Journal of Oil Palm Research Vol. 22 August 2010 p. 814-821

SOMATIC INCOMPATIBILITY AND AFLP ANALYSIS OF FOUR SPECIES OF *Ganoderma* ISOLATED FROM OIL PALM

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ABSTRACT

Amplified fragment length polymorphism (AFLP) analysis and somatic incompatibility were used to assess inter- and intra-specific variations among 12 Ganoderma *isolates from four different species isolated from oil palm (*Elaeis guineensis*). Scorable bands (646) were obtained with 10 AFLP primer combination markers. Cluster analysis by the unweighted pair-group method with arithmetic averaging (UPGMA) using genetic distances showed that the isolates were of two main groups. The somatic incompatibility test gave results congruent to AFLP analysis where somatic incompatibility also occurred within the different isolates of the same species which indicated two different genotypes in one species. In general, pathogenic isolates were found to group together in the same cluster. Isolates from the same species were also more closely related to each other. The results indicate that molecular identification provides support to the morphological identification of the isolates studied.*

Keywords: *Ganoderma*, inter-intra specific variation, AFLP, somatic incompatibility.

Date received: 13 November 2009; **Sent for revision:** 27 January 2010; **Received in final form:** 7 June 2010; **Accepted:** 9 July 2010.

INTRODUCTION

Malaysia is one of the world's largest producers and exporters of palm oil. In fact, oil palm is the most important agriculture crop in Malaysia and contributes significantly to her economy. In the year 2008, Malaysia's export earnings increased by 44% to around USD 19.6 billion (MPOB, 2009). Increasing competition from other countries (mainly Indonesia) as well as from other vegetable oils (namely soyabean, rapeseed, sunflower seed, corn and peanut) dictates that Malaysian plantations have to improve yield continuously.

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Managing disease is an important aspect of maintaining and subsequently increasing oil palm yields. One of the most significant diseases is basal stem rot (BSR) that causes significant losses in production (Ho and Nawawi, 1985; Khairudin, 1990; Idris, 1999). To date very little information is available on the establishment of the causal fungus, and the mode of infection is still not clear. Furthermore, there is a lack of information on the identity of the pathogenic *Ganoderma* that causes BSR as well as the environmental conditions that help to spread BSR disease, adding to the confusion. Such a lack of understanding and knowledge on the inter- and intra-relationships of *Ganoderma* populations in oil palm is slowing down the development of effective disease control strategies (Miller, 1995).

The taxonomy of *Ganoderma* was initiated by Karsten in 1881 and continued by other mycologists (Miller, 1995; Moncalvo and Ryvarden, 1997; Gottlieb and Wright, 1999a, b; Idris, 1999; Latiffah, 2001; Pilotti, 2001, Nusaibah, 2007). However to date, the taxonomy is still incomplete and remains unclear. This is because most of the identification was done

based on morphological characteristics that were found to be inconsistent and confusing, especially at the species level (Adaskaveg and Gilbertson, 1986; 1987). Moncalvo and Ryvarden (1997) stated that taxonomic synonyms still exist because a large number of species were distinguished using characters that depend on the growth conditions and developmental stages rather than other more reliable morphological characters such as spore size and shape. Spore size is currently used and is the most reliable morphological character in identification at the species level (Miller, 1995; Gottlieb and Wright, 1999a, b; Idris, 1999; Latiffah *et al.*, 2005; Nusaibah, 2007). In the mid-1990s, *Ganoderma* taxonomists started to use molecular tools for identification as they were thought to be more reliable than using morphological traits. Moncalvo *et al.* (1995a, b) and Hseu *et al.* (1996) were among the first taxonomists to use ribosomal DNA for analysing the phylogenic relationships of *Ganoderma lucidum*. The somatic incompatibility test can also be used to determine whether fungal isolates are from different genotypes or are clones of a single genotype (Pilotti, 2001). Work by Worral (1997) indicated that somatic incompatibility maintains the individuality of confronting mycelia, and usually prevents genetic exchange between them. Somatic incompatibility in basidiomycetous fungi can be found in the secondary mycelia (heterokaryotic) stage.

In this study, both the somatic incompatibility test and amplified fragment length polymorphism (AFLP) markers were used to analyse *Ganoderma* isolates from four different species that have been distinguished morphologically. In the first experiment, AFLP analysis was used to measure inter- and intra-species variations among all the isolates, while in the second experiment the relationships between the genotypes were studied using the somatic incompatibility test. Both experiments were compared to determine if they gave a congruent result.

MATERIALS AND METHODS

Sample Collection

The species name, isolate code, host, host status and origin of the *Ganoderma* sp. used in this study are given in *Table 1*. All the isolates used in the study were identified morphologically using the criteria described by Khairudin (1990). Three different isolates of each species were studied. These isolates were maintained at 27 ± 2°C on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI).

Dikaryotic and Single Culture Isolates

Each isolate was examined under the microscope to confirm the presence of clamp connections. The somatic incompatibility group (SIG) test was carried out as described by Worral (1997) with a slight modification. A standard cork borer was used to obtain the plugs from 8-day-old actively growing *Ganoderma* cultures. Each plug in all combinations was paired in a 10 mm-square PDA plastic petri plate. Each combination was prepared in three replicates. The plates were then incubated in the dark at 26ºC, and hyphal interaction was observed daily until day 10. The degree of antagonism of the hyphal interaction was rated according to Adaskaveg and Gilbertson (1986): 0 = compatible, + = weak antagonism, ++ = moderate antagonism and +++ = strong antagonism.

DNA Extraction

Ganoderma mycelia grown on PDA plates were used for DNA extraction. Four pieces of a 2×2 cm dialysis membrane were cut and sterilized. These

TABLE 1. SPECIES NAME, ORIGIN AND HOST PLANT OF THE ISOLATES

Isolate code	*Ganoderma* species	Host	Host status	Origin
PER71	*G. boninense*	*Elaeis guineensis*	Living	Teluk Intan, Perak
POR69	*G. boninense*	*Elaeis guineensis*	Living	Batu Pahat, Johor
JOH200	*G. boninense*	*Elaeis guineensis*	Living	Batu Pahat, Johor
POR73	*G. zonatum*	*Elaeis guineensis*	Living	Sepang, Selangor
POR74	*G. zonatum*	*Elaeis guineensis*	Living	Sepang, Selangor
POR75	*G. zonatum*	*Elaeis guineensis*	Living	Sepang, Selangor
337035	*G. miniatocinctum*	*Elaeis guineensis*	Living	Klang, Selangor
337036	*G. miniatocinctum*	*Elaeis guineensis*	Living	Klang, Selangor
337037	*G. miniatocinctum*	*Elaeis guineensis*	Living	Klang, Selangor
POR54	*G. tornatum*	*Elaeis guineensis*	Living	Bangi, Selangor
POR57	*G. tornatum*	*Elaeis guineensis*	Living	Bangi, Selangor
NPG1	*G. tornatum*	*Elaeis guineensis*	Living	Bangi, Selangor

pieces of membrane were placed on PDA plates using sterile forceps, and one drop of liquid PDA was subsequently placed on each membrane in order to enhance the growth of mycelia. *Ganoderma* pure cultures were 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. To prepare for DNA extraction, mycelia which had grown on the dialysis membrane were torn out using forceps and placed in a mortar. An adequate amount of liquid nitrogen was added, and the mycelia were ground to a fine powder. The 20 to 25 mg were weighed in a 1.5-ml eppendorf tube for DNA extraction. DNA extraction was carried out using the phenol-chloroform method as described by Reader and Broda (1985). The DNA was dissolved in TE buffer and stored at -20ºC until use. The DNA concentration was determined by nanodrop readings at A_{260} and A_{280}.

AFLP Analysis

AFLP analysis was carried out using the *Eco*RI/*Mse*I enzyme pairs. The *Eco*RI/*Mse*I assay was carried out using the GIBCO BRL AFLP® microorganism primer kit (INVITROGEN, USA), essentially as described in the manufacturer's manual with some modifications. The 250 ng of genomic DNA were digested with *Eco*RI and *Mse*I at 37ºC for 4 hr in a final volume of 25 µl. After heat inactivation of the enzymes at 70ºC, the fragments were ligated to the *Eco*RI and *Mse*I adapters in the presence of T4 DNA ligase (1 U) at 20ºC for 3 hr. A pre-selective amplification was then carried out by amplifying a 10-fold dilution of the ligation mixture. The pre-selective amplification was carried out with *Eco*RI and *Mse*I primers with no selective nucleotides. PCR was carried out for 20 cycles using a Perkin Elmer 9600 thermocycler as follows: 94ºC for 30 s, 56ºC for 60 s and 72ºC for 60 s. A 50-fold dilution was performed on the pre-amplified PCR products for subsequent use in selective amplification.

For selective amplification, a selected *Eco*RI primer was labelled with γ-^{33}PdATP using T4 polynucleotide kinase. The labelled *Eco*RI primer was mixed with a selected *Mse*I primer (containing dNTPs) in the ratio of 1:9 to form a primer master mix. The PCR contained 5 µl of the 10-fold diluted pre-amplified DNA, 5 µl of the primer master mix, 0.5 U of *Taq* DNA polymerase, and 2 µl of a 10× PCR buffer in a final volume of 20 µl. PCR conditions were one cycle at 94ºC for 30 s, 65ºC for 30 s and 72ºC for 60 s. The annealing temperature was lowered by 0.7ºC for each cycle during the subsequent 12 cycles, giving a touchdown phase of 13 cycles. Twenty-three cycles were then performed as follows: 94ºC for 30 s, 56ºC for 30 s and 72ºC for 60 s.

Gel Analysis

Aliquots of the post-PCR mixture were heated with an equal volume of formamide dye (98% (v/v) formamide, 10 mM EDTA, 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol) at 90ºC for 3 min. The 7 µl of the sample were electrophoresed in a 6% (w/v) polyacrylamide sequencing gel with 7.5 M urea at a constant power (85 W) and at approximately 1600 V and 40 mA for 2-3 hr. The gel was dried and exposed to X-ray film (Kodak XK-1) at -80ºC for two to three days.

Ten primer combinations (*Table 2*) were used in the study. These 10 primer combinations were selected by pre-screening a sub-set of the samples with 30 primer combinations. The 10 selected primer pairs produced clear and scorable polymorphic bands.

Data Analysis

The resulting binary matrix was used as the input file for a computer program that computes distances from qualitative data by a simple matching coefficient (SIMQUAL) in the Numerical Taxonomy System of Multivariate programme software (NTSYS) (Rohlf, 2000). A dendrogram was constructed using UPGMA to infer genetic relatedness of the *Ganoderma* isolates from different species.

RESULTS

Somatic Incompatibility Test

Clamp connections (*Figure 1*) were found in all isolates from day 5 to day 7. Interactions of all combinations in the somatic incompatibility test are shown in *Table 3*. In all self-pairings, the mycelia merged on the PDA medium forming a single colony indicating somatic compatibility

TABLE 2. LIST OF SELECTED PRIMER COMBINATIONS

Selected primer combination	No. of bands
1. EAC/MO	56
2. EO/MT	70
3. EA/MG	53
4. ET/MG	59
5. EO/MC	76
6. EA/MC	65
7. ET/MA	61
8. EAA/MA	66
9. EAA/MC	74
10. EA/MA	66
Total	646

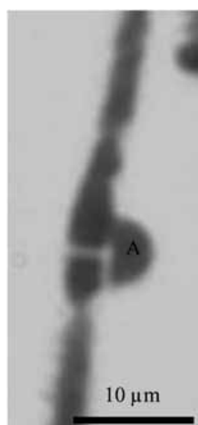

Figure 1. Light microscope (LM) photograph of a fully developed clamp connection (A) on the hypha of a dikaryotic Ganoderma *culture. Magnification (40 × 10).*

(*Figure 2A*). The presence of a demarcation line was not observed in all self-pairing plates. However, the presence of a demarcation line (arrowed) occurred in different isolates of the same species, and in plates paired with two different species, indicating somatically incompatible pairings (*Figures 2B* and *2C*).

AFLP Analysis

Out of 30 primer combinations screened, 10 primer combinations (*Table 2*) amplified the DNA well, producing clear and scorable bands (*Figure 3*). A total of 646 scorable markers were generated from the amplification products. These primers could generally differentiate between the pathogenic and non-pathogenic isolates. The size of the AFLP bands produced was between 0.089 and 0.33 kb in size for all the isolates analysed.

Cluster analysis with UPGMA using genetic distances showed that the isolates were divided into two main groups, cluster I and II. Cluster I comprised all the pathogenic *Ganoderma* species, namely *G. boninense* and *G. zonatum*, while cluster II contained *G. tornatum*, a species non-pathogenic to oil palm (*Figure 4*).

DISCUSSION

The results show that clamp connections appeared in all cultures used in the study. Clamp connections can be detected from day five onwards (Pilotti *et al.*, 2003). These structures are found in many basidiomycetes and they operate to ensure the maintenance of the dikaryotic condition in compartments of the secondary mycelia.

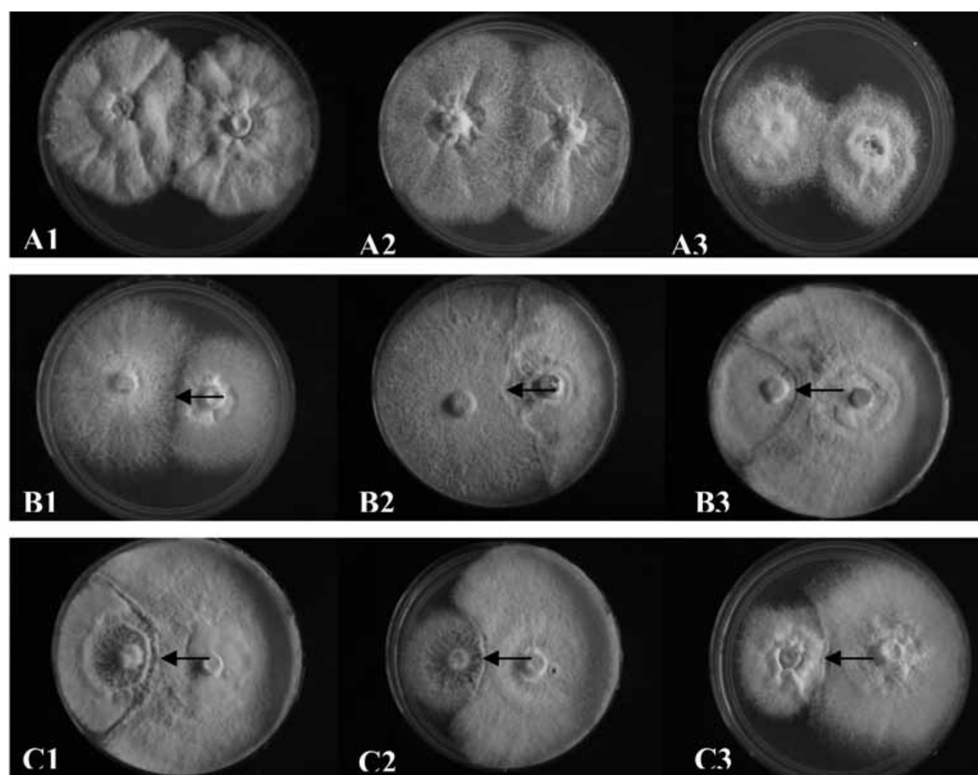

Figure 2. Somatic incompatibility reactions of Ganoderma *sp. isolates used in this study. A: Self-pairing (control), showing somatic compatibility. B: Somatic incompatibility observation among the same species but different isolates C: Somatic incompatibility among different* Ganoderma *species observed. Note the presence of a demarcation line (arrowed) in somatically incompatible pairings.*

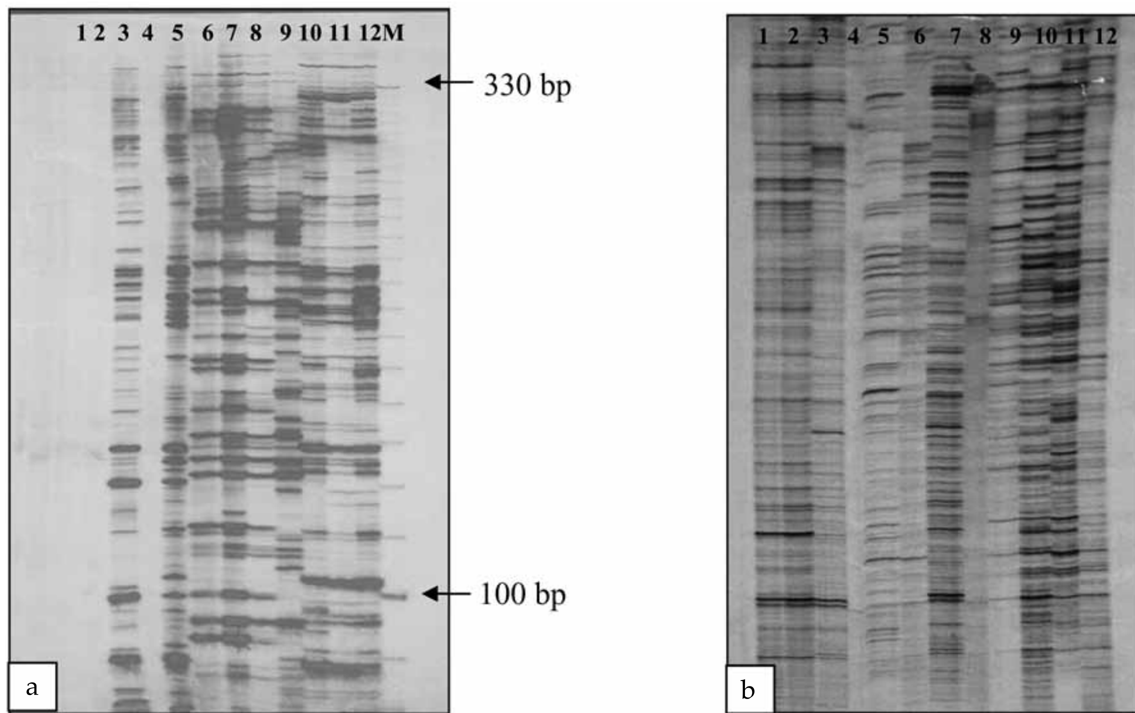

Figure 3. Close-up of AFLP profile: (a: primer EAC/MO) and (b: primer EO/MO), Lane 1-3: G. boninense*; lane 4-6:* G. zonatum*; lane 7-9;* G. miniatocinctum*; lane 10-12:* G. tornatum *and M-marker (30-330 bp AFLP ladder).*

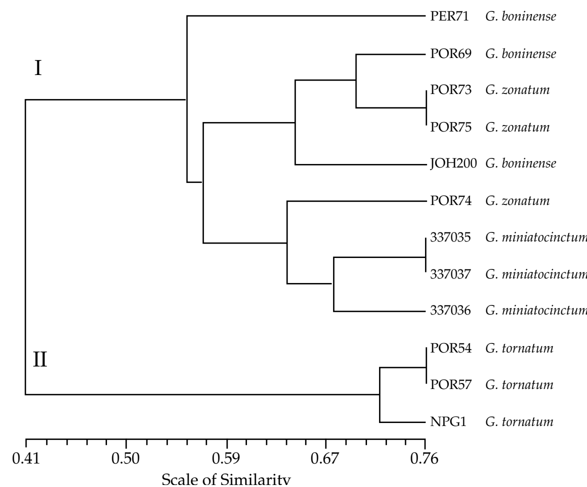

Figure 4. Dendrogram generated from pooled AFLP fingerprints of Ganoderma *dikaryon DNA. Groupings were based on UPGMA analysis using simple matching coefficient and the UPGMA method of cluster analysis and were based on 10 selected AFLP primers.*

In this study, we used the somatic incompatibility mechanism to identify different genets (numerous distinct individuals) among the *Ganoderma* isolates identified at the species level. This mechanism prevents or restricts non-self anastomosis somatic incompatibility (SI) or non-self rejection. In the cultures, incompatibility was observed from the occurrence of demarcation zones or barrage lines between colonies which were compared against the control, *i.e.* self-pairings. The results obtained indicate that genets were detected in all the samples that showed demarcation lines even between the same species. A total of 144 combinations of pairings gave 10 different genets in all the four species studied. In all cases, incompatibility occurred between paired isolates among different as well as the same species except for isolates POR75 and POR 73 (*G. zonatum*), 337035 and 337037 (*G. miniatocinctum*), and POR54 and POR57 (*G. tornatum*). These isolate pairings gave poor demarcation lines (weak antagonism) and merged into one culture. AFLP also gave the same results for these isolates as they showed 100% genetic similarity between the pairs of isolates from

TABLE 3. HYPHAL INTERACTION RATE OBSERVED IN SOMATIC INCOMPATIBILITY TEST

	PER71	POR69	JOH200	POR73	POR74	POR75	337035	337036	337037	POR54	POR57	NPG1
PER71	0	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
POR69	++	0	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
JOH200	++	++	0	+++	+++	+++	+++	+++	+++	+++	+++	+++
POR73	+++	++++	+++	0	++	+	+++	+++	+++	+++	+++	+++
POR74	+++	++++	+++	++	0	++	+++	+++	+++	+++	+++	+++
POR75	+++	+++	+++	+	++	0	+++	+++	+++	+++	+++	+++
337035	+++	+++	+++	+++	+++	+++	0	++	+	+++	+++	+++
337036	+++	+++	+++	+++	+++	+++	++	0	++	+++	+++	+++
337037	+++	+++	+++	+++	+++	+++	+	++	0	+++	+++	+++
POR54	+++	+++	+++	+++	+++	+++	+++	+++	+++	0	+	++
POR57	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	0	++
NPG1	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	0

Note: 0 = compatible.
+ = weak antagonism.
++ = moderate antagonism.
+++ = strong antagonism.

the same species mentioned above. The results indicate the close relatedness of these isolates. A possible explanation for this phenomenon includes root-to-root contact and perhaps mycelial spread as reported by Miller (1995) and Pilotti (2001). Although the role of basidiospores as one of the causal agents for this phenomenon was denied by many *Ganoderma* mycologists, a study by Lim and Fong (2005) showed that the fusion of two single basidiospores will form dikaryotic mycelia which can also be a causal explanation for the phenomenon stated above.

Genetic variation among all four species from oil palm, namely *G. boninense, G. zonatum, G. miniatocinctum* and *G. tornatum*, were examined using AFLP data and two major clusters of isolates were detected with UPGMA using NTSYS. All three isolates of the pathogenic species, namely *G. boninense, G. zonatum* and *G. miniatocinctum*, were clustered together while *G. tornatum*, a species non-pathogenic to oil palm (Idris, 1999), was clustered separately. According to the genetic similarity percentage, it was also observed that variations not only appeared between species but also between isolates within a species. These results might indicate that different genets occur even within a species. Somatic incompatibility supported this observation.

The ability of the AFLP technique to determine the genets in all the isolates studied as compared to the somatic incompatibility test, and analysing the genetic variations among these *Ganoderma* species were the goals of this study. The results obtained show congruency between the two methods.

CONCLUSION

The congruency of the somatic incompatibility test and the genotypes derived using AFLP found in this study agrees with earlier comparisons between somatic incompatibility and individual delimitation made using other genetic markers (Miller *et al.*, 1994; Latiffah, 2001; Pilotti *et al.*, 2003).

ACKNOWLEDGEMENT

We wish to thank the Director-General of MPOB for permission to publish this article. Thanks also to the staff of the Genomic and Pathology Group of MPOB for their assistance in conducting the study. This work was financed by the *Ganoderma* Special Grant (Code No. R003805000 RB01) of MPOB.

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