Jurnal Penelitian Kelapa Sawit, 2005, 13(2): 65 - 75

PHYLOGENETIC ANALYSIS OF PATHOGENIC *GANODERMA* IN OIL PALM BASED ON MANGANESE SUPEROXIDE DISMUTASE (Mn-SOD) GENE

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ABSTRACT

Primer sets of Mn-SOD 1 (5`CTCCACCACAAGAAGCACCAC`3) and Mn-SOD 2 (5`GAAGGCGTGCTCCCAGATGTC`3) were designed from nucleotide sequences of amino acid sequence regions LHHKKHH and DIWEHAF in the Mn-SOD gene of G. boninense *RSH RS was used to amplify Mn-SOD gene of pathogenic* Ganoderma *isolated from oil palm. Polymerase chain reaction (PCR) with these primers amplified a single PCR product of about 700 bp for pathogenic* Ganoderma *isolated from oil palm. 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. 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.*

Keywords: *Phylogenetic tree, oil palm,* Ganoderma, *Mn-SOD gene*

ABSTRAK

Pasangan primer Mn-SOD 1 (5`CTCCACCACAAGAAGCACCAC`3) and Mn-SOD 2 (5`GAAGGCGTGCTCCCAGATGTC`3) yang didesain dari sekuen nukleotida pada asam amino LHHKKHH and DIWEHAF dari gen Mn-SOD dari G. boninense *RSH RS digunakan untuk mengisolasi gen Mn-SOD* Ganoderma *asal kelapa sawit. Primer ini dalam polymerase chain reaction menghasilkan produk PCR berukuran kira-kira 700 pb. Metoda multiple sequence alignment digunakan untuk mengkonstruksi pohon kekerabatan dan sebagai hasilnya menunjukkan bahwa* Ganoderma *asal kelapa sawit mengelompok pada* G. boninense *LKM. Identitas dari ketiga* Ganoderma *asal kelapa sawit jika dibandingkan dengan* G. boninense *LKM berkisar antara 96.5 - 98.2 %. Secara keseluruhan identitas dari ketiga* Ganoderma

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asal kelapa sawit berkisar antara 84.2-90.2 % jika dibandingkan dengan seluruh Ganoderma *yang diuji.*

Kata Kunci: *Pohon kekerabatan, kelapa sawit,* Ganoderma, *gen Mn-SOD*

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is one of the most important estate crops in Indonesia. 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 the early 1916, in North Sumatra recorded 1,272 ha, in 1984 it was reported 479,048 ha and in 2003 total area of oil palm in North Sumatra was 3,712,878 ha or 75 % of total area of oil palm in Indonesia (4,926,080 ha).

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 (13), Bazzalo and Wright (6), and Adaskaveg and Gilbertson (2,3), but these attempts caused more confusion because they were often quite different from classical identifications based on morphological features. For example, Nobles (13) 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* (14). Biochemical and molecular parameters like isozymes (8,10,16) and random amplification of polymorphic DNA (RAPD) (1,15) were applied. Results of isozymes and RAPDs are difficult to interpret for differentiation among *Ganoderma* species because they produced variable electrophoretic patterns.

Although Mn-SOD genes 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 of oil palm pathogenic *Ganoderma* with other *Ganoderma*, Mn-SOD genes have been chosen for taxonomic and phylogenetic studies because sequence data are available through public access databases such as GenBank and European Molecular Biology Laboratory (EMBL).

The objective of this research was to develop molecular characterization for pathogenic *Ganoderma* in oil palm based on phylogenetic analyses in Manganese superoxide dismutase (Mn-SOD) gene.

MATERIALS AND METHODS

Ganoderma isolates and DNA extraction

Three *Ganoderma* of oil palm isolated from Aek Pancur (AP), Bah Jambi (BJ 8) and Bukit Sentang (BS)

estates were used in this study. All isolates were grown in malt-yeast medium (7) 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 Möller *et al.* (11) 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.

Primers design and PCR conditions

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. PCR procedure was carried out according to the method described by White *et al.* (17). 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 61 °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. The PCR products were either analysed immediately or stored at -20 °C.

Cloning and Sequencing

To clone the Mn-SOD gene, three *Ganoderma* isolated from oil palm were used. PCR products were purified using QIAquick PCR purification kit (Qiagen, Germany) according to the manufacturer`s instructions. After purification, PCR products were cloned in plasmid vector of PCR$^R$ 2.1-TOPO from TOPO TA cloning kit (Invitrogen, Netherlands) according to the manufacturer`s instructions. The cloned DNA fragments were sequenced on both strands using forward and reverse universal primers M13. Ready Reaction BigDye Terminator Cycle Sequencing kit (perkin Elmer Corp., USA) was used to sequence of the IGS1 regions. The sequence was determined using an ABI prism 310 DNA sequencer (Applied Biosystem Inc., USA).

Data analysis and phylogeny construction

For the Mn-SOD genes, computer-assisted comparisons of the nucleotide sequences were made to find the similarities of nucleotide sequences in NCBI/GenBank, using BLASTN program (4). Nucleotide sequences that encoded amino acid (exons) for 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 1 for the Mn-SOD gene. *Amauroderma rude* JMM ASP.1 (U56109) was used as outgroup for phylogeny reconstruction based on the Mn-SOD genes. Alignments of sequences were done using Clustal V algorithm method (MegAlign; DNAstar, Madison, USA). Percent identities, alignment reports and phylogenetic trees of Mn-SOD gene were calculated and constructed using MegAlign program (DNAstar, Madison, USA).

Table 1. 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)

RESULTS

Primer design

Primer Mn-SOD 1 (5` CTCCACCACAAGAAGCACCAC`3) and Mn-SOD 2 (5`GAAGGCGTGC TCCCAGATGTC`3) were designed from nucleotide sequences of amino acid sequence regions LHHKKHH and DIWEHAF in the Mn-SOD gene of *G. boninense* RSH RS. 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 Figure 1. The first intron showed more conserved and shorter nucleotide sequences compared to the second intron.

Deduced exons 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 (5,9). For a better visualization, the deduced exon and intron sequences are presented as capital and lowercase characters, respectively, as shown in Figure 2. The deduced exon sequences were translated to the predicted amino acid sequences by using EditSeq program (DNAstar, Madison, USA).

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 (Figure not shown).

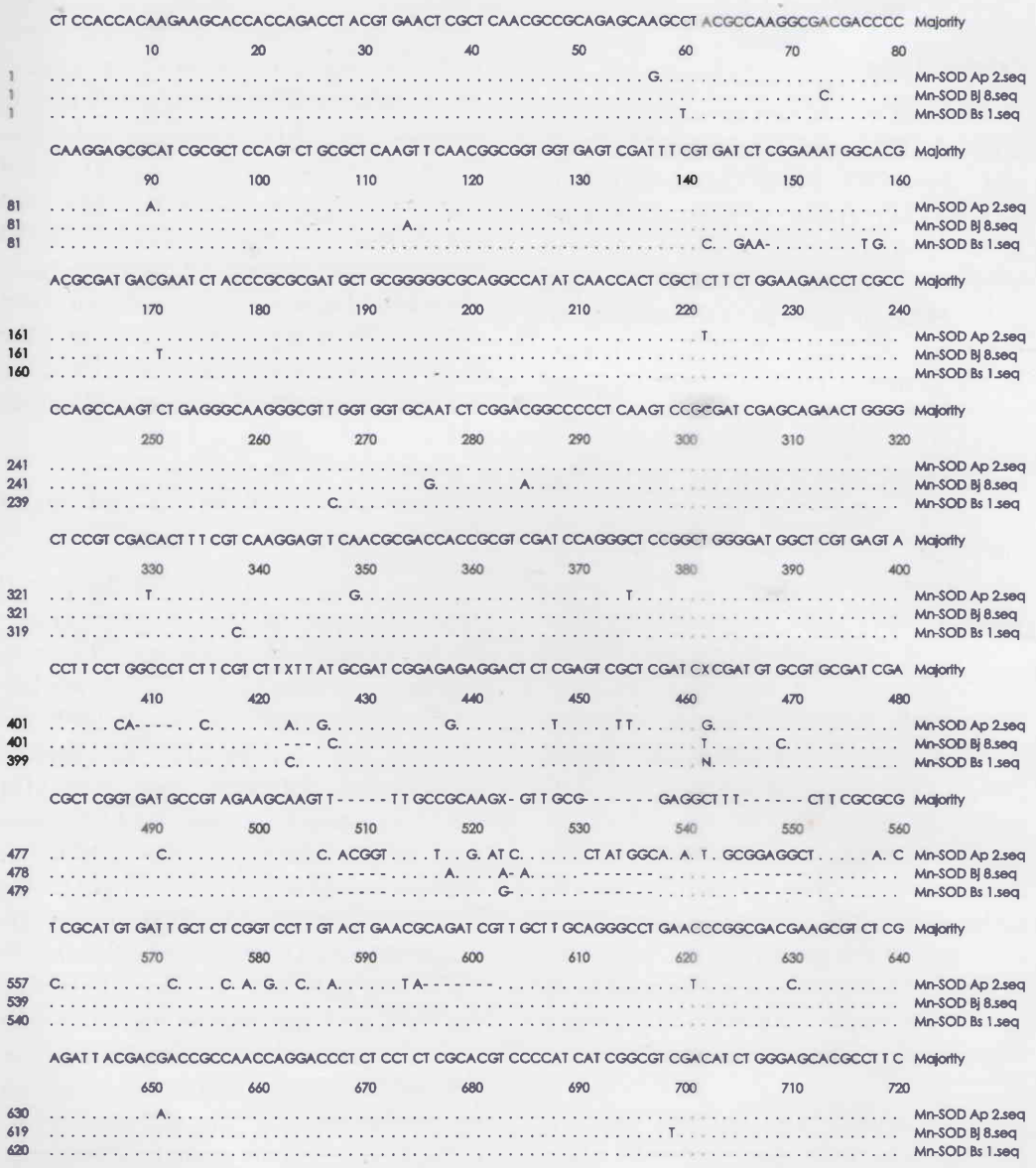

Figure 1. Nucleotide sequence alignment of the partial Mn-SOD gene from 3 oil palm trees *Ganoderma* (isolates AP, BJ 8 and BS). Dashes (-) indicate gaps and X indicates a base that could not be determined

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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

ATCCAGGGCTCCGGCTGGGGATGGCTC gtgagtaccttcctggccctcttcgtcttcttatgcgatcggagagaggactctcgagtcgctc  458
I  Q  G  S  G  W  G  W  L

gatcncgatgtgcgtgcgatcgacgctcggtgatgccgtagaagcaagttttgccgcaagggttgcggaggctttcttcgcgcgtcgcatgtgattgctctcgg  562

tccttgtactgaacgcagatcgttgcttgcagGGCCTGAACCCGGCGACGAAGCGTCTCGAGATTACGAC GACCGCCAAC  642
                                G  L  N  P  A  T  K  R  L  E  I  T  T  T  A  N

CAGGACCCTCTCCTCTCGCACGTCCCCATCATCGGCGTCGAC ATCTGGGAGCACGCCTTC                699
Q  D  P  L  L  S  H  V  P  I  I  G  V  D  I  W  E  H  A  F
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Figure 2. 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

Multiple sequence were used to infer a phylogenetic tree and the generated tree showed that all oil palm *Ganoderma* isolates clustered with G. *boninense* LKM (Figure 3). 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 2).

Table 2. 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

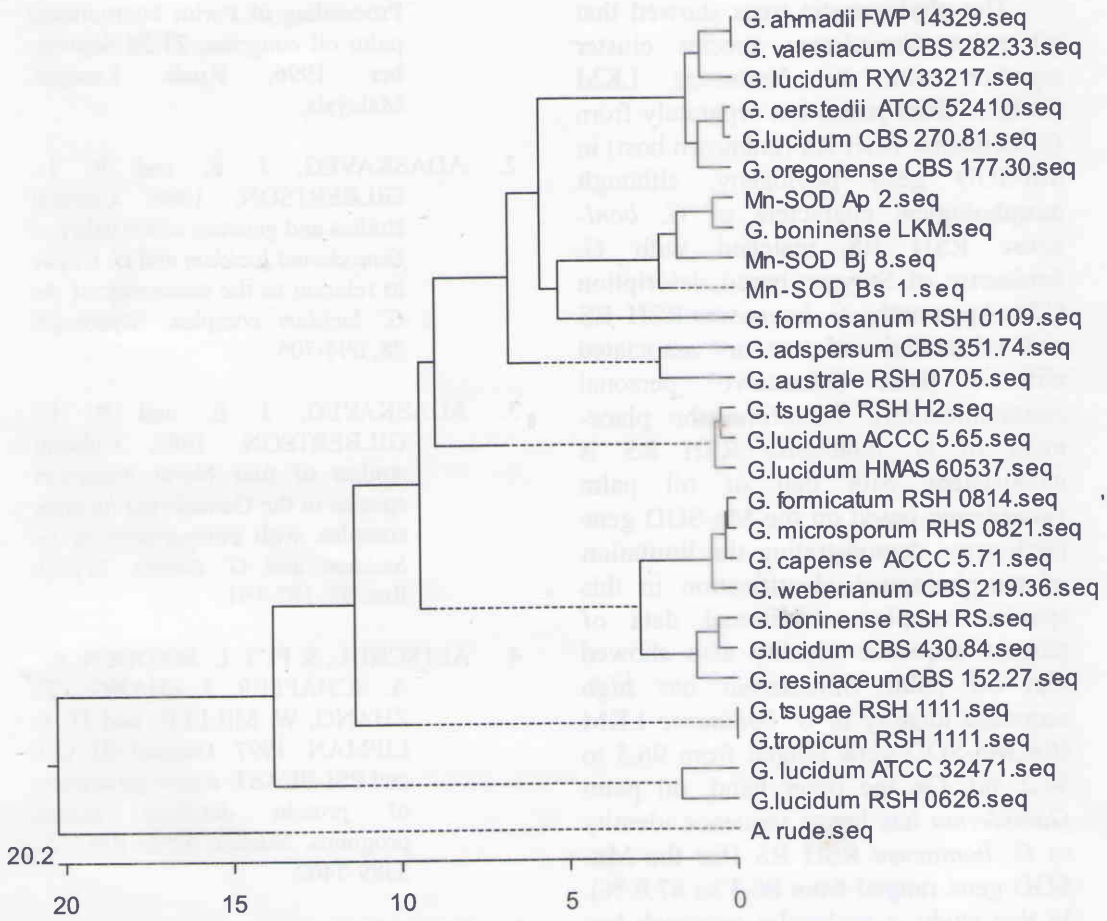

Figure 3: Phylogenetic relationships of *Ganoderma* species inferred from the nucleotide sequences of the Mn-SOD genes.

DISCUSSION

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.

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 Mn-SOD gene phylogeny, although morphological characters of *G. boninense* RSH RS matched with *G. boninense* of Steyaert-based description (12). 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 Mn-SOD gene phylogeny, 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 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 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.

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