CHARACTERIZATION OF *FUSARIUM* ISOLATES FROM RICE, SUGARCANE AND MAIZE USING RFLP-IGS

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Abstract: Isolates of *Fusarium* from rice, sugarcane and maize were identified as *F. verticillioides, F. sacchari*, *F. proliferatum F. subglutinans, F. fujikuroi* and *F. oxysporum*. The species were then characterized by restriction analysis of intergenic spacer (RFLP-IGS) using *Alu*I, *Eco*88I, *Rsa*I and *Xho*I. Twenty-five haplotypes were identified among the isolates of *Fusarium* which indicated high levels of variations. UPGMA cluster analysis was conducted to cluster the isolates and to estimate the intraspecific and interspecific variability. Isolates of *F. fujikuroi* from rice were clustered together with isolates of *F. proliferatum* from rice and maize with a similarity value of 88–100%. Isolates of *F. verticilliodes* from maize and sugarcane were clustered together with a similarity value ranging from 92–100%, and two isolates from rice formed another cluster. Isolates of *F. oxysporum* from maize and rice were clustered together with a similarity value ranging from 87–95%. Isolates of *F. subglutinans* from rice and maize, and *F. sacchari* from rice and sugarcane were also clustered together with a similarity value of 77–100%. Based on RFLP-IGS analysis, variability was observed within and between species of *Fusarium* from rice, maize and sugarcane and the technique could be use to complement morphological characterization and to determine genetic relationships between the species.

Key words: *Fusarium*, rice, sugar cane, maize, RFLP-IGS

INTRODUCTION

Rice (*Oryza sativa*), sugarcane (*Saccharum* sp.) and maize (*Zea mays*) are among the important agricultural crops in Malaysia. Rice is the staple food of Malaysians and mainly planted for domestic consumption. Sugarcane is cultivated on a small-scale. Cultivation of improved varieties have produced yields which have increased steadily. Sugarcane in Malaysia is often associated with the food processing industry which has increased over the years because the demand for sugar has increased. Maize is planted as a cash crop to generate extra income for farmers. It is planted on a small-scale.

Species of *Fusarium* are among the plant pathogenic fungi which commonly infected the three crops. The most common species of *Fusarium* associated with rice, sugarcane and maize were from the section *Liseola* although other species have also been reported on these crops (Leslie and Summerell 2006).

For identification of *Fusarium* species, morphological characteristics are often used, however it is sometimes difficult and requires considerable expertise. Therefore, molecular approaches such as: combination of PCR and restriction analysis (RFLP) are widely used in taxonomic studies of *Fusarium*. PCR-RFLP has often been used to analyze both the conserved and variable regions of rDNA. One of the variable regions is the intergenic spacer (IGS) which separate the repeated ribosomal units and appears to be the most rapidly evolving spacer region (Hillis and Dixon 1991). Restriction analysis of IGS (RFLP-IGS) has been used to provide useful information on the intraspecific variation (Patino *et al*. 2006) and also to discriminate species of *Fusarium* at different taxonomic levels (Edel *et al*. 1995). Therefore, the present study was conducted to characterize isolates of *Fusarium* isolated from rice, sugarcane and maize using a morphological approach and RFLP-IGS analysis.

MATERIALS AND METHODS

Isolation of *Fusarium* isolates

Rice and maize samples were collected from Gurun, Kedah and sugarcane from Chuping, Perlis, in the northern Malay Peninsula. Isolation of *Fusarium* isolates from rice was made from roots showing bakanae symptoms. From maize, isolation was made from different parts of the plant showing disease symptoms; such as leaves with lesions, stunted flowers and infected kernels. Isolations from sugarcane were made from leaves with pokkah boeng symptoms of crumpled, stunted, twisted leaves and chlorosis near the base.

The diseased plant parts were cut to approximately 1 cm^2 and the surface was sterilized using 10% sodium hypochlorite for 1 min. The plant parts were then transferred into sterile distilled water and soaked for 30 s and put onto Peptone PCNB Agar (PPA). The PPA plates were incubated at 25°C for 3 days. After mycelial growth was

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observed, the mycelia were transferred to potato dextrose agar (PDA). Flowers and kernels from maize were directly plated on PPA and incubated at 25°C until mycelial growth was observed from the samples. The mycelia were then transferred onto PDA and potato sucrose agar (PSA).

To obtain pure culture, single spore technique was conducted. Mycelia were scraped from 5-day-old colony and suspended in 10 ml sterilized distilled water. A sterilized inoculation loop was used to take a small portion of the conidial suspension and streaked on water agar (WA). The inoculated plates were incubated at 25°C for 18 hours to allow the conidia to germinate. The single spore cultures were used for morphological characterization.

Morphological characterization

Species of *Fusarium* were identified by using the descriptions in The *Fusarium* Laboratory Manual (Leslie and Summerell 2006). Carnation Leaf Agar (CLA) was used to observe the morphological characteristics of *Fusarium* species. The morphological characteristics used for species identification were the shapes, sizes and formation of macroconidia and microconidia, formation of conidiophores and, presence or absence of chlamydospores. Pigmentation was observed using PDA.

RFLP-IGS

All the isolates successfully recovered from rice, maize, sugarcane, and 10 isolates from the *Fusarium* Culture Collection, School of Biological Sciences, Universiti Sains Malaysia were used in RFLP-IGS analysis (Table 1). The 10 isolates from the culture collection were also isolated from the three hosts and were morphologically identified.

For DNA extraction, mycelia were harvested from PDA plates after 7 days of incubation at 25°C. The DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturers' instructions. IGS region was amplified using primer pair described by Appel and Gordon (1995) i.e. CNL12 (CTGAACGCCTCTAAGTCAG) and CNS1 (GAGACAAGCATATGACTACTG). PCR amplification reactions were conducted in a 50 µl reaction mixture containing 1X PCR buffer, 3.5 mM $MgCl_2$, 0.16 mM of dNTP mix, 1.75 unit GoTaq® DNA polymerase (Promega), 0.3 µM of each primers and 0.35 µl template DNA up to a total volume of 50 µl with deionized distilled water. Twenty-five µl of paraffin oil was added to overlay each reaction.

PCR amplification was performed in 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 final extension for 7 min at 72°C. Negative controls were used to test for the presence of nonspecific reaction. The PCR product was detected in 1.5% agarose (Promega) gel electrophoresis, run in TBE buffer at 100 min, 80 V and 400 mA. The gel was stained with ethidium bromide and visualized under UV transalluminator. The size of the amplified IGS band was estimated by comparison to 1 kb marker (Fermentas).

Four restriction enzymes, *Alu*I, *Rsa*I, *Eco*88I and *Xho*I (Fermentas) were used to digest the PCR products in a total volume of 15 µl reaction. The digestion procedure was according to the manufacturers' instructions. Digested PCR product was run in 2% agarose (Promega) 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 were 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

Numerical Taxonomy and Multivariate Analysis System (NT-SYS) software package Version 2.0 (Rohlf 2000) was used to analyze the data. All reproducible restriction fragments were scored on the basis of absence (0) and presence (1) of particular bands. The binary data was then constructed to generate a similarity matrix using simple matching coefficient. Cluster analysis was performed using the Unweighted Pair-group Method with arithmetic Mean (UPGMA). Each of the isolates was also assigned to a composite haplotype which is defined by a combination of the restriction patterns obtained using the four restriction enzymes: *Alu*I, *Rsa*I, *Eco*88I and *Xho*I.

RESULTS

A total of 30 isolates of *Fusarium* were successfully isolated from samples collected from the field. Three species from rice were identified as *F. proliferatum, F. oxysporum* and *F. sacchari.* From maize, *F. subglutinans, F. proliferatum, F. verticillioides* and *F. oxysporum* were recovered from different parts of the plant and two species, *F. sacchari* and *F. verticillioides* were isolated from infected leaves of sugarcane (Table 1).

A fragment of 2.6 kb was amplified from all the isolates of *Fusarium*. After digestion using *Alu*I, *Eco*88I, *Rsa*I and *Xho*I, estimated restriction fragment size and the restriction patterns (A–N) are summarized in table 2. Two to six fragments ranging from 0.2 to 1.7 kb were produced, however fragments less than 100 bp were not taken into consideration as the fragments were not clearly resolved. The total sizes of the restriction fragments did not always add up to the undigested 2.6 kb fragment which could be due to the difficulty in detecting very small or co-migrating fragments. Highly variable restriction patterns were observed among the isolates (Table 2). *Rsa*I showed the highest variability with 14 patterns, followed by *Eco*88I with 11 patterns. Both *Alu*I and *Xho*I produced six patterns.

*Alu*I patterns differentiate between isolates of *F. fujikuroi* and *F. proliferatum* from the other species of *Fusarium*, while *Rsa*I patterns differentiate *Fusarium* species section Liseola from *F. oxysporum*. IGS region of *F. oxysporum*, some isolates of *F. proliferatum,* and most of the *F. verticillioides* isolates did not have restriction sites for *Xho*I. All the four isolates of *F. subglutinans,* produced the same *Xho*I patterns. It was also noted that isolates of *F. verticillioides* from maize and sugarcane produced the same *Eco*881 and *Xho*I patterns whereas isolates from rice

Table 1. Morphologically identified species of *Fusarium* from rice, sugarcane and maize from the field and stock culture used in RFLP-IGS analysis

Isolate	Host	Species
Fusarium isolates from the field		
F3	maize (flower)	*F. oxysporum*
B7	maize (kernel)	*F. oxysporum*
B8	maize (kernel)	*F. oxysporum*
P3	rice (root)	*F. oxysporum*
JF5	maize (kernel)	*F. proliferatum*
P1	rice	*F. proliferatum*
P2	rice	*F. proliferatum*
P4	rice	*F. proliferatum*
T5	sugarcane	*F. sacchari*
T6	sugarcane	*F. sacchari*
T7	sugarcane	*F. sacchari*
T8	sugarcane	*F. sacchari*
P5	rice	*F. sacchari*
B10	maize (kernel)	*F. subglutinans*
F2	maize (kernel)	*F. subglutinans*
B1	maize (kernel)	*F. verticillioides*
B11	maize (kernel)	*F. verticillioides*
B3	maize (kernel)	F. verticillioides
B4	maize (kernel)	*F. verticillioides*
B5	maize (kernel)	*F. verticillioides*
B6	maize (kernel)	*F. verticillioides*
F1	maize (kernel)	*F. verticillioides*
JB1	maize (kernel)	*F. verticillioides*
JB2	maize (kernel)	*F. verticillioides*
JB3	maize (kernel)	*F. verticillioides*
JB4	maize (kernel)	*F. verticillioides*
JD4	maize (kernel)	*F. verticillioides*
B2	maize (kernel)	*F. verticillioides*
T1	sugarcane	*F. verticillioides*
T2	sugarcane	*F. verticillioides*
Fusarium isolates from the stock culture		
0621	rice	*F. fujikuroi*
3132	rice	*F. fujikuroi*
3074	rice	*F. proliferatum*
3095	rice	*F. proliferatum*
3262	sugarcane	*F. sacchari*
3084	rice	*F. sacchari*
3308	sugarcane	*F. subglutinans*
3077	rice	*F. subglutinans*
3061	rice	*F. verticillioides*
3137	rice	*F. verticillioides*

Table 2. Restriction patterns (A–N) and estimated restriction band sizes after digestion with four restriction enzymes

Restriction pattern	Restriction bands [bp]			
	*Alu*I	*Eco 88*I	*Rsa*I	*Xho*I
A	800, 500, 400, 380, 220, 200	1 500, 1 100	900, 600, 400, 380	2 600
B	800, 500, 400, 380, 200	1 100, 400	900, 700, 400, 380	1 300
C	800, 500, 400, 380, 300, 200	1 100, 250	900, 750, 500, 400, 380	1 700, 950
D	800, 500, 400, 220, 200	1 500, 900	750, 500, 400, 380	1 300, 950, 400
E	700, 500, 400, 380, 200	1 500, 900, 250	900, 750, 400, 380	1 300, 950
F	1 000, 300, 280, 220	1 300, 900	1 350, 600, 400	800, 400
G	–	1 300, 1 100, 200	700, 500, 400, 380	–
H	–	1 100, 900, 400, 250, 200	800, 700, 400, 350	–
I	–	1 200, 900, 250, 200	600, 500, 400, 380	–
J	–	1 300, 900, 250, 200	600, 500, 400	–
K	–	1 500, 900, 200	700, 600, 550, 400, 380	–
L	–	–	700, 600, 550, 400	–
M	–	–	700, 600, 550, 400, 250	–
N	–	–	900, 600, 550, 400	–

showed different *Eco*881 and *Xho*I patterns, from isolates of maize and sugarcane. Based on the restriction patterns, 25 haplotypes were identified among the 40 isolates (Table 3). Intraspecific and interspecific variations were observed within and between the six species of *Fusarium*. The two isolates of *F. fujikuroi* showed the same haplotypes which were different from isolates of *F. proliferatum* in which the isolates showed five haplotypes. Four haplotypes were found among the isolates of *F. oxysporum* and *F. subglutinans*. For isolates of *F. verticillioides*, five haplotypes were obtained in which isolates from rice showed slightly different haplotypes from isolates of maize and sugarcane.

As the *Fusarium* species showed variable restriction patterns, UPGMA cluster analysis was performed to cluster the isolates and to estimate the intraspecific and interspecific variability. In the dendrogram (Fig. 1), isolates of *F. fujikuroi* from rice were clustered together with isolates of *F. proliferatum* from rice and maize with similarity values of 88–100%. Isolates of *F. verticilliodes* from maize and sugarcane were clustered in sub-sub-cluster A1b with similarity values ranging from 92–100%. Sub-cluster A2 comprised isolates of *F. oxysporum* from maize and rice (P3). Isolates of *F. subglutinans* from rice and maize and isolates of *F. sacchari* from rice and sugarcane, were clustered together in sub-cluster B with similarity values of 77–100%. Main Cluster II consisted of only two isolates of *F. verticillioides* from rice.

Table 3. Morphologically identified and RFLP-IGS haplotypes of *Fusarium* isolates from rice, sugarcane and maize

Isolate	Host	Species	*Alu*I	*Eco88*I	*Rsa*I	*Xho*I	IGS haplotype
0621	rice	*F. fujikuroi*	A	A	A	A	1
3132	rice	*F. fujikuroi*	A	A	A	A	1
F3	maize (flower)	*F. oxysporum*	A	E	L	A	2
B7	maize (kernel)	*F. oxysporum*	C	K	K	A	3
B8	maize (kernel)	*F. oxysporum*	D	K	M	A	4
P3	rice	*F. oxysporum*	B	D	L	A	5
JF5	maize (kernel)	*F. proliferatum*	B	A	A	F	6
3074	rice	*F. proliferatum*	B	A	A	A	7
3095	rice	*F. proliferatum*	B	A	F	A	8
P1	rice	*F. proliferatum*	B	A	B	A	9
P2	rice	*F. proliferatum*	B	A	B	A	9
P4	rice	*F. proliferatum*	B	A	N	A	10
T6	sugarcane	*F. sacchari*	B	K	I	C	11
T7	sugarcane	*F. sacchari*	B	J	I	D	12
T8	sugarcane	*F. sacchari*	B	J	I	D	12
3262	sugarcane	*F. sacchari*	E	J	G	D	13
T5	sugarcane	*F. sacchari*	E	C	B	D	14
3084	rice	*F. sacchari*	E	I	D	D	15
P5	rice	*F. sacchari*	E	F	J	E	16
B10	maize (kernel)	*F. subglutinans*	B	F	I	D	17
F2	maize (kernel)	*F. subglutinans*	E	J	G	D	18
3308	sugarcane	*F. subglutinans*	B	J	I	D	19
3077	rice	*F. subglutinans*	E	H	C	D	20
3061	rice	*F. verticillioides*	B	G	H	B	21
3137	rice	*F. verticillioides*	F	G	H	B	22
B1	maize (kernel)	*F. verticillioides*	E	B	E	A	23
B11	maize (kernel)	*F. verticillioides*	E	B	E	A	23
B3	maize (kernel)	*F. verticillioides*	E	B	E	A	23
B4	maize (kernel)	*F. verticillioides*	E	B	E	A	23
B5	maize (kernel)	*F. verticillioides*	E	B	E	A	23
B6	maize (kernel)	*F. verticillioides*	E	B	E	A	23
T1	sugarcane	*F. verticillioides*	E	B	E	A	23
F1	maize (kernel)	*F. verticillioides*	E	B	B	A	24
JB1	maize (kernel)	*F. verticillioides*	E	B	B	A	24
JB2	maize (kernel)	*F. verticillioides*	E	B	B	A	24
JB3	maize (kernel)	*F. verticillioides*	E	B	B	A	24
JB4	maize (kernel)	*F. verticillioides*	E	B	B	A	24
JD4	maize (kernel)	*F. verticillioides*	E	B	B	A	24
T2	sugarcane	*F. verticillioides*	E	B	B	A	24
B2	maize (kernel)	*F. verticillioides*	F	B	E	A	25

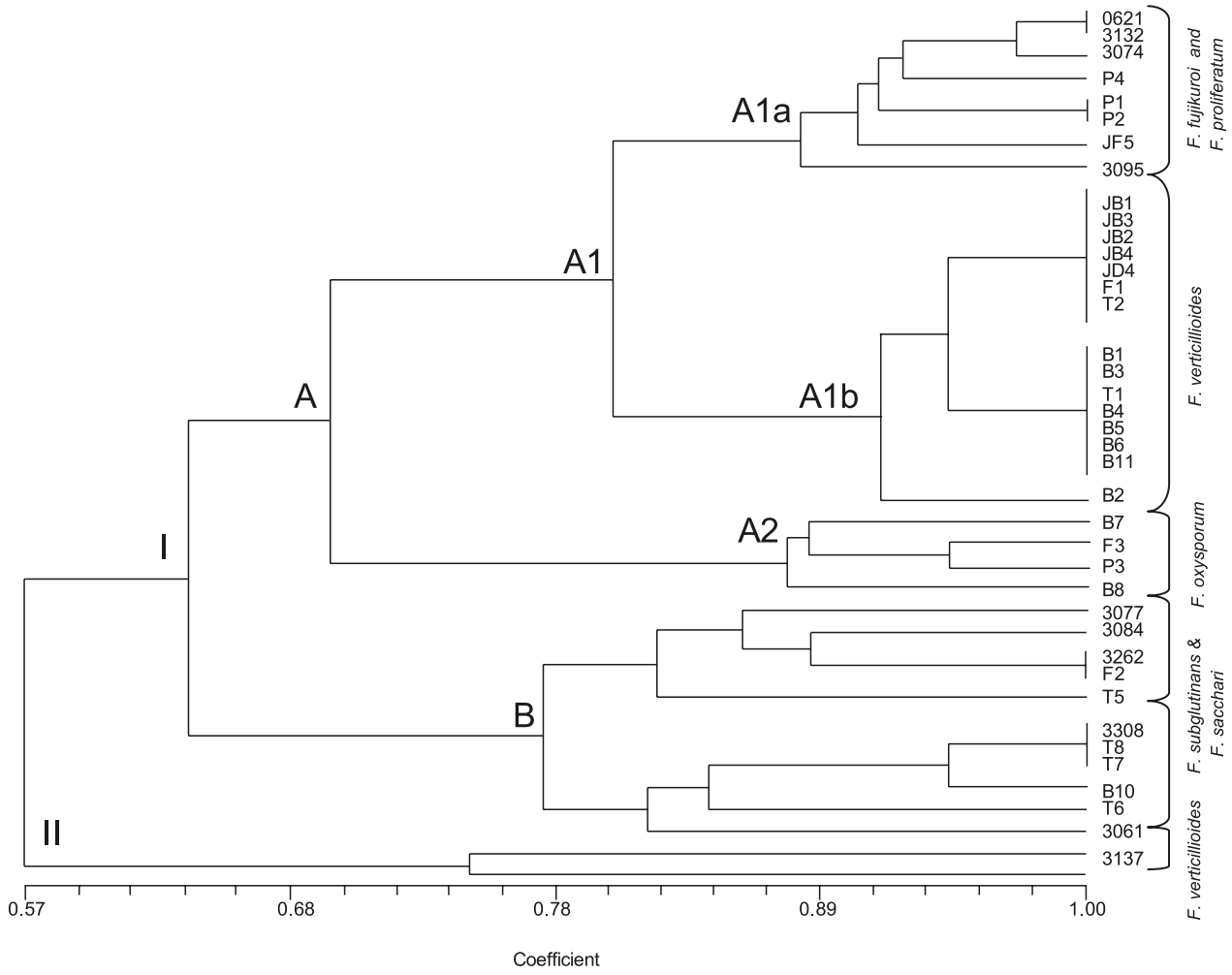

Fig. 1. Dendogram generated by UPGMA cluster analysis of restriction bands of IGS region using *Alu*I, *Eco*88I, *Rsa*I and *Xho*I showing relationships among 40 isolates of *Fusarium* from rice, sugarcane and maize

DISCUSSION

Although, some species of *Fusarium* were difficult to identify morphologically, identification based on morphological characteristics is necessary, as it allows the sorting of *Fusarium* isolates before applying other methods of identification and characterization (Leslie and Summerell 2006).

Morphological characteristics of *F. proliferatum* were similar with *F. verticillioides*. The primary characters used to differentiate the two species was their form of microconidia chains. *F. proliferatum* formed microconidia in a chain from polyphialides and the chain was shorter than *F. verticillioides* (Burgess *et al.* 1988; Leslie and Summerell 2006). *F. verticillioides* produced a long chain of microconidia by the monophialides.

Morphological characteristics of *F. subglutinans* and *F. sacchari* were also very similar, and to differentiate both species was difficult. However, based on *Xho*I patterns, isolates of *F. subglutinans* from different hosts produced the same patterns which were different from *Xho*I patterns of *F. sacchari*.

F. verticillioides, *F. sacchari*, *F. proliferatum*, *F. fujikuroi* and *F. subglutinans* are members of the *Gibberella fujikuroi* species complex, which are morphologically similar and phylogenetically closely related (Nirenberg and O'Donnell 1998; O'Donnell *et al.* 1998). Therefore, after identification using morphological characteristics, the isolates were characterized using restriction analysis of IGS region. The region is useful for early characterization of *Fusarium* species as IGS region was reported to be the most evolving spacer region (Hills and Dixon 1991), due to its larger size and lack of selective constrain. Thus, IGS region is suitable for studying relationships at intraspecies levels and among closely related species (Appel and Gordon 1995).

Variable restriction patterns were obtained from RFLP of IGS region. Variations in IGS region could be due to minor changes in the nucleotide composition which may generate different restriction patterns even within the same species (Konstantinova and Yli-Mattila 2004). Variations could also be caused by length and sequence variations especially among closely related species (Martin 1990; Hillis and Dixon 1991). RFLP-IGS of several species of *Fusarium* have also revealed variable restriction patterns such as variability among 22 strains of *F. oxysporum* (Kim *et al.* 2001), 33 isolates of *F. verticillioides* (Patinõ *et al.* 2006) and among 27 isolates of *F. equiseti* (Kosiak *et al.* 2005).

From the dendrogram generated using UPGMA cluster analysis, isolates of *F. oxysporum* (section Elegans) from rice and maize were grouped in main cluster I which also grouped isolates of *F. fujikuroi, F. proliferatum* and *F. verticillioides*. The results suggest that *F. oxysporum* was related to the species in the section Liseola which is not unexpected as species in the section Liseola are known to be related to species in section Elegans. Guadet *et al*. (1989) reported that based on sequence analysis part of 28S rRNA, several species of *Fusarium* in section Elegans were closely related to species of *Fusarium* in the section Liseola.

F. proliferatum and *F. fujikuroi* from rice were clustered together in sub-cluster A1a which is not surprising as both species are regarded as sibling species (Leslie and Summerell 2006) and often associated with bakanae disease of rice.

F. subglutinans and *F. sacchari* were also clustered together. *F. sacchari* was recognized as a species by Gerlach and Nirenberg (1982) but Nelson *et al.* (1983) regarded the species to be synonomous with *F. subglutinans*. *F. subglutinans* was commonly found on several host plants such as maize, sugarcane and mango (Booth 1971) and has been associated with disease on sugarcane (Muramoto *et al.* 1993) and maize (Munkvold 2003; Gortz *et al*. 2008).

Isolates of *F. verticillioides* were clustered in two clusters in which sub-cluster A1b comprised isolates from maize and sugarcane, and isolates from rice were clustered in the main cluster II. The separate grouping of *F. verticillioides* isolates could be due to different levels of fumonisin production. In a study by Steenkamp *et al*. (2000), *F. verticillioides* isolates formed two distinct clusters, one cluster corresponding to fumonision producing isolates from cereals and the other cluster corresponding to non-fumonisin producing isolates from banana. In conclusion, six species of *Fusarium* were isolated from rice root, different parts of maize plant and sugarcane leaves. Although highly variable restriction patterns were produced, the technique is useful for early characterization of *Fusarium* species, as it could be used to complement morphological characterization.

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POLISH SUMMARY

CHARAKTERYSTYKA IZOLATÓW *FUSARIUM* Z RYŻU, TRZCINY CUKROWEJ I KUKURYDZY PRZY WYKORZYSTANIU RFLP-IGS

Izolaty *Fusarium* z ryżu, trzciny cukrowej i kukurydzy, określono jako *F. verticillioides*, *F. sacchari*, *F. proliferatum*, *F. subglutinans*, *F. fujikuroi* oraz *F. oxysporum*. Gatunki te scharakteryzowano przy pomocy analizy restrykcyjnej międzygenowego niekodującego odcinka genu (RLFP-IGS), wykorzystując *Alu*I, *Eco*881, *Rsa*I i *Hoi*. Określono dwadzieścia pięć haplotypów wśród izolatów Fusarium, co wskazywało na wysoki poziom zmienności. Przeprowadzono analizę skupień w celu pogrupowania izolatów i określenia intraspecyficznej interspocyficznej zmienności. Izolaty *F. fujikuroi* z ryżu zgrupowano razem z izolatami *F. proliferatum* z ryżu i kukurydzy wykazującymi podobieństwo 88–100%. Izolaty *F. verticillioides* z kukurydzy i trzciny cukrowej zgrupowano w ramach wartości podobieństwa od 92 do 100%, a dwa izolaty z ryżu tworzyły inne zgrupowanie. Izolaty *F. oxysporum* z kukurydzy i ryżu zgrupowano również razem, z wartością podobieństwa od 87 do 95%. Izolaty *F. subglutinans* z ryżu i kukurydzy i *F. sacchari* z ryżu i trzciny cukrowej zostały też zgrupowane razem i miały wartość podobieństwa 77–100%. W oparciu o analizę RLFP–IGS obserwowano zmienność w ramach i pomiędzy gatunkami *Fusarium* z ryżu, kukurydzy i trzciny cukrowej. Technika RLFP–IGS mogłaby być wykorzystywana w celu uzupełnienia charakterystyki mikroskopowej i do określenia genetycznych związków pomiędzy gatunkami.