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Molecular Fingerprint of *Ganoderma* spp. from Sabah, Malaysia

Khim-Phin Chong[1*], Syahriel Abdullah[1] and Tong-Leong Ng[2]
[1]*Sustainable Palm Oil Research Unit (SPOR), School of Science and Technology, Universiti Malaysia Sabah, 88400 Kota Kinabalu, Sabah, Malaysia*
[2]*School of Sustainable Agriculture, Universiti Malaysia Sabah, Sandakan Campus, Mile 10, Sg. Batang, 90000, Sandakan, Sabah, Malaysia*
*For correspondence: chongkp@ums.edu.my

Abstract

Basal Stem Rot (BSR) of oil palm (OP) (*Elaeis guineensis*) caused by *Ganoderma boninense* is the most devastating disease in South East Asia. High incidence of BSR results in economic losses, due to zero yield from dead palms and significantly reduced weight and number of fruit bunches from infected living palms. Although BSR is a very important disease in OP, molecular information on *Ganoderma* isolates from Sabah, however is not well documented. There is limited information on the use of molecular techniques in identification of *G. boninense* available in current literature for *G. boninense* isolates from Sabah. The only report on this pathogen isolate in Sabah is based solely on their morphology and pathogenicity. In conjunction with the morphological similarities between the different isolates, there are also varied opinions on the aggressiveness of the pathogen in Sabah. In this paper we report on the identity of *Ganoderma* isolates from several OP Estates in Sabah, Malaysia. The identities of these isolates were confirmed using DNA sequence analysis after PCR amplification. The latter method showed that the Sabah isolates were very similar to *Ganoderma* sp with a maximum similarity of 99%. © 2013 Friends Science Publishers

Keywords: *Ganoderma boninense*; Basal stem rot; *Ganoderma* sp BRIUMS a; *Ganoderma* sp BRIUMS b

Introduction

Basal Stem Rot (BSR) disease in oil palm (OP) is caused by *Ganoderma boninense*. This fatal disease is considered the most serious disease affecting OP in Malaysia and some estates in South East Asia (Chong *et al*., 2012), where losses can reach up to 80% after repeated planting cycles. Almost 90% of the estates in West Malaysia reported the presence of *G. boninense* compared to only 4% in Sabah (Khairuddin and Chong, 2008). However, this is far from the actual situation in Sabah estates. Many methods have been used to try and control the BSR infection, but to date there is no record of any method achieving good control of *Ganoderma* infection in established plantations and some methods also have technical limitations in terms of their application.

In Malaysia, the pathogen attacking OP was originally identified as *G. lucidum* (Thompson, 1931), however after decades of research, the general consensus now appears to be that *G. boninense* is the main species pathogenic to the oil palm, especially in South East Asia (Moncalvo, 2000). *In vitro* studies on the morphological characteristics of *G. boninense* by Idris *et al*. (2000) found the colonies of *G. boninense* were white in color on the surface, while the reverse side was dark in color (pigmented). Cultures of *G. boninense* had an undulating surface in the darkened regions. The first indication of basidiomata formation was the appearance of a white mycelium after one to three weeks of incubation on rubber wood blocks, which then developed into a small, white, button-like structure. The apical end began expanding rapidly giving rise to bracket-like structures which were generally white when first formed, but as their length and width increased rapidly, the upper surface developed various yellowish-brown coloration with concentric zonations (Idris, 2009).

To date, although, several methods based on the biochemistry approach were used to detect *Ganoderma* infection, such as the use of the *Ganoderma* Selective Medium (GSM) or molecular DNA based technique and polymerase chain reaction (PCR) amplification (Miller *et al*., 2000; Idris *et al*., 2003; Latifah *et al*., 2005; Kandan *et al*., 2009), however the arguments on the differences in disease intensity due to aggressiveness of pathogen isolates between Peninsular Malaysia and Sabah remain unclear. There is scarce information on molecular identification of *G. boninense* isolates from Sabah. The only report on Sabah isolates was based solely on their morphology and pathogenicity (Idris *et al*., 2001) and isolates from Langkon, Sabah (Chong *et al*., 2011). Due to the importance of the OP industry to Malaysia's economy, the transfer of any materials that are related to *Ganoderma* from Peninsular Malaysia to Sabah and Sarawak is strictly prohibited. In conjunction with the morphological similarities between the

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different isolates, there are varied opinions on the aggressiveness of the pathogen in Sabah. The isolates of *G. boninense* from Sabah were claimed to be less aggressive compared to those from Peninsular Malaysia. This paper presents the report on the molecular identity of *Ganoderma* isolates from five different areas in Sabah.

Materials and Methods

Isolation and Classification of *Ganoderma*

A total of 106 fruiting bodies samples were collected from Beaufort (Kimanis), Kota Marudu (Langkon), Sandakan (Gomantong), Lahad Datu (Sandau), and Tawau (Sungai Balong) OP estates of Sabah, Malaysia. The fruiting bodies were grouped according to their morphology as described by Seo and Krik, 2000, then the internal tissues of fruiting bodies were excised and cultured on *Ganoderma* Selective Medium (GSM). The media was prepared as described by Ariffin and Idris (1992). The cultures were incubated at room temperature (27±2^{o}C) for two weeks and after that the mycelia were collected and transferred onto potato dextrose agar (PDA) plates and maintained for a week at similar temperature. Several subcultures were carried out until pure cultures were obtained. The cultures were maintained at similar conditions until further use.

DNA Extraction

Cetyltrimethylammonium bromide (CTAB)-based DNA extraction as described by Doyle and Doyle (1987) was used to extract DNA from the isolates, albeit with a little modification. Before extraction was carried out, 2% CTAB solution, Tris-EDTA (TE) buffer and 5 M potassium acetate were autoclaved at 121°C for 20 min. Under aseptic conditions, 20-200 mg of fresh isolates mycelium were transferred into an 1.5 mL sterile centrifuge tube, followed by the addition of 300 µL of 2% CTAB solution. The sample was ground using a sterile micropestle until a white bulk of tissues formed in the solution. Then, 120 µL of 10% SDS solution was added into the tube. The sample was ground again for 10 min and incubated in an oven at 60°C for 30 min, until the white bulk dissolved in the solution. After the incubation, 300 µL of 5 M potassium acetate was added and mixed with the solution. The mixture was cooled on ice for 10 min. Subsequently, 600 µL of phenol:chloroform:isoamyl alcohol (P:C:IA) (25:24:1) was added into the mixture. The tube was gently inverted 40 to 50 times and centrifuged at 14,000 rpm for 10 min. Without disturbing the lower layer, 500 µL of the aqueous layer at the upper part was transferred into a new 1.5 mL sterile centrifuge tube. Once again, 500 µL P:C:IA was added into the solution and centrifuged at 14,000 rpm for 10 min. This time, 300 µL of the aqueous layer was transferred into a new sterile 1.5 µL centrifuge tube. For DNA precipitation, 750 µL of ice-cold absolute ethanol was added into the new centrifuge tube. The tube was gently inverted for approximately 30 times and centrifuged at 14,000 rpm for 10 min. Ethanol was expelled from the tube, leaving behind a small patch of white pellets. One mL of ice-cold 70% ethanol was added to the tube and centrifuged again at the same speed. The tube was air dried at room temperature before 50 µL of TE buffer solution was added to the white pellets. The pellets were left at room temperature until it was completely suspended in the solution. To detect the presence of DNA, 5 µL of the solution was used in 1% 1x TBE agarose gel at 100V for 30 min, with 1kb DNA ladder as guidance.

PCR Amplification

PCR amplification of the fungal DNA was done on ITS 1 and ITS 2 regions and the 5.8S gene using primers ITS 1 (forward primer) and ITS 4 (reverse primer) described by Latifah *et al*. (2005). The primer sequences used were ITS 1: 5'- TCC GTA GGT GAA CCT GCG G-3' and ITS 4: 5'- TCC GCT TAT TGA TAT GC -3'. PCR amplification was carried out using TopTaq Master Mix Kit (Qiagen), as described in the manual. The reaction was performed in 25 µL TopTaq Master Mix, 0.2 µM forward primer, 0.2 µM reverse primer, 1 µg template DNA, and 22 µL RNase-free water. Thermalcycler was programmed for initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30s, annealing at 55°C for 40 s, and extension at 72°C for one min. The final extension was set to 72°C for 10 min. Detection of the PCR product was conducted by aliquoting 5 µL of the solution into 1% 1x TBE agarose gel at 100V for 30 min, with 100 bp DNA ladder as guidance. PCR product purification was performed using QIAquick PCR Purification Kit (Qiagen) and the protocol used as stated in the manual according to the protocol proposed by the manufacturer.

Cloning, DNA Sequencing and Sequence Analysis

Cloning and DNA sequencing were done at First BASE Laboratories Sdn Bhd in Selangor, Malaysia. BLAST search was conducted for the obtained sequences for closest matches in the NCBI GenBank database. The sequence was trimmed for a better BLAST search in the NCBI gene bank. Furthermore, a phylogenetic tree was constructed using BLAST pairwise alignments with Fast Minimum Evolution tree method to show the relationships among the homologous microorganisms.

Results

Fruiting Bodies

All the fruiting bodies collected from Sandakan (Gomantong), Lahad Datu (Sandau), and Tawau (Sungai

Table 1: Fruiting bodies collected from the different infected estates in Sabah

Estate Location	No. of samples	Types of samples
Beaufort (Kimanis)	23	A, B
Kota Kinabalu (Langkon)	50	A, C
Sandakan (Gomantong)	5	A
Lahad Datu (Sandau)	8	A
Tawau (Sungai Balong)	20	A

(A). Gradual hues of brown rings, and smooth margin pattern of the pileus;
(B). Brown reddish color, and waved margin pattern of the pileus.
(C). Brown reddish color and smooth margin pattern of the pileus

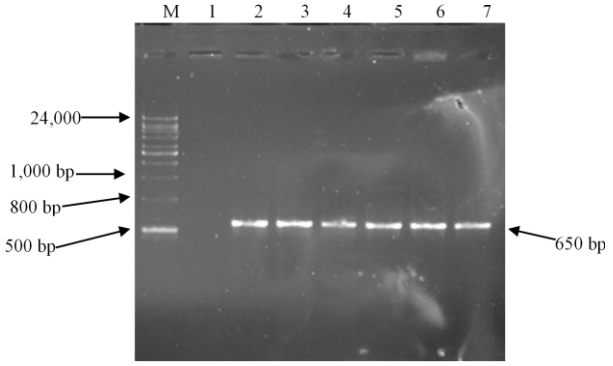

Fig. 1: M = 1 kb ladder (Vivantis), 1 = negative control, 2 = Kimanis type B, 3 = Langkon type A, 4 = Kimanis type A, 5 = Sandau type A, 6 = Gomantong type A, 7 = Balong type A. The size of the target band is 650 bp

Balong) are seen to belong to Type A (Table 1). Both Types A and B were collected from Beaufort (Kimanis) while Types A and C were collected from Kota Marudu (Langkon). Different climatic conditions (e.g. rainfall, humidity, altitude etc.) may contribute to the different morphology of the fruiting bodies including the *Ganoderma* species in Sabah. The different morphology of fruiting bodies is illustrated in Fig. 3.

PCR Amplification

Polymerase chain reaction (PCR) using ITS 1 and ITS 4 as primers showed that *Ganoderma* isolated from infected OP stumps produced the same PCR fragment size of 650 bp in accordance to the size of the 5.8S rDNA gene as described by Latiffah *et al.* (2005) and Zheng *et al*. (2006) (Fig. 1). These data provided stronger evidence that the isolated fruiting bodies from OP stump were from *Ganoderma* species. Though the sizes of PCR products among the samples were approximately the same, it may not represent similarity of the sequence. Therefore, the genomic sequence obtained must be further distinguished using analysing tools such as the Random Amplification Polymorphic DNA (RAPD) or genomic sequence analysis (e.g. BLAST, Phylogenetic tree, etc.). For the identification purpose, the sequences obtained were BLAST analysed to identify the homologous sequences.

Sequence and Phylogenetic Tree Analysis of the three Different Types (A, B and C) Isolated Ganoderma

For each type; A, B and C, the seven possible homologous from the NCBI GenBank database were identified as shown in Table 2a, b and c, respectively. In Table 2a, it was suggested that the Type A isolate was the *Ganoderma sp.* since the most similar homology to the sequence is *Ganoderma sp.* BRIUMSa, based on BLAST maximum score followed by *Ganoderma sp.* BRIUMSc and *Ganoderma sp.* BRIUMSb species with 99% maximum identity for each of the homologous sequence. Meanwhile, the nearest identified subspecies of the *Ganoderma* from sample A, based on the NCBI Genbank database, was *Ganoderma aff. Steyaertanum* with maximum identity of 99%. Type B isolate was also identified as *Ganoderma sp.* as shown in Table 2b with 99% maximum identity for the first three BLAST results. Type C isolate was also suggested belong to *Ganoderma sp.* as shown in Table 2c with 99% maximum identity for the first two BLAST results. In contrast to type A isolate, the maximum identity for *Ganoderma aff. Steyaertanum* were slightly lower with 98% identity for both type B and C isolates. Fig. 2a, b and c show the phylogenetic tree constructed on the relationships among the homologous microorganisms.

Discussion

The samples of fruiting bodies were collected from the five incident areas and divided based on their morphologies. They were further divided based on their context colour and the pileus margin patterns. Context colour of *Ganoderma* varies from white to deep brown and this is considered as a useful character in classification (Seo and Krik, 2000). From the 106 fruiting bodies collected from different areas in Sabah, three major groups were identified (indicated as type A: which shows gradual hues of brown rings and smooth margin pattern of the pileus; B: Brown reddish color and waved margin pattern of the pileus; and C: Brown reddish color, and smooth margin pattern of the pileus) as shown in Fig. 3.

In recent years, progress has been made in the early detection and identification of this pathogen through more reliable methods such as enzyme-linked immunosorbent assays (ELISA) (Utomo and Niepold, 2000; Idris and Rafidah, 2008; Kandan *et al*., 2009) as well as polymerase chain reaction (PCR) based techniques involving certain non-specific *Ganoderma* primers (Miller *et al*., 2000; Idris *et al*., 2003; Latifah *et al*., 2005; Kandan *et al*., 2009). Molecular techniques exploiting variations in the ribosomal DNA (rDNA) have been used extensively for systematic and phylogenetic studies of fungal pathogens. Different regions of the rDNA diverged at different rates allowing the regions to be exploited at different taxonomic levels (Bruns *et al*., 1991; Latifah *et al*., 2005).

Table 2a: The most homologous seven microorganisms from the NCBI gene bank in comparison to the Type A isolate

Accession	Description	Max. score	Total score	Query coverage (%)	E value	Max. ident (%)
JN234427.1	Ganoderma sp. BRIUMSa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1164	1164	99%	0.0	99%
JN234429.1	Ganoderma sp. BRIUMSc internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1160	1160	99%	0.0	99%
JN234428.1	Ganoderma sp. BRIUMSb internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1158	1158	99%	0.0	99%
EF016754.1	Ganoderma sp. STK-2006a internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1146	1146	100%	0.0	98%
EU239386.1	Ganoderma aff. steyaertanum C16452 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	1059	1059	90%	0.0	99%
HM138671.1	Ganoderma sp. HKAS58053 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1048	1048	100%	0.0	96%
HM138670.1	Ganoderma sp. HKAS58055 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1048	1048	100%	0.0	96%

Table 2b: The most homologous seven microorganisms from the NCBI gene bank in comparison to the Type B isolate

Accession	Description	Max. score	Total score	Query coverage (%)	E value	Max. ident (%)
JN234428.1	Ganoderma sp. BRIUMSb internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1173	1173	99%	0.0	99%
JN234427.1	Ganoderma sp. BRIUMSa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1168	1168	99%	0.0	99%
JN234429.1	Ganoderma sp. BRIUMSc internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1153	1153	99%	0.0	99%
EF016754.1	Ganoderma sp. STK-2006a internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1146	1146	100%	0.0	98%
HM138671.1	Ganoderma sp. HKAS58053 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1053	1053	100%	0.0	96%
HM138670.1	Ganoderma sp. HKAS58055 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1053	1053	100%	0.0	96%
EU239386.1	Ganoderma aff. steyaertanum C16452 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	1046	1046	90%	0.0	98%

Table 2c: The most homologous seven microorganisms from NCBI gene bank in comparison to the Type C isolate

Accession	Description	Max. score	Total score	Query coverage (%)	E value	Max. ident (%)
JN234427.1	Ganoderma sp. BRIUMSa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1173	1173	99%	0.0	99%
JN234428.1	Ganoderma sp. BRIUMSb internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1168	1168	99%	0.0	99%
JN234429.1	Ganoderma sp. BRIUMSc internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1147	1147	99%	0.0	98%
EF016754.1	Ganoderma sp. STK-2006a internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1140	1140	100%	0.0	98%
HM138671.1	Ganoderma sp. HKAS58053 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1048	1048	100%	0.0	96%
HM138670.1	Ganoderma sp. HKAS58055 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1048	1048	100%	0.0	96%
EU239386.1	Ganoderma aff. steyaertanum C16452 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	1040	1040	90%	0.0	98%

Note: The *Ganoderma sp*. BRIUMSa with maximum identification of 99% to this isolate

Fig. 2a: Evolutionary relationship of nine taxa among the Type A samples (with NCBI query ID: lcl|23611, highlighted in yellow) with other eight homologous microorganisms. The evolutionary history was inferred using the Fast-Minimum Evolution method with maximum sequence difference at 0.05. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic. The evolutionary distances were self-computed by BLAST pairwise alignment for distance tree result and are in the units of the number of base substitutions per site

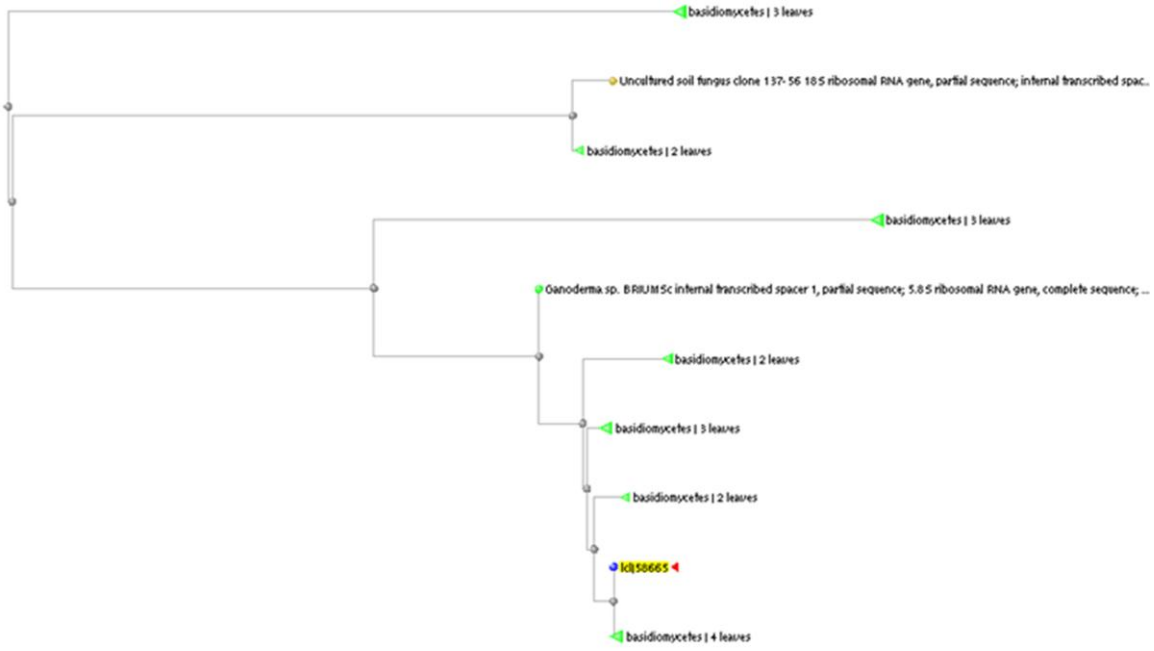

Fig. 2b: Evolutionary relationship of nine taxa among the Type B samples (with NCBI query ID: lcl|58665 highlighted in yellow) with other eight homologous microorganisms. The evolutionary history was inferred using the Fast-Minimum Evolution method with maximum sequence difference at 0.05. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenies. The evolutionary distances were self-computed by BLAST pairwise alignment for distance tree result and are in the units of the number of base substitutions per site

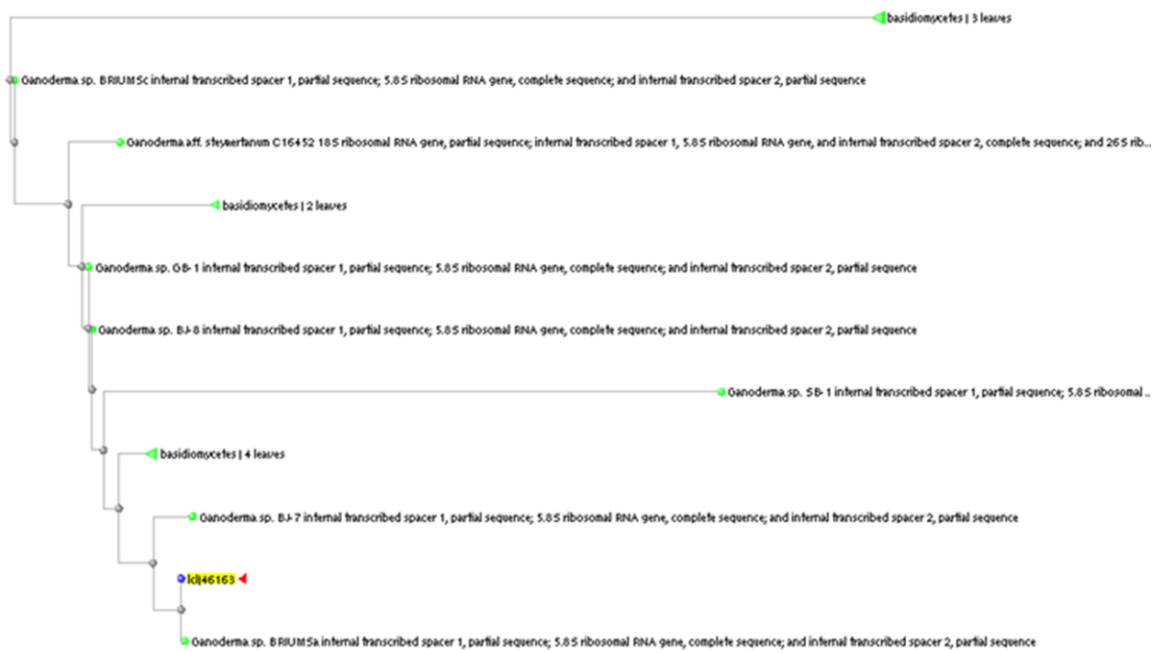

Fig. 2c: Evolutionary relationship of nine taxa among the Type C samples (with NCBI query ID: lcl|46163, highlighted in yellow) with other eight homologous microorganisms. The evolutionary history was inferred using the Fast-Minimum Evolution method with maximum sequence difference at 0.05. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenies. The evolutionary distances were self-computed by BLAST pairwise alignment for distance tree result and are in the units of the number of base substitutions per site

Fig. 3: Fruiting bodies of basidiomycetes collected from infected oil palms at five different locations in Sabah. The fruiting bodies were morphologically classified into three types; A (Gradual hues of brown rings, and smooth margin pattern of the pileus), B (Brown reddish color, and waved margin pattern of the pileus) and C (Brown reddish color, and smooth margin pattern of the pileus). **i:** Type A (fruiting bodies collected from Kimanis); **ii:** Type B (fruiting bodies collected from Kimanis),**iii:** Type A (fruiting bodies collected from Langkon estate,. **iv:** Type C (fruiting bodies collected from Langkon estate),**v:** Type A (fruiting bodies collected from Sandau, Lahad Datu),**vi:** Type A(fruiting bodies collected from Gua Gomantong, Sandakan),**vii:** Type A (fruiting bodies collected from Sungai Balong, Tawau)

The phylogenetic tree gives stronger evidence for the three samples (type A, B and C isolates) to being classified as *Ganoderma* species. Although from the phylogenetic tree, no specific subspecies identified are closely related to the three samples, however the homologous sequence database shows that the nearest subspecies for the sample is *Ganoderma aff. Steyaertanum,* which is mostly associated with root rot disease of *Acacia mangium* plantations in Indonesia and Malaysia (Glen *et al*., 2007). The samples were collected from infected oil palm, so it is strongly believed that the species are a type of *Ganoderma*, which usually infect oil palm such as *G.*

boninense (Chong *et al*., 2011). Further identification in terms of morphology, invasion or infection at molecular level must be conducted to identify the subspecies of these collected fruiting bodies.

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