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THE USE OF LACCASE GENE INTRONS TO DESIGN SPECIFIC PRIMER FOR *GANODERMA* ISOLATED FROM OIL PALM IN ORDER TO DIFFERENTIATE OTHER *GANODERMA*

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ABSTRACT

Evaluation of the primer pairs designed from the nucleotide sequence corresponding to the amino acid sequence ILHCHI of laccase gene as reverse primer Lac 2b 2 (5`GATGTGGCAGTGGAGGAT3`) and the introns of laccase gene as forward primer was tested for the specificity for Ganoderma *isolated from oil palm. PCR amplification by using primer pairs of Lac 2b 2-Intron 5 (5`TATTCCCTCCTCCCCTGT3`) and Lac 2b 2-Intron 2 (5`GAAGGCCTCCAAGA CAAG 3`) produced a single band with PCR product about 1012 bp and 213 bp for* Ganoderma *isolated from oil palm and no PCR product any other* Ganoderma *species tested, indicating that they are species-specific primers for oil palm* Ganoderma. *The primer pairs Lac 2b 2- Intron 1 (5`AGGGTCCAGGTACAGCAG 3`) and Lac 2b 2-Intron 9 (5`CTCATGGTAGT GCGCAG 3`) 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 Amplification with primer pair Lac 2b 2-Intron 4 (5`GTCCCCTTCGGATAA CAG 3`) indicated a cross-reaction by producing a faint band of about 508 bp for* G. applanatum *BAFC 2552 and* G. applanatum *BAFC 2408.*

Keywords: *specific primer*, *laccase gene*, Ganoderma

ABSTRAK

Evaluasi dari pasangan primer yang didesain dari daerah sekuen asam animo ILHCHI pada gen laccase sebagai primer reverse Lac 2b 2 5`GATGTGGC AGTGGAGG T3`) dan intron dari gen laccase sebagi primer forward dilakukan untuk menguji ke spesifikan terhadap Ganoderma *yang berasal dari kelapa sawit. Amplifikasi PCR dengan menggunakan pasangan primer Lac 2b 2-Intron 5 (5`TATTCCCTCCTCCCCTGT3`) dan Lac 2b 2-Intron 2 (5`GAAGGCCTCCAA GACAAG 3`) masing-masing menghasilkan produk PCR tunggal berukuran kira-kira 1012 bp dan 213 bp bila DNA dari* Ganoderma *yang berasal dari kelapa sawit digunakan dan tidak ada menghasilkan produk PCR bila spesies* Ganoderma *lainnya digunakan (non-kelapa sawit), hal ini menunjukkan kekhususan pasangan primer-primer tersebut terhadap* Ganoderma *asal kelapa sawit. Pasangan primer lainnya Lac 2b 2- Intron 1 (5`AGGGTCCAGGTACAGCAG 3`) and Lac 2b 2-Intron 9 (5`CTCATGGTAGTGCGCAG 3`) tidak menghasilkan PCR produk yang sama dengan* Ganoderma *yang berasal dari kelapa sawit tetapi menghasilkan produk PCR lainnya*

bila Ganoderma *non-kelapa sawit diamplifikasi, tetapi pasangan primer Lac 2b 2-Intron 4 (5`GTCCCCTTCGGATAA CAG 3`) menghasilkan produk PCR yang sama baik untuk* Ganoderma *yang berasal dari kelapa sawit maupun* Ganoderma *non-kelapa sawit seperti* G. applanatum *BAFC 2552 dan* G. applanatum *BAFC 2408 dengan produk PCR berukuran kira-kira 508 bp.*

Kata kunci: *primer spesifik, gen laccase*, Ganoderma

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is one of the most important estate crops in Indonesia, comprising 62 % of the total plantation which contributes 1.5 % to the GDP in 2004. In 1968, the total area under oil palm cultivation was only about 120,000 hectares but in 1999 the area had extended to 2.8 million hectares, an increase of almost 24 times in 32 years and by 2006, no less than 5.6 million ha oil palm has been planted and produced more than 13 million tones of crude palm oil (CPO) a year.

Basal stem rot (BSR) caused by *Ganoderma boninense* remains a major threat to sustainable oil palm production in South East Asia with significant yield losses through direct loss of the stand, reduced yield of diseased palms and requirement for early replanting. The BSR disease was first reported in Malaysia in 1930 and the causal agent was identified as *G. lucidum* (W.Curt.:Fr.) Karst (19)., Steyaert (17) in 1967 identified *Ganoderma* species associated with BSR in oil palms in Malaysia and Indonesia namely *G. boninense* Pat. At present, BSR disease spread over oil palm plantation in Indonesia especially in the second and subsequent planting cycles. 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.

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 (10). Another molecular approach was to develop a molecular diagnostic tool by exploiting the available sequence data of laccase gene from various basidiomycete fungi. Previous studies indicated that intron sequences of laccase genes in basidomycete fungi show a low homology within the genus (3). Laccase gene is one of the attractive targets for the construction of species-specific primers for molecular diagnosis of oil palm *Ganoderma* due to the genes contain relatively large number of introns, for example, the laccase genes from *Phlebia radiata* (9 introns) (15), *Coriolus hirsutus* (10 introns) (7), *Agaricus bisporus* (14 introns) (14), *Trametes villosa* (10 introns) (21), *Pycnoporus cinnabarinus* (10 introns) (4), *Coprinus cinereus* (10 introns) (22) and *Lentinula edodes* (13 introns) (23).

The aim of this research is to design specific primer from the conserved copper-binding region and the introns of the laccase gene of *Ganoderma* isolated from oil palm.

MATERIALS AND METHODS

Fungal isolates and DNA extraction

Ganoderma isolates from oil palm (from Bukit Sentang estate/BS) and other *Ganoderma* (Table 1) were grown in malt-yeast medium (2) for a month at 30 °C. 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. Total genomic DNA of each *Ganoderma* isolate was extracted according to the method of Moeller *et al.* (9) with an additional phenol/chloroform extraction. The extracted DNA was quantified by UV spectrophotometry (Beckman DU-50 Spectrophotometer, Germany) and checked by agarose gel electrophoresis.

Primer design and polymerase chain reaction (PCR) conditions

To amplify partial laccace gene of *Ganoderma,* Primers were designed based on the conserved amino acid sequence in copper-binding region I 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* (accession number AAC41686), *T. villosa* (L49377), *T. villosa* (L78077), Basidiomycete CECT 20197 (AAB63443), Basidiomycete PM 1 (CAA78144), *Trametes versicolor* (CAA59161). Two 17- or 18-base sequences designed from DNA sequences of the conserved amino acid sequences of copper-binding region are primers Lac 2a (5`TGGCACGGCTTCT TCCAG 3`) and Lac 2r (5`CACTGCCA CATCGACTTC 3`). To clone and sequence of partial laccase gene of *Ganoderma* isolated from oil palm is as described by Utomo (20). PCR conditions to test the specificity of the primers were carried out as described by Niepold and Schoeber-Butin (12). Composition of buffer, nucleotide mix, primers and *Taq* polymerase was described as follow: for PCR amplification, 5 µl of the extracted DNA (100 ng) was added to 20 ∝l 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 µCR products were either analysed immediately or stored at -20 °C.

Table 1. Source and Host of *Ganoderma* used in this study

Ganoderma species	Source	Host
Oil palm *Ganoderma* (Indonesia), 20 isolates	IOPRI	Oil palm
G. boninense (oil palm, PNG), 10 isolates	OPRA	Oil palm
G. boninense (coconut stump, PNG), 7 isolates	OPRA	Coconut
G. tornatum (hardwood, PNG), 11 isolates	OPRA	Hardwood
G. oerstedii BAFC 178	U.B.A	Unknown
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 BAFC 2353	,,	*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 DSMZ 9612	DSMZ	Dead wood
G. applanatum DSMZ 3800	DSMZ	*Salix humboldtiana*
G. tsugae	FAL	Unknown
G. applanatum 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
G. australe DAR 73781	CSIRO	Unknown
G. incrassatum DAR 73783	CSIRO	Stump rainforest
G. cupreum DFP 4336	CSIRO	*Banksia seminuda*
*Ganoderma sp.*Group 6.3 DAR 73779	CSIRO	*Albizia lebbeck*
G. weberianum DFP 4483	CSIRO	*Casuarina* sp

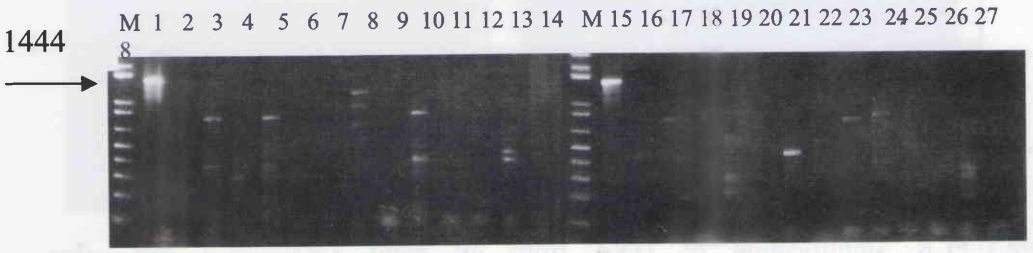
Source: IOPRI Indonesian Oil Palm Research Institute, Medan, Indonesia
U.B.A Universidad De Buenos Aires, Argentina (gifts of Dr. A. M.Gottlieb)
DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany
FAL Bundesforschungsanstalt für Landwirtschaft, Braunschweig, Germany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

RESULTS

By using the primer pair Lac 2a-Lac 2r, *Ganoderma* isolated from oil palm produced a single PCR product of about 1,650 bp, sequence of the PCR product as described by Utomo (20). Primer Lac 2b 2 (5`GATGTGGCAGTGGAGG AT3`) 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`CTCATGGTAGTTGCG CAG 3`), Intron 1 (5`AGGGTCCA GGTACAGCAG 3`), Intron 2 (5`GAAG GCCTCCAAGACAAG 3`), Intron 4 (5`GTCCCCTTCGGATAACAG 3`) and Intron 5 (5`TATTCCCTCCTCCCCTGT 3`). 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* (Figure 1). 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 (Figure 1). 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 (Figure 1). The results of the PCR amplification using five primer pairs for oil palm *Ganoderma* and other *Ganoderma* species studied are summarized in Table 2.

A



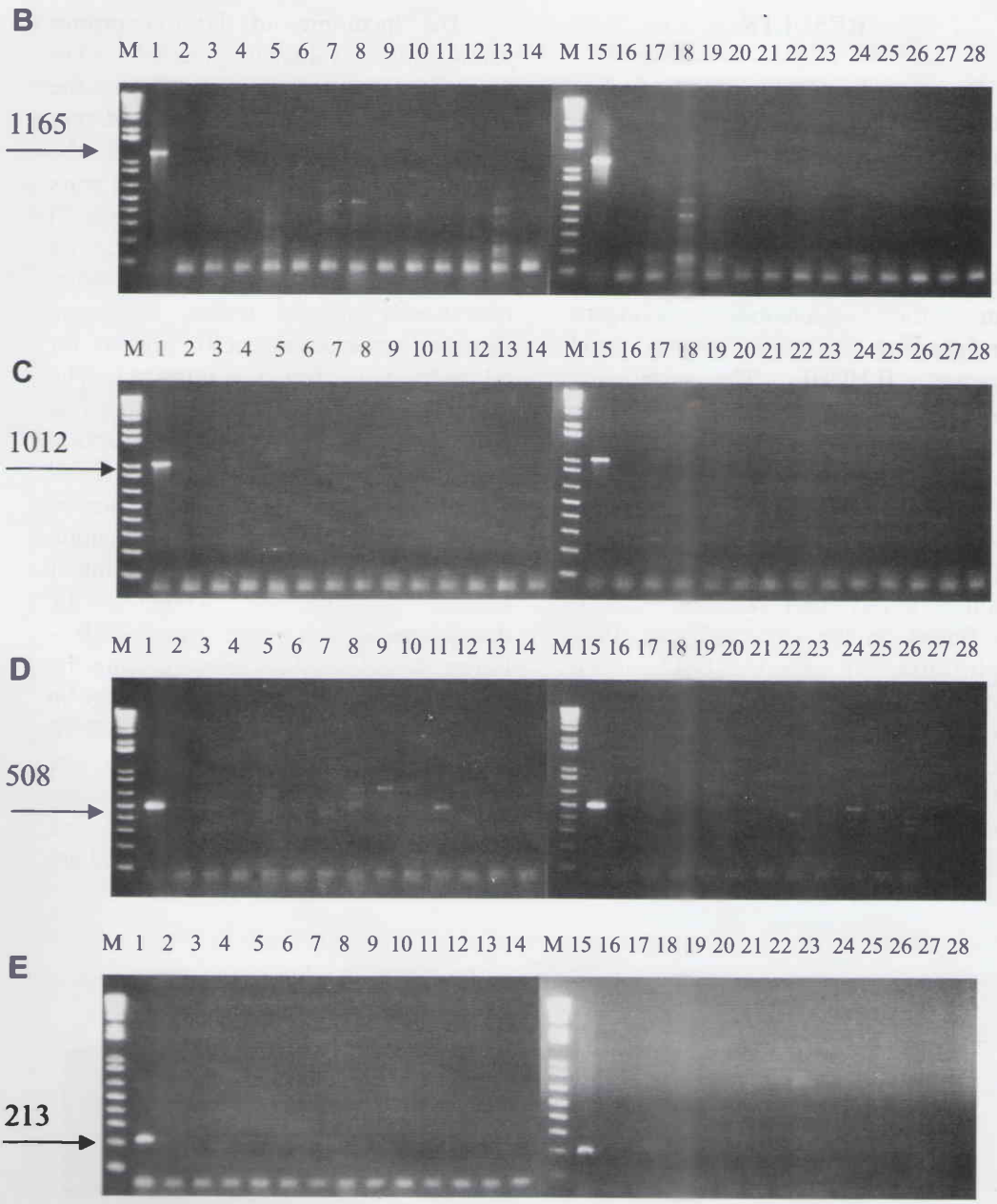


Figure 1: PCR amplification of DNA from oil palm *Ganoderma* and other *Ganoderma* species using the primer Lac 2b 2 combined with primers designed from intron A, B, C, D and E: Results of PCR amplification using the primer pairs Lac2b2 with Intron 9, Intron 1, Intron 2, Intron 4 and Intron 5

Table 2: 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

DISCUSSION

Since introns have potentially high rates of sequence evolution, their analysis has become an important tool in studies of evolutionary relationships among species (16). 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 (11,13,18). The reason for the existence and distribution of introns is debated continuously and whether these intervening sequences have a function remains unclear (5,8).

In this study, introns in laccase gene fragments of *Ganoderma* isolated from oil palm were targeted to discriminate between oil palm *Ganoderma* and other *Ganoderma* species studied. From the introns of the laccase gene of *Ganoderma* isolated from oil palm, 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.

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