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

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## 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. chalconeum* (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<sub>2</sub> 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 basidiomycete 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.