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Characterization of *Fusarium* species section Liseola by restriction analysis of the IGS region

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ABSTRACT. *Fusarium* species section Liseola namely *F. fujikuroi*, *F. proliferatum*, *F. andiyazi*, *F. verticillioides*, and *F. sacchari* are well-known plant pathogens on rice, sugarcane and maize. In the present study, restriction analysis of the intergenic spacer regions (IGS) was used to characterize the five *Fusarium* species isolated from rice, sugarcane and maize collected from various locations in Peninsular Malaysia. From the analysis, and based on restriction patterns generated by the six restriction enzymes, *Bsu*151, *Bsu*RI, *Eco*RI, *Hin*6I, *Hin*fI, and *Msp*I, 53 haplotypes were recorded among 74 isolates. *Hin*fI showed the most variable restriction patterns (with 11 patterns), while *Eco*RI showed only three patterns. Although a high level of variation was observed, it was possible to characterize closely related species and isolates from different species. UPGMA cluster analysis showed that the isolates of *Fusarium* from the same species were grouped together regardless of the hosts. We conclude that restriction analysis of the IGS regions can be used to characterize *Fusarium* species section Liseola and to discriminate closely related species as well as to clarify their taxonomic position.

Key words: *Fusarium*; Intergenic spacer regions; Liseola

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INTRODUCTION

Section Liseola or *Gibberella fujikuroi* species complex was established by Wollenweber and Reinking (1935) in which the species in the section produced microconidia in chains as well as microconidia in false heads but did not produce chlamydospores. Snyder and Hansen (1945) adopted the name *Fusarium moniliforme* as the only member of the *Fusarium* species in the section Liseola. In later studies, based on morphological characters, various workers recognized that section Liseola consisted of a number of species, from 4 to 29 (Booth, 1971; Nelson et al., 1983; Nirenberg, 1989; Nirenberg and O'Donnell, 1998). Therefore, morphological characters alone are not sufficient to identify and characterize the species of *Fusarium* in the section Liseola.

One of the most common methods to characterize plant pathogenic fungi is by using a combination of PCR and restriction analysis, and the region commonly used in the analysis is the intergenic spacer (IGS) of ribosomal DNA (rDNA). The IGS region has been used to differentiate fungal isolates at the intraspecific level (Hillis and Dixon, 1991; Edel et al., 1995) and to compare inter- and intraspecific variations in several species of *Fusarium* (Appel and Gordon, 1995; Lee et al., 2000; Hinojo et al., 2004; Konstantinova and Yli-Mattila, 2004; Llorens et al., 2006a,b; Masratul Hawa et al., 2010).

In Malaysia, studies on characterization using molecular methods of *Fusarium* species section Liseola are limited. In most studies, morphological characteristics and mating studies were used to identify and characterize the species (Siti Nordahliawate et al., 2008; Zainudin et al., 2008). Therefore, the objective of the present study was to characterize *Fusarium* species section Liseola by using restriction analysis of the IGS region.

MATERIAL AND METHODS

Fungal isolates

The *Fusarium* isolates used in this study are listed in Table 1. The isolates were isolated from rice, maize and sugarcane.

Restriction analysis of the IGS region

For DNA extraction, mycelia were harvested from PDA plates after 7 days of incubation at 25°C. The DNA was extracted using a Qiagen DNeasy® Plant Mini Kit (Qiagen, USA) according to instructions provided by the manufacturer.

The IGS region was amplified using primers CNL12 (5'-CTGAACGCCTCTAAGT CAG-3') and CNS1 (5'-GAGACAAGCATATGACTACTG-3') as described by Appel and Gordon (1995). PCR amplifications were conducted in a 50-µL reaction mixture containing 1X PCR buffer, 3.5 mM $MgCl_2$, 0.16 mM dNTP mix, 1.75 U GoTaq® DNA polymerase (Promega), 0.3 µM of each primers CNL12 and CNS1 and 0.35 µL template DNA up to a total volume of 50 µL with deionized distilled water.

PCR amplification was performed in a DNA Engine™ Peltier Thermal Cycler Model PTC-100 with an initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 59°C for 55 s and extension at 72°C for 2 min, followed by a

final extension for 7 min at 72°C. Negative controls were used to test for the presence of non-specific reactions. The PCR product was detected on 1.5% agarose gel electrophoresis, run in Tris-borate-EDTA (TBE) buffer at 80 V and 400 mA for 100 min. The gel was stained with ethidium bromide and visualized under a UV transilluminator. The size of the amplified IGS band was estimated by comparison to a 1-kb marker (Fermentas).

Six restriction enzymes, namely *Bsu*151, *Bsu*RI, *Eco*RI, *Hin*6I, *Hin*fI, and *Msp*I (Fermentas), were used to digest the PCR products in a total reaction volume of 15 µL. The digestion procedure was according to manufacturer instructions. Digested PCR product was run on 2% agarose gel in TBE buffer for 140 min at 80 V and 400 mA, stained with ethidium bromide and visualized on a UV transalluminator. The size of the restriction fragments was estimated and analyzed by comparison to 100-bp DNA marker (Fermentas) with the Discovery Series™ Quantity One® 1-D Analysis software, version 4.6.5.

Data analysis

Each isolate was assigned to a composite IGS haplotype defined by the combination of restriction patterns generated by the six restriction enzymes.

The Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) version 2.2 (Rohlf, 2005) was used to analyze the data. Restriction fragments produced were scored as present (1) or absent (0) for particular fragments. The binary data were then used to generate a similarity matrix using the simple matching coefficient. The similarity values obtained were then utilized to construct a dendrogram based on UPGMA cluster analysis.

In order to measure the goodness of fit of the cluster analysis to the data, a cophenetic value matrix was constructed from the dendrogram to obtain a cophenetic correlation coefficient (r) (Rohlf and Sokal, 1981), which measures the degree of correlation between the similarity matrix and the cophenetic value matrix. The r value was interpreted based on Rohlf (2005) in which $r > 9.0$ is considered to be a very good fit and $0.8 < r < 0.9$ is considered to be a good fit.

RESULTS

From the PCR of the IGS region using CNS and CNL primers, a single fragment of 2600 bp was amplified from all 74 isolates of *Fusarium*. The PCR products were digested independently using six restriction enzymes. Depending on the isolates and the restriction enzyme, the PCR products were digested into one to six fragments. Restriction fragments of less than 100 bp were not clearly resolved by electrophoresis. Therefore, for some of the isolates, the PCR products estimated by adding the size of the restriction fragments were less than the size of the undigested PCR products. Three to 11 restriction patterns were generated by the six restriction enzymes. *Hin*fI showed the most variable patterns with 11 patterns followed by *Hin*61 and *Msp*I with nine patterns. *Bsu*RI produced eight patterns and *Bsu*151 produced four patterns. Figure 1 shows the restriction patterns produced using *Hin*61. The least variable patterns were generated by *Eco*RI with three patterns (Figure 2). Isolates of *F. fujikuroi* showed the same restriction patterns as *Hin*fI and *Msp*I, and isolates of *F. verticillioides* produced the same *Bsu*RI restriction patterns.

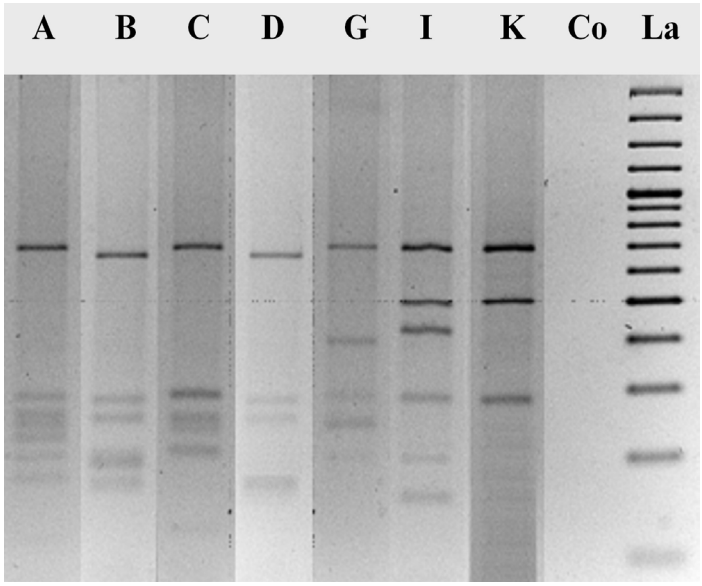

Figure 1. Restriction patterns obtained after digestion with *Hin*fI. *Lane A* = 3067 (*Fusarium fujikuroi*); *lane B* = JB2 (*F. verticillioides*); *lane C* = B1 (*F. verticillioides*); *lane D* = 3055 (*F. andiyazi*); *lane G* = P4 (*F. proliferatum*); *lane I* = 3081 (*F. sacchari*); *lane K* = T9 (*F. sacchari*); *lane Co* = control; *lane La* = 100-bp DNA marker.

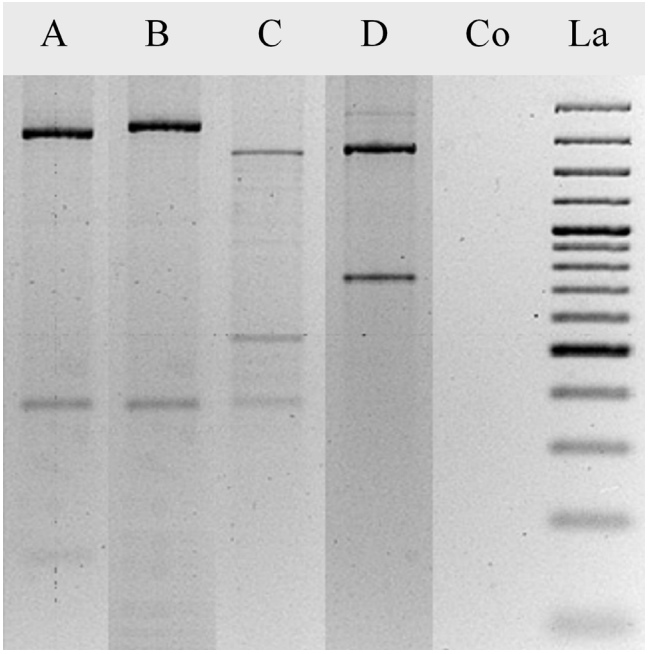

Figure 2. Restriction patterns obtained after digestion with *Eco*RI. *Lane A* = 3308 (*Fusarium sacchari*); *lane B* = T4 (*F. sacchari*); *lane C* = B2 (*F. verticillioides*); *lane D* = 3055 (*F. andiyazi*); *lane Co* = control; *lane La* = 100-bp DNA marker.

Based on restriction patterns generated by the six restriction enzymes, 53 haplotypes were recorded among 74 isolates (Table 1). In general, none of the haplotypes assigned were shared between different species except for two isolates of *F. proliferatum* (3151 and 3170) and one isolate of *F. fujikuroi* (3122), which shared the same haplotype. Haplotypes 1-7 were assigned to isolates of *F. fujikuroi*, haplotypes 8-13 to *F. proliferatum*, haplotypes 14-17 to *F. andiyazi*, haplotypes 18-31 to *F. verticillioides*, and haplotypes 32-53 to isolates of *F. sacchari*.

Table 1. *Fusarium* isolates used in this study and the haplotypes generated by using restriction analysis of the IGS region among the isolates of *Fusarium* spp section Liseola.

Host/isolate	Species	Restriction patterns						Haplotype
		*Bsu*15I	*Bsu*RI	*Eco*RI	*Hin*6I	*Hin*fI	*Msp*I	
0621 (R)	*F. fujikuroi*	A	A	A	A	A	A	1
3132 (R)	*F. fujikuroi*	A	A	A	A	A	A	1
3067 (R)	*F. fujikuroi*	A	A	B	A	A	A	2
3099 (R)	*F. fujikuroi*	A	A	B	A	A	A	2
3101 (R)	*F. fujikuroi*	A	B	A	I	A	A	3
3105 (R)	*F. fujikuroi*	A	B	C	A	A	A	4
3208 (R)	*F. fujikuroi*	A	E	B	A	A	A	5
3122 (R)	*F. fujikuroi*	D	B	B	A	A	A	6
JF5 (M)	*F. fujikuroi*	D	A	A	A	A	A	7
P4 (R)	*F. proliferatum*	A	A	B	F	G	H	8
3074 (R)	*F. proliferatum*	A	G	B	D	H	G	9
3075 (R)	*F. proliferatum*	A	G	B	D	H	G	9
3151 (R)	*F. proliferatum*	D	B	B	A	A	A	6
3170 (R)	*F. proliferatum*	D	B	B	A	A	A	6
P1 (R)	*F. proliferatum*	D	G	B	D	H	H	10
P2 (R)	*F. proliferatum*	D	G	B	D	H	H	10
3095 (R)	*F. proliferatum*	D	A	B	D	H	D	11
3238 (SC)	*F. proliferatum*	A	E	C	I	G	I	12
3324 (SC)	*F. proliferatum*	A	E	C	H	G	I	13
3055 (R)	*F. andiyazi*	C	E	B	C	C	D	14
3061 (R)	*F. andiyazi*	C	E	B	C	C	D	14
3086 (R)	*F. andiyazi*	C	E	B	C	C	D	14
3073 (R)	*F. andiyazi*	D	G	C	F	C	A	15
3088 (R)	*F. andiyazi*	D	E	B	C	C	D	16
3137 (R)	*F. andiyazi*	C	E	B	I	C	E	17
T1 (SC)	*F. verticillioides*	C	C	C	E	D	C	18
JB1 (M)	*F. verticillioides*	C	C	C	E	D	C	18
B1 (M)	*F. verticillioides*	C	C	C	E	D	C	18
JB4 (M)	*F. verticillioides*	C	C	C	E	D	C	18
B2 (M)	*F. verticillioides*	C	C	C	E	B	B	19
B3 (M)	*F. verticillioides*	C	C	C	E	B	B	19
B5 (M)	*F. verticillioides*	C	C	C	E	B	B	19
B6 (M)	*F. verticillioides*	C	C	C	E	B	B	19
B9 (M)	*F. verticillioides*	C	C	C	E	B	B	19
JB2 (M)	*F. verticillioides*	C	C	C	E	B	B	19
JD4 (M)	*F. verticillioides*	C	C	C	E	B	B	19
3124 (R)	*F. verticillioides*	C	C	C	E	B	B	19
JF1 (M)	*F. verticillioides*	C	C	C	E	B	C	20
B4 (M)	*F. verticillioides*	C	C	C	E	B	E	21
F1 (M)	*F. verticillioides*	C	C	C	E	A	A	22
3257 (SC)	*F. verticillioides*	C	C	C	B	K	E	23
3277 (SC)	*F. verticillioides*	C	C	C	B	B	B	24
T2 (SC)	*F. verticillioides*	C	C	C	E	D	B	26
0654 (R)	*F. verticillioides*	C	C	C	B	B	C	27
3063 (R)	*F. verticillioides*	C	C	B	B	B	D	28
T5 (SC)	*F. verticillioides*	D	C	B	E	D	D	29
3068 (R)	*F. verticillioides*	D	C	C	B	C	A	30
JB3 (M)	*F. verticillioides*	D	C	C	E	B	B	31

Continued on next page

Table 1. Continued.

Host/Isolate	Species	Restriction patterns						Haplotype
		*Bsu*15I	*Bsu*RI	*Eco*RI	*Hin*6I	*Hin*fI	*Msp*I	
3054 (R)	*F. sacchari*	C	F	B	G	C	D	32
3078 (R)	*F. sacchari*	C	H	B	G	E	F	33
3081 (R)	*F. sacchari*	A	H	B	H	I	E	34
3327 (SC)	*F. sacchari*	A	H	B	H	G	F	35
3343 (SC)	*F. sacchari*	A	H	B	H	F	F	35
3350 (SC)	*F. sacchari*	A	D	A	H	I	G	36
3358 (SC)	*F. sacchari*	A	D	A	H	J	C	37
3308 (SC)	*F. sacchari*	A	D	A	H	J	C	37
T8 (SC)	*F. sacchari*	A	D	B	H	J	C	37
T6 (SC)	*F. sacchari*	A	H	B	H	J	G	38
3281 (SC)	*F. sacchari*	A	H	B	H	J	G	38
3082 (R)	*F. sacchari*	B	H	B	H	J	F	39
3087 (R)	*F. sacchari*	B	H	B	H	I	D	41
B10	*F. sacchari*	B	D	A	H	I	C	42
3084 (R)	*F. sacchari*	C	F	B	G	E	F	43
3246 (SC)	*F. sacchari*	C	F	B	G	F	F	44
3262(SC)	*F. sacchari*	C	G	A	G	F	F	45
3310 (SC)	*F. sacchari*	C	G	A	G	F	F	45
T4 (SC)	*F. sacchari*	C	D	B	G	F	F	46
T9 (SC)	*F. sacchari*	C	D	A	H	E	F	47
3295 (SC)	*F. sacchari*	C	F	B	I	F	F	48
3349 (SC)	*F. sacchari*	C	F	B	D	E	F	49
P5 (R)	*F. sacchari*	D	H	B	H	J	G	50
F2 (M)	*F. sacchari*	D	D	A	G	J	F	51
T7 (SC)	*F. sacchari*	D	D	B	H	J	C	52
T3 (SC)	*F. sacchari*	D	D	B	H	J	F	53

Since highly variable restriction patterns were generated by the six restriction enzymes and the isolates were divided into different haplotypes, UPGMA cluster analysis was performed to group the isolates and to estimate the levels of intra- and interspecies variability among the isolates.

The dendrogram constructed from the similarity matrix using UPGMA cluster analysis is presented in Figure 3. The cophenetic correlation coefficient (r) obtained was 0.91, which indicated a good fit between the cluster analysis and the data. Based on the dendrogram, the isolates from the same species were clustered in the same cluster and the grouping of the isolates can be divided into two major clusters, 1 and 2, and several sub-clusters (Figure 3).

Major cluster 1 consisted of isolates from four species, namely *F. fujikuroi*, *F. verticillioides*, *F. proliferatum*, and *F. andiyazi*. Although, *F. fujikuroi* and *F. proliferatum* are reported to be sibling species, both species produced different haplotypes except for isolate 3122 (*F. fujikuroi*), 3151 (*F. proliferatum*) and 3170 (*F. proliferatum*), which shared the same haplotype. Isolates of *F. sacchari* and five isolates of *F. proliferatum* were grouped in major cluster 2.

Isolates of *F. fujikuroi* were grouped in sub-cluster 1A with a similarity value of 82-100%. Sub-clusters 1C and 2B consisted of isolates of *F. proliferatum*. Isolates of *F. verticillioides* and six isolates of *F. andiyazi* formed sub-cluster 1B with similarity values ranging from 73-100%. Isolates of *F. sacchari* were clustered in sub-cluster 2A with 72-100% similarity.

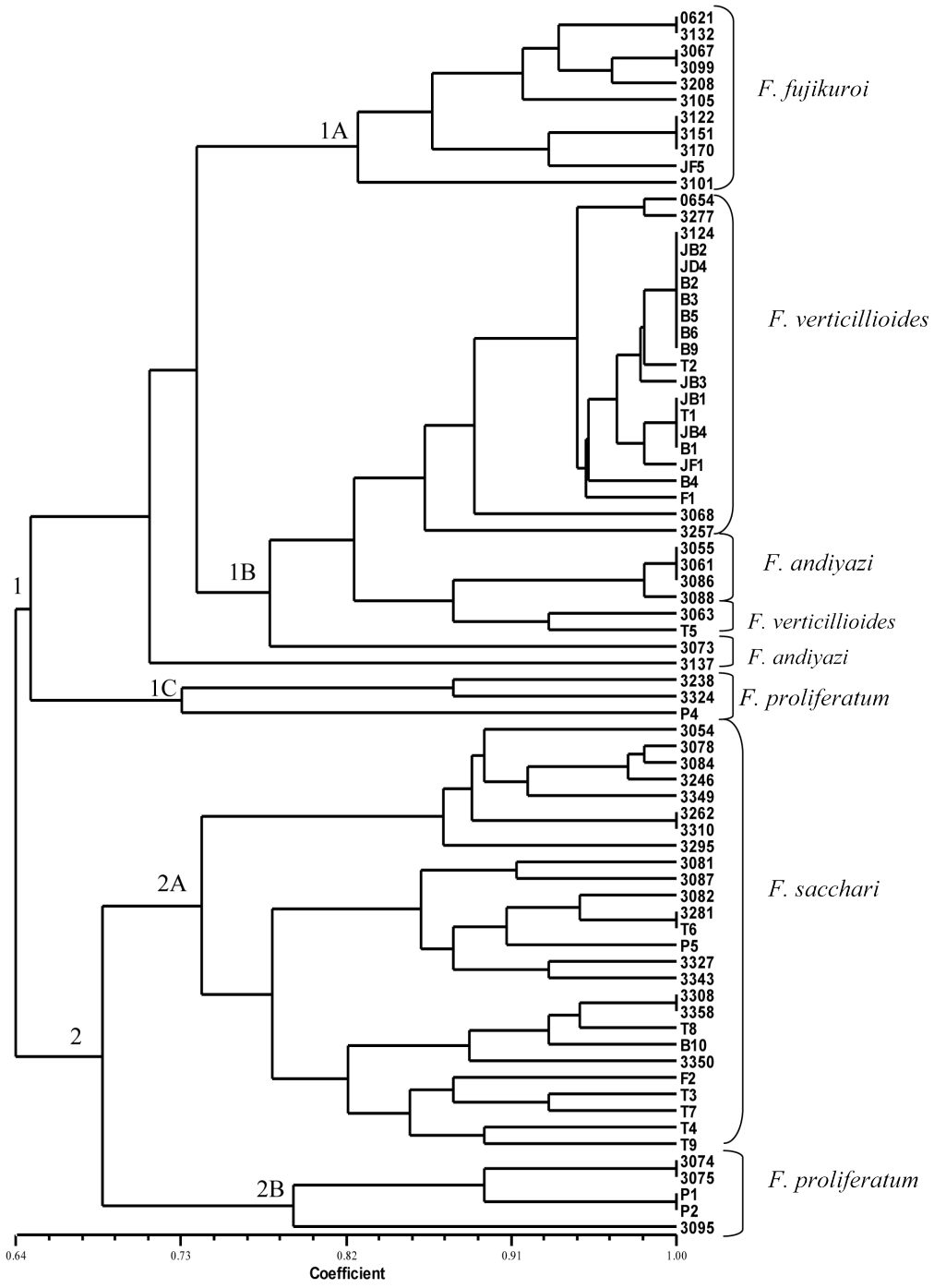

Figure 3. Dendrogram generated using UPGMA cluster analysis based on restriction fragments of the IGS region.

DISCUSSION

A single fragment of 2600 bp was amplified from all the 74 isolates of *Fusarium* spp, which was similar to the results obtained from several studies of *Fusarium* spp in which the size of the IGS region varied from 2200 to 2600 bp (Konstantinova and Yli-Mattila, 2004; Patino et al., 2006; Llorens et al., 2006a,b).

Among the six restriction enzymes used in this study to digest the PCR products, *Hin*fI showed the most variable restriction patterns with 11 patterns while *Eco*RI produced only three patterns. In general, the restriction patterns produced by the isolates of *Fusarium* spp showed highly variable restriction patterns. The high levels of variability of the IGS region could be due to insertions or deletions in the arrays of sub-repeat units within the IGS region and unequal cross-over, which indicated that this region might be evolving intensively (Coen et al., 1982; Hillis and Davis, 1988).

High levels of inter- and intraspecific variability were also observed among the five *Fusarium* species as indicated by different haplotypes produced by different restriction enzymes. The results obtained were similar to findings by Patino et al. (2006), in which intraspecific variability was detected within the *F. verticillioides* species complex based on PCR-RFLP of the IGS region using seven restriction enzymes and a study from Hinojo et al. (2004) on *G. fujikuroi* isolates from pine, maize and banana fruits. Edel et al. (1996) also found that the intraspecific variability of *Fusarium* spp was greater than interspecific variability using PCR-RFLP analysis of rDNA with eight restriction enzymes.

The high degree of intraspecific variability could also be caused by species complex in which the species in section Liseola are grouped in the *Gibberella fujikuroi* species complex. The species complex consisted of sexual stage or *Gibberella* teleomorph, which has been found in *F. fujikuroi*, *F. proliferatum*, *F. verticillioides*, and *F. sacchari*.

As the isolates of *Fusarium* spp showed variable restriction patterns, UPGMA cluster analysis was performed to cluster the isolates and estimate the intra- and interspecific variability. From UPGMA cluster analysis based on the restriction bands, most of the isolates of *Fusarium* spp from the same species were generally clustered in the same cluster. The restriction patterns and the haplotypes obtained did not show any correlation to the host.

From the present study, although a high level of variation was observed, the variations were found to be sufficient to characterize closely related species and isolates from different species. Isolates from the same species produced similar haplotypes and isolates from different species did not produce the same haplotype except for an isolate of *F. fujikuroi* (3122) and two isolates of *F. proliferatum* (3151, 3170). The most variable restriction patterns were shown by isolates of *F. sacchari* followed by isolates of *F. verticillioides*. However, for both species, the number of isolates was higher than the number of isolates for the other species.

Isolates of *F. fujikuroi* and *F. proliferatum* were grouped in separate clusters. Both species are very closely related and are regarded as sibling species (Leslie et al., 2007). Moreover, both teleomorphs (*G. fujikuroi and G. intermedia*) have been reported to be interfertile (Leslie et al., 2004). In the present study, isolates of *F. fujikuroi* can be differentiated from isolates of *F. proliferatum* based on *Hin*fI and *Msp*I patterns in which the restriction patterns of *Hin*fI and *Msp*I produced by isolates of *F. fujikuroi* were different from isolates of *F. proliferatum*. In Malaysia, *F. fujikuroi* is commonly associated with bakanae disease on rice and *F. proliferatum* has a wide host range, infecting various agricultural crops, such as rice, asparagus and

maize. The variability shown by isolates of *F. proliferatum* was similar to a study by Edel et al. (1996) using restriction analysis of the internal transcribed spacer region.

Although isolates of *F. verticillioides* and *F. andiyazi* were clustered in the same sub-cluster (sub-cluster 1B), the restriction patterns and the haplotypes produced by both species were different. Morphological characters of both species are very similar but *F. andiyazi* produced pseudochlamydospore instead of chlamydospore (Marasas et al., 2001). *F. andiyazi* has been isolated from sorghum (Marasas et al., 2001) and rice seed (Wulff et al., 2010), whereas *F. verticillioides* is widely distributed worldwide and causes various types of diseases on a wide host range, including maize and rice.

Isolates of *F. sacchari* were grouped together and showed variable restriction patterns, but the haplotypes produced were different from the other *Fusarium* species. *Fusarium sacchari* was the most common species associated with pokkah boeng disease of sugarcane (Gerlach and Nirenberg, 1982; Egan et al., 1997) and the species has been isolated from maize and rice in Malaysia.

The results of the present study showed that restriction analysis of the IGS region can be used to characterize *Fusarium* species section Liseola as the technique was found to allow discrimination of closely related species. The technique can also be used to assign new isolates to a species or closely related species as well as to clarify their taxonomic position.

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