Advanced Journal of Microbiology Research Vol. 2016
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Genetic diversity of *Fusarium oxysporum* f. sp. *cubense* isolates from Malaysia

S. K. Leong, Z. Latiffah* and S. Baharuddin

School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia.

Accepted 2 April, 2016

Genetic diversity of *Fusarium oxysporum* f. sp. *cubense* (FOC) isolates were analyzed using enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), random amplified microsatellites (RAMS) and restriction fragment length polymorphism of Intergenic Spacer (RFLP-IGS) . The three molecular techniques showed intraspecific variations and the banding patterns generated using each technique were highly variable. UPGMA cluster analysis of the combined data of the three techniques showed 35.7 - 100% genetic similarity among the FOC isolates. Based on the dendrogram, most of the isolates including four isolates from Indonesia were clustered together in two main clusters. Sequence analysis of TEF-1 gene of some of the isolates of FOC using parsimony and distance methods also showed intraspecific variations. The highly variable banding patterns shown by using molecular methods supported the hypothesis that the isolates of FOC co-evolved with the edible bananas and dissemination of the pathogen was probably through infected rhizomes.

Key words: *Fusarium oxysporum* f.sp*. cubense*, enterobacterial repetitive intergenic consensus PCR, random amplified microsatellites, restriction fragment length polymorphism of Intergenic Spacer.

INTRODUCTION

Fusarium wilt or Panama disease caused by *Fusarium oxysporum* f. sp *cubense* (FOC) is the most destructive disease of banana (*Musa* spp.) worldwide. The disease occurs essentially in every banana-growing region except in the South Pacific Islands, Somalia, Mediterranean and Melanesia (Stover and Simmonds, 1987; Ploetz, 1992).

Panama disease has been a threat to banana industry since 1900s, especially in the developing countries where banana is one of the important staple foods. In 1940s, banana industry based on Gros Michel banana cultivar was almost crippled due to it's susceptibility to the pathogen in which almost 40,000 ha of the plantation were affected (Ploetz and Pegg, 2000) . As a result, virgin lands had been developed to ensure continuous supply of the fruit. However, poor handling and management led to the infestation of these new plantations and Gros Michel cultivar was replaced by resistant Cavendish cultivar. Unfortunately, a new race of FOC, that is, Race 4 emerged and was found to be virulent towards Cavendish in addition to the existing race 1, 2 and 3 (Su et al., 1986).

Among the 4 races, races 1 and 2 are virulent to Gros Michel and Bluggoe banana cultivars while race 3 is a pathogen of *Heliconia* sp. and only has mild effects on *Musa* sp. Race 4 is the most virulent and affects Cavendish and other banana cultivars susceptible to races 1 and 2 (Stover, 1972). Race 4 was first reported in 1980s in Taiwan and subsequently in South Queensland, Australia and Africa. In 1990s, the pathogen was found in Malaysia. However, different variants of Race 4 have been recognize and designated as tropical race 4 and subtropical race 4 (Ploetz, 2006). Tropical race 4 infected Cavendish bananas in the tropical regions such as Southeast Asia and Australia (Bentley et al., 1998, Pegg et al., 1994) and sub-tropical race 4 infected Cavendish bananas in South Africa, Australia, Taiwan and Canary Island (Su et al., 1986; Ploetz et al., 1990).

In Malaysia, banana is a popular crop, planted for local consumption and for export, with cultivated area of 25 710 hectares and production of 254 440 tonnes in 2008 (Agriculture Statistical Handbook, 2008). Almost half of

*Corresponding author. E-mail: Lfah@usm.my. Tel: 604 - 6533506. Fax: 604 - 6565125.

the cultivated area is planted with 'pisang berangan' and Cavendish types (Jamaluddin et al., 1999). In recent years, areas cultivated with banana have decreased and among the contributing factors is the effect of Fusarium wilt. The most susceptible cultivar is 'pisang berangan' and 'pisang rastali'.

As many banana cultivars in Malaysia are susceptible to infection by Fusarium wilt, there is a need to develop the most suitable disease management strategies and to develop resistant or tolerant banana cultivars. Therefore, a thorough understanding of genetic diversity of FOC is essential. With the development of PCR, molecular methods such as random amplified polymorphic DNA (RAPD) (Bentley et al., 1995; Bentley et al., 1998), restriction fragment length polymorphism (RFLP) of Internal Transcribed Spacer (ITS) (Edel et al., 1996; Bao et al., 2002) and Intergenic Spacer (IGS) (Appel and Gordon, 1995), sequencing analysis of IGS regions, Translation Elongation Factor-1 gene (TEF-1) and mitochondrial small subunit (mtSSU) (O'Donnell et al., 1998) have been used to study genetic diversity of *Fusarium* species as well as FOC isolates. Besides using molecular methods, biochemical methods of isozyme and protein analyses have been applied to study genetic diversity of FOC in India (Kumar et al., 2010).

Some FOC isolates from Malaysia have been studied using RAPD (Bentley et al., 1995; Bentley et al., 1998). However, further studies need to be conducted to elucidate their genetic diversity which will benefit the effort of disease control as well as breeding for resistant or tolerant banana cultivars. Furthermore, molecular markers for rapid and efficient identification of FOC are needed to answer any confusion in morphological identification. In the present study, Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR), Random Amplified Microsatellites (RAMS), Restriction Fragment Length Polymorphism of Intergenic Spacer (RFLP-IGS) and sequence analysis of TEF-1 gene were conducted to assess the genetic diversity within and between FOC isolates in Malaysia.

MATERIALS AND METHODS

FOC isolates

A total of 57 FOC isolates from different banana cultivars were used in this study (Table 1). Twenty-one isolates were isolated from infected banana and another 36 isolates were obtained from USM *Fusarium* culture collection. Two isolates of *F. solani* from soil and two isolates of *Fusarium solani* were also included in this study (Table 1). The isolates from the culture collection were re-identified to confirm the identity.

DNA extraction

For extraction of genomic DNA, mycelium was grown on potato dextrose agar, overlaid with dialysis membranes and incubated for 7 days. The mycelium was then ground into fine powder with liquid nitrogen using mortar and pestle. Genomic DNA was extracted using DNeasy MiniPlant Kit (QIAGEN) according to the manufacturer's instructions and stored at -20°C until use.

ERIC-PCR analysis

PCR amplifications were carried out twice according to the reaction conditions described by Edel et al. (1995) with some modifications using primer pair ERIC1R (5'-ATG-TAA-GCT-CCT-GGG-GAT-TCA-C-3') and ERIC2 (5'-AAG-TAA-GTG-ACT-GGG-GTG-AGC-G-3') (Versalovic et al ., 1991). ERIC-PCR amplification was carried out in a 25 µl reaction mixture containing 1X PCR buffer, 4.0 mM $MgCl_2$, 120 mM of each dNTPs, 0.5 µM of each primer, 1.3 U of *Taq* polymerase (Promega) and 5 ng of genomic DNA. PCR amplification was performed using Peltier Thermal Cycler (PTC-200, MJ Research) with 7 min initial denaturation at 95°C followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 30 s, extension at 65°C for 8 min and final extension at 65°C for 16 min to ensure complete extension of the amplification products.

RAMS analysis

Genomic DNA was amplified using four microsatellite primers namely: M1 $(GT)_7$, M2 $(AAC)_5$, M4 $(CAT)_5$ and M5 $(ACTG)_5$. PCR amplification was carried out in a 25 µl reaction mixture comprised 1X PCR buffer, 2.0 mM $MgCl_2$, 160 µM of each dNTPs, 8 mM primer, 1.75 U *Taq* polymerase (Promega) and 6 ng genomic DNA, and performed using Peltier Thermal Cycler (PTC-200, MJ Research) with initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing for 45 s and the temperature depends on the primers used [$(GT)_7$ and $(ACTG)_5$ at 44°C, $(AAC)_5$ and $(CAT)_5$ at 43°C], extension at 72°C for 2 min and completed with a final extension at 72°C for 7 min.

Gel electrophoresis for ERIC-PCR and RAMS analyses

The amplification products were separated in 1.5% agarose gel (Amresco) using 1X Tris Borate EDTA (TBE) as running buffer. Electrophoresis was conducted at 80 V, 500 mA for 160 min after which, the gels were stained in ethidium bromide for 10 min, visualized under UV transilluminator and photographed using SnapGene Photo Imaging System (SynGene). The sizes of the amplified products were estimated using 1kb marker (MBI Fermentas).

RFLP-IGS analysis

The Intergenic Spacer (IGS) was amplified using primer pair CNL12 (5'-CTG-AAC-GCC-TCT-AAG-TCA-G-3') and CNS1 (5'-GAG-ACA-AGC-ATA-TGA-CTA-CTG- 3') (Appel and Gordon, 1995) according to the reaction conditions described by Llorens et al. (2006) with some modifications. The amplification reaction was conducted in a 50 µl reaction mixture containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 0.1 mM of each primers, 2.25 U of *Taq* polymerase (Promega) and 3 ng genomic DNA, and performed using a Peltier Thermal Cycler(PTC-100, MJ Research). PCR amplification was carried out with the following profiles: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 55s, annealing at 59°C for 30 s and extension at 72°C for 2 min and a final extension at 72°C for 7 min.

For restriction analysis, the PCR products were digested with *Alu* I, *Eco* 88I, *Eco* RI, *Hin* 6I, *Hin f*I, *Msp* I and *Taq* I in a 15 µl reaction mixture in separate reactions. The reaction mixture contained 1X reaction buffer, 1 U of restriction enzyme, 6 - 10 µl of PCR products and distilled water and incubated for 1 h at 37°C for

Table 1. Isolates of *Fusarium* used in ERIC-PCR, RAMS and RFLP-IGS.

Code	Isolate	Host/Banana cultivar	Location
I2130N^{S}*	FOC	Berangan	Pancur Batu, Medan, Indonesia.
I2238N^{S}	FOC	Emas	Peningsurat, Indonesia.
I2240N^{S}	FOC	Kepok (Abu)	Peningsurat, Indonesia.
I2244N^{S}	FOC	Uter	Pekalongan, Indonesia.
A1452N^{S}	FOC	Raja	Trong, Perak.
A2279N^{S}	FOC	Emas	KM4, Changkat Jering, Perak.
A2280N^{S}	FOC	Awak	Sri Iskandar, Perak.
A2281N^{S}	FOC	Awak	Padang Ragut, Bota Kanan, Perak.
A2282N^{S}	FOC	Berangan	Titi Gantung, Perak.
A2295N^{S}	FOC	Kepok (Abu)	Bukit Nangka, Lenggong, Perak.
A2296N^{S}	FOC	Berangan	Changkat Jambu, Kati, Kuala Kangsar, Perak.
A2306N^{S}	FOC	Awak	Sri Iskandar, Perak.
A2309N^{S}	FOC	Awak	Sri Iskandar, Perak.
A1BN^{T}	FOC	Emas	Taiping, Perak.
A2BN^{T}	FOC	Emas	Taiping, Perak.
A5BN^{T}	FOC	Berangan	Taiping, Perak.
A6AN^{T}	FOC	Berangan	Taiping, Perak.
A7AN^{T}	FOC	Berangan	Taiping, Perak.
A7BN^{T}	FOC	Berangan	Taiping, Perak.
A8AN^{T}*	FOC	Berangan	Taiping, Perak.
A9AN^{T}	FOC	Berangan	Taiping, Perak.
A9BN^{T}*	FOC	Berangan	Taiping, Perak.
A10BN^{T}*	FOC	Berangan	Taiping, Perak.
K1AN^{T}	FOC	Berangan	Gurun, Kedah.
K1BN^{T}	FOC	Berangan	Gurun, Kedah.
K4AN^{T}*	FOC	Berangan	Gurun, Kedah.
K5AN^{T}	FOC	Berangan	Gurun, Kedah.
K5BN^{T}	FOC	Berangan	Gurun, Kedah.
K6AN^{T}	FOC	Berangan	Gurun, Kedah.
K6BN^{T}	FOC	Berangan	Gurun, Kedah.
K7AN^{T}*	FOC	Berangan	Gurun, Kedah.
K8AN^{T}	FOC	Berangan	Gurun, Kedah.
K8BN^{T}	FOC	Berangan	Gurun, Kedah.
K9AN^{T}*	FOC	Berangan	Gurun, Kedah.
B1456N^{S}	FOC	Awak	Tiram Buruk, Tanjong Karang, Selangor.
B2286N^{S}*	FOC	Kepok (Abu)	Kancong Darat, Banting, Selangor.
B2287N^{S}*	FOC	Tanduk	Batu Laut, Selangor.
B2469N^{S}	FOC	Emas	Serdang, Selangor.
B2471N^{S}*	FOC	Cavendish cv. William	Serdang, Selangor.
B2473N^{S}	FOC	Abu Keling	Serdang, Selangor.
D2291N^{S}*	FOC	Berangan	Wakaf Stan, Kota Bharu, Kelantan.
D2293N^{S}	FOC	Kepok (Abu)	Wakaf Stan, Kota Bharu, Kelantan.
D2294N^{S}*	FOC	Berangan	Kampung Berangan, Tumpat, Kelantan.
J2327N^{S}*	FOC	Unknown	JTP, Johor.
J2329N^{S}	FOC	Unknown	JTP, Johor.
J2330N^{S}*	FOC	Unknown	JTP, Johor.
PU1N^{S}*	FOC	Unknown	USM, Penang.
PU2N^{S}	FOC	Unknown	USM, Penang.
PU3N^{S}	FOC	Unknown	USM, Penang.
M2300N^{S}*	FOC	Nangka	Merlimau, Melaka.
M2322N^{S}*	FOC	Berangan	Pulai, Merlimau, Melaka.

Table 1. Contd.

T2290N^{S}*	FOC	Awak	Lak Luk, Terengganu.
T2304N^{S}*	FOC	Awak	Kampung Penjara, Terengganu.
3755^{S}	FO(Soil)	Coconut crop soil	Pantai Semerak, Marang, Terengganu.
4275^{S}*	FO(Soil)	Orchard soil	Baling, Kedah.
P2091N^{S}*	FS	Awak	Bumbung Lima, Penang.
3667^{S}*	FS	Angsana	Cheras Pokok 2, Kuala Lumpur.

FOC = *F. oxysporum* f. sp *cubense,* FO = *F. oxysporum,* FS = *F. solani.* * = Isolates used in sequence analysis of TEF-1 gene. ^{F} = Isolates from the field. ^{S} = Isolates from USM *Fusarium* culture collection.

B2471N (isolated from William variety) which was clustered separately from the other isolates of FOC. One isolate of *F. oxysporum* from soil and two isolates of *F. solani* were also included as comparison and to root the phylogenetic trees (Table 1).

Translation Elongation Factor-1 gene was amplified using primer pair EF1 (5'-ATG-GGT-AAG-GAG-GAC-AAG-AC-3') and EF2 (5'-GGA-AGT- ACC-AGT-GAT-CAT-GTT-3') (O'Donnell, 1998). PCR amplification was carried out in a 50 µl reaction mixture containing 1X PCR buffer, 1 mM $MgCl_2$, 1 mM of each primers, 200 µM of each dNTPs, 1 U of *Taq* polymerase (Promega), 3 ng genomic DNA and performed using Peltier Thermal Cycler (PTC-100, MJ Research) . PCR amplification started with an initial denaturation at 94°C for 85 s, followed by 35 cycles of denaturation at 95°C for 35 s, annealing at 46°C for 55 s, extension at 72°C for 90 s and final extension at 72°C for 10 min. The PCR products were purified using QIAquick PCR Purification Kit (QIAGEN) according to the manufacturers' instructions.

DNA sequence analysis of TEF-1

The sequences of TEF-1 were aligned using BioEdit version 7.0.5. The alignments were edited manually where needed. All TEF-1 sequences were compared with sequences of *Fusarium* species available in the GenBank database using BLAST.

Multiple sequence alignment was performed using ClustalW 1.6 and phylogenetic analyses were performed using maximum parsimony and distance analysis using MEGA version 4 (Tamura et al., 2007) . Maximum parsimony analysis was conducted by employing Close-Neighbour-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). For distance analysis, the evolutionary distances were computed using Kimura 2-parameter method. To evaluate the stability of each branch, bootstrap analysis with 1000 bootstrap replicates was performed.

RESULTS

Primer pair ERIC1R and ERIC2 amplified 8 – 22 bands for all isolates of FOC. The banding patterns were complex with varying intensities. However, the amplified bands were reproducible and ranging from 100 – 3750 bp. Although faint bands were present, the bands were reproducible and consistently present.

A total of 29 ERIC banding patterns were identified among 53 isolates of FOC from different banana cultivars (Table 2). Two ERIC patterns (E15 and E17) were identified for isolates of *F. oxysporum* from soil and isolates of *F. solani* (E32 and E33). None of the banding patterns could differentiate the FOC isolates according to banana cultivars. Figure 1 shows ERIC banding patterns of some of the FOC isolates used in this study.

Microsatellite primers of $(GT)_7$, $(AAC)_5$, $(CAT)_5$ and $(ACTG)_5$ generated reproducible banding patterns for all the isolates of FOC. The four primers produced 1 - 12 bands ranging from 500 - 6000 bp with 12 – 25 haplotypes (Table 3). The banding patterns produced by the primers were highly similar and differences were due to the presence or absence of a few bands of different molecular sizes. The banding patterns generated showed considerable variation. Figure 2 shows the banding patterns of some FOC isolates generated using primer $(CAT)_5$.

For restriction analysis of the IGS region, an approximately 2.6 kb IGS region was successfully amplified from all isolates of FOC and two isolates of *F. oxysporum* from soil. For the two isolates of *F. solani*, an approximately 3.0 kb IGS region was amplified.

Using *Alu* I, *Eco* RI, *Eco* 88I, *Hin* 6I, *Hin f*I, *Msp* I and *Taq* I, 10 - 15 restriction patterns were obtained. Based on the restriction patterns, isolates of FOC were assigned into 27 haplotypes, in which 15 haplotypes were represented by a single representative. Each haplotype comprised 1 – 7 isolates (Table 4). UPGMA cluster analysis showed that isolates of FOC, *F. oxysporum* from soil and *F. solani* were grouped in four clusters, with similarity value ranging from 11.0 - 100% (Figure 3). Isolates of FOC showed 35.7 - 100% genetic similarity and were not clustered according to the banana cultivars and locations. Most of the isolates of FOC were clustered in clusters I and II except isolate B2471N which was clustered in cluster III. The overall similarity in cluster I ranged from 60.4 - 100% while cluster II showed 60 - 100% genetic similarity.

An approximately 700 bp TEF-1 gene was successfully amplified from isolates of FOC from different banana cultivars, *F. oxysporum* from soil and two isolates of *F. solani*. Most of the isolates of FOC showed 97 - 100% sequence similarity with sequences of FOC 50 (AY217146), FOC 57 (AY217149), FOC 58 (AY217150), FOC 59 (AY217151) and FOC 60 (AY217152) from the GenBank. Isolate B2471N (William variety) showed 95% homology with *F. oxysporum* 1517 from Australian native gossypium soil.

Table 2. ERIC banding patterns generated by isolates of FOC from different banana cultivars, *F. oxysporum* from soil and *F. solani.*

ERIC patterns	Banana cultivars : isolates
E1	Emas: A2279N Berangan: A2282N, A2296N, M2322N
E2	Kepok: B2286N
E3	Awak: A2280N
E4	Kepok: A2295N
E5	Awak: A2306N
E6	Tanduk: B2287N
E7	Berangan: D2291N
E8	Awak: T2290N Berangan: D2294N Kepok: D2293N, I2240N
E9	Awak: A2281N, A2309N, T2304N Uter: I2244N
E10	Nangka: M2300N Unknown: J2327N, J2329N, J2330N
E11	Raja: A1452N
E12	Emas: B2469N
E13	Unknown: PU1N, PU3N
E14	Unknown: PU2N
E15	*F. oxysporum* from soil: 4275
E16	William Honduras: B2471N
E17	*F. oxysporum* from soil: 3755
E18	Awak: B1456N
E19	Emas: I2238N Berangan: K5BN, K6BN, K7AN, K8BN
E20	Berangan: K1AN, K1BN
E21	Berangan: I2130N
E22	Emas: A1BN, A2BN
E23	Berangan: A5BN, A6AN, A7AN, A7BN, K4AN
E24	Berangan: K8AN
E25	Berangan: A9BN
E26	Berangan: A9AN
E27	Berangan: K9AN
E28	Berangan: K5AN, K6AN
E29	Berangan: A8AN
E30	Berangan: A10BN
E31	Abu keling: B2473N
E32	Awak: P2091N (*F. solani*)
E33	Angsana: 3667 (*F. solani*)

The most parsimonious (MP) tree was inferred based on 625 informative characters with 119 parsimony-informative sites. Figure 4 shows the MP tree obtained with 1000 bootstrap value. The tree length was 176 steps with a consistency index (CI) of 0.9459 and retention index (RI) of 0.9757. Isolate B247N was grouped separately from the other FOC isolates. The clusters were well supported with bootstrap values of 61 - 100%.

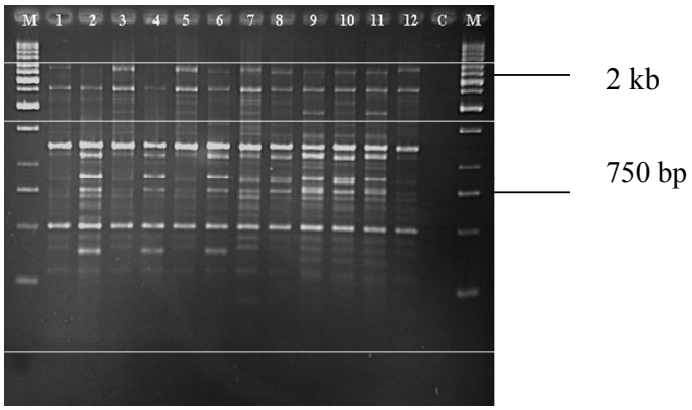

Figure 1. ERIC banding patterns of FOC isolates from different banana cultivars amplified using primer pair ERIC1R and ERIC2. Lane 1-12: 1: A2279N (E1), 2: A2280N (E3), 3: A2282N (E1), 4: A2295N (E4), 5: A2296N (E1), 6: A2306N (E5), 7: B2286N (E2), 8: B2287N (E6), 9: T2290N (E8), 10: D2291N (E7), 11: D2294N (E8) and 12: M2322N (E1). C: control. M: 1 kb ladder.

Table 3. Microsatellite primers used in RAMS, length of RAMS products and number of heliotypes.

Primer	Length of RAMS product (bp)	Number of haplotypes of FOC
$(GT)_7$	650 – 4000	13
$(AAC)_5$	850 – 5000	12
$(CAT)_5$	600 – 6000	25
$(ACTG)_5$	500 – 4000	12

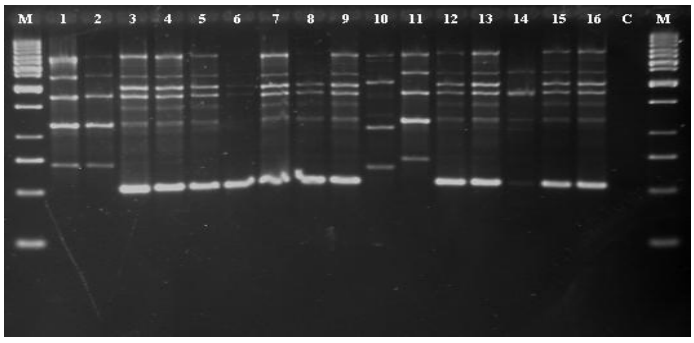

Figure 2. RAMS banding patterns of FOC isolates from different banana cultivars obtained by using primer $(CAT)_5$. Lane 1-16: 1: A1BN (CAT8), 2: A2BN (CAT8), 3: A5BN (CAT17), 4: A6AN (CAT17), 5: A7AN (CAT17), 6: A7BN (CAT17), 7: A9BN (CAT17), 8: K1AN (CAT17), 9: K1BN (CAT17), 10: K3BN (CAT21), 11: K4AN (CAT22), 12: K5AN (CAT17), 13: K6AN (CAT17), 14: K7BN (CAT24), 15: K8AN (CAT17) and 16: K9AN (CAT25). C: control. M: 1kb ladder.

Using distance method, a total of 625 nucleotides with equal rates were included. Figure 5 shows the neighbour joining (NJ) tree generated based on 1000 bootstrap

Table 4. Restriction patterns of isolates of FOC from different banana cultivars, *F. oxysporum* from soil and *F. solani* generated using seven restriction enzymes and haplotypes obtained.

IGS Haplotypes	*Alu* I	*Eco* RI	*Eco 881*	*Hin f*I	*Hin* 6I	*Msp* I	*Taq* I	Banana cultivars and Isolates
1	A1	ER1	E1	HF1	H1	M1	T1	Emas: A2279N Berangan: A2282N, A2296N, M2322N Kepok: B2286N
2	A1	ER1	E1	HF3	H1	M1	T1	Emas: I2238N
3	A1	ER1	E1	HF3	H2	M1	T1	Nangka: M2300N
4	A1	ER3	E1	HF4	H1	M1	T5	Berangan: K5BN, K6BN, K8BN
5	A1	ER6	E1	HF3	H1	M1	T5	Berangan: A9AN
6	A1	ER4	E5	HF4	H5	M1	T5	Berangan: K7AN
7	A2	ER1	E2	HF1	H2	M1	T2	Awak: A2280N, A2281N, A2306N, A2309N Kepok: A2295N, D2293N, I2240N
8	A3	ER1	E1	HF1	H4	M1	T2	Awak: T2290N Berangan: D2294N
9	A3	ER1	E1	HF5	H4	M1	T2	Raja: A1452N Awak: T2304N
10	A3	ER1	E2	HF1	H2	M1	T2	Berangan: D2291N
11	A3	ER4	E2	HF3	H2	M1	T3	Awak: B1456N
12	A3	ER5	E2	HF7	H2	M1	T3	Berangan: A8AN
13	A3	ER1	E3	HF1	H1	M3	T6	Berangan: I2130N
14	A3	ER5	E6	HF7	H2	M1	T3	Berangan: A10BN Emas: B2469N
15	A4	ER1	E1	HF3	H1	M1	T1	Unknown: J2327N, J2329N, J2330N, PU1N, PU2N, PU3N: Uter: I2244N
16	A4	ER1	E1	HF3	H1	M1	T5	Berangan:A7BN
17	A4	ER3	E1	HF4	H1	M1	T5	Berangan: K6AN, K8AN
18	A4	ER3	E1	HF6	H1	M1	T5	Abu Keling: B2473N
19	A4	ER4	E1	HF3	H1	M1	T5	Berangan: A5BN, A6AN, A7AN
20	A4	ER4	E1	HF4	H1	M1	T5	Berangan: K5AN, K9AN
21	A4	ER6	E1	HF3	H1	M1	T5	Berangan: A9BN
22	A4	ER1	E7	HF4	H1	M1	T5	Berangan: K1BN
23	A5	ER1	E1	HF4	H1	M1	T3	Berangan: K1AN
24	A5	ER5	E2	HF4	H2	M1	T3	Berangan: K4AN
25	A6	ER4	E2	HF3	H2	M1	T3	Emas: A1BN
26	A6	ER4	E6	HF3	H2	M1	T3	Emas: A2BN
27	A8	ER7	E8	HF9	H6	M5	T1	William : B2471N
28	A6	ER5	E2	HF13	H2	M1	T2	Soil: 4275 (*F. oxysporum*)
29	A6	ER1	E2	HF12	H2	M1	T10	Soil: 3755 (*F. oxysporum*)
30	A12	ER11	E11	HF14	H10	M9	T11	Awak: P2091N (*F. solani*)
31	A13	ER12	E12	HF15	H11	M10	T12	Angsana: 3667 (*F. solani*)

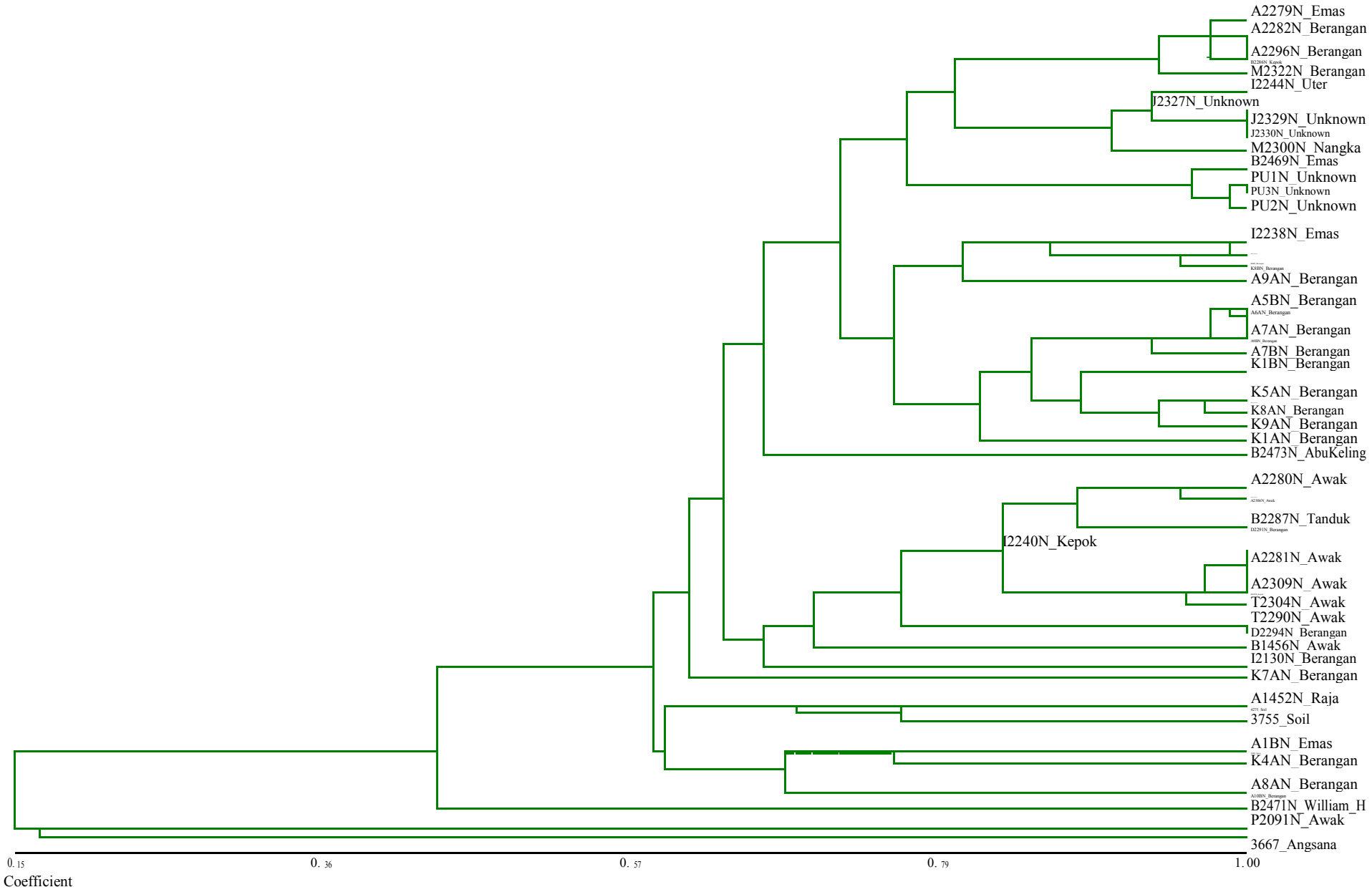

Figure 3. Dendrogram generated using Jaccard's Coefficient based on combined data of ERIC-PCR, RAMS and RFLP-IGS of isolates of FOC, *F. oxysporum* and *F. solani*.

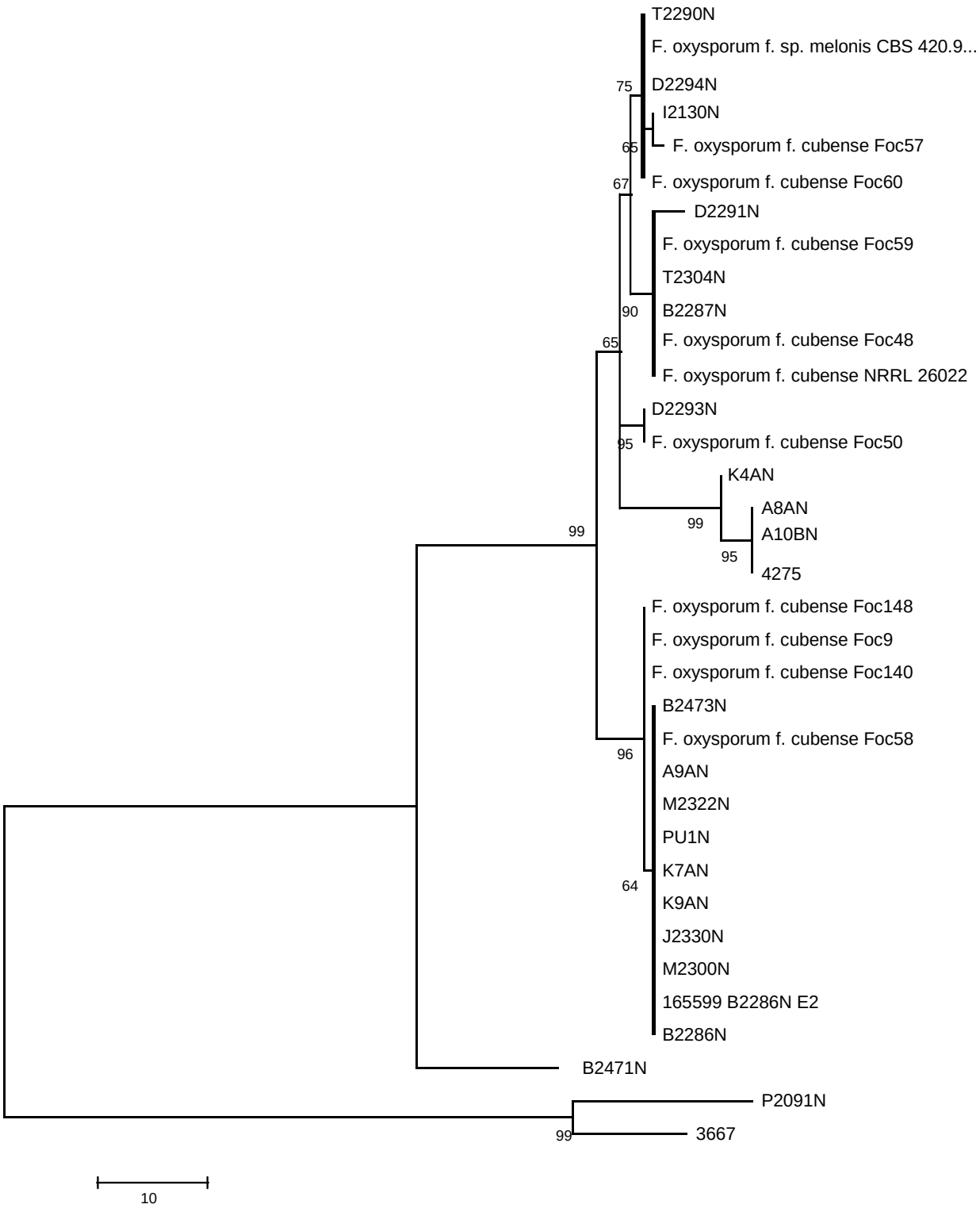

Figure 4. Most Parsimonious tree generated from TEF-1 sequences of isolates of FOC from different banana cultivars and the GenBank, isolates of *F. oxysporum* from soil and *F. solani*. Tree length = 176 steps, Consistency Index (CI) = 0.9489, Retention Index (RI) = 0.9757.

replicates. The topology of the NJ tree and clustering of the isolates obtained from distance matrix were similar to that of the MP tree. Isolate B2471N was also clustered separately from the other FOC isolates. However, the bootstrap values obtained from distance method (23 - 100%) was slightly lower than the bootstrap values

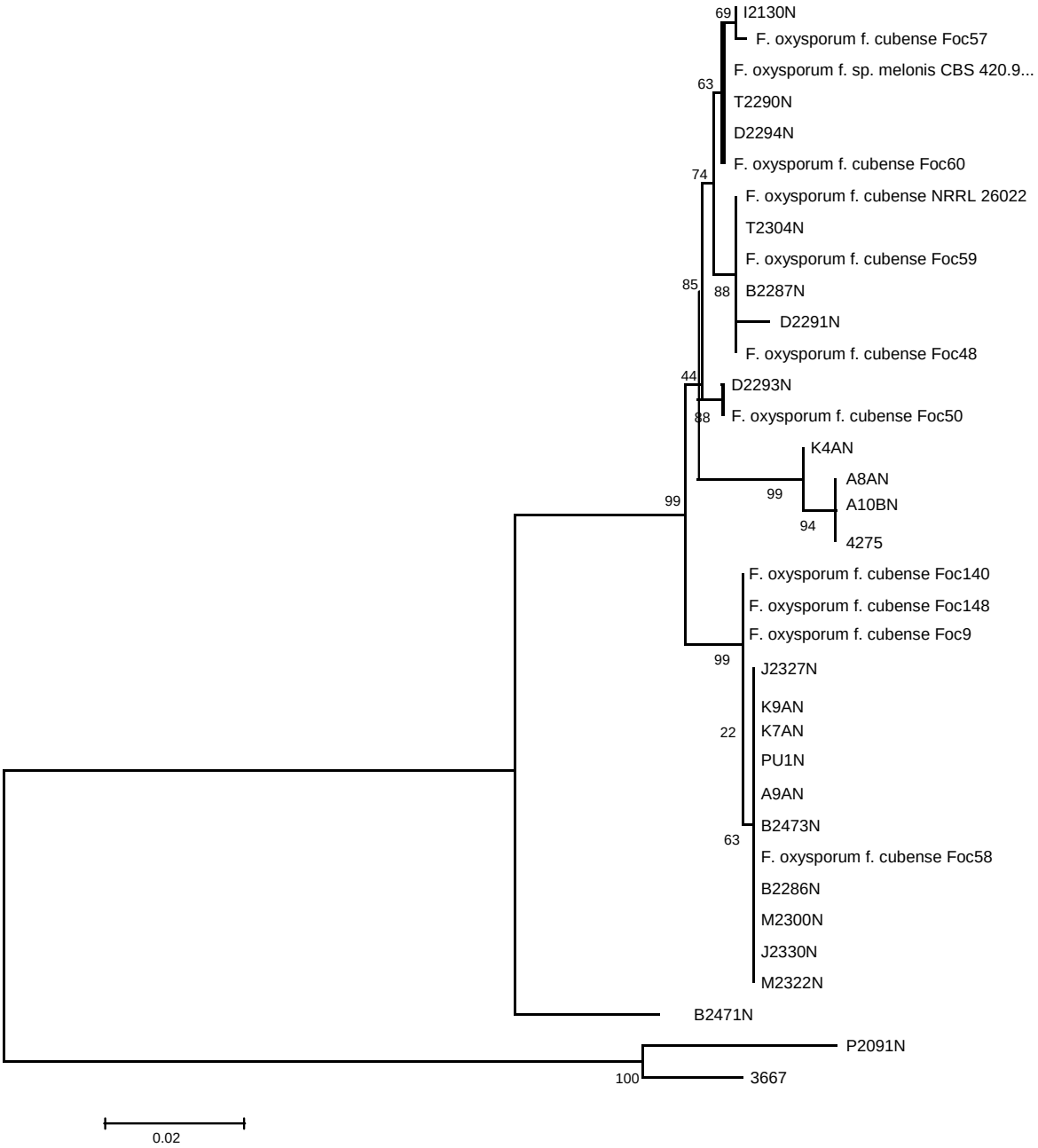

Figure 5. Neighbour Joining tree generated from TEF-1 sequences of FOC isolates from different banana cultivars and from the GenBank, isolates of *F. oxysporum* from soil and *F. solani*.

obtained from the parsimony method (61 - 100%).

DISCUSSION

ERIC banding patterns were successfully generated from all isolates of FOC which indicated that the repetitive elements could be found in abundance in the FOC genome. The results were similar with a study by Edel et al. (1995) which also obtained bands with sizes ranging from 100 - 4000 bp. In this study, the isolates of FOC were more variable with 29 ERIC banding patterns compared to only 19 types detected by Edel et al. (1995) using 60 strains of *F. oxysporum* from rhizoplane of flax, melon, tomato and wheat.

In a study by Godoy et al. (2004) using ERIC-PCR, high levels of genetic variation were also observed among 44 isolates of *F. solani* causing keratitis, in which 39 banding patterns were generated. However, Jureen et al. (2008) reported only 7 ERIC types among 62 isolates

of *F. solani* responsible for fungal keratitis in Singapore.

Four microsatellite primers namely: $(GT)_7$, $(AAC)_5$, $(CAT)_5$ and $(ACTG)_5$ were able to generate banding patterns for all the isolates of FOC as well as isolates of *F. oxysporum* from soil and *F. solani*. This could indicate that the four microsatellite motifs exist abundantly in the genome of all the isolates of FOC used in this study. Previous studies also demonstrated the abundance of microsatellite motifs in the genome of *F. oxysporum* (Barve et al., 2001; Balmas et al., 2005) and *F. solani* (Zaccardelli et al., 2008).

Using the four microsatellite primers, 12 - 25 haplotypes were obtained among the isolates of FOC, indicating high levels of genetic variation. Similar with the genetic variation of FOC, studies of *F. culmorum* (Prashant et al., 2003), *F. oxysporum* species complex (Bogale et al., 2005) and *F. oxysporum* f. sp. *ciceris* (Dubey and Singh, 2008) also showed high levels of genetic variation using microsatelite primers.

PCR amplification using CNL12 and CNS1 primers showed that the isolates of FOC produced an approximately 2.6 kb IGS region which was in agreement with Appel and Gordon (1995), Edel et al. (1995) and Cai et al. (2003) in which a 2.6 kb IGS region was generated from isolates of *F. oxysporum* using the same primer pairs.

Based on the restriction patterns of IGS, isolates of FOC generated 27 haplotypes in which 15 haplotypes were represented by only a single isolate, indicating high levels of genetic variation among the isolates. This was in agreement with Koenig et al. (1997) who reported that 72 haplotypes were produced by 165 isolates of FOC using genomic RFLP. Almost half of the isolates were assigned into five most common haplotypes and 50 of the haplotypes were represented by a single isolate. RFLP-IGS analysis of several species of *Fusarium* have also revealed intraspecific variation among the isolates in which nine IGS haplotypes were identified among 22 strains of *F. oxysporum* (Kim et al., 2001), 14 IGS haplotypes among 33 isolates of *F. verticillioides* (Patinõ et al., 2006) and four IGS haplotypes among 27 isolates of *F. equiseti* (Kosiak et al., 2004).

UPGMA cluster analysis of the combined data showed 35.7 - 100% genetic similarity using JC which indicated high levels of genetic variation among the FOC isolates. Most of the isolates were clustered together except isolates B2471N which formed separate cluster. The high levels of genetic variation among the isolates of FOC supported the hypothesis that FOC isolates co-evolved with the edible bananas (Stover, 1962). According to Bentley et al. (1995), greatest diversity within the population of the pathogen detected at the centre of the origin of the host would not be unexpected if the hypothesis of co-evolution is correct. Bentley et al. (1998) reported that greatest diversity of FOC isolates was observed in clonal lineages which consisted pre-dominantly of isolates from Indonesia, Malaysia and the Philippines. Therefore, the variations within the FOC isolates supported the hypothesis that banana originated from Southeast Asia. Four isolates of FOC from Indonesia were clustered together with isolates of FOC from Malaysia, and shared high genetic similarity. This was supported by the sequence analysis of TEF-1 which showed that isolates of FOC T2290N and D2294N from Malaysia showed100% homology with FOC 60 (AY217152) from Indonesia. Additionally, sequences of TEF-1 of a few isolates from Malaysia also showed 100% homology to isolates of FOC from Thailand, Australia and South Africa, suggesting the dispersal of isolates of FOC between Malaysia, Thailand, Australia and South Africa. The results suggested that the fungus was probably spreading through infected planting materials. Similarly, Pegg et al. (1995) demonstrated that the FOC population in Carnavon might have been introduced through windbreak plants from South-East Asia, rather than evolving independently from the existing FOC population.

Isolate B2471N isolated from William banana variety showed different banding patterns and shared limited genetic similarity with the other FOC isolates. Similar results was also reported by Bentley et al. (1995) in which two FOC isolates showed different banding patterns from the other FOC isolates and 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. Studies by O'Donnell et al. (1998) using TEF-1 and mtSSU genes showed that some of the FOC isolates were more closely related to the other *F. oxysporum* forma specialis such as *F. oxysporum* f. sp. *gladioli* and *F. oxysporum* f. sp. *melonis*, and *Fusarium Fusarium inflexum*. Groenewald et al. (2006) also reported the same phenomena in which several isolates of FOC were grouped closer to *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *dianthi*.

Phylogenetic analysis using TEF-1 gene of 21 isolates of FOC also revealed high levels of variation among the isolates which could indicate the polyphyletic nature of the isolates which was in agreement with the other three molecular techniques. The results obtained suggested multiple origins of the isolates based on the high levels of genetic variation of the isolates and the close relationship between isolates of FOC from Malaysia and Indonesia as well as isolates of *F. oxysporum* from soil. The polyphyletic origin of FOC isolates have been previously indicated by other molecular methods such as RAPD (Bentley et al., 1995) and DNA fingerprinting analysis (DAF) (Bentley et al., 1998). Based on a study by Fourie et al. (2009) on the relationship among VCGs and races of FOC, there is polyphyletic trait of the pathogen and the ability to cause vascular wilt on banana which has emerged multiple times. Among nine clonal lineages identified by Bentley et al. (1998), five of the lineages supported the hypothesis of co-evolution while

clonal lineages VI, VII, VIII and IX probably evolved independently from local *F. oxysporum* populations in different countries. These early findings were well supported by O'Donnell et al. (1998) and Groenewald et al. (2006) through phylogenetic studies using sequence analysis of TEF-1 gene and mtSSU of rDNA gene and AFLP analysis.

In conclusion, by using three molecular methods, ERIC-PCR, RAMS and RFLP- IGS and sequencing of TEF-1 gene, high levels of genetic variation were observed within isolates of FOC which supported the hypothesis that the isolates of FOC co-evolved with edible bananas and probably disseminated through infected rhizome.

ACKNOWLEDGEMENT

The study was supported by the Ministry of Science, Technology and Innovation, Malaysia, grant 305/PBIOLOGY/612113.

REFERENCES

Agriculture Statistical Handbook (2008). Ministry of Agriculture and Agro-based Industry.

Appel DJ, Gordon TR (1995). Intraspecific variation within populations of *Fusarium oxysporum* based on RFLP analysis of the intergenic spacer region of the rDNA. Exp. Mycol. 19: 120-128.

Balmas V, Scherm B, Di Primo P, Rau D, Marcello A, Migheli Q (2005). Molecular charaterisation of vegetative compatibility groups in *Fusarium oxysporum* f. sp. *radicis-lycopersici* and f. sp. *lycopersici* by random amplification of polymorphic DNA and microsatellite-primed PCR. E J. Pl Path. 111: 1-8.

Bao JR, Fravel DR, O'Neill NR, Lazarovits G, van Berkum, P (2002). Genetic analysis of pathogenic and nonpathogenic *Fusarium oxysporum* from tomato plants. Can. J. Bot. 80: 271-279.

Barve MP, Haware MP, Sainani MN, Ranjekar PK, Gupta VS (2001). Potential of microsatellites to distinguish four races of *Fusarium oxysporum* f. sp. *ciceri* prevalent in India. Theor. Appl. Genet. 102: 138-147.

Bentley S, Bassam BJ (1996). A robust DNA amplification fingerprinting system applied to analysis of genetic variation within *Fusarium oxysporum* f. sp. *cubense*. J. Phytopath. 144: 207-213.

Bentley S, Pegg KG, Dale JL (1995). Genetic variation among a world-wide collection of isolates of *Fusarium oxysporum* f. sp. *cubense* analysed by RAPD-PCR fingerprinting. Mycol. Res. 99(11): 1378-1384.

Bentley S, Pegg KG, Moore NY, Davis RD, Buddenhagen IW (1998). Genetic variation among vegetative compatibility groups of *Fusarium oxysporum* f. sp. *cubense* analyzed by DNA fingerprinting. Phytopathol. 88: 1283-1293.

Bogale M, Wingfield BD, Wingfield MJ, Steenkamp ET (2005). Simple sequence repeat markers for species in the *Fusarium oxysporum* complex. Mol. Ecol. Notes. 5(3): 622-624.

Cai G, Gale R, Schneider RW, Kistler HC, Davis RM, Elias KS, Miyao EM (2003). Origin of race 3 of *Fusarium oxysporum* f. sp. *lycopersici* at a single site in California. Phytopathol. 93: 1014-1022.

Dubey SC, Singh SR (2008). Virulence analysis and oligonucleotide fingerprinting to detect diversity among Indian isolates of *Fusarium oxysporum* f. sp. *ciceris* causing chickpea wilt. Mycopathol. 165: 389-406.

Edel V, Steinberg C, Gautheron N, Alabouvette C (1996). Evaluation of restriction analysis of polymerase chain reaction (PCR)-amplifies ribosomal DNA for the identification of *Fusarium* species. Mycol. Res. 101 (2): 179–187.

Edel V, Steinberg C, Avelange I, Langueere G, Alabouvette C. (1995). Comparison of three molecular methods for the identification of *Fusarium oxysporum* strains. Phytopathol. 85: 579-585.

Edel V, Steinberg C, Gautheron N, Recorbet G, Alabouvette C (2001). Genetic diversity of *Fusarium oxysporum* populations isolated from different soils in France. FEMS Microbiol. Ecol. 36: 61-71.

Fourie G, Steenkamp ET, Gordon TR, Viljoen (2009). Evotutionary relationships among *Fusarium oxysporum* f.sp *cubense* vegetative compatibility groups. Appl. Environ. Microbiol. 75(14): 4770-4781.

Godoy P, Cano J, Gené J, Guarro J, Höfling-Lima AL, Colombo AL (2004). Genotyping of 44 isolates of *Fusarium solani* , the main agent of fungal keratitis in Brazil. J. Clin. Microbiol. 42(10): 4494-4497.

Groenewald S, Van Den Berg N, Marasas WFO, Viljoen A (2006). The application of high-throughput AFLPs in assessing genetic diversity in *Fusarium oxysporum* f. sp. *cubense*. Mycol. Res. 110: 297-305.

Holiday P (1980). *Fungus diseases of tropical crops*. Cambridge University Press. pp. 171-173.

Jamaluddin SH, Nik Masdek NH, Ibrahim O (1999). Preliminary survey of banana wilt disease in Malaysia – Implications on production and the industry.. In : Banana Fuarium wilt Management : Towards sustainable cultivation. Eds: Molina A.B., Nik Masdek N.H., and Liew K.W. Proceedings of the International Workshop on the Banana Fusarium wilt Disease. Genting Highlands Resort, Malaysia. 18 – 20 October 1999. pp. 76 – 85

Jureen R, Koh TH, Wang G, Chai LY, Tan AL, Chai T, Wong YW, Wang Y, Tambyah PA, Beuerman R, Tan D (2008). Use of multiple methods for genotyping *Fusarium* during an outbreak of contact lens associated funga keratitis in Singapore. BMC Infect. Dis. 8: 92-97.

Kim HJ, Choi YK, Min BR (2001). Variation of the Intergenic Spacer (IGS) region of ribosomal DNA among *Fusarium oxysporum* formae speciales. J. Microbiol. 39(4): 265-272.

Koenig RL, Ploetz RC, Kistler HC (1997). *Fusarium oxysporum* f. sp. *cubense* consists of a small number of divergent and globally distributed clonal lineages. Phytopathol. 87: 915-923.

Kosiak EB, Holst-Jensen A, Rundberget T, Jaen MTG, Torp M (2005). Morphological, chemical and molecular differentiation of *Fusarium equiseti* isolates from Norwegian cereals. Int. J. Food Microbiol. 99: 195-206.

Kumar B, Udaya Shankar AC, Chandra Nayaka S, Ramachandra Kini S, Shetty HS, Prakash HS (2010). Biochemical characterization of *Fusarium oxysporum* f.sp *cubense* isolates from India. Afr. J. Biotech. 9(4): 523 – 530.

Llorens A, Hinojo MJ, Mateo R, González-Jaén MT, Valle-Algarra FM, Logrieco A, Jiménez M (2006). Characterization of *Fusarium* spp. isolates by PCR-RFLP analysis of the intergenic spacer region of the rRNA gene (rDNA). Int. J. Food Microbiol. 106: 297-306.

O'Donnell K, Kistler HC, Cigelnik E, Ploetz RC (1998). Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Appl. Biol. Sci. 95: 2044-2049.

Patiño B, Mirete, S, Vázquez C, Jiménez M, Rodríguez MT, González-Jaén MT (2006). Characterization of *Fusarium verticillioides* strains by PCR-RFLP analysis of the intergenic spacer region of the rDNA. J. Sci .Food Agric. 86: 429-435.

Pegg KG, Moore NY, Sorensen S (1994). Variability in populations of *Fusarium oxysporum* f.sp *cubense* from Asia/Pacific region. In: The Improvement and Testing of *Musa*: A Global Partnership. Ed. DR Jones. Proceedings of the First Global Conference of the International Musa Testing Program. Honduras. pp. 70 – 82.

Pegg KG, Shivas RG, Moore NY, Bentley S (1995). Characterization of a unique population of *Fusarium oxysporum* f. sp. *cubense* causing *Fusarium* wilt in Cavendish bananas at Carnavon, Western Australia. Aust. Agric. Res. 46: 167-178.

Prashant KM, Fox RTV, Culham A (2003). Inter-simple sequence repeat and aggressiveness analyses revealed high genetic diversity, recombination and long-range dispersal in *Fusarium culmorum*. Ann. Appl. Biol. 143: 291-301.

Ploetz RC, Herbert J, Sebasigari K, Hernandez JH, Pegg KG, Ventura JA, Mayato LS (1990). Importance of Fusarium wilt in different banana-growing regions. In : Fusarium wilt of banana. Ed. RC Ploetz. The Am. Phytopathol. Soc. pp. 9-26.

Ploetz RC (1992). *Fusarium* wilt of banana (Panama disease). *In*: Plant diseases of International Importance. Diseases of fruit crops. (*Eds*: Kumar J, Chaube HS, Singh US, Mukhopadhyay AN) Prentice Hall, Englewood Cliffs, New Jersey. 3: 270-281.

Ploetz RC (2006). *Fusarium* wilt of banana is caused by several pathogens referred to as *Fusarium oxysporum* f. sp. *cubense*. Phytopathol. 96: 653-656.

Ploetz RC, Pegg KG (2000). Fungal diseases of the root, corm and pseudostem: Fusarium wilt. In: Jones DR (ed). Diseases of banana, abaca and enset. CABI Publishing, UK. pp. 143-159.

Rohlf FJ (2000). NTSYS-pc Numerical Taxonomy and Multivariate Analysis System. Version 2.1. Exeter Publishing Ltd. Setauket. New York, USA. p. 38.

Su MJ, Hwang SC, Ko WH (1986). Fusarial wilt of Cavendish bananas in Taiwan. Plant Dis. 70: 814-818.

Stover RH (1962). Studies of *Fusarium* wilt of bananas: IX. Competitive saprophytic ability of *F. oxysporum* f. *cubense*. Can. J. Bot. 40: 1473-1481.

Stover RH (1972). *Banana, plantain and abaca diseases*. Commonwealth Mycological Institute, Kew, Surrey, England. pp. 167-179.

Stover RH, Simmonds NW (1987). Bananas. 3rd edition. Longmans, London, UK.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. (Publication PD http://www.kumarlab.net/publications). Mol. Biol. Evol. 24: 1596-1599.

Trujillo EE, Snyder WC (1963). Uneven distribution of *Fusarium oxysporum* f. *cubense* in Honduras soils. Phytopathol. 53: 167-170.

Versalovic J, Koeuth T, Lupski JR (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acid Res. 19: 6823-6831.

Zaccardelli M, Vitale S, Luongo L, Merighi M, Corazza L (2008). Morphological and molecular characterization of *Fusarium solani* isolates. J. Phytopath. 156: 534-541.