

## Molecular Characterization of *Fusarium Oxysporum* F. Sp. *Cubense* of Banana

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**Abstract: Problem statement:** Morphological characterization of *Fusarium* species which emphasize on microscopic and cultural characteristics are not sufficient to characterize *Fusarium Oxysporum* F. sp. *Cubense* (FOC) from banana as these characteristics could easily influence by environmental factors. As an alternative molecular methods were used to characterize and to assess genetic variation of FOC from different banana cultivars. Knowledge on the genetic variation is important to determine the genetic relationship between FOC isolates from different banana cultivars. **Approach:** Two PCR-based methods, Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and restriction analysis of the Internal Transcribed Spacer and 5.8S regions (ITS+5.8S regions) were used to characterize *Fusarium Oxysporum* F. sp. *Cubense* (FOC) isolates from different banana cultivars. The genetic relationship of the FOC isolates were analyzed using Un-weighted Pair-Group Method with Arithmetic Averages (UPGMA) cluster analysis based on Jaccard Similarity Coefficient. **Results:** Restriction patterns of the ITS+5.8S regions using nine restriction enzymes namely, Alu I, Eco RI, Eco 88I, Bsu RI, Bsu 15I, Hin fI, Hin 6I, Msp I and Taq I and ERIC-PCR showed low variation among the FOC isolates studied, indicating close relationship among the isolates. Un-weighted Pair-group Method with Arithmetic Averages (UPGMA) cluster analysis based on Jaccard Similarity Coefficient showed that the FOC isolates were grouped into two main clusters with similarity value of 41.4-100% in PCR-RFLP of ITS + 5.8S and 45-100% similarity based on ERIC-PCR analysis, respectively. Cluster analysis of the combined data also showed that the FOC isolates were grouped into two clusters, sharing 42.9-100% similarity. **Conclusion/Recommendations:** The results of the present study indicate that the FOC isolates were closely related regardless of banana cultivars and location.

**Key words:** PCR-RFLP, ERIC-PCR, *Fusarium oxysporum* F. sp. *Cubense*

### INTRODUCTION

*Fusarium Oxysporum* F. sp. *Cubense* (FOC) is the causal agent of banana wilt or Panama disease. It is a cosmopolitan soil-borne fungus which colonizes the vascular system of the host plant. When the fungus infected the host plant, it triggers the self-defense mechanisms whereby secretion of gel occurs followed by formation of tylose in the vascular vessels, thus, blocking the movement of water to the upper part of the host plant which in turn causes yellowing, wilting and eventually death to the host. At present, there are four identified races of FOC in which only races 1, 2 and 4 attacked banana cultivars. Race 4 is the most virulent strain which attacks Cavendish cultivar as well as Gros Michel and Bluggoe which is susceptible to race 1 and 2, respectively. Race 3 only attacks *Heliconia* sp. and only has mild effects on banana<sup>[13]</sup>.

Since the Panama disease outbreak in 1950s, the susceptible Gros Michel has been replaced with the more resistant Cavendish cultivar. However, the emergence of race 4 in Taiwan, South Africa and South Queensland, Australia<sup>[14]</sup>, followed by the disease incidence in the tropics sparked the urgency in formulating control methods especially in breeding resistant banana cultivar. In Malaysia, FOC race 4 was first reported in Johor in 1992.

Due to the inconsistency in some of the control methods used to control banana wilt such as flood fallowing and chemical fumigation, planting of resistant banana cultivar remain as the most promising option to solve banana wilt problem. However, breeding process to produce resistant banana cultivar is not an easy task as understanding of fungal genetics is essential. Morphological characterization alone is not enough as genetics information is not provided. Furthermore,

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expertise and experience are important in accurate morphological identification of *Fusarium* species since the process emphasized on differences of the morphological features such as the shapes and sizes of the macro and micro-conidia as well as the conidiogenous cells, pigmentation and growth rates which could readily be altered by environmental and cultural factors<sup>[5]</sup>. To compensate for the weaknesses, molecular methods using PCR-based techniques such as Random Amplified Polymorphic DNA (RAPD), PCR-RFLP of Internal Transcribed Spacer (ITS) and Intergenic Spacer (IGS), Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Random Amplified Microsatellites (RAMS) analysis have been used in characterization of *Fusarium* species. These techniques served as supportive means which provide genetic insight of *Fusarium* species.

Therefore, in this preliminary study, PCR-RFLP of ITS+5.8S regions and ERIC-PCR were conducted to characterize and assess genetic variation among FOC isolates from different banana cultivars in Malaysia.

## MATERIALS AND METHODS

**Fungal isolates and DNA extraction:** Thirteen FOC isolates from Malaysia and two isolates from Indonesia were used in this study (Table 1). For DNA extraction, cultures derived from single spores were plated on potato sucrose agar and incubated for 7 days at 28°C. Genomic DNA was extracted using DN easy Mini Plant kit (QIAGEN) according to the manufacturer's instructions.

**PCR amplification of ITS+5.8S regions:** The ITS+5.8S regions were amplified using ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primer pair<sup>[17]</sup>. Amplification was carried out in 25 µL reaction mixture containing 1X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 0.6 mM of each dNTPs, 0.25 µM of each primers, 1.25 U Taq polymerase and 4 ng genomic DNA. The reagents were obtained from Promega.

PCR amplification was performed in a Peltier Thermal Cycler (MJ research, PTC-100). The amplification starts with initial denaturation at 95°C for 5 min, followed by 35 cycles of 1 min denaturation at 94°C, 1 min of annealing at 55°C and 2 min extension at 72°C with a 10 min final extension at 72°C. The amplification products were separated by electrophoresis in 1.0% agarose gel (Amresco) using Tris Borate EDTA (TBE) as running buffer. After electrophoresis, the gels were stained with ethidium bromide, visualized under UV light and photographed using GeneSnap photo imaging system (SynGene). The approximate size of the amplified product was estimated using a 100 bp marker (MBI fermentas).

Table 1: List of banana cultivars of the FOC isolates

Code	Location	Banana cultivar/ common name
I2240N	Peningsurat, Indonesia	Kepok (Abu)
I2244N	Pekalongan, Indonesia	Uter
A2279N	KM 4, Changkat Jering, Perak	Emas
A2282N	Titi Gantung, Perak	Berangan
A2281N	Padang Ragut, Bota Kanan, Perak	Awak
A2295N	Bukit Nangka, Lenggong, Perak	Kepok (Abu)
A2296N	Changkat Jambu, Kati, Kuala Kangsar, Perak	Berangan
A2306N	Sri Iskandar, Perak	Awak
B2286N	Kancong Darat, Banting, Selangor	Kepok (Abu)
D2293N	Wakaf Stan, Kota Bharu, Kelantan	Kepok (Abu)
D2294N	Kg Berangan, Tumpat, Kelantan	Berangan
M2300N	Merlimau, Melaka	Nangka
P2305N	Sg Semambu, Kubang Semang, Penang	Awak Masam
T2290N	Lak Luk, Terengganu	Awak
T2304N	Kampung Penjara, Terengganu	Awak

**Restriction analysis:** About 5-10 µL of PCR products were digested using nine restriction enzymes, namely Alu I, Eco RI, Eco 88I, Bsu RI, Bsu 15I, Hin fI, Hin 6I, Msp I and Taq I (MBI fermentas) in separate reactions according to the manufacturer's instructions. The restriction fragments were separated by electrophoresis in 1.7% agarose gel (Amresco) using 1X TBE as running buffer. The analysis of restriction analysis after electrophoresis was the same as those described for PCR product analysis.

**Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR):** PCR amplifications were carried out in 25 µL reaction mixtures using ERIC1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3')<sup>[16]</sup>. The reaction mixture consists of 1X PCR buffer, 4.0 mM MgCl<sub>2</sub>, 120 mM of each dNTPs, 0.5 µM of each primer, 1.3 U of Taq polymerase, 5 ng of genomic DNA and de-ionized distilled water using a Peltier thermal cycler (PTC-200, MJ research). PCR amplification conditions were as follow: Initial denaturation at 95°C for 7 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 30s and extension at 65°C for 8 min and final extension was conducted at 65°C for 16 min. The analysis of ERIC-PCR banding patterns was the same as those described for restriction patterns except that the amplification products were separated in 1.5% agarose gel (Amresco) and the approximate sizes of the fragments were estimated by comparing with 1 kb ladder (MBI fermentas).

**Data analysis:** The restriction bands and ERIC-PCR bands were scored as presence (1) or absence (0) of a particular band to generate a binary matrix. The binary

matrix data were then subjected to Un-weighted Pair-Group Method with Arithmetic Averages (UPGMA) cluster analysis and genetic similarity matrices were constructed. For both analyses, Jaccard Similarity Coefficient was applied. UPGMA cluster analysis based on the combined data of restriction analysis and ERIC-PCR were also conducted using Jaccard Similarity Coefficients.

The binary matrices were analyzed using numerical taxonomy and multivariate analysis system (NT-SYSp) software version 2.1<sup>[12]</sup>. The genetic relationship of the 15 FOC isolates were inferred from the dendrogram constructed based on UPGMA cluster analysis.

## RESULTS

**PCR-RFLP of ITS+5.8S regions:** For all FOC isolates, DNA fragments approximately 550 bp were amplified using ITS1 and ITS4 primer pair. No length variation was observed for the amplified ITS + 5.8S regions. The ITS + 5.8S of all the FOC isolates could be digested using Taq I, Msp I, Alu I, Bsu 15I, Bsu RI, Hin fl, Hin 6I and Eco RI indicating that the ITS + 5.8S regions of the FOC isolates contain recognition sites for these enzymes. Figure 1 shows the restriction patterns of FOC isolates using Taq I. Only one isolate (P2305N) contains Eco 88I restriction site within the ITS + 5.8S regions. Each restriction enzyme generated two types of restriction patterns which were highly similar for the isolates studied, indicating close relationship between the FOC isolates. Estimated sizes of the restriction fragments generated using nine restriction enzymes are summarized in Table 2.

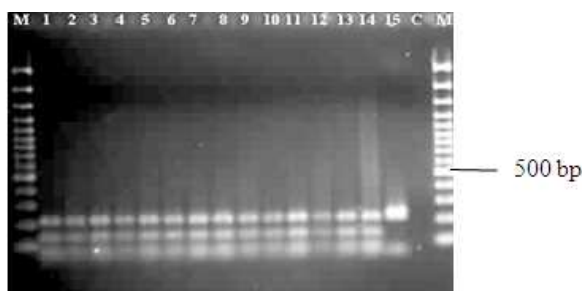


Fig 1: Restriction patterns of ITS+5.8S regions of FOC isolates from different banana cultivars, digested with Taq I. (Lane 1-15): 1: I2240N; 2: I2244N; 3: A2279N; 4: A2281N, 5: A2282N; 6: B2286N; 7: T2290N; 8: D2293N; 9: D2294N; 10: A2295N; 11: A2296N; 12: M2300N; 13: T2304N; 14: A2306N and 15: P2305N. C: Control; M: 100 bp marker

Cluster analysis based on Jaccard similarity coefficient separated the FOC isolates into two main clusters, sharing 41.4-100% genetic similarity (Fig. 2). Most of the FOC isolates were clustered in cluster A, except isolate P2305N which formed cluster B. Within cluster A, the FOC isolates were further divided into sub-cluster A1 and A2 with 90% similarity. Isolates in sub-cluster A1 (I2244N, A2279N, A2282N, B2286N, A2296N and M2300N) and isolates in sub-cluster A2 (I2240N, A2281N, T2290N, A2306N, T2304N, A2295N, D2293N and D2294N) showed 100% similar. Isolate P2305N in cluster B shared 41.4-44.4% similarity with other FOC isolates in cluster A.

Table 2: Estimated sizes of restriction fragments of FOC isolates

Restriction enzymes	Estimated sizes of restriction fragments (bp)
Taq I	80, 120, 220 (most) 220, 250 (P2305N)
Msp I	100, 450 (most) 100, 150, 300 (P2305)
Alu I	150, 350 (most) 150, 450 (P2305N)
Bsu 15I	210, 350 (most) 210, 380 (P2305N)
Bsu RI	100, 350 (most) 100, 300 (P2305N)
Hin FI	100, 180, 280 (I224N, A2281N, T2290N, T2304, D2293N, D2294N, A2295N and A2306N) 280 (2244, A2279N, A2282N, B2286N, A2296N, M2300N, P2305N)
Hin 6I	250, 300, 550 (most) 100, 180, 300 (P2305N)
Eco RI	550, 280 (most) 280, 300, 550 (P2305N)
Eco 88I	550 (most) 200, 350 (P2305N)

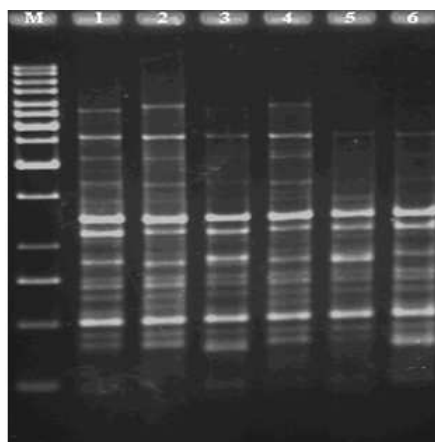


Fig. 2: Banding patterns obtained using ERIC1R and ERIC2 primers of several FOC isolates. (Lane 1-6) 1: I2240N, 2: I2244N, 3: D2293N, 4: M2300N, 5: T2304N and 6: A2281N. M: 100 bp marker

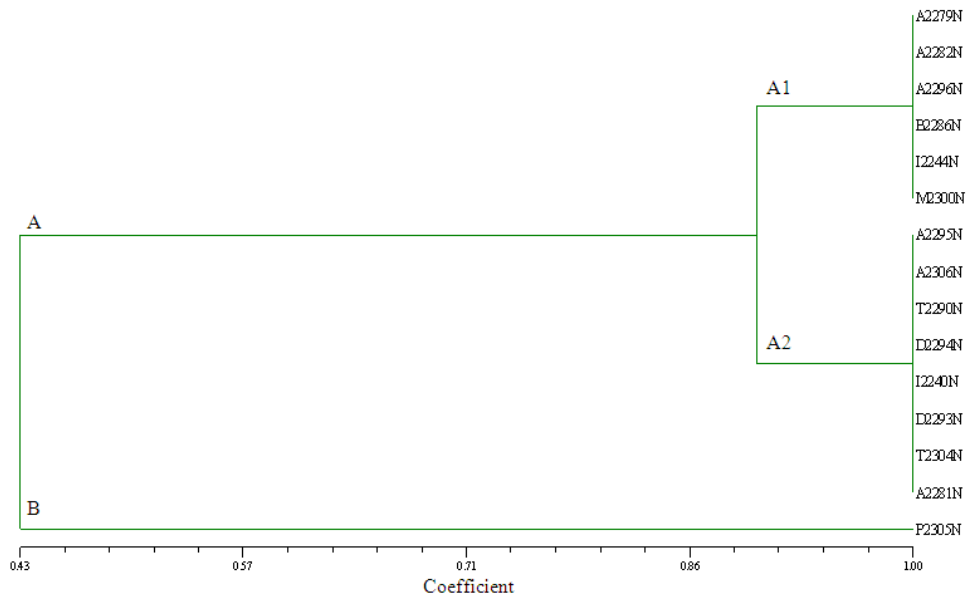


Fig. 3: Dendrogram generated from UPGMA cluster analysis using Jaccard Similarity Coefficient based on PCR-RFLP of ITS + 5.8S regions of rDNA

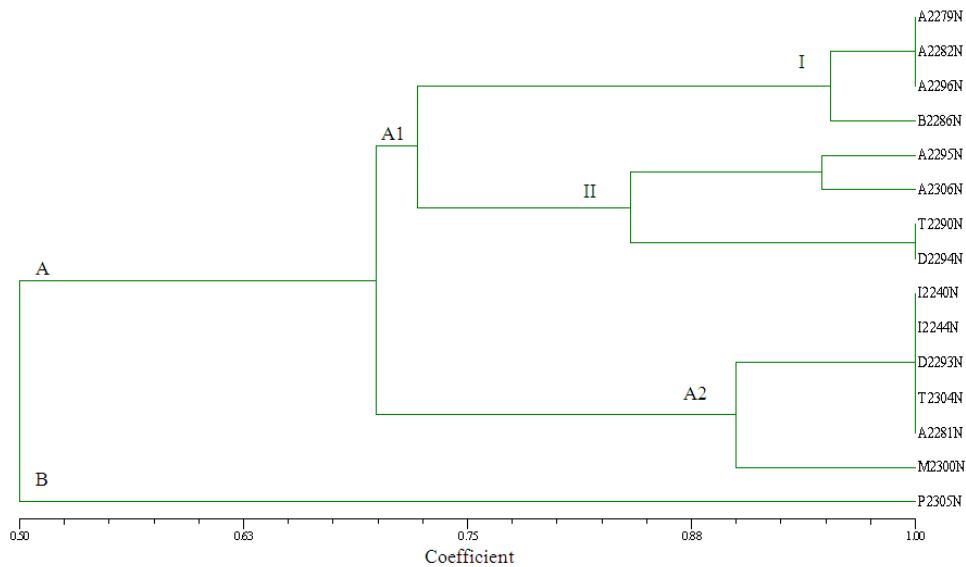


Fig. 4: Dendrogram generated from UPGMA cluster analysis using Jaccard Similarity Coefficient based on ERIC-PCR analysis

**Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR):** All FOC isolates generated 8-21 fragments ranging from 100-3750 bp using ERIC1R and ERIC2 primer pair. The FOC isolates shared highly similar banding patterns and four bands with sizes approximately 500, 750, 1125 and 1250 bp were present in all FOC isolates. Figure 3 shows ERIC-PCR banding patterns for several FOC isolates.

Cluster analysis of ERIC-PCR showed that the FOC isolates were clustered into two main clusters, sharing 45-100% similarity (Fig. 4). Like PCR-RFLP of ITS + 5.8S, most of the isolates were clustered in cluster A and only isolate P2305N was grouped into cluster B which shared 45-52.6% similarity with other FOC isolates.

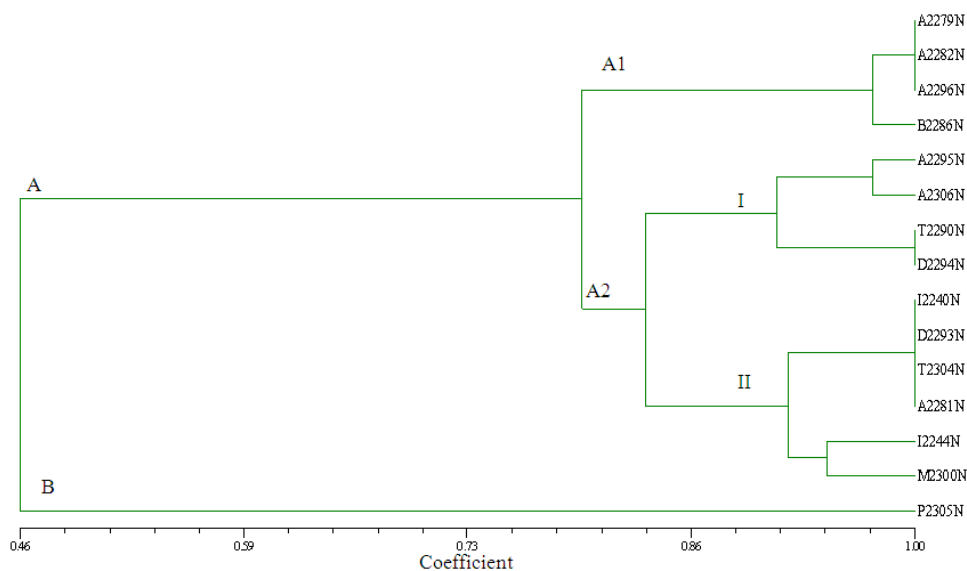


Fig. 5: Dendrogram generated from UPGMA cluster analysis using Jaccard Similarity Coefficient based on combined data of PCR-RFLP of ITS + 5.8S regions and ERIC-PCR

**Combined analysis of PCR-RFLP of ITS + 5.8S regions and ERIC-PCR:** Cluster analysis based on combined data, separated the FOC isolates into two main clusters, showing 42.9-100% similarity (Fig. 5). Most of the isolates were grouped into cluster A and only P2305N formed cluster B. Cluster A was further separated into sub-cluster A1 and A2 which shared 65.2-79.2% similarity. The isolates in sub-cluster A1 showed 97.6-100% similarity. In sub-sub-cluster I, the isolates (A2295N, A2306N, T2290N and D2294N) showed 92.7-100% similarity. Within sub-sub-cluster II which consists of isolates from Indonesia (I2240N and I2244N) and Malaysia (D2293N, T2304N, A2281N and M2300N) showed similarity from 94.7-100%.

## DISCUSSION

For all FOC isolates, 550 bp band of ITS + 5.8S was amplified using ITS1 and ITS4 primer pair which corresponded with the expected size of ITS + 5.8S for *F. oxysporum* as reported by<sup>[11]</sup>. A 550 bp of ITS + 5.8S was amplified for three special forms of *F. oxysporum*, namely *F. oxysporum* F. sp. *conglutinans*, *F. oxysporum* F. sp. *fragariae* and *F. oxysporum* F. sp. *raphani*.

Restriction analysis of the ITS + 5.8S revealed limited genetic variation among the FOC isolates. This was also in concordance with<sup>[11]</sup> which showed that the formae species of *F. oxysporum* were 100% genetically similar. In their study, restriction analysis of ITS + 5.8S with seven restriction enzymes yielded identical banding patterns for *F. oxysporum* F. sp. *conglutinans*,

*F. oxysporum* F. sp. *fragariae* and *F. oxysporum* F. sp. *raphani*. The results of the present study showed that the FOC isolates were closely related regardless of banana cultivars and locations.

In the restriction analysis, only Msp I and Eco 88I produced restriction fragments that were equivalent to the approximate size of the undigested fragment of the ITS + 5.8S regions. The total sizes of the restriction fragments were smaller than the undigested PCR product when digested using Taq I, Bsu RI, Hin fl and Alu I. This could be attributed to the difficulties in visualizing fragments smaller than 50 bp or co-migrating of same-sized fragments on the gel<sup>[3]</sup>. Another possible reason could be small fragments may be unresolved or lost during gel electrophoresis<sup>[8]</sup>. The total sizes of the restriction fragments were larger than the undigested fragment when digestions were conducted using Bsu 15I, Hin 6I, Eco RI and Eco 88I. This probably due to the presence of a mixed rDNA type<sup>[9]</sup> or the polymorphisms in the recognition sites<sup>[3]</sup>.

ERIC-PCR produced bands with approximately 100-3750 bp and the banding patterns for all FOC isolates were highly similar. The banding patterns generated using ERIC-PCR were more variable compared to restriction analysis of the ITS + 5.8S. Dendrograms generated based on ERIC-PCR data successfully separated the FOC isolates which showed 100% genetic similarity based on PCR-RFLP of ITS+5.8S analysis. ERIC-PCR analyzed the entire genome, whereas PCR-RFLP only analyzed a specific region of FOC genome. This suggests that ERIC-PCR

is more informative in characterizing closely related strains. Godoy *et al.*<sup>[7]</sup> also found that ERIC-PCR was more discriminatory compared to PCR-RFLP of ITS + 5.8S regions in their effort to characterize *F. solani* associated with mycotic keratitis. From their study, 39 ERIC groups and 13 restriction patterns were identified from 44 *F. solani* isolates. In addition, ERIC-PCR is also faster and less tedious compared to PCR-RFLP of ITS + 5.8S and a large number of samples could be analyzed in a short time<sup>[6]</sup>.

The highly similar banding patterns generated in both analyses showed close relationship among the FOC isolates, as no correlation was found between the banding patterns and the banana cultivars and locations. A study by<sup>[2]</sup> found that DNA fingerprinting revealed that the FOC isolates were generally VCG specific and neither correlates with location nor banana cultivar. In another study<sup>[1]</sup>, also pointed out that genetic profile of *F. oxysporum* from tomato plants did not correlate with locations. This suggests that the fungus was probably spreading through infected planting materials<sup>[10,15]</sup>.

Cluster analysis based on PCR-RFLP of ITS + 5.8S and ERIC-PCR data showed that the FOC isolates were clustered into two main clusters. Most of the isolates were clustered in cluster A which indicates close relationship among these FOC isolates. Isolate P2305N occupied cluster B and often showed distinct banding patterns in both analyses. It is difficult to determine the observed dissimilarity of isolate P2305N and further work such as sequencing need to be carried out to clarify the relationships of the isolate with other FOC isolates.

Adaptation to the host might have contributed to genetic variation of FOC isolates. Bentley *et al.*<sup>[2]</sup> suggested that genetic variation in FOC might be due to adaptation and co-evolution of the fungus with the host and environmental factors of the location.

The topology of the dendrogram generated based on ERIC-PCR analysis closely resembled the dendrogram based on the combined data, compared to the dendrogram based on PCR-RFLP of ITS + 5.8S regions. This shows that combined analysis of the data gave better insight into the genetic variation among the FOC isolates. More thorough analysis could be conducted by employing more than one molecular technique.

In this preliminary study, the FOC isolates were found to be closely related regardless of the location and banana cultivar. ERIC-PCR was more informative and less time consuming in studying closely related strains compared to PCR-RFLP of ITS + 5.8S analysis. The results were similar with the results obtained by<sup>[5,7]</sup>. The genetic variation of ITS + 5.8S within the closely

related isolates was low and sometimes no variation was observed and therefore was not informative to determine the genetic variation within FOC isolates. The results were in accordance with<sup>[11]</sup> in which they suggested that the ITS + 5.8S is more useful in differentiation of *Fusarium* species.

## CONCLUSION

Combined analysis of the PCR-RFLP of ITS+5.8S analysis and ERIC-PCR suggests that more than one molecular technique should be used to infer the intra-specific variation among FOC isolates. Therefore, further studies with more isolates and additional techniques such as sequencing of the Translation Elongation Factor-1 $\alpha$  (TEF-1 $\alpha$ ) and Random Amplified Microsatellites (RAMS) need to be conducted to reveal the genetic variation within these closely related isolates.

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