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ITS-PCR-RFLP Analysis of *Ganoderma* sp. Infecting Industrial Crops

Nusaibah, S.A.[1*], Latiffah, Z.[2] and Hassaan, A.R.[3]
[1]Institute of Tropical Agriculture, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
[2]School of Biological Sciences, School of Pharmaceutical Sciences,
Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia
[*]E-mail: nus_ali@yahoo..com

ABSTRACT

Ganoderma is a disastrous pathogen that has been causing tremendous losses to economically important crops in many countries. Vast genetic variations have been observed among several *Ganoderma* species, even from the same host. In this study, genetic variation was assessed among 44 isolates of *Ganoderma* sp. isolated from the basidiocarps of four different hosts (oil palm, rubber, tea, and forest trees) collected from selected areas of Peninsular Malaysia. Restriction Fragment Length Polymorphism (RFLP) technique, using ITS1 and ITS4 primers, was used to amplify Internal Transcribed Spacer (ITS) regions. Amplified products were further digested using *Bsu* 151, *Hind* III and *Taq* I restriction enzymes. Cluster analysis with UPGMA using genetic distances clustered all the isolates studied into four main groups. Generally, *Ganoderma* isolates from the same host were clustered together. The isolates from tea and rubber were more closely related compared to oil palm and forest trees. Similarly, the *Ganoderma* isolates from the same host were also clustered together, and three species were identified, namely, *G. boninense* (from oil palm and coconut stumps), *G. philippii* (rubber) and *G. australe* (forest trees). The results obtained from the analysis showed that host preference was a possible factor in the differentiation of *Ganoderma* species.

Keywords: *Ganoderma*, ITS region, PCR-RFLP

INTRODUCTION

Ganoderma is a basidomycetous fungus that causes disease in many crops, such as rubber, tea, palms and forest trees. Among other, *Ganoderma* also causes basal stem rot (BSR) disease in oil palm (*Elaeis guineensis*), red rot in rubber (*Hevea brasiliensis*) and white rot in forest trees. The BSR disease in oil palm creates symptoms like large lesions at the early stage and at the foliar stage. One-half of the cross-sectional areas at the stem base necroses by the pathogen causing restriction of water supply and nutrients to the aerial part that lead external symptoms such as wilting and malnutrition (Turner, 1981). Meanwhile, wood or forest trees under decay by white rotters show symptoms where the wood appears stringy in later stages of decay and it also gets bleached, exhibiting lighter pigmentation than sound wood (Alexopoulos *et al*., 1979).

Genetic studies on the population and host range of *Ganoderma* species showed that inter- and intra-genetic variations occur specifically in this fungus (Miller, 1995; Idris, 1999; Moncalvo *et al*., 1995b; Utomo and Niepold, 2000; Latiffah, 2001; Pilotti *et al*., 2003; Nusaibah *et al*., 2007). Nonetheless, the identification of *Ganoderma* at the species level is difficult and a reliable identification key does not exist

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[*]Corresponding Author

for pre-adult stages based on the morphological characteristics. The use of restriction fragment length polymorphism (RFLP) has the advantage of combining highly conserved sequences in the Internal transcribed spacer (ITS) - 5.8S- ITS4 rDNA regions with variable sequences in the ITS regions at species level (Moritz *et al.*, 2000), whereby, the ITS shows a high inter-specific variability and an extremely low intra-specific variability. ITS-PCR-RFLP is a power tool that has been proven to facilitate in genetic variation studies among *Ganoderma*. A similar tool was used by Moncalvo *et al*. (1995b), Utomo and Niepold (2000), and Latiffah (2001) in their *Ganoderma* population work.

The aim of the present study was to collect the *Ganoderma* basidiocarps from different hosts (oil palm, rubber, tea and forest trees) from some selected geographical locations (states in Peninsular Malaysia), isolate the pure cultures of *Ganoderma* from its basidiocarps, as well as identify it at species level using the molecular approach, the PCR-RFLP on ITS - 5.8S- ITS4 rDNA regions.

MATERIALS AND METHODS

Sampling and Isolation of Ganoderma sp. *from Its Basidocarps*

The species name, isolates code, host and origin of the *Ganoderma sp*. used in this study are given in Table 1. Meanwhile, the pure cultures of *Ganoderma sp*. from all the hosts were isolated from its basidiocarps following the method proposed by Latiffah (2001) with a slight modification. These isolates were maintained at 27± 2°C on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI).

Genomic DNA Extraction Protocol

In this study, the *Ganoderma* mycelium, grown on the PDA plates, was used for DNA extraction. Four pieces of 2 × 2 cm^2 dialysis membrane were cut and sterilized. These pieces of membrane were placed on the PDA plates using sterile forceps and one drop of liquid PDA was subsequently placed on each membrane. The pure cultures of *Ganoderma* were then sub-cultured on each membrane on the PDA plates until the growth covered the whole plate, a process which took about seven to eight days. In order to prepare the samples for DNA extraction, mycelium which had grown on dialysis membrane was thorn using the forceps and placed in a mortar. Later, an adequate amount of liquid nitrogen was added before the mycelium was ground into fine powder. For DNA extraction, 20 to 25 mg of the powdered sample was weighed in a 1.5 ml Eppendorf tube. The DNA extraction was carried out using the phenol-chloroform method described by Reader and Broda (1985). This was followed by dissolving the DNA TE buffer and storing it at -20°C until further use. The extracted genomic DNA was checked for its concentration and purity using a spectrophotometer (Ultrospec 2000, UV/Visible Spectrophotometer, Pharmacia Biotech).

DNA Amplification

The ITS regions were amplified using primer ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White *et al.*, 1991). Amplification was performed in 25µl of reaction mixture containing 0.25 µl of DNA template, 2.5 µl 10X PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP (dATP, dTTP, dGTP, and dCTP), 0.4 µM of both primers, 1 unit *Taq* polymerase and ddH_2O. Each PCR tube was overlaid with two drops of paraffin oil to prevent evaporation during the process. Meanwhile, DNA Engine™ Peltier Thermal Cycler Model PTC-100 was used to run the Polymerase Chain Reaction (PCR). The PCR started with denaturation for 2 min at 95°C. This was followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 30s at 63°C and extension for 2 min at 72°C. The final step of extension was then carried out for 10 min at 72°C, before it was maintained at 10°C. The PCR product was run on 1.7% agarose gel, stained with ethidium bromide (EtBr) and visualized under a UV transilluminator (BioRad).

TABLE 1
The isolate code, origin and host of *Ganoderma* sp. sampled from 2004 – 2006.

No.	Isolate code	Origin	Host
1	TPS	Pondok Tanjung, Perak	*Shorea* tree
2	BG2	Botanical Garden, Pulau Pinang	Kedondong tree
3	BG10	Botanical Garden, Pulau Pinang	Forest tree
4	BG12	Botanical Garden, Pulau Pinang	Forest tree
5	BG9	Botanical Garden, Pulau Pinang	Forest tree
6	BG21	Botanical Garden, Pulau Pinang	Forest tree
7	BG22	Botanical Garden, Pulau Pinang	Forest tree
8	BG23	Botanical Garden, Pulau Pinang	Forest tree
9	BW10	Bukit Wang, Kedah	Forest tree
10	BW3	Bukit Wang, Kedah	Forest tree
11	BW11	Bukit Wang, Kedah	Forest tree
12	BW5	Bukit Wang, Kedah	Forest tree
13	BW2	Bukit Wang, Kedah	Forest tree
14	BW1	Bukit Wang, Kedah	Forest tree
15	BW8	Bukit Wang, Kedah	Forest tree
16	FP589	FRIM- *Ganoderma philippii*	Unknown
17	FP152	FRIM- *Ganoderma lucidum*	Unknown
18	FP104	FRIM- *Ganoderma australe*	Unknown
19	O9BS3	Bukit Serampang Estate, Tangkak Johor	Oil palm
20	38PL2	Paya Lang Estate, Segamat, Johor	Oil palm
21	BKS1	Bertam Estate, Seberang Prai	Oil palm
22	BKS2	Bertam Estate, Seberang Prai	Oil palm
23	BKS5	Bertam Estate, Seberang Prai	Oil palm
24	BKS7	Bertam Estate, Seberang Prai	Oil palm
25	BKS10	Bertam Estate, Seberang Prai	Oil palm
26	BKS11	Bertam Estate, Seberang Prai	Oil palm
27	BKS12	Bertam Estate, Seberang Prai	Oil palm
28	BKS13	Bertam Estate, Seberang Prai	Oil palm
29	BKS14	Bertam Estate, Seberang Prai	Oil palm
30	BKS15	Bertam Estate, Seberang Prai	Oil palm
31	BKS16	Bertam Estate, Seberang Prai	Oil palm
32	TTKS26	Trong, Taiping, Perak	Oil palm
33	PRBKS27	Padang Rotan, Bruas, Perak	Oil palm
34	LPOP	Ladang Pelam, Perak	Oil palm
35	UPKS	Teluk Intan, Perak	Oil palm
36	BIO	Rumah Tumbuhan, USM	Oil palm
37	TRKS	Teluk Ramunia, Johor	Oil palm
38	SEL28	MPOB- *Ganoderma boninense*	Oil palm
39	PER71	MPOB- *Ganoderma boninense*	Oil palm
40	CTEA1	Rubber Research Institute Malaysia	Tea
41	CTEA2	Rubber Research Institute Malaysia	Tea
42	CTEA3	Rubber Research Institute Malaysia	Tea
43	JPG2	Kluang, Johor	Rubber
44	JPG1	Kluang, Johor	Rubber
45	TH1	Titi Hayun, Kedah	Rubber

Enzyme Digestions

The amplified products were digested for 2 hours at 37°C, using the following restriction enzymes, *Bsu* 151, *Taq* I, and *Hind* III. The digested DNA fragments were then separated on 1.7% agarose gel and stained with EtBr before visualizing them under UV transilluminator.

Data Analysis

The molecular size of each fragment was estimated using a 100bp ladder. Meanwhile, the software NTSYS-PC (Numerical Taxonomy System of Multivariable Program) version 2.0 (Rohlf, 2000) was used to analyze the electrophoretic data. The presence and absence of restriction bands were scored as 1 or 0. The similarity of Simple Matching Coefficient (SMC) was used to generate the similarity matrix and coefficient, as described by Romesburg (1984), using the following formula:

SMC = (a + d) / (a + b + c + d), where:
a = number of bands present in two isolates
b = total number of bands unique in isolate 1
c = total number of bands unique in isolate 2
d = number of bands absent in two isolates

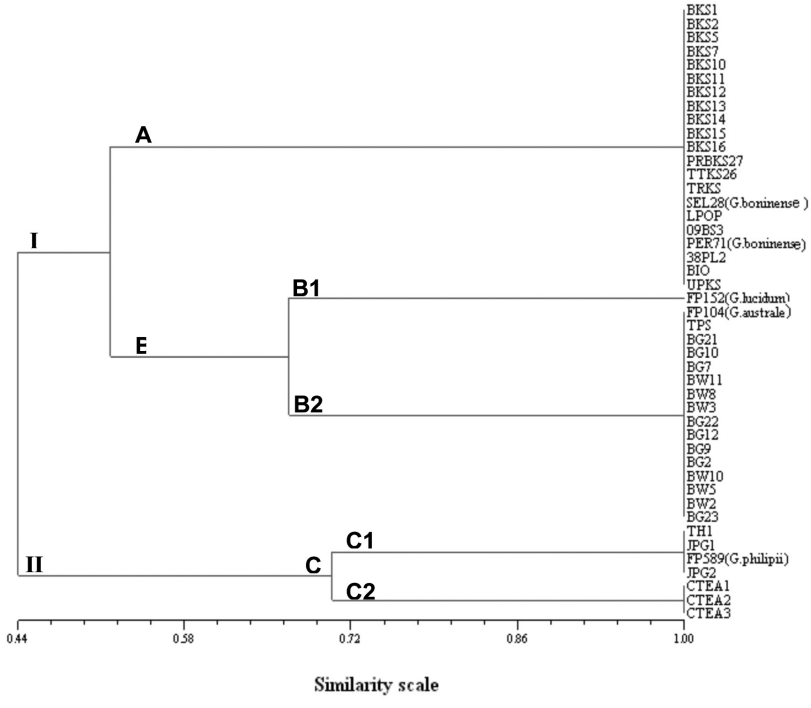

Fig. 1: Dendrogram from the UPGMA analysis using Simple Matching Coefficient based on the PCR-RFLP restriction patterns of ITS-5.85S-ITS2 region of Ganoderma *samples from various host*

Restriction Pattern of Hind *lll*

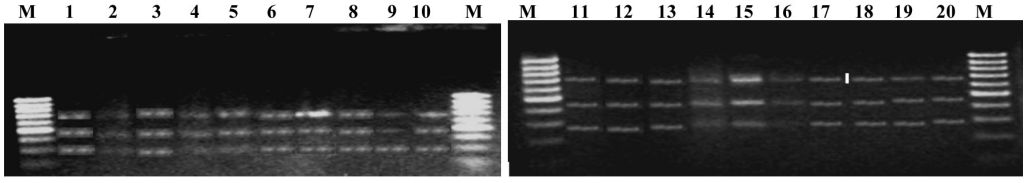

Fig. 2a: Restriction patterns of the amplified ITS regions + 5.8S gene of oil palm isolates digested using Hind *lll restriction enzyme Lane 1: BKS1; Lane 2: BKS2; Lane 3: BKS5; Lane 4: BKS7; Lane 5: BKS10; Lane 6: BKS11; Lane 7: BKS12; Lane 8: BKS13; Lane 9: BKS14; Lane 10: BKS16; Lane 11: PBRKS; Lane 12: TTKS; Lane 13: TRKS; Lane 14: SEL 28* (G. boninense)*; Lane 15: UPKS; Lane 16: PER 71* (G. boninense)*; Lane 17: 09BS3; Lane 18: 38PL2; Lane 19: BIO; Lane 20: LPOP; M=Marker 100 bp* (Fermentas)

Restriction Patterns of Bsu *151*

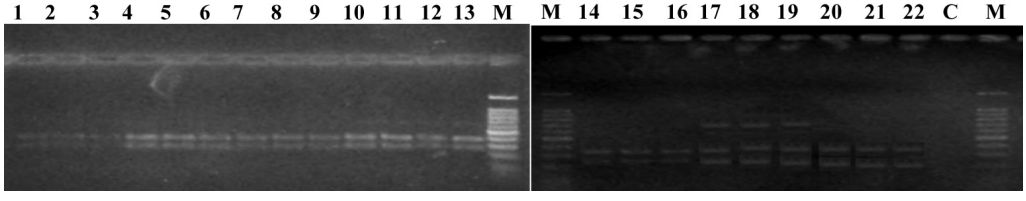

Fig. 2b: Restriction patterns of the amplified ITS regions + 5.8S gene of forest tree isolates digested using Bsu *151 restriction enzyme. Lane 1: BW2; Lane 2: BW3; Lane 3: BW5; Lane 4: BW8; Lane 5: BW10; Lane 6: BW11; Lane 7: BG2; Lane 8: BG7; Lane 9:BG9; Lane 10: BG10; Lane 11: BG12; Lane 12: BG21; Lane 13: BG22; Lane 14-16: isolates from rubber (1: PG1, 2: PG2, 3: PG3); Lane 17-19: isolates from tea (4: CTEA1, 5: CTEA2, 6: CTEA3); Lane 20: FP152* (G. lucidum)*; Lane 21: FP589* (G. philippii)*; Lane 22: FP104* (G. australe)*; Lane C: Control; M= Marker 100 bp* (Fermentas)

Restriction Patterns of Taq *I*

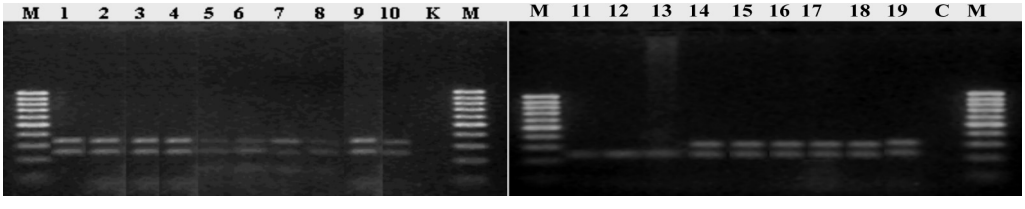

Fig. 2c: Restriction patterns of the amplified ITS regions + 5.8S gene of oil palm isolates digested using Taq *I restriction enzyme. Lane 1: PBRKS; Lane 2: TTKS; Lane 3: TRKS; Lane 4: SEL 28* (G. boninense)*; Lane 5: UPKS; Lane 6: PER71 (*G. boninense*); Lane 7: 09BS3; Lane 8: 38PL2; Lane 9: BIO; Lane 10: LPOP; Lane 11-13: isolates from rubber (1: PG1, 2: PG2, 3: PG3); Lane 14-16: isolates from tea (4: CTEA1, 5: CTEA2, 6: CTEA3); Lane 17: FP152* (G. lucidum)*; Lane 18: FP589* (G. philippii)*; Lane 19: FP104* (G. australe)*; Lane C: Control; M=Marker 100 bp* (Fermentas)

RESULTS AND DISCUSSION

Fig. 1 shows a dendrogram from the UPGMA cluster analysis of the ITS1-5.8S-ITS4 restriction fragments from all the samples analyzed. The dendrogram can be separated into two major clusters. Major cluster I comprises of sub-clusters A and B, while major cluster II is formed by only one sub-cluster that is labelled as sub-cluster C. Major Cluster II was linked to major cluster I with approximately 44% similarity. The sub-cluster A consisted of 21 isolates of *Ganoderma* from oil palm (BKS1, BKS2, BKS5, BKS7, BKS10, BKS11, BKS12, BKS13, BKS14, BKS 15, BKS16, PBRKS27, TTKS 26, TRKS, SEL28, LPOP, 09BS3, PER71, 38PL2, BIO, and UPKS), whereas sub-cluster B was divided into sub-sub cluster B1 and B2. The sub-sub cluster B1 contained only one isolate, FP152 (*G. lucidum*), whereas the sub-sub cluster B2 comprised all the isolates from the forest trees (TPS, BG21, BG10, BG7, BW11, BW8, BW3, BG22, BG12, BG9, BG2, BW10, BW5, BW2, and BG23) and FP104 (*G. australe*). In the major cluster II, sub-cluster c was divided to sub-sub cluster C1 and C2. Sub-sub cluster C1 comprised of all the isolates from rubber THI, JPG1, JPG2) and the isolate FP589 (*G. philippii*). Meanwhile, all the isolates from tea (CTEA1, CTEA2, and CTEA3) were clustered under the sub-sub cluster C2. The overall matrix similarity obtained among all the isolates, from the different hosts, was found to be between 44-100%. A 100% similarity was obtained from all the isolates within the same host. The highest similarity of 70% from the different hosts was observed between rubber and tea. A similarity of 52% was obtained between oil palm and both rubber and forest trees, followed by 49% of the matrix similarity between rubber and forest trees. Finally, the lowest similarity of 30% was observed among the *Ganoderma* isolates from oil palm and tea.

Table 2 indicates the estimated restriction fragment sizes of the *Ganoderma* isolates from oil palm, rubber, tea, and forest trees, FP152, FP589 and FP104 digested using *Bsu* l5l, *Taq* l and *Hind* lll restriction enzymes. The total PCR-RFLP size for all the *Ganoderma* isolates was about 600 bp. A similar amplification result was obtained by Bridge *et al*. (2000) in their *Ganoderma* phylogenetic study. Nonetheless, the total restriction fragment sizes recorded in Table 2 did not give 600 bp as a sum. A smaller sum of fragment sizes was observed in *Taq* l (*Fig. 2a*) restriction enzymes fragments for forest tree *Ganoderma* isolates. According to Gottlieb *et al*. (2000), this phenomenon might have occurred because the size of the developed fragments was too small (10 bp to 50 bp) and it could have been lost during the electrophoresis procedure. The other phenomenon that was detected was the larger sum of fragments from the total PCR-RFLP product. This was observed in the fragments developed by *Bsu* 151 (*Fig. 2b*) in all its restricted *Ganoderma* isolates from tea plant and by *Hind* lll (*Fig. 2a*) in all its *Ganoderma* isolates from oil palm. The explanation for this phenomenon was probably the presence of heterocaryon in the mycelium of *Ganoderma* that had been prepared for sexual reproduction (Hibbett, 1992). In this study, not all restriction enzymes managed to digest the PCR-RFLP amplified products. For instance, *Taq* l (*Fig. 2c*) restriction enzyme could digest all the PCR products of *Ganoderma* isolates from all the hosts. However, restriction enzyme *Hind* lll (*Fig. 2a*) only managed to digest the *Ganoderma* isolates from oil palm, SEL 28, and PER 71 (*G. boninense*). This was because these restriction enzymes did not find recognition sites on the ITS1-5.8S-ITS4 regions of the undigested isolates.

A previous study by Miller (1995) revealed that the use of PCR-RFLP had proven variability could be detected between the *Ganoderma* isolates in a population that differs in terms of the isolate field-related characters. Nusaibah *et al*. (2007) reported that by using the AFLP as a molecular marker on the *Ganoderma* isolates from oil palm could reveal both the inter- and intra-specific variations between the same species of *Ganoderma* which had made the fungus difficult to be characterized at the species level. Moreover, Nusaibah *et al*. (2007) have also managed to cluster the pathogenic *Ganoderma* in a cluster other than the non-

TABLE 2
Estimated restriction fragment sizes of *Ganoderma* isolates from oil palm, rubber, tea, and forest trees, FP152, FP589 and FP104 digested using *Bsu* l5l, *Taq* l and *Hind* lll restriction enzymes

Isolate	Estimated restriction fragment size (bp)								
	Bsu 151			*Taq* I			*Hind* III		
Oil palm									
O9BS3	150	200	350	150	250	350	200	350	600
38PL2	150	200	350	150	250	350	200	350	600
BKS1	150	200	350	150	250	350	200	350	600
BKS2	150	200	350	150	250	350	200	350	600
BKS5	150	200	350	150	250	350	200	350	600
BKS7	150	200	350	150	250	350	200	350	600
BKS10	150	200	350	150	250	350	200	350	600
BKS11	150	200	350	150	250	350	200	350	600
BKS12	150	200	350	150	250	350	200	350	600
BKS13	150	200	350	150	250	350	200	350	600
BKS14	150	200	350	150	250	350	200	350	600
BKS15	150	200	350	150	250	350	200	350	600
BKS16	150	200	350	150	250	350	200	350	600
TTKS26	150	200	350	150	250	350	200	350	600
PRBKS27	150	200	350	150	250	350	200	350	600
LPOP	150	200	350	150	250	350	200	350	600
BIO	150	200	350	150	250	350	200	350	600
TRKS	150	200	350	150	250	350	200	350	600
SEL28 (*G. boninense*)	150	200	350	150	250	350	200	350	600
PER71(*G. boninense*)	150	200	350	150	250	350	200	350	600
UPKS	150	200	350	150	250	350	200	350	600
Forest tree									
TPS		300	400		150	250			
BG2		300	400		150	250			
BG10		300	400		150	250			
BG12		300	400		150	250			
BG9		300	400		150	250			
BG21		300	400		150	250			
BGBG23		300	400		150	250			
BW10		300	400		150	250			
BW3		300	400		150	250			
BW11		300	400		150	250			
BW5		300	400		150	250			
BW2		300	400		150	250			
BW1		300	400		150	250			
BW8		300	400		150	250			
Tea									
CTEA1	250	300	600	100	250	300			
CTEA2	250	300	600	100	250	300			
CTEA3	250	300	600	100	250	300			
Rubber									
JPG2		250	350	100	250	350			
JPG1		250	350	100	250	350			
TH1		250	350	100	250	350			
FP152 (*G. lucidum*)		250	350		250	350			
FP589 (*G. philippii*)		250	350	100	250	350			
FP104 (*G. australe*)		300	400		150	250			

pathogenic ones. The ITS region was used by Bridge *et al.* (2000) to develop a genetic marker that could differentiate the pathogenic and non-pathogenic *Ganoderma* isolates from oil palm.

A cluster analysis carried out on the banding patterns, obtained from the amplified ITS1-5.8S-ITS4 regions digested by the restriction enzymes, showed that the isolates were clustered according to the host. A similar study by Gottlieb *et al.* (2000) also proved that the *Ganoderma* isolates which were collected from South America could be identified at the species level using the ITS1-5.8S-ITS4 regions as they managed to identify the *Ganoderma* isolates collected as *G. lucidum*, *G. zonatum*, *G. sessiliformen*, *G. platense*, and *G. praelongum.* Another study by Smith and Sivasithamparam (2000a, b) used ITS region for species level characterization of *Ganoderma* sp. in Australia. An internal transcribed spacer region analysis was also found to be a useful tool in a phylogenetic study for *Ganoderma sp*. characterization. Moncalvo *et al.* (1995c) stated that the ITS1 region of *Ganoderma* is small enough to be easily amplified by PCR and is flanked by highly conserved sequences. Meanwhile, a phylogenetic study by Moncalvo *et al*. (1995a, c) managed to characterize *Ganoderma* from the temperate and tropical locations to its species level.

CONCLUSIONS

Based on the restriction fragment banding pattern analysis on all the isolates used in this study, three different species of *Ganoderma*, namely *G. boninense, G. philippii* and *G. australe*, gave different banding patterns, which were grouped under different clusters. This finding indicates that the PCR-RFLP on ITS1-5.8S-ITS4 region is a reliable technique to discriminate the *Ganoderma* species from different hosts. Nonetheless, further research on genetic variation is important to understand how the disease caused by *Ganoderma* on industrial crops is spread.

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