

***Ganoderma* Species Associated with Basal Stem Rot Disease of Oil Palm**

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Abstract: Problem statement: Basal Stem Rot disease (BSR) is one of the most serious diseases that have been causing major losses in the oil palm industry in Southeast Asia, especially in Malaysia and Indonesia. Several species of *Ganoderma* have been reported pathogenic to oil palm, however, the diversity and differentiation of the *Ganoderma* species were not widely studied and the identity of these species are still unclear which may lead to inaccurate and inefficient decision-making in disease management. **Approach:** In this study, several isolates of *Ganoderma* were collected in Sarawak, Malaysia and the Multiplex Polymerase Chain Reaction was carried out to differentiate the isolates into species level. This was followed by morphological studies of basidiocarp of the *Ganoderma* isolates cultivated via artificial cultivation whereby parameters, such as basidiocarp and spore size, color and physical morphology were recorded. **Results:** Multiplex PCR could be used to differentiate the *Ganoderma* isolates, however, optimization had to be done to obtain convincing results. Morphology studies of the *Ganoderma* isolates showed that spore length could be used to distinguish among the *Ganoderma* species. **Conclusion:** Three *Ganoderma* species viz., *G. boninense*, *G. zonatum* and *G. miniatocinctum* are associated with the basal stem rot disease in Sarawak. Further studies on *Ganoderma* morphological traits is suggested so that immediate identification method can be developed to give guidance in deciding the most suitable way for control measures without any delay, leading to reduced palm deaths and disease control cost, thus, reducing losses in the oil palm industry.

Key words: *Ganoderma boninense*, *Ganoderma zonatum*, *Ganoderma miniatocinctum*, basal stem rot disease, multiplex PCR-DNA, oil palm

INTRODUCTION

One of the major diseases causing losses in the oil palm industry is the Basal Stem Rot disease (BSR) which was recognized in Malaya since 1928 when the disease was reported to attack mainly palms aging 30 years and above. Infections in younger palms of 10-15 years become more apparent after 1957, followed by spreading of the disease in oil palms at nursery stage. This trend demonstrates the ability of the pathogens to adapt to the environmental conditions and food source from plantations to nursery and from old oil palms to its seedlings. Immature palms are also attacked by this disease. Due to this reason, the total area of oil palm plantations infected by BSR disease has been

increasing, therefore instilling fear in planters as currently, there is still no methods to address the disease effectively. Presently, it is considered to be the most serious and deadly oil palm disease in the Southeast Asian countries, especially in Malaysia and Indonesia, which are the major producers and exporters of palm oil in the world.

Initially, BSR disease had been thought to be caused by *Ganoderma boninense* alone, however, in recent studies, several species had been reported to be responsible for BSR disease of oil palm in Malaysia, namely, *G. boninense*, *Ganoderma zonatum* and *Ganoderma miniatocinctum* respectively, while *Ganoderma tornatum* is non-pathogenic, infecting only dead trunks of oil palms. *Ganoderma boninense* is

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considered to be the most aggressive pathogen known. However, studies had shown that dominant *Ganoderma* spp. for BSR disease in oil palms can differ based on locality. As different species of *Ganoderma* exhibit different characteristics and aggressiveness, the identity of the pathogen is crucial in determining the appropriate disease management. Unfortunately, the identification and differentiation of *Ganoderma* spp. were not widely studied, causing confusion in the identification process, thus, leading to inefficient disease management. Evidently, the identity of the pathogen is crucial in deciding the most efficient and economic disease management, hence, more studies on the identification of *Ganoderma* spp. should be conducted.

Over the last few decades, only several studies have been done on the molecular aspect of these *Ganoderma* spp. Studies had shown high level of genetic variation among monokaryons of *G. boninense*, suggesting that *G. boninense* is genetically heterogenous which could be caused by different geographical locations or outcrossing of the isolates over generations (Miller *et al.*, 1999; Pilloti, 2003) whereby the pathogen could originate from the same species containing wide genetic variation or from closely related species (Zakaria *et al.*, 2005).

Monoclonal and polyclonal antibodies had also been used to detect pathogenic *Ganoderma* spp, however, the assay using polyclonal antibodies showed cross reaction with saprophytic fungi commonly found on diseased oil palm roots and trunk although the percentage of cross reaction were lower (Utomo and Niepold, 2000; Shamala *et al.*, 2006; Idris and Rafidah, 2008). In addition, no distinction was found between pathogenic *Ganoderma* spp. isolated from oil palms and other pathogenic *Ganoderma* spp, therefore, indicating that ELISA test cannot be used to distinguish among different *Ganoderma* species (Utomo and Niepold, 2000).

Polymerase Chain Reaction (PCR) techniques had also been used to identify *Ganoderma* species (Moncalvo *et al.*, 1995; Idris *et al.*, 2003; Chong *et al.*, 2011). Primers Gan1 and Gan2 which were designed from ITS region 1 from *G. boninense* DNA cross-reacted with saprophytic fungi found on diseased oil palm. It was later found out that no amplifications were observed when the DNA extracts of saprophytic fungi were diluted in the sap of healthy oil palm roots (Utomo and Niepold, 2000). The use of Random Amplified Polymorphic DNA (RAPD)-PCR and Internal Transcribed Spacer (ITS) showed different results whereby RAPD was found to be suitable for systematic of lower taxonomy level that cannot be resolved by using ITS sequence data. The RAPD analysis showed variations despite the high degree of similarity for *G. boninense* isolates (Zakaria *et al.*, 2009). Thus, RAPD can be used to differentiate different isolates of

Ganoderma spp. that have identical ITS sequences (Hseu *et al.*, 1996). However, accurate identification of *Ganoderma* spp. is still not possible via RAPD analysis.

A novel technique developed recently, the Multiplex Polymerase Chain Reaction (MPCR), has the ability to differentiate between *G. boninense*, *G. zonatum*, *G. miniatocinctum* and *G. tornatum* in a single reaction. This technique utilizes Dual Priming Oligonucleotide (DPO) technology which can block non-specific priming, thus, increases sensitivity and specificity of PCR reactions. In addition, an 8-Methoxypsoralen (8-MOP) system and internal control were integrated to eliminate template activity of contaminating DNAs and identify samples that may contain substances that can interfere with the PCR amplification reaction (Idris *et al.*, 2010). However, this technique requires some time before the final result is obtained. Therefore, more studies should be carried out to distinguish *Ganoderma* spp. morphologically as this method requires the shortest time for identification.

Different species of *Ganoderma* require different combinations of nutrients for growth, exhibiting different infection rates and pathogenicity, thus, appropriate method should be used to manage infections by different species. The ability to differentiate pathogenic and non-pathogenic *Ganoderma* species can assist in decision making of whether to treat the palms; if the infection is determined to be caused by non-pathogenic *Ganoderma* species, then, it is unnecessary to take drastic control measures as it only infects dead palms, thus saving enormous budget and man power used for control and preventive measures. On the other hand, if the infection is caused by pathogenic *Ganoderma* spp., prompt measures can be taken to suppress infection and prevent further spreading of the disease. Indeed, the characterization of *Ganoderma* species is crucial in achieving effective disease control management for basal stem rot disease (Zakaria *et al.*, 2009). Therefore, studies on morphological identification of *Ganoderma* species should be carried out so that identification can be done without any delay followed by prompt actions in managing the disease, thus, preventing the spread of the disease and reducing losses caused by palm deaths and reduced yields.

MATERIALS AND METHODS

Culture source and maintenance: *Ganoderma* was isolated from fruiting bodies obtained from infected oil palm planted on peat and mineral soils in Sarawak, Malaysia. The *Ganoderma* Selective Medium (Ariffin and Idris, 1991) which was able to inhibit growth of bacteria and other saprophytic fungi, was used to isolate the *Ganoderma* spp. Potato Dextrose Agar (PDA) was used for culture maintenance.

Molecular analysis: The *Ganoderma* isolates were grown on PDA for one week before mycelia plugs of eight millimeter were inoculated into Potato Dextrose Broth (PDB). The broth cultures were incubated in static and dark condition at room temperature. After two weeks, mycelia mats that were formed on top of the broth were harvested and deep-frozen overnight at -20°C before taken out for freeze-drying process using Freeze dryer (ilShin model TFD5505).

The dried mycelia were then ground into a fine powder with liquid nitrogen using mortar and pestle (Karakousis *et al.*, 2006). DNA extraction was done using DNeasy Mini Plant kit (Qiagen) and the eluted DNA was stored at -20°C until further use. The MPCR was done using Ganoderma4 Genotyping kit (Seplex) which was able to distinguish between four species of *Ganoderma*, namely *G. boninense*, *G. zonatum*, *G. miniatocinctum*, *G. tornatum* and *G. tornatum* (Idris *et al.*, 2010). The Dual Priming Oligonucleotide (DPO) primers provided along with the kit can increase sensitivity and specificity of PCR reactions by blocking non-specific priming. Furthermore, 8-methoxypsoralen (8-MOP) in the kit can eliminate template activity of contaminated DNAs. Internal control was also introduced into the amplification activity to identify samples that may contain substances that can interfere with the PCR amplification reaction. The multiplex PCR was done with one cycle of initial denaturation at 94°C for 15 minutes and 40 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 90 sec and elongation at 72°C for 90 sec, followed by one cycle of further elongation at 72°C for 10 min (Idris *et al.*, 2010).

Gel electrophoresis was done using 5 µL of the PCR products and 5 µL of Ganoderma4 Marker on a 2% agarose gel containing ethidium bromide and visualized Under Ultraviolet (UV) light for imaging process. The PCR products displayed bands with sizes of 900, 656, 415 and 331 bp for *G. boninense*, *G. zonatum*, *G. tornatum* and *G. miniatocinctum* respectively and 242 bp for internal control.

Morphological study: For morphological studies, the isolates were grown in the laboratory. Spawn was prepared using paddy grain and spawn run was done at room temperature in dark condition until full colonization in about two weeks. Planting media were prepared using a combination of rubber wood sawdust, calcium carbonate (1%) and water (60%). These were autoclaved and later inoculated with the grain spawns followed by incubation at room temperature in dark condition. After full colonization, the planting spawns were exposed to light. The caps of the planting bags were opened and water was sprayed twice a day to

initiate primordia formation in the pinning stage. The primordia that grew into fruiting bodies were harvested and oven-dried upon maturation. The morphological characteristics of the fruiting bodies were recorded. Spores were also collected for morphological studies.

RESULTS

Ganoderma markers were used in MPCR whereby the marker clearly yielded bands for *G. boninense*, *G. zonatum* and *G. miniatocinctum* in a single lane (Fig. 1: Lane 2). The gel electrophoresis for the multiplex analysis clearly showed that isolate G25 and G47 were *G. boninense*, each showing clear bands at 900 bp while bands at 656 bp indicates that isolate G46 and G50 were *G. zonatum*. Bands at 242 bp showed that isolate G43 and G30 were *G. miniatocinctum* (Fig. 1). None of the isolates were *G. tornatum* as no bands were detected at 415 bp. This indicated that *G. boninense*, *G. zonatum* and *G. miniatocinctum* were present in the infected oil palms in Sarawak, Malaysia.

For the assessment of basidiocarp colour, Munsell chart was used. Normally, colours were identified through visual assessment. However, visual assessment may give varying and subjective results because of difference in perception. Thus, a standard colour chart was used to accurately identify the colour of the specimen. Table 1 show that isolate G25, G46 and G43 had lighter colour from yellowish red to strong brown while G47, G50 and G30 had darker colour ranging from dusky red to black. The surface of G25 and G43 was smooth while G50 and G30 had slightly wrinkled surface, followed by G47 and G46 which had deep wrinkled surface (Fig. 2). As for concentric zones, G50 had multiple shallow wrinkled zones while G30 had multiple shallow slightly wrinkled zones. Isolate G25 had well developed multiple smooth concentric zones, followed by G43 with well developed multiple smooth wavy concentric zones, G46 with well developed multiple wrinkled concentric zones and G47 with well developed multiple deep wrinkled concentric zones (Table 1).

All of the isolates had smooth margin except for G47 which had incurved wavy margin. Isolates G25 and G47, both *G. boninense* had short stipe which was similar to G30. Isolate 50 was sessile while G46 and G43 were either sessile or short-stiped. The attachment for G2 and G30 was lateral and in the case of multiple fruiting bodies, they exhibited divided and parallel-lateral attachment. Isolate G47 showed bent attachment, bending upwards before forming fruiting bodies. Isolates G46 and G43 sometimes had stipes that exhibited lateral attachment (Table 1).

Table 1: Descriptions of basidiocarp morphologies for *Ganoderma* isolated from infected oil palms in Sarawak

Parameters	Isolates of <i>Ganoderma</i>					
	G25	G47	G46	G50	G43	G30
Color (Munsell Chart)	7.5YR 5/8 strong brown to 5YR 4/6 yellowish red	10R 3/6 dark red to 10YR 2/1 black	7.5R 5/8 strong brown	5R 3/6 dark red to 10R 2.5/1 reddish black	10 YR 5/8 Yellowish brown	7.5R 2.5/2 dusky red to 10YR 2/1black
Surface	Smooth	Wrinkled	Wrinkled	Slightly wrinkled	Smooth	Slightly wrinkled
Concentric zone	Well developed multiple smooth	Well developed multiple deep wrinkled	Well developed multiple wrinkled	Multiple shallow wrinkled	Well developed multiple smooth wavy	Multiple shallow slightly wrinkled
Margin	Smooth	Incurved, wavy	Smooth	Smooth	Smooth	Smooth
Stipe	Short	short	Sessile or short stiped	Sessile	Sessile or short stiped	Short
Attachment	Lateral, parallel and divided	bent upwards into	Sessile or lateral	Sessile	Sessile or lateral	Lateral, parallel and divided
Spore shape	Ellipsoid	Ellipsoid	Ellipsoid	Clavate	Ellipsoid	Ellipsoid

Table 2: Morphological descriptions of basidiocarps for *Ganoderma* isolated from infected oil palms in Sarawak

Parameters	Isolates of <i>Ganoderma</i>					
	G25	G47	G46	G50	G43	G30
Length (cm)	4.700 ^b	6.500 ^a	7.100 ^a	5.900 ^{ab}	6.100 ^{ab}	6.000 ^{ab}
Width (cm)	5.800 ^b	8.100 ^a	8.400 ^a	6.600 ^{ab}	5.400 ^b	6.500 ^{ab}
Dry weight of basidiocarp (g)	20.433 ^{ab}	27.127 ^a	27.275 ^a	22.072 ^{ab}	15.750 ^b	25.009 ^{ab}
Spore length (µm)	10.0032 ^a	9.7380 ^a	8.6402 ^b	10.0073 ^a	9.8006 ^a	8.5933 ^b
Spore width (µm)	4.3965 ^a	4.1149 ^a	4.2433 ^a	4.2433 ^a	4.0950 ^a	4.2936 ^a

Means with the same alphabets within the same row are not significantly different at $p < 0.05$ by New Duncan's New Multiple Range test

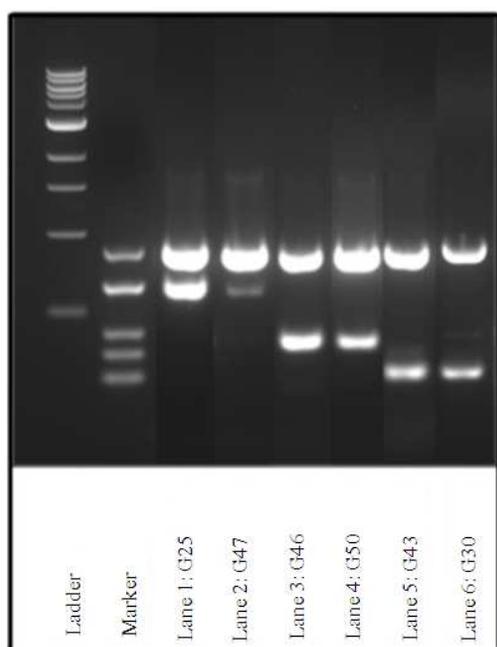


Fig. 1: Results of Multiplex Polymerase Chain Reaction (MPCR) for identification of *Ganoderma* species isolated from infected oil palm in Sarawak. Notes: Isolates G25 and G47 were identified as *G. boninense*, isolates G46 and G50 were *G. zonatum* and isol

As for the size of basidiocarp, G25 had significantly the shortest length, followed by G43 and G30 (both *G. miniatocinctum*) which was not significantly different from G50. Isolate G47 and G46 exhibited both the longest and widest basidiocarp. This was followed by

G50 and G30 while G25 and G43 recorded the shortest width. Generally, the results showed that isolate G47 and G46 produced the biggest basidiocarp, thus giving the highest basidiocarp dry weight. On the other hand, isolate G25 gave the smallest basidiocarp although G25 belong to the same species as G47, indicating that there is variation of basidiocarp sizes even within the same species. Isolate G43 had the lowest dry weight which was significantly lower than G25, G50 and G30 (Table 2).

Isolate G25, G47, G50 and G43 exhibited significantly bigger spore size compared to G46 and G30. While there was difference in spore length of different isolates, there was no significant difference in the spore width for all isolates, indicating that spore length can be used to distinguish *Ganoderma* spp. The shape of the spore was similar for all isolates which was ellipsoid except for G50 exhibiting clavate spores (Table 1 and 2).

DISCUSSION

The Multiplex PCR-DNA analysis clearly differentiated the isolates into different *Ganoderma* species as shown in the gel electrophoresis (Fig. 1). Although not all of the isolates produced very clear bands, the presence of visible bands were sufficient to determine the species of the isolates. The visible bands showed that the extracted DNA genomes were successfully amplified by the specific primers, indicating the high specificity of the primers used in the multiplex analysis.

Despite that, isolate G47 showed a faint band which can be due to the low concentration of DNA genome extracted compared to the other isolates.



Fig. 2: Basidiocarp morphology of *Ganoderma* isolated from infected oil palms in Sarawak

Apart from that, preliminary test for the multiplex DNA analysis in this study showed isolates exhibiting one clear band with one or more faint bands in one single lane. This indicates that the Multiplex PCR-DNA may not be specific enough to produce a single clear band for different isolates or strains due to the heterogeneity of *Ganoderma* spp.

The presence of multiple faint bands in the same lane along with one clear band is not an uncommon occurrence. Generally, simple PCR-DNA analysis involves the amplification by a single primer, whereby the conditions for the PCR cycles are adjusted to optimize the performance of that particular primer alone. However, in the case of Multiplex PCR-DNA analysis, several primers are involved in a single reaction whereby each primers have different optimized temperatures for denaturation, annealing and elongation process respectively. Thus, by carrying out the Multiplex PCR-DNA with only one set of denaturation, annealing and elongation temperatures, it is possible that only the amplification of one of the primers is

optimized or none of the primer amplifications is optimized leading to the occurrence of faint bands. Moreover, unspecific binding may also occur during the process due to the different temperatures.

Therefore, it is suggested to repeat the Multiplex PCR-DNA analysis under different sets of temperatures; one set using the recommended set of denaturation, annealing and elongation temperature to get the general idea of the species of the *Ganoderma* isolates whereby they are then grouped into species indicated in the results. Multiplex PCR-DNA is then repeated for each group using the same set of primers but with optimized set of temperature for the specific primer of the *Ganoderma* species indicated by each group to obtain single clear band. This is done to further confirm the identity of the *Ganoderma* spp.

Based on the characteristics recorded in Table 1 and 2, similarities can be detected between species, while differences were also found between isolates from the same species. Thus, detailed morphological characteristics had to be taken into consideration to identify the differences among the *Ganoderma* isolates. One of the obvious differences among the three *Ganoderma* spp. were the presence of stipe as most of the isolates of *G. zonatum* and *G. miniatocinctum* were sessile while *G. boninense* had short stipes.

As for the basidiocarp, there were differences even within species, shown by basidiocarp length and width of isolates G25 and G47, whereby both originated from *G. boninense*. Furthermore, isolates G50, G43 and G30, consisting of *G. zonatum* and *G. miniatocinctum* showed similarities in terms of length, width and dry weight. This indicated that *G. zonatum* and *G. miniatocinctum* were closely related in terms of morphological traits compared to *G. boninense*. Thus, *G. boninense* can be differentiated more easily compared to the other two species respectively as there are more distinct differences between *G. boninense* and the other species. Variations within species could also occur, it had been reported that there was difference in morphology of the *Ganoderma* spp. obtained from coconut stumps and oil palm, suggesting that the morphology may vary due to environmental factors and growing substrate (Pilotti *et al.*, 2004). As for the pore size of basidiocarp, several studies had been conducted; however, some studies showed that the pore size varied with the age of the fruiting body, thus, in this study, the parameter for pore size was not taken (Pilotti *et al.*, 2004).

The most obvious differences between the three *Ganoderma* species were the spore length whereby *G. boninense* (G25 and G47) exhibited longer spore length compared to the other two species. The spore shape and size for the samples of *G. boninense* strains were within

the range of spores size 9.0-11.0×4.2-5.6 µm, as reported by Pilotti *et al.* (2004). However, the spore width was shorter compared to the range 5.2-6.5 µm reported by Ho and Nawawi (1985) although the spore length was within the range described. As for isolates G46 and G50 (*G. zonatum*), the spore shape and size was similar and within the range 8-10×4-6 µm as reported by Murill (2010). It was quite difficult to differentiate between *G. zonatum* and *G. miniatocinctum* as there was no difference in the spore length of isolates 46, 50 and 43. Moreover, differences could not be detected through spore width and shape as there was no difference in the spore width among the three species while only isolate 50 showed difference in the spore shape. The three *Ganoderma* species can be easily differentiated from non-pathogenic *Ganoderma tornatum* as the spore size reported, 6.4-8.6×4.4-5.8 µm, were significantly different from the pathogenic species studied (Pilotti *et al.*, 2004).

CONCLUSION

Three *Ganoderma* species, namely *G. boninense*, *G. zonatum* and *G. miniatocinctum* were isolated and identified to be consistently associated with Basal Stem Rot disease of oil palm in Sarawak, Malaysia. A fast and simple way of identifying the fungi was deemed important in developing effective management strategies for the disease. The study concluded that the simplest way in differentiating the 3 species of was to compare the spore length. Basidiocarp morphology can be used as a preliminary tool for general identification, taking into consideration that environmental factors and genetic heterogeneity may hinder its use in specific identification purpose. Further confirmation of species can be done using Multiplex PCR-DNA method which is more time consuming and tedious.

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