Sains Malaysiana 46(12)(2017): 2425–2432
http://dx.doi.org/10.17576/jsm-2017-4612-18

Characterization of *Fusarium proliferatum* and *Fusarium verticillioides* based on Species-Specific Gene and Microsatellites Analysis

(Perincian *Fusarium proliferatum* dan *Fusarium verticillioides* Berdasarkan Gen Khusus Spesies dan Analisis Mikrosatelit)

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ABSTRACT

Fusarium *species are known to cause various diseases on plantations including fruits and vegetables. The most common* Fusarium *that can cause plant diseases are* Fusarium proliferatum *and* Fusarium verticillioides. *Ear rot disease on maize, wilt disease on cucurbits and fruit rot disease on tomato as well as banana are example of diseases caused by these two species. The objectives of this study were to identify* F. proliferatum *and* F. verticillioides *based on species-specific primers and polymerase chain reaction (PCR) amplification and to evaluate the genetic diversity of both species based on microsatellite markers. Fifty isolates of* Fusarium *species that were previously collected throughout Malaysia from different hosts were identified by using species-specific PCR amplification. Twenty-nine isolates were identified as* F. proliferatum *and 21 isolates were identified as* F. verticillioides *based on species-specific primer. The genetic diversity of all the fungal isolates was evaluated by using microsatellite analysis with six established primers. Five out of six primers amplified polymorphic bands with the most effective primer showing high polymorphism were (AG)7C and (TCC)5 meanwhile one primer (TTTC)4 gave negative result with no band amplified. The phylogenetic tree that was constructed showing two different clades distinguished between* F. proliferatum *and* F. verticillioides.

Keywords: Fusarium proliferatum; Fusarium verticillioides*; microsatellite*; *species-specific gene*

ABSTRAK

Spesies Fusarium *dikenali sebagai penyebab pelbagai penyakit terhadap tumbuhan termasuk buah-buahan dan sayur-sayuran. Antara spesies* Fusarium *yang paling kerap menyebabkan penyakit pokok adalah* Fusarium proliferatum *dan* Fusarium verticillioides. *Penyakit reput tongkol pada jagung, penyakit layu pada mentimun dan penyakit buah reput pada tomato dan pisang adalah contoh penyakit disebabkan oleh dua spesis ini. Objektif kajian ini adalah untuk mengenal pasti* F. proliferatum *dan* F. verticillioides *berdasarkan khusus spesies reaksi rantai polimerase dan untuk menilai kepelbagaian genetik kedua-dua spesies berdasarkan mikrosatelit. Lima puluh isolat spesies* Fusarium *yang terlebih dahulu dipencilkan daripada pelbagai perumah yang diperoleh dari serata Malaysia telah diuji dan dikenal pasti dengan menggunakan gen khusus spesies reaksi rantai polimerase. Dua puluh sembilan isolat telah dikenal pasti sebagai* F. proliferatum *dan dua puluh satu isolat telah dikenal pasti sebagai* F. verticillioides *berdasarkan primer gen khusus spesies. Setelah proses pengenalpastian dilakukan, kepelbagaian genetik telah dinilai dengan menggunakan analisis mikrosatelit menggunakan enam primer. Lima daripada enam primer menghasilkan band polimorfik dan primer yang paling tinggi kadar polimorfisme adalah (AG)7C dan (TCC)5 manakala satu primer (TTTC)4 memberikan hasil negatif dan tiada amplifikasi. Pohon filogenetik yang telah dihasilkan menunjukkan dua klad yang membezakan antara* F. proliferatum *dan* F. verticillioides.

Kata kunci: Fusarium proliferatum; Fusarium verticillioides; *gen khusus spesies; mikrosatelit*

INTRODUCTION

Fusarium proliferatum and *Fusarium verticillioides* can cause various diseases and have become potential pathogens on maize causing Fusarium ear rot disease, on tomato causing fruit rot disease and on luffa and pumpkin causing wilt disease. In Malaysia, *Fusarium* species such as *F. proliferatum, F. verticillioides, F. subglutinans, F. sacchari* and *F. fujikuroi* have been found to infect agricultural crops such as maize, rice and sugarcane (Nur Ain Izzati et al. 2011; 2010; 2009). Another occurrence in Malaysia was *F. proliferatum, F. oxysporum, F. nygamai, F. semitectum, F. solani* and *F. verticillioides* were successfully isolated from corn in four different states of Malaysia namely Perlis, Pulau Pinang, Sabah and Sarawak (Darnetty et al. 2008). Factors leading to contamination of *Fusarium* species might be influenced by environmental condition such as temperature and humidity, susceptibility of the species and cultural practices such as crop rotation (Reid et al. 2001).

Identification of *F. proliferatum* and *F. verticillioides* only by morphological characteristics is very difficult due to their similar characteristics like mycelial pigmentation

and shape of the conidia. Hence, development of molecular markers based on the Polymerase Chain Reaction (PCR) has become the most effective method for species identification and these species-specific primers will result in sensitive and rapid identification (Zheng & Ploetz 2002).

Microsatellites or simple sequence repeats (SSRs) can be found abundantly and ubiquitous in all eukaryotic genome (Yogeshwar et al. 2010). Since it has high level of polymorphism, microsatellites have become genetic markers frequently used in population genetics and diversity studies (Ellegren 2000). Genetic studies by Ren et al. (2012) on *F. verticillioides* suggested that the population grouped by geographical area were genetically similar and have very low extent of genetic differentiation among the populations. Microsatellite analysis has become one of the most reliable genetic tools in population and conservation genetic studies. This technique can be used for analysis of phylogenetic relationship among populations and to detect the genetic variation. The objectives of this study were to identify *F. proliferatum* and *F. verticillioides* based on species-specific PCR assays and to evaluate the genetic diversity of both species based on microsatellite markers.

Materials and Methods

Fungal purification and preservation

Fifty isolates of *F. proliferatum* and *F. verticillioides* were obtained from Mycology Laboratory, Department of Biology, Universiti Putra Malaysia and Mycology Laboratory, School of Biological Sciences, Universiti Sains Malaysia. All of the isolates were previously collected throughout Malaysia from different hosts. The isolates were re-purified on Potato Dextrose Agar (PDA), incubated for 5 days and then were transferred on Spezieller Nahrstoffarmer Agar (SNA) and filter paper for preservation. The isolates were incubated at room temperature (27 ± 2°C) for 7 days and then the filter papers were transferred into cryovial tube, dried using silica gels in a desiccator and kept in -20°C.

DNA extraction and species-specific PCR amplification

All isolates were cultured on PDA for 7 days and genomic DNA were extracted by using UltraClean® Microbial DNA isolation kit (MO BIO, Carlsbad, CA, USA) according to the protocols provided by the manufacture.

Species-specific PCR amplifications were carried out using primers ProF1 (5'-CTTTCCGCCAAGTTTCTTC-3') and ProR1 (5'-TGTCAGTAACTCGACGTTGTTG-3') for detection of *F. proliferatum* (Jahan Quazi et al. 2013); while VertF1 (5'-GTCAGAATCCATGCCAGAACG-3') and VertR1 (5'-CACCCGCAGCAATCCATCAG-3') for the detection of *F. verticillioides* (Patino et al. 2004). PCR reactions for both primer pairs performed in a final volume of 20 µl consisting of 1 PCR buffer, 0.5 µM primer, 0.2 mM of each deoxynucleotide triphosphate (dNTPs), 2.5 mM magnesium chloride ($MgCl_2$), 0.125 U Go*Taq* DNA Polymerase, nuclease free water and 20 ng DNA template. PCR condition for species-specific identification of *F. proliferatum* follows Jahan Quazi et al. (2013); initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 30 s, extension at 72°C for 1 min and a single cycle of final extension at 72°C for 5 min while identification of *F. verticillioides* follows Patino et al. (2004); initial denaturation at 94°C for 1.25 min, 25 cycles of denaturation at 95°C for 35 s, annealing at 66°C for 30 s, extension at 72°C for 2 min and final extension at 72°C for 5 min.

Gel electrophoresis was performed by using 1.5% agarose gel and immersed in 1X Tris Borate-acid EDTA (TBE) buffer amended with FloroSafe DNA stain according to manufacturer's instructions (1st BASE, Asia). Approximately 5 µL for each DNA ladder 100 bp (Thermo Scientific) and PCR products were loaded and electrophoresed for 35 min at 90 V. The gel was viewed and analysed using Syngene software by a gel documentation system under UV light visualisation (Syngene, Germany).

Microsatellite analysis

Six established microsatellite markers as listed in Table 1 were selected to perform the analysis on genetic diversity of *F. proliferatum* and *F. verticillioides* isolates. Standard PCR master mix for all reactions were each 20 µL comprises 1 PCR buffer, 0.5 µM primer, 0.2 mM of each deoxynucleotide triphosphate (dNTPs), 2.5 mM magnesium chloride ($MgCl_2$), 0.125 U Go*Taq* polymerase, nuclease free water and 20 ng DNA. The following PCR amplification was followed as: initial denaturation at 94°C for 2 min, 39 cycles of denaturation at 94°C for 1 min, annealing as follow the optimal Tm for each primer

TABLE 1. Primers for microsatellite analysis

Nucleotide repeats	Primer	Tm (°C)	GC content (%)	Expected alleles size range (bp)
Di-	(AG)7C	50	53.3	200-2500
	(CA)7T	52	46.7	300-1700
Tri-	(CTG)5	52	66.7	450-2000
	(TCC)5	52	66.7	400-2000
Tetra-	(TAGG)4	50	50.0	200-2000
	(TTTC)4	50	25.0	300-2000

(Table 1) for 90 min, extension at 72°C for 2 min and a final extension at 72°C for 6 min (Bahkali et al. 2012).

Gel electrophoresis was performed by using 1.5% agarose gel and immersed in 1X Tris Borate-acid

TABLE 2. Species identification of *F. proliferatum* and *F. verticillioides* based on species-specific primer of ProF1/ProR1 and VertF1/VertR1

Isolate	Host	Locality	Zone	ProF1/ProR1	VertF1/VertR1
A2358	*Musa acuminata*	Hutan Melintang, Perak	Central	-	+
A2359	*Musa acuminata*	Hutan Melintang, Perak	Central	-	+
B68	*Zea mays*	Serdang, Selangor	Central	+	-
B92	*Zea mays*	Serdang, Selangor	Central	+	-
B106	*Zea mays*	Serdang, Selangor	Central	-	+
B146	*Zea mays*	Serdang, Selangor	Central	-	+
B1371	*Zea mays*	Semenyih, Selangor	Central	-	+
B1777	*Luffa acutangula*	Tanjung Karang, Selangor	Central	+	-
B1778	*Luffa acutangula*	Tanjung Karang, Selangor	Central	+	-
B1779	*Luffa acutangula*	Tanjung Karang, Selangor	Central	+	-
B1780	*Luffa acutangula*	Tanjung Karang, Selangor	Central	+	-
B1781	*Luffa acutangula*	Tanjung Karang, Selangor	Central	+	-
B1784	*Luffa acutangula*	Tanjung Karang, Selangor	Central	+	-
B2377	*Musa acuminata*	Tanjung Karang, Selangor	Central	+	-
B2433	*Musa acuminata*	Serdang, Selangor	Central	+	-
C116	*Zea mays*	Cameron Highland, Pahang	Central	-	+
C121	*Zea mays*	Cameron Highland, Pahang	Central	-	+
F286	*Cosmos caudatus*	Puchong, Selangor	Central	+	-
J44	*Zea mays*	Senggarang, Johor	South	-	+
J1361	*Zea mays*	Sri Medan, Johor	South	-	+
J1362	*Zea mays*	Sri Medan, Johor	South	-	+
J1363	*Zea mays*	Senggarang, Johor	South	-	+
J1364	*Zea mays*	Senggarang, Johor	South	-	+
J1789	*Cucurbita pepo*	Tangkak, Johor	South	+	-
J1790	*Cucurbita pepo*	Tangkak, Johor	South	+	-
J1791	*Cucurbita pepo*	Tangkak, Johor	South	+	-
J1792	*Cucurbita pepo*	Tangkak, Johor	South	+	-
J1793	*Cucurbita pepo*	Tangkak, Johor	South	+	-
K2344	*Musa acuminata*	Bukit Kayu Hitam, Kedah	North	-	+
M2396	*Musa balbisiana*	Masjid Tanah, Melaka	South	+	-
M2399	*Musa paradisiaca*	Merlimau, Melaka	South	+	-
N1387	*Cucumis sativus*	Rembau, Negeri Sembilan	South	-	+
N2215	*Cucumis sativus*	Rembau, Negeri Sembilan	South	+	-
P202	*Zea mays*	Seberang Prai, Pulau Pinang	North	+	-
P204	*Zea mays*	Seberang Prai, Pulau Pinang	North	+	-
P1366	*Zea mays*	Seberang Prai, Pulau Pinang	North	+	-
P1367	*Zea mays*	Seberang Prai, Pulau Pinang	North	-	+
680	*Oryzae sativa*	Haji Kudung, Kedah	North	+	-
901	*Asparagus officinalis*	Kundasang, Sabah	East M.	+	-
971	*Triticum aestivum*	Teluk Kumbar, Pulau Pinang	North	+	-
1007	*Sorghum bicolor*	Sri Aman, Sarawak	East M.	+	-
1380	*Dendrobium* sp.	Kuala Lumpur	Central	+	-
3240	*Saccharum officinarum*	Padang Terap, Kedah	North	+	-
3244	*Saccharum officinarum*	Padang Terap, Kedah	North	+	-
7696	*Zingiber officinale*	Gelugor, Pulau Pinang	North	-	+
7697	*Zingiber officinale*	Gelugor, Pulau Pinang	North	-	+
7703	*Zingiber officinale*	Batu Uban, Pulau Pinang	North	-	+
7704	*Zingiber officinale*	Gelugor, Pulau Pinang	North	-	+
7705	*Zingiber officinale*	Gelugor, Pulau Pinang	North	-	+
7706	*Zingiber officinale*	Batu Uban, Pulau Pinang	North	-	+

EDTA (TBE) buffer amended with FloroSafe DNA stain according to manufacturer's instructions (1st BASE, Asia). Approximately 5 µL for each 1 kb DNA ladder (Thermo Scientific) and PCR products were loaded and electrophoresed for 90 min at 90 V. The gel was viewed and analysed using Quantity One® 1-D Analysis Software version 4.6.5 by a gel documentation system under UV light using Bio-Rad Molecular Imager® Gel Doc™ XR System.

The results of band pattern obtained were compared for polymorphism by visual observation. Visible bands among isolates with the same migration distance were considered no differences. Every presence band was scored (1) or absence band was scored (0) among all the isolates. Only reproducible bands in PCR amplifications were considered for analyses. All the band scoring were analysed by using GenAlEx 6.5 (Peakall & Smouse 2012) and phylogenetic tree was computed by using NEXUS formatted data and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) on PAUP 4.0 (Cummings 2004).

RESULTS AND DISCUSSION

SPECIES-SPECIFIC GENE OF *F. PROLIFERATUM* AND *F. VERTICILLIOIDES*

Based on 50 isolates of *Fusarium* species from all around Malaysia and from diverse hosts, 29 were *F. proliferatum*; while 21 were *F. verticillioides*. Table 2 indicates the identification of all isolates of both *Fusarium* species by using species-specific primers ProF1/ProR1 and VertF1/VertR1. PCR amplification using primer VertF1/VertR1 was conducted to identify *F. verticillioides*. Twenty-one isolates of *F. verticillioides* have amplified fragments ranging from 700-800 bp. According to Patino et al. (2004), amplification of the target DNA from *F. verticillioides* was successful by using primer VertF1/VertR1 and single fragment of 800 bp was amplified in all strains. Meanwhile, there were no amplifications produced when using another species including a closely related species from the *Gibberella fujikuroi* species complex.

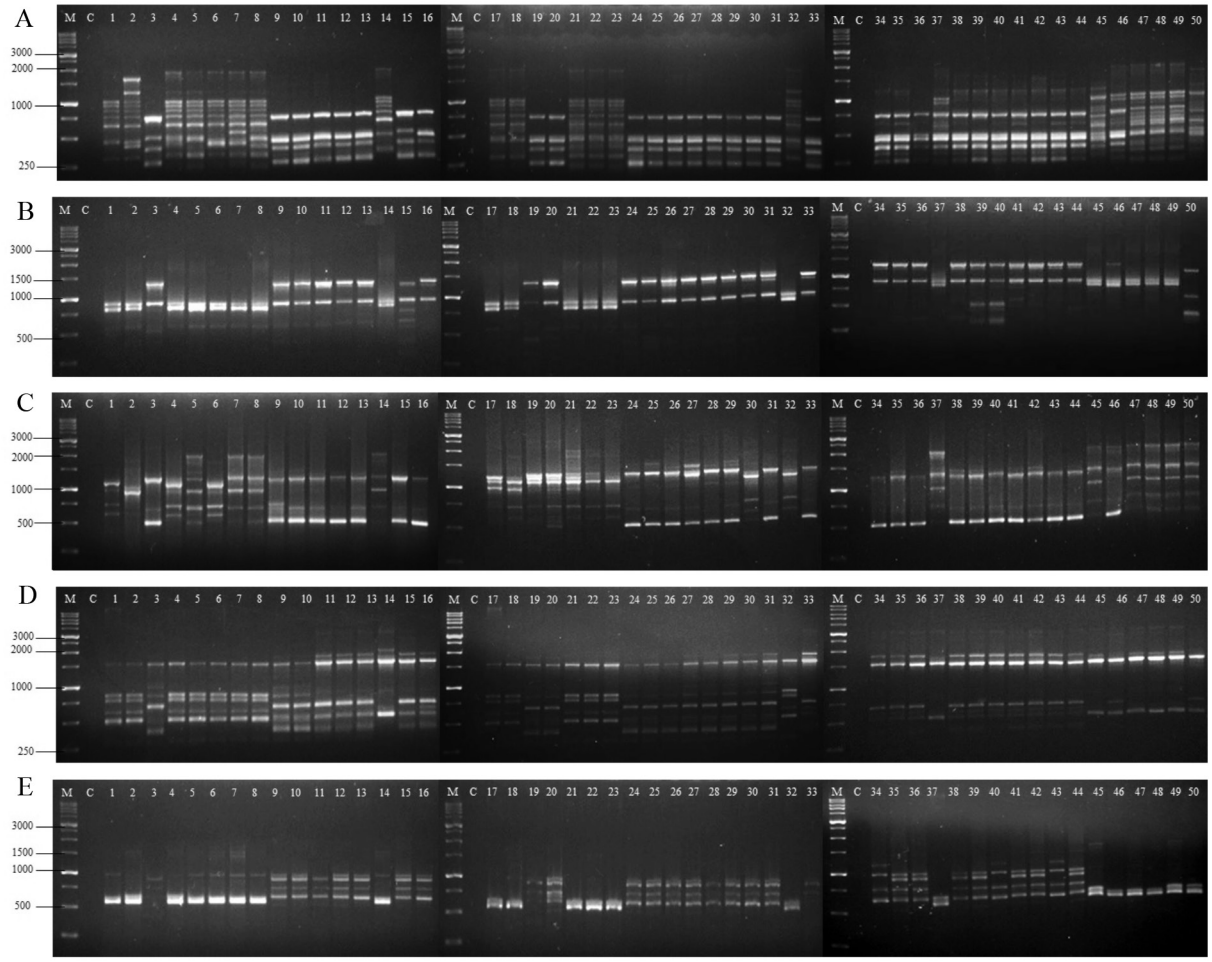

FIGURE 1. DNA amplifications of 50 *Fusarium* isolates by using primer A:(AG)7C, B:CA(7)T, C:(CTG)5, D:(TCC)5 and E:(TAGG)4 respectively. Lane M: 1 kb ladder. Lane C: control. Lane (1-16): C116, C121, F286, J44, J1361, J1362, J1363, J1364, J1789, 71790, J1791, J1792, J1793, K2344, M2396, M2399. Lane (17-33): A2358, A2359, B68, B92, B106, B146, B1371, B1777, B1778, B1779, B1780, B1781, B1784, B2377, B2433, N1387, N2215. Lane (34-50): P202, P204, P1366, P1367, 680, 901, 971, 1007, 1380, 3240, 3244, 7696, 7697, 7703, 7704, 7705, 7706

Identification of *F. proliferatum* was conducted by using species-specific primer of ProF1/ProR1. A single fragment ranging from 500-600 bp was clearly amplified in all twenty-nine isolates. Based on Jahan Quazi et al. (2013), all PCR products from *F. proliferatum* have amplified a single fragment with size approximately 550 bp. Negative results obtained when identifying *F. verticillioides* with ProF1/ProR1 primer pairs. Both *F. proliferatum* and *F. verticillioides* have similar morphological characteristics including macroscopic and microscopic. The size of macroconidia and microconidia between both species are found to be similar as reported by Gohari et al. (2007). Thus, identification by species-specific PCR amplification can confirmed and distinguished between both species.

MICROSATELLITE ANALYSIS AND GENETIC DIVERSITY

To validate the polymorphism and genetic diversity among all the *Fusarium* isolates, 6 established primers were selected from Bahkali et al. (2012). Five primers have successfully amplified bands while one primer showed negative result. Three types of nucleotides repeats were chosen which are di-, tri- and tetranucleotide. Since microsatellites in fungal genome are shorter than other higher organism, choosing shorter nucleotide repeats will give better result because fungal microsatellites are predominated by mono-, di- and trinucleotides (Toth et al. 2000). From the results of our study (Figure 1), sizes of PCR fragments amplified from primers (AG)7C and (TCC)5 were as expected ranging from 250-2000 bp and 300-2000 bp, respectively. Meanwhile for primer (TAGG)4, the fragments amplified ranged from 500-1500 bp in range, however, as reported by Bahkali et al. (2012) the expected size ranged from 200-2000 bp. For primer (TTTC)4, there are negative results and no amplifications produced even though modifications were made on the annealing temperature and PCR reaction volume. It can be concluded that this primer is not suitable for analysis on *F. verticillioides* and *F. proliferatum* and from all the results, only partial agreement can be made with previous study by Bahkali et al. (2012).

Between all 6 primers, the most successful primer is dinucleotide (AG)7C with total 259 number of alleles produces on both *Fusarium* species (Table 3). Primer (TCC)5 is the most successful trinucleotide primer that amplified 213 alleles in both *Fusarium* species. Both of these primers are suitable interspecies comparison as it shows high level of polymorphisms. In fact, fungi have shorter genome as compared to the other higher organisms. Dinucleotide and trinucleotide repeat primers are the most frequent motifs and most successful motifs in fungi (Jany et al. 2006; Karaoglu et al. 2005; Lu et al. 2004).

Analysis of Molecular Variance (AMOVA) was analysed from GenAlEx 6.5 and showed the variation among populations and within populations. From the chart (Figure 2), the variation among populations was 22% while variation within populations was 78%. The variation and diversity within the population were high may be due to genetic drift and mutation that occurred within the population. The genetic drift happened when the allele frequencies can change over time randomly meanwhile the mutation happened when there is an error in the replication of DNA that causes structural change in a gene. Mutation might occurred due to long exposures of fungicide and pesticide that change and alter the DNA of fungi to develop a new resistant. As mention by Rampersad et al. (2013), areas of high biodiversity are due to the emergence of a new genotype that caused by the changes in pathogen resistance to a certain fungicide. Four populations were differentiated based on zone, which is north, central, south and east Malaysia. The percentage of polymorphic loci (Table 5) was given based on populations. Central population have the highest polymorphic loci (84.62%), followed by North (76.92%), South (64.10%) and while east Malaysia has the lowest polymorphic loci (23.08%). The differences of percentage between these two populations are due to different number of samples for each population.

From the phylogenetic tree that was constructed using UPGMA (Figure 3), two clades were formed that distinguished between *F. proliferatum* and *F. verticillioides*. The similarity among all *F. verticillioides* was 70% while similarity among all *F. proliferatum* was 98%. However, for each clade more differentiation and subclades formed and this proved that there were genetic diversity occurred within the species. Case study by Abd-Elsalam et al. (2011), 19 isolates of *Fusarium* species were analysed by UPGMA have revealed a high degree of interpopulation differentiation.

TABLE 3. Total number of alleles amplified from *F. proliferatum* and *F. verticillioides* with six established microsatellite primers

Primers	Number of alleles from different species		Total
	F. verticillioides	F. proliferatum	
(AG)7C	139	120	259
(CA)7T	44	77	121
(CTG)5	55	65	120
(TCC)5	88	125	213
(TAGG)4	51	106	157
(TTTC)4	0	0	0

TABLE 4. Total number of alleles amplified from five successful primers according to isolates

Species	Isolates	Number of alleles amplified from primers				
		(AG)7C	(CA)7T	(CTG)5	(TCC)5	(TAGG)4
F. verticillioides	A2358	7	2	3	5	2
	A2359	7	2	3	5	2
	B106	7	2	3	5	2
	B146	7	2	2	5	2
	B1371	7	2	2	5	2
	C116	6	2	3	5	3
	C121	7	2	2	5	2
	J44	7	2	3	5	2
	J1361	7	2	4	5	3
	J1362	7	2	3	5	3
	J1363	8	2	3	5	3
	J1364	7	2	3	5	3
	K2344	7	2	3	3	3
	N1387	7	2	2	5	2
	P1367	6	2	3	2	2
	7696	6	2	2	3	3
	7697	7	3	3	3	2
	7703	6	2	2	3	2
	7704	6	2	2	3	2
	7705	6	2	2	3	3
	7706	4	3	2	3	3
Total		139	44	55	88	51
F. proliferatum	B68	4	2	4	3	3
	B92	4	3	4	3	4
	B1777	4	2	2	3	4
	B1778	4	2	2	3	4
	B1779	4	3	2	3	4
	B1780	4	3	3	3	4
	B1781	4	3	2	3	2
	B1784	4	3	2	4	4
	B2377	4	3	2	4	4
	B2433	4	3	2	4	4
	F286	4	3	2	3	1
	J1789	4	3	3	5	4
	J1790	4	3	2	5	4
	J1791	4	3	2	5	3
	J1792	4	3	2	5	4
	J1793	4	3	2	5	4
	M2396	4	2	2	5	4
	M2399	4	3	2	6	4
	N2215	4	3	2	3	2
	P202	5	2	2	5	4
	P204	5	2	2	5	4
	P1366	3	2	2	5	4
	680	4	2	2	5	4
	901	5	4	2	5	4
	971	4	4	2	5	3
	1007	4	2	3	5	4
	1380	5	2	2	5	4
	3240	5	2	2	5	4
	3244	4	2	2	5	4
Total		120	77	65	125	106

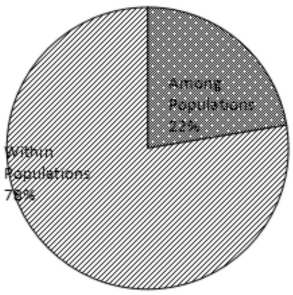

FIGURE 2. Analysis of Molecular Variance (AMOVA) from GenAlEx 6.5 showing percentage of variation among populations and within populations

TABLE 5. Percentage of polymorphic loci according to population from GenAlEx 6.5

Population	Polymorphic Loci (%)
North	76.92
Central	84.62
South	64.10
East Malaysia	23.08

CONCLUSION

From all 50 isolates of *Fusarium* species that were collected throughout Malaysia from different range of hosts, 29 isolates were *F. proliferatum* and 21 isolates were *F. verticillioides*. Species-specific primer on PCR is quite identification of both species. The available microsatellite primers, five gave positive results while one of it gave negative result with no amplification. From these primers, phylogenetic tree was constructed and AMOVA have provided genetic diversity among population, which is 22% and within population, which is 78%. From this study, we have confirmed the efficacy of the species-specific primer to identify both species of *Fusarium*. Furthermore, the diverse strains of these species in the population warrant further study on their resistance towards fungicide used in the field.

ACKNOWLEDGEMENTS

This study was partially supported by the IPS Putra Grant Universiti Putra Malaysia (UPM) (Vot number: 9496600) to Nur Ain Izzati M. Z. and Najihah A. received scholarship from the Graduate Research Fund (GRF), UPM.

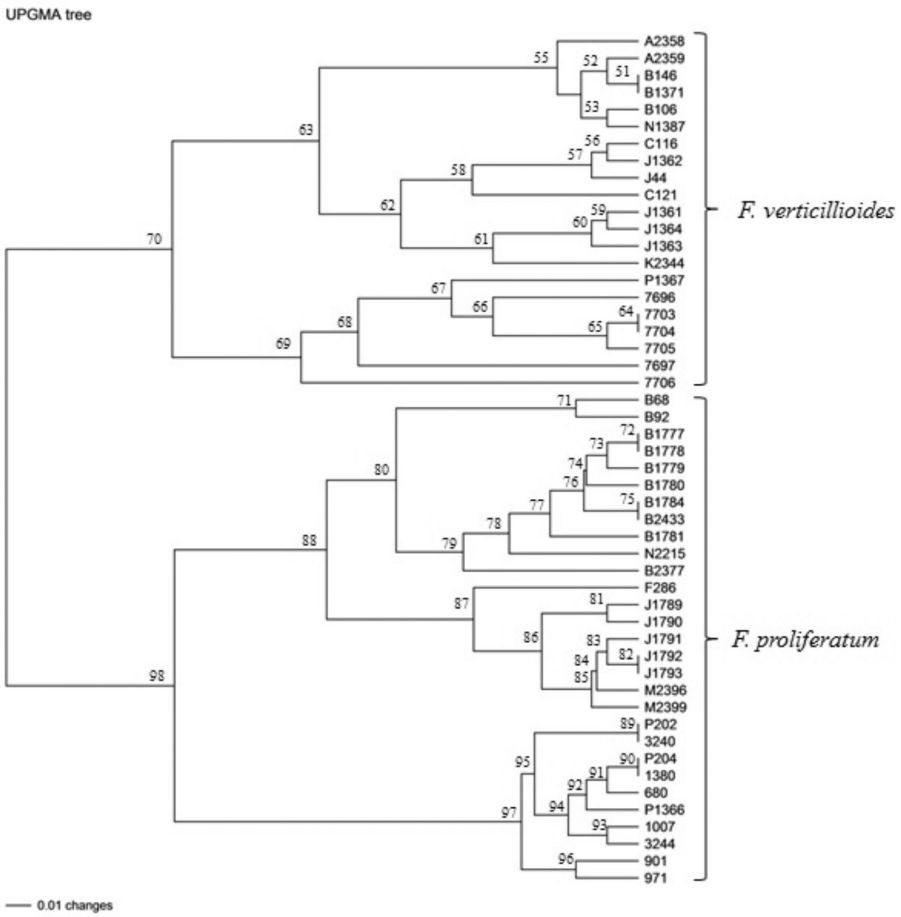

FIGURE 3. Phylogenetic tree of *Fusarium* species by Unweighted Paired Group Method with Arithmethic Averages (UPGMA) showing two distinct clades between *F. verticillioides* and *F. proliferatum*. Phylogenetic tree was constructed from microsatellite analysis of primer (AG)7C, (CA)7T, (CTG)5, (TCC)5 and (TAGG)4

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Received: 14 November 2016
Accepted: 23 May 2017