The First Report on Basal Stem Rot Disease Causal Pathogen in Asian Agri Group, North Sumatra, Indonesia

Dedek Haryadi[1,2], Hadi Hendra[2], Manjit Singh Sidhu[2], Tumpal Panjaitan[2], Khim Phin Chong[1,3#]

1 Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, MALAYSIA.
2 Asian Agri Research and Development Centre, Bahilang, Tebing Tinggi, North Sumatra, INDONESIA.
3 FGV Chair of Sustainable Oil Palm Management, Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, UMS Sandakan Campus, Mile 10, Sg. Batang, 90000 Sandakan, Sabah, MALAYSIA.
Corresponding author. E-Mail: chongkp@ums.edu.my; Tel: +6088-320000 Ext. 5655; Fax: +6-088-435324.

ABSTRACT Basal stem rot (BSR) is the most serious oil palm disease in Asian Agri Group's estates in North Sumatra, Indonesia. Up to 2017, a total of 30 palms per hectare (22% per hectare) in Asian Agri Group have been killed by the disease and caused a serious threat to the oil palm sustainability. Whether, BSR is caused by the well known reported *Ganoderma boninense,* the causal pathogen of BSR is uncertain as no basic molecular identification of the causal pathogen has ever conducted in Asian Agri Group. The reports on the causal pathogen of this disease in Asian Agri Group were merely based on their morphology and pathogenicity. This paper is the first comprehensive report on the identification of the BSR causal pathogen from Asian Agri Group in North Sumatra. The identifcation was done using morphological characterization and DNA sequence analysis. The result showed that BSR causal pathogen of Asian Agri Group in North Sumatra was identified as *G. boninense* and closely related to the aggressive strain of *G. boninense* isolate GB001 with 99% of maximum silimarity based on phylogenetic analysis.

KEYWORDS: Basal stem rot; *Ganoderma boninense;* molecular identification, Asian Agri Group, North Sumatra Indonesia
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INTRODUCTION

Basal stem rot (BSR) is one of the most pathogenic diseases causing significant losses of oil palms in South-East Asia (Alexander *et al.,* 2014). BSR caused by several species of *Ganoderma* (Idris, 2009), it is widely spreads in both temperate and tropical regions of the world (Pilotti *et al*., 2004) and occurs predominantly as saprophytes on dead wood in the natural environment. Turner (1981) reported that *Ganoderma* spp. has a world-wide distribution of over 200 species and at least 15 species have been recorded to affect oil palm. Several species such as *G. boninense*, *G. zonatum* and *G. miniatocinctum* have been reported pathogenic to oil palm while *G. tornatum* is non-pathogenic (Moncalvo, 2000). As different species of *Ganoderma* exhibit different characteristics and aggressiveness, Idris and Ariffin (2004) reported that *G. boninense* was found to be the most common and virulent species than the other two (*G. zonatum* and *G. miniatocinctum*) in estates with high disease incidence of BSR in Malaysia. In Indonesia, Susanto (2009) also reported *G. boninense* to be the major *Ganoderma* species attacking oil palm.

BSR is also the most serious disease of oil palm in Asian Agri Group's estates in North Sumatra, Indonesia. The disease has been particularly severe in first generation of oil palm plantings established on volcanic and peat soils. Up to 2017, a total of 30 palms per hectare (22% per hectare) have been killed by the BSR disease in Asian Agri Group plantation (Asian Agri R&D internal report, 2018). Apart from a significant decline in yield, the high loss of palm stand has significantly shortened the economic life-span of the oil palm plantings necessitate premature replanting. This has becomes a serious threat to the oil palm plantations sustainability of Asian Agri Group. Identification of *Ganoderma* species that attack oil palm is important in order to understand and overcome BSR disease with a better strategy. However, Asian Agri Group in North Sumatra,

Indonesia has never perform molecular identification of the pathogen that caused BSR in its plantation. This paper is the first report on the identification of BSR disease causal pathogen from Negeri Lama Estate of, Asian Agri Group at North Sumatra, Indonesia.

METHODOLOGY

Isolation of BSR causal pathogen on Ganoderma selective medium (GSM)

The causal pathogen of BSR was obtained from *Ganoderma*-suspected fruiting bodies that found on the infected stem of oil palms from Negeri Lama Estate, Asian Agri Group, North Sumatra, Indonesia following the method as described by Ariffin and Idris (1992) with modification of the antibiotic involved. The internal tissue of fruiting bodies was excised and cultured on GSM. The GSM composition was prepared in two parts (part A and B). Part A consisted of 5 g of bacto peptone, 20 g of agar, 0.25 g of $MgSO_4.7H_2O$ (Merck), 0.5 g of K_2HPO_4 (Merck) and 900 ml of distilled water with pH 5.5. Part B comprised of 300 mg of streptomycin sulphate, 100 mg of chloramphenicol, 285 mg of pentachloronitrobenzene (PCNB), 130 mg of ridomil (25% WP), 150 mg of benlate T20, 20 ml of ethanol 95%, 2 ml of lactic acid 50%, 1.25 g of tannic acid and 80 ml of distilled water with pH 5.5. Part A was stirred on a hot plate at 100 °C until dissolved, and then autoclaved for 15 minutes. Meanwhile, part B was stirred for about two hours at room temperature (27 ± 2 °C). Subsequently, part B was added to part A when the autoclaved medium had cooled down to approximately 45-50 °C. The sterilized mixture was then poured into the petri dishes. The isolates were incubated at room temperature (27 ± 2 °C) for ± 7 days. Isolates which survived from GSM were transferred and maintained on potato dextrose agar (PDA) in petri dishes. The daily growth of mycelium was measured up to 11 days. For long term storage, the cultures were stored on PDA slant at 6 °C . The content of fungicides and antibiotics in GSM is optimal to control growth of bacteria and other contaminating fungi, while allowing *Ganoderma* to thrive.

Observation of BSR causal pathogen morphology

Morphological observations of the colonies and conidium-bearing structures were based on isolates grown on PDA. The typical morphological structures was observed under microscope (Olympus CX31) with 100x and 1000x magnifications and recorded with digital camera (Canon D30). In order to collect the spores, fruiting bodies were put on the sterile filter papers and left at room temperature (27 ± 2 °C) for one day. Spores which fallen on the sterile filter papers were then collected and mounted on the glass slides and added with 3% KOH. The spores were then observed under light microscope (Jargalmaa *et al.*, 2017).

DNA extraction of BSR causal pathogen

The BSR causal pathogen DNA was isolated using the modified mini protocol *Invitrogen* DNA isolation kit for purification of total DNA from fungi tissues. Mycelia samples of about ±100 mg were added into 1.5 ml tubes with 25 µL of re-suspension buffer (R2), then grinded with plastic pestle until tissues were destroyed. 15 µl of 20% SDS and 15 µl of RNase A (20 mg/ml) were added to the samples. The samples were incubated for 15 minutes at 55 °C and then centrifuged at speed of 13,000 rpm for five minutes. The supernatant was taken and put in a new tube of 1.5 ml and 100 µl of precipitation buffer (N2) was added to the supernatant, mixed vigorously and incubated on ice for five minutes. Samples were centrifuged at 13,000 rpm for five minutes. The supernatant 25 µl was taken and pipetted into a new 1.5 ml tube, then added with 375 µl of binding buffer (B4) and mixed.

The mixtures were introduced into the column. Samples were centrifuged at 13,000 rpm for one minute. Collection tube sample was then removed and replaced with new ones. The samples were

added to 500 µl wash buffer (W4), and centrifuged at 13,000 rpm for one minute, flow through, removed from the tube and reinstalled with collection tube. The samples were then added to 500 µl wash buffer (W5) and centrifuged at 13,000 rpm for one minute, flow through in the tube, removed and reinstalled with collection tube and re-added to 500 µl W4 buffer. The samples were centrifuged at a speed of 13,000 rpm for two minutes. Collection tube was removed and placed on a column of 1.5 ml new tube. Then the samples were added with 50 µl elution buffer (E1) and incubated for four minutes. Samples were centrifuged at 13,000 rpm for one minute. DNA samples were eluted and the integrity of DNA (qualitative) was observed with agarose gel electrophoresis. DNA samples were stored at -20 °C.

PCR amplification

The PCR protocol used was as described by Boyle *et al.* (2008) with internal transcribed spacer (ITS) region as the chosen universal primers. ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') as forward primer and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as the reverse primer. The concentration of PCR reagents was 2x KAPA ready mixed, dH_2O, 0.4 µM forward and reverse primers and 18 ng of DNA template. Amplification was performed in a 2720 Thermal Cylcer using Applied Biosystems thermocyler. The reaction began with an initial temperature at 95 °C pre-denaturation for five minutes, followed by 30 cycles of denaturation at 95 °C for one minute, annealing at 55 °C for 45 seconds and extension at 72 °C for one minute. A final extension step at 72 °C for five minutes allowed all amplicons to be fully extended.

DNA sequencing and sequence analysis

DNA sequencing was performed at Bioneer Laboratory, South Korea. The sequences obtained were further used for basic local alignment search tool (BLAST) analysis to determine the closest matches sequence based on National Center for Biotechnology Information (NCBI) GenBank database. A phylogenetic tree was constructed using BLAST pairwise alignments and phylogenetic construction with Neighbor-Joining method using molecular evolutionary genetics analysis (MEGA 5.0) to show the relationships among the homologous microorganisms.

RESULT AND DISCUSSION

Isolation and morphological identification of BSR causal pathogen

Figure 1. Fruiting bodies of BSR causal pathogen from Negeri Lama Estate.

The causal pathogen of BSR was collected from the infected oil palms (Figure 1) in Negeri Lama Estate, Asian Agri Group, North Sumatra, Indonesia. The fruiting body of the causal pathogen of BSR was identified based on their morphological and microscopic characteristics. Rakib *et al*. (2014) stated that morphological characteristic variation could be closely related to the heterogenity of *Ganoderma* species. Identification of *Ganoderma* species begins with isolation of *Ganoderma* fruiting

body and cultured onto *Ganoderma* selective medium (GSM). *Ganoderma* selective medium allowed only *Ganoderma* spp. of basidiomycetes to strive and survive on it (Ariffin & Idris, 1992). Result showed that the margins of the fruiting body were wide, thick and yellowish white and the context was brown with concentric zonations and thinner towards the margin (Figure 2). The tube layer was yellowish brown and hymenophore was round and cream.

The fruiting body of BSR causal pathogen was excised and incubated on GSM for 5-7 days. GSM provides a useful tool for isolation of *Ganoderma* which free of bacteria and other fungi contamination. The addition of tannic acid into the medium induced the formation of a brown halo around the colony. The surface texture of BSR causal pathogen isolate on PDA culture was moderately wavy with rough mycelia texture. Surface of pigmentation colour of the isolate was white, while reverse pigmentation colour was brownish yellow. *Ganoderma* isolate has dense mycelia and colony concentric rings which were presence in the culture (Figure 2). Several species of *Ganoderma* cause root rot disease on various hosts that affect productivity due to death of hosts. The common *Ganoderma* species in oil palm plantations are *G. boninense*, *G. zonatum*, and *G. miniatocinctum* (Idris, 1999; Wong *et al*., 2012). Among them, *G. boninense* is the most common and aggressive causal agents of basal stem rot (BSR) disease infecting oil palms (Goh *et al*., 2014). A study on biology, detection and control of BSR disease conducted by Idris and Ariffin (2004) also found that *Ganoderma* to be the most common and pathogenic species caused BSR. Khairudin (1990) in his research on controlling BSR in oil palm confirmed that the most common species which related to BSR was *G.boninense*.

Figure 2. BSR causal pathogen from Negeri Lama Estate cultured on PDA media (after eleven days of incubation)

Spores collected from fruiting body and examined under microscope were brown in colour, narrowly ellipsoidal with rounded apices, sized 10.4 μm in length and 5.2 μm in width (Figure 3). The characteristics of spore from the isolate observed were very similar to basidiospores of the basidiomycetes. Rakib *et al.* (2014) reported the *Ganoderma* isolate from the same species varied in term of their cultural and basidiospore morphological characteristics. Genetic variation induced the appearance of *Ganoderma* with genetically heterogenous caused by outcrossing over generations and different geographical origins (Miller *et al.*, 1999; Pilotti *et al*., 2003). High level of intraspecies variation, apparently, exists in some species as a result of this morphological plasticity. Some isolates were thought to represent different species until it was later shown, based on the biological and phylogenetic species concepts, that they belong to the same species (Adaskaveg & Gilbertson, 1986; Moncalvo *et al.*, 1995; Hong & Jung, 2004). Gams and Bissett (2002), also reported that colony appearance does not provide sufficient information for characterization due to the difficulties to establish a precise description. Hence, there is a necessity to complete the limitation of morphological identification with molecular approach such as through polymerase chain reaction

(PCR) and sequence analysis to identify *Ganoderma* species which is more rapid and precise (Wong *et al.*, 2012).

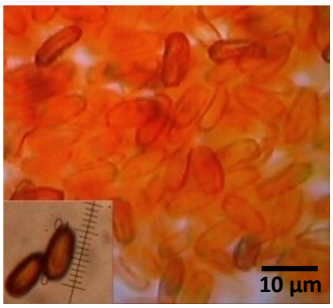

Figure 3. Spores of BSR causal pathogen from Negeri Lama Estate under light microscope observation with 1000x magnification.

DNA Amplification of BSR causal pathogen

The gel electrophoresis result of PCR amplicon of fungal at ITS1 and 5.8S gene is shown in Figure 4. Internal transcribed spacer (ITS) region was chosen because it is easy to review and amplify although from the small amount of DNA (due to the high copy number of sRNA genes) (Gomes, 2002; Das & Deb, 2015) and also has a high degree of variation even between closely related species or inter species in one genus (White *et al*, 1990). ITS region has the highest probability of successful identification for the broadest range of fungi, with the most clearly defined barcode gap between inter and intraspecific variation (Schoch *et al.*, 2012). ITS region also reported to have highly conserved and consistent sequences (Gomes, 2002). Sukmaningrum (2015) used the ITS region as primer for the molecular identification of *Ganoderma* spp. and *Trichoderma* spp. In this study, PCR amplicon size of the isolate was approximately 650 bp, the same size of amplicon also being reported by Rakib *et al*. (2014) for *Ganoderma* spp.

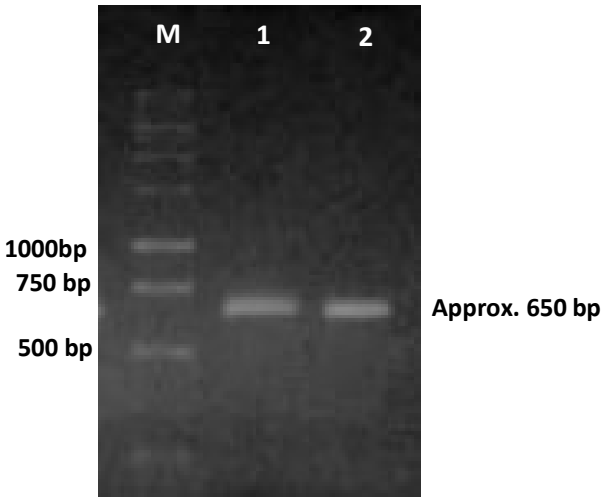

Figure 4. PCR amplification of the fungal samples using primers ITS1 and ITS4. M = Promega 1kb DNA Ladder (Lane 1 & 2 : BSR causal pathogen isolates of Negeri Lama Estate).

BLAST search and phylogenetic tree construction

The DNA product of the BSR causal pathogen isolate was further identified using online basic local alignment search tools (BLAST) to match the closest related organism in National Center for Biotechnology Information (NCBI) GenBank database. The size of DNA and their homology sequence was determined. The similarity analysis of the sample with Genebank database is shown in Table 1. The most homologous sequence obtained from BLAST result acquires 99% maximum identity which suggests the isolate originated from the species of *G. boninense*.

Table 1. The most homologous microorganism from NCBI gene bank in comparison to isolate from Negeri Lama Estate, North Sumatra.

No.	Accession No.	Description	Max Score	Total Score	Query Value	E Value	Max Ident
1.	KX092000.1	*Ganoderma boninense* isolate GB001 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and ITS2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1136	1136	97%	0.0	99%
2.	JN400510.1	*Ganoderma* sp. BL-9 18S ribosomal RNA gene, partial sequence; 5.8S ribosomal RNA gene, and ITS2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1122	1122	97%	0.0	98%
3.	KF164430.1	*Ganoderma boninense* strain GBLS internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and ITS2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1120	1120	94%	0.0	99%
4.	JN234429.1	*Ganoderma* sp. BRIUMSc internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and ITS2, partial sequence	1118	1118	97%	0.0	98%
5.	JN234427.1	*Ganoderma* sp. BRIUMSa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and ITS2, partial sequence	1116	1116	97%	0.0	98%
6.	KM015454.1	*Ganoderma boninense* strain PER71 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and ITS2, partial sequence	1072	1072	89%	0.0	99%
7.	AB985729.1	*Ganoderma boninense* genes strain Tr1 for 18S rRNA, internal transcribed spacer 1, 5.8S ribosomal RNA gene, ITS2, 28S rRNA, partial and complete sequence	1046	1046	90%	0.0	98%

Based on Table 1, it is suggested that *Ganoderma* isolate from Negeri Lama Estate had closest homology with *G. boninense* GB001 with 99% of similarity. Phylogenetic tree was constructed to show the relationships between the *Ganoderma* isolated from Negeri Lama estate, North Sumatra, Indonesia with other *Ganoderma* species (Figure 5.). The identity of the causal pathogen of BSR from Negeri Lama estate was identified and further confirmed as *G. boninense*. The causal pathogen which usually infecting oil palm and caused BSR.

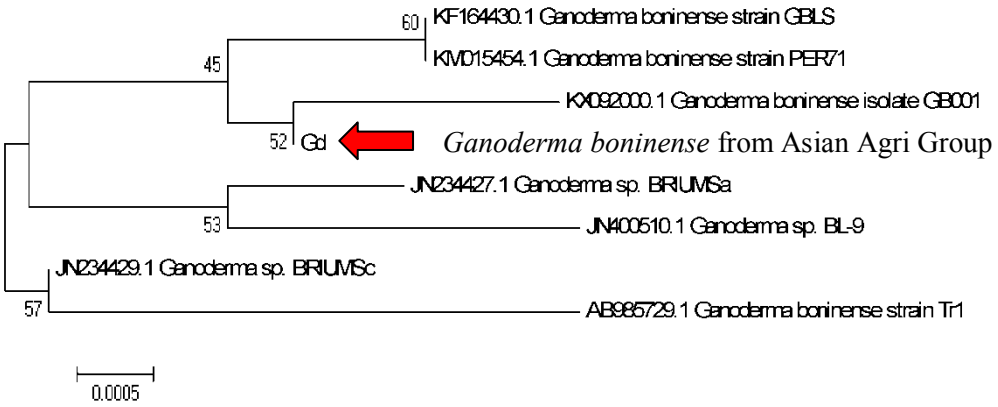

Figure 5. Phylogenetic tree of seven species of *Ganoderma* including the isolate from Negeri Lama estate (with NCBI query ID:lcl|Query_15105). The evolutionary history was inferred using Neighbor-Joining method with 1000 of bootstrap and maximum sequences difference at 0.0005.

Phylogenetic tree construction is a strong evidence for the BSR causal pathogen from Asian Agri Group in North Sumatra to be classified as *G. boninense*. The isolate is closely related to *G. boninense* isolate GB001 that obtained from the infected oil palm. The phylogenetic tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic. Studies have revealed that population of *G. boninense* are predominantly comprised of distinct individuals, a number of isolates were also found to share single mating alleles (Pilotti *et al*., 2003; Pilotti *et al.*, 2004). This indicates that outcrossing had occurred over several generations in the resident or wild population of *G. boninense* prior to colonization of oil palm.

CONCLUSION

The BSR causal pathogen of oil palm from Asian Agri Group, North Sumatra, Indonesia was successfully identified using morphological characterization and molecular approaches. Based on the morphology characteristics, the isolate from Negeri Lama Estate, Asian Agri Group was identified as *Ganoderma* species. However, the morphological characteristics could not precisely determine the species of *Ganoderma* due to its diversity. Subsequently, molecular approach through PCR amplification and DNA sequencing analysis was conducted, which confirmed the isolate as *G. boninense* and had closest related strain with *G. boninense* isolate GB001 based on the phylogenetic analysis.

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