
*G.zo.*BAFC2374	78.2	79.7	79.7	78.2	80.2	78.2	80.2	78.7
Gano PNG	99.5	99.0	99.5	99.5	99.5	99.5	99.5	94.7
G.bo LKM	98.6	98.1	98.6	98.6	98.6	98.6	98.6	94.2
Gano Ad	--	98.1	99.0	100.0	99.0	100.0	99.0	93.8
Gano AP		--	98.6	98.1	98.6	98.1	98.6	93.8
Gano BJ 7			--	99.0	99.0	99.0	99.0	94.7
Gano BJ 8				--	99.0	100.0	99.0	93.8
Gano BS					--	99.0	100.0	94.2
Gano GB						--	99.0	93.8
Gano Mt							--	94.2

Percent identities of ITS1 region among other *Ganoderma* species were not stated in this table

Table 3A: Percent identities of the ITS2 region of oil palm *Ganoderma* compared with other *Ganoderma* species

Ganoderma	Percent Identity of ITS 2 (%)							
	Gano Ad	*Gano* AP	*Gano* BJ 7	*Gano* BJ 8	*Gano* BS	*Gano* GB	*Gano* Mt	*Gano* SB
*G.ad.*CBS 351.74	74.2	73.2	74.2	74.2	70.5	74.2	74.2	70.5
*G.ah.*FWP14329	69.5	70.0	70.5	70.5	61.6	70.5	69.5	62.6
*G.au.*0705	75.3	74.2	76.3	76.3	68.9	76.3	75.3	69.9
G.bo. RS	72.1	72.6	73.2	73.2	61.6	73.2	72.1	62.6
*G.co.*CBS216.36	70.7	71.2	70.2	70.2	62.3	70.2	70.7	61.8
*G.cu.*DFP3896	76.8	75.8	76.8	76.8	72.4	76.8	76.8	72.4
*G.cu.*DFP4336	76.3	75.3	76.3	76.3	71.2	76.3	76.3	71.2
*G.cu.*QFRI8678	77.3	76.3	77.3	77.3	72.2	77.3	77.3	72.2
*G.fo.*RSH0814	71.4	72.0	70.9	70.9	61.9	70.9	71.4	61.4
*G.fo.*0109	72.7	71.6	72.2	72.2	65.8	72.2	72.7	65.3
*G.gi.*ACCC5.151	71.1	70.1	71.6	71.6	69.5	71.6	71.1	70.1
*G.li.*BAFC2424	82.0	81.4	83.0	83.0	72.7	83.0	82.0	73.7
*G.lo.*BAFC2411	74.0	73.4	75.0	75.0	64.6	75.0	74.0	65.6
*G.lu.*ATCC32471	66.8	67.4	67.4	67.4	56.7	67.4	66.8	57.2
*G.lu.*BAFC2419	67.7	68.3	67.2	67.2	60.3	67.2	67.7	59.8
*G.mi.*0821	71.4	72.0	71.4	71.4	61.4	71.4	71.4	61.4
*G.oe.*ATCC52410	74.3	74.9	75.4	75.4	64.4	75.4	74.3	65.4
*G.or.*CBS177.30	74.5	75.0	75.5	75.5	65.1	75.5	74.5	66.1
*G.pf.*CBS747.84	66.3	66.8	67.4	67.4	58.5	67.4	66.3	59.6
*G.pl.*BAFC384	68.0	68.6	69.1	69.1	58.8	69.1	68.0	59.8
*G.re.*CBS194.76	70.8	71.4	71.9	71.9	60.9	71.9	70.8	62.0
*G.se.*BAFC2373	68.6	69.1	69.6	69.6	59.3	69.6	68.6	60.3
*G.se.*BAFC2580	69.6	70.2	70.7	70.7	60.2	70.7	69.6	61.3
*G.si.*Zhang1734	73.7	72.7	73.2	73.2	66.2	73.2	73.7	65.7
*G.su.*ATCC52419	69.3	69.8	70.4	70.4	59.8	70.4	69.3	60.8
*G.to.*BAFC1139	82.8	82.3	83.9	83.9	73.4	83.9	82.8	74.5
*G.to.*BAFC1172	82.0	81.4	83.0	83.0	72.7	83.0	82.0	73.7
*G.tr.*HK93-8	70.1	70.6	69.6	69.6	60.2	69.6	70.1	59.7
*G.ts.*CBS428.84	74.5	75.0	75.5	75.5	65.1	75.5	74.5	66.1
*G.tu.*BAFC2414	68.9	69.5	70.0	70.0	60.0	70.0	68.9	61.1
*G.va.*CBS282.33	69.1	69.6	69.1	69.1	58.4	69.1	69.1	58.4
*G.we.*CBS219.36	71.6	72.2	71.6	71.6	60.9	71.6	71.6	60.9
*G.zo.*BAFC2374	72.1	72.6	73.2	73.2	61.6	73.2	72.1	62.6
Gano PNG	96.4	95.9	96.9	96.9	92.8	96.9	96.4	93.3
G.bo LKM	99.0	98.5	99.5	99.5	90.2	99.5	99.0	90.7
Gano Ad	--	99.5	99.5	99.5	91.2	99.5	100.0	90.7
Gano AP		--	99.0	99.0	90.7	99.0	99.5	90.2
Gano BJ 7			--	100.0	90.7	100.0	99.5	91.2
Gano BJ 8				--	90.7	100.0	99.5	91.2
Gano BS					--	90.7	91.2	99.5
Gano GB						--	99.5	91.2
Gano Mt							--	90.7

Percent identities of ITS2 region among other *Ganoderma* species were not stated in this table

Table 4A: Percent identities of partial Mn-SOD gene of oil palm *Ganoderma* compared with other *Ganoderma* species

Ganoderma species	Percent identity of Mn-SOD gene		
	Ganoderma AP	*Ganoderma* BJ 8	*Ganoderma* BS
G. adspersum CBS 351.74	87.0	88.1	87.4
G. ahmadii FWP 14329	87.0	87.0	88.8
G. australe RSH 0705	86.3	87.0	87.4
G. capense ACCC 5.71	85.3	86.3	86.7
G. formicatum RSH 0814	84.9	86.0	86.3
G. formosanum RSH 0109	88.8	90.2	89.8
G. lucidum ATCC 32471	84.2	84.9	85.6
G. lucidum RYV 33217	87.4	86.7	88.4
G. lucidum ACCC 5.65	86.0	86.0	87.0
G. lucidum CBS 270.81	86.0	86.0	87.0
G. lucidum CBS 430.84	86.0	87.0	87.4
G. lucidum HMAS 60537	87.0	87.0	88.1
G. lucidum RSH 0626	84.6	85.3	86.0
G. microsporum RSH 0821	85.3	86.3	86.7
G. oerstedii ATCC 52410	86.0	86.0	87.0
G. oregonense CBS 177.30	85.6	85.6	86.7
G. resinaceum CBS 152.27	86.0	87.0	87.4
G. tropicum RSH 1111	85.6	86.3	86.7
G. tsugae RSH 1109	85.6	86.3	86.7
G. tsugae RSH H2	87.4	87.4	88.4
G. valesiacum CBS 282.33	87.0	87.0	88.8
G. weberianum CBS 219.36	86.3	87.4	87.7
G. boninense RSH RS	86.3	87.0	87.0
G. boninense LKM	98.2	96.5	96.8
Ganoderma AP	-	95.8	96.1
Ganoderma BJ 8	-	-	96.8

Percent identities of partial Mn-SOD gene among other *Ganoderma* species were not stated in this table

Table 5A: Percent identities of IGS1 of oil palm *Ganoderma* compared with other *Ganoderma* species

Ganoderma species	Percent identity of IGS1 (%)			
	Ganoderma BJ8	*Ganoderma* Lo	*Ganoderma* PM	*Ganoderma* SB
G. adspersum G224	64.7	62.2	65.2	65.5
G. applanatum DSM 3800	65.7	72.7	63.7	71.0
G. applanatum G211	66.0	59.2	61.7	67.0
G. australe DAR 73781	71.9	66.9	71.6	72.6
G. cupreum DFP 4336	73.5	72.2	70.8	73.5
G. cupreum QFRI 8678	73.5	72.9	70.5	72.9
G. pfeifferi G225	66.3	66.3	65.9	66.6
G. incrassatum DAR 78783	70.1	67.9	68.8	66.1
Ganoderma sp. DAR 73779	68.1	59.8	60.9	68.9
Ganoderma BJ8		97.1	93.3	95.6
Ganoderma Lo			94.3	94.3
Ganoderma PM			-	96.6

Percent identities of IGS1 among other *Ganoderma* species were not stated in this table

Curriculum Vitae

Name	Condro Utomo
Date of birth	19. 01. 1959
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Education	1975 – 1977	St. Albertus High School, Malang, Indonesia
	1978 – 1983	Bachelor of Agricultural Sciences, Department of Plant Pests and Diseases, Faculty of Agriculture, Bogor Agricultural University, Bogor, Indonesia
	1995 – 1997	Master of Agricultural Sciences, The Faculty of Agricultural Sciences, Georg-August-University Göttingen, Germany
	1998 – 2001	Doctoral research in Biologische Bundesanstalt, Braunschweig and Institute of Plant Breeding and Plant Protection, Martin-Luther-University Halle-Wittenberg, Germany
	2001 – present	Postgraduate studies at the Institute of Plant Breeding and Plant Protection, Martin-Luther-University Halle-Wittenberg, Germany

Research Institute Appointment

1985 – present	Researcher, Indonesia Oil Palm Research Institute (IOPRI), Medan, Indonesia

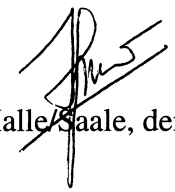
Eidesstattliche Erklärung

Ich versichere hiermit, dass ich die vorliegende Dissertation

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

selbständig angefertigt und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Condro Utomo



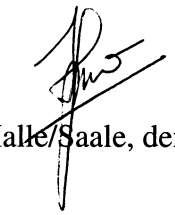
Halle/Saale, den 6 Mai 2002

Erklärung

Hiermit erkläre ich, dass mit dieser wissenschaftlichen Arbeit noch keine vergeblichen Promotionsversuche unternommen wurden.

Des weiteren erkläre ich, dass keine Strafverfahren gegen mich anhängig sind.

Condro Utomo



Halle/Saale, den 6 Mai 2002