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MOLECULAR CHARACTERIZATION AND MATING STUDY OF *Fusarium proliferatum* FROM VARIOUS HOSTS IN MALAYSIA

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ABSTRACT

Fusarium proliferatum is a well-known plant pathogenic fungus-infected many crops. The present study was carried out to molecularly identified and characterized morphologically identified *F. proliferatum* from various hosts and substrates. The species identity of the isolates was verified as *F. proliferatum* based on TEF-1α sequences and phylogenetic analysis indicated high intraspecific variations. RFLP-IGS analysis also indicated high intraspecific variations of which the isolates were clustered into three RFLP Groups (I, II, and III) comprising 67 IGS haplotypes. Seventy isolates were crossed-fertile and proven to be members of mating population D (MP-D) of *Gibberella fujikuroi* while four isolates were infertile. A high level of intraspecific variations is vital for *F. proliferatum* adaptation and survival in the host and environment. Correct species identification of *F. proliferatum* is important as the fungus is a well-known plant pathogen and mycotoxin producer. Correct species identity is also essential to strategize suitable disease control methods as well as to predict their host range and mycotoxin production.

Key words: *Fusarium proliferatum*, mating study, RFLP-IGS, TEF-1α

INTRODUCTION

Fusarium proliferatum is members of a species complex known as *Fusarium fujikuroi* species complex (FFSC) which is widespread and can be isolated from various host plants as well as from agricultural and non-agricultural soils. *Fusarium proliferatum* is one of the well-known pathogens causing rot, blight, dieback and wilt on various types of host plants including asparagus, corn, date palm, ornamental palm, rice, and wheat (Proctor *et al*., 2010). The species is also mycotoxigenic species contaminating agriculture products particularly cereal grains. In addition, *F. proliferatum* can also infected human causing onchomycosis (Hattori *et al*., 2005; Hong *et al*., 2019), ungual hyalohyphomycosis (Noguche *et al*., 2017), keratitis (Sun *et al*., 2018) and urinary tract infection (Su *et al*., 2016).

Identification based only on morphological characteristics is not sufficient to differentiate many *Fusarium* spp. especially isolates within a species complex as the microscopic characteristics are similar. Morphological identification of *Fusarium* isolates is mainly used to sort the groups before other methods of identification and characterization are applied (Summerell *et al*., 2003; Leslie & Summerell, 2006). Hence, molecular identification and phylogenetic analysis using Translation Elongation Factor1α (TEF-1α) gene were chosen to confidently identify *F. proliferatum* isolates from various hosts and substrates.

The TEF-1α gene is the recommended marker for the identification of *Fusarium* spp. This protein-coding gene occurs as a single-copy, and non-orthologous copies were not found which provide highly sufficient phylogenetic information particularly among closely related *Fusarium* species (Geiser *et al*., 2004). Furthermore, many universal primers have been designed from this gene. TEF-1α sequences can also be used to determine the genetic variations as well as provide information on phylogenetic relationships among isolates of *F. proliferatum* from various hosts and substrates.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) combines PCR and RFLP techniques. For observation of genetic variability among *Fusarium* isolates, Intergenic Spacer Region (IGS) is commonly applied in PCR-

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RFLP analysis. The IGS region is used to differentiate isolates within the same species or distinguish isolates at intraspecies levels, as it evolved rapidly. Moreover, in a large portion of the IGS region, it lacks selective constraints (Appel & Gordon, 1996; Edel *et al*., 1997).

The mating study was further applied to verify the species identity of *F. proliferatum*. Isolates of *F. proliferatum* were crossed with tester strains of mating population D (MP-D) which is the mating population of *F. proliferatum.* Successful crosses produce perithecia with eight ascospores and the crosses are considered as fertile progeny (Nirenberg & O'Donnell, 1998). For crosses to occur, it requires two mating-type alleles (*MAT-1* & *MAT-2* alleles) of which one isolate must carry *MAT*-1 allele and the other carry *MAT*-2 allele (Leslie & Summerell, 2006). Characterization of *F. proliferatum* by the mating study would place the isolates in biological species.

Morphologically identified *Fusarium* species in a species complex are usually misidentified as the morphological characteristics observed are very similar. Thus, the present study was carried out to verify the species identity of morphologically identified *F. proliferatum* in stock culture collection using TEF-1α gene sequences, RFLP-IGS analysis, and mating study. A correct identification of *F. proliferatum* associated with economically important crops as well as from environmental samples is essential as this will assist in the formulation of suitable management of plant diseases, prediction on the host range and mycotoxins production.

MATERIALS AND METHODS

Fungal isolates

Isolates of *F. proliferatum* were attained from the stock culture of the Plant Pathology Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang. The isolates were labelled using a coding system based on the host/substrate and location of the isolates collected (Table 1). Initially, all the isolates were tentatively identified as *F. proliferatum* using microscopic and macroscopic characteristics, based on species description by Nelson *et al*. (1983).

DNA extraction and PCR amplification

For DNA extraction, the fungal isolates were grown in Potato Dextrose Broth (PDB) and then incubated for 6 days, after which, the mycelia were harvested and lyophilized. The lyophilized mycelia were ground in a sterile mortar and pestle using liquid nitrogen with to a fine powder. Approximately 0.22 – 0.25 g of the mycelia fine powder were used for DNA extraction. Genomic DNA was extracted using DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

PCR reaction was carried out in a total volume of 25 µL containing 4 µL 5× Green GoTaq® Flexi Buffer, 4 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTP mix (Promega, Madison, WI, USA), 0.75 U/µL GoTaq® DNA polymerase (Promega®, USA), and 4 µL of 5 µM each primer EF1 (5'-ATGGGTAA GGAGGACAAGAC-3') and EF2 (5'-GGAAGTA CCAGTGATCATGTT-3') (O'Donnell *et al*., 1998b) and 6 ng genomic DNA.

PCR amplification was carried out in a thermal cycler (Bio-Rad MyCycler, Hercules, CA, USA) with the following conditions: initial denaturation at 94°C for 85 sec, 35 cycles of denaturation at 95°C at 35 sec, annealing at 59°C for 55 sec and extension at 72°C for 2 min and final extension at 72°C for 10 min.

Agarose gel electrophoresis (1.5%) was carried out at 80 V, 400 mA for 90 min. The estimated bands were visualized under UV light using Bio-Rad Molecular Imager® Gel Doc™ XR System, and the bands were estimated based on 1 kb DNA marker (GeneRuler™ Plus DNA ladder, ready-to-use, Thermo Fisher Scientific, USA) using Quantity One® 1-D Analysis Software version 4.6.5. The PCR products were then sent for sequencing to a service provider.

Sequence analysis of TEF-1α gene

The DNA sequences were pairwise aligned using Molecular Evolution Genetic Analysis (MEGA) version 5.0 (Tamura *et al*., 2011) and where necessary, the sequences were edited manually. The aligned sequences were then BLAST against sequences in the Fusarium-ID (http://isolate.fusariumdb.org/index.php) and GenBank databases to obtain the maximum identity of the isolates.

Multiple sequence alignments were generated to produce a phylogenetic tree and Maximum-Likelihood (ML) method was used to construct the tree using MEGA5. Kimura 2-parameter model was found to be suitable to generate the ML tree with 1,000 replicates. Missing data and gaps were treated as complete deletion. The heuristic method used in ML was Nearest-Neighbour-Interchange (NNI) and the initial tree for the ML was generated automatically. *Fusarium proliferatum* NRRL22944, *F. fujikuroi* NRRL13566 and *F. globosom* NRRL26131 were included in phylogenetic analysis. *Fusarium oxysporum* and *F. inflexum* served as an outgroup to root the tree.

PCR of IGS region

PCR amplification of IGS region was carried out in a total volume of 50 µL reaction containing 5 µL of 25 mM $MgCl_2$, 0.8 µL of 10 mM dNTP mix (Promega), 10 µL of 5× Colourless GoTaq® Flexi Buffer (Promega), 8 µL of 5 µM each primer CNL12

Table 1. *Fusarium proliferatum* isolates used in this study, and their host/substrate

No	Isolate Code	Disease	Host / Substrate	Location
1	P48N	Crown rot	Banana (Berangan)	Gelugor, Penang
2	P57G	Leaf rot	Onion	Sungai Ara, Penang
3	P64N	Crown rot	Banana (Berangan)	Penang
4	P70N	Crown rot	Banana (Berangan)	Penang
5	P195N	Crown rot	Banana (Berangan)	Penang
6	K680R	Bakanae	Rice, stem	Haji Kudung, Kedah
7	K687R	Bakanae	Rice, stem	Kampung Paya, Kedah
8	P761M	Malformation	Mango (Magolba), inflorescence	Air Hitam, Penang
9	P770M	Malformation	Mango (Epal), inflorescence	Teluk Kumbar, Penang
10	P821M	Malformation	Mango (Epal), inflorescence	Teluk Kumbar, Penang
11	S901A	Crown rot	Asparagus (var. MW 500), stem	Kundasang, Sabah
12	S902A	Crown rot	Asparagus (var. MW 500), stem	Kundasang, Sabah
13	P970F		Wheat, grain	Teluk Kumbar, Penang
14	P971F		Wheat, grain	Teluk Kumbar, Penang
15	P972F		Wheat, grain	Teluk Kumbar, Penang
16	P976O		Corn, seed	Teluk Kumbar, Penang
17	P977O		Corn, seed	Teluk Kumbar, Penang
18	Q1006Q	Root rot	Sorghum	Sri Aman Sarawak
19	Q1007Q	Root rot	Sorghum	Sri Aman Sarawak
20	Q1009Q	Root rot	Sorghum	Sri Aman Sarawak
21	Q1012Q	Root rot	Sorghum	Sri Aman Sarawak
22	Q1019Q	Root rot	Sorghum	Sri Aman Sarawak
23	Q1032Q	Root rot	Sorghum	Sri Aman Sarawak
24	C1051=	Infected	flower	Cameron Highland, Pahang
25	C1053=	Infected	flower	Cameron Highland, Pahang
26	C1054=	Infected	flower	Cameron Highland, Pahang
27	P1233A	Crown rot	Asparagus (var. Trio), stem	Sungai Ara, Penang
28	B1380=	Infected	Orchid (*Dendrobium*)	ASEAN PLANTI, Serdang, Selangor
29	P1693A	Crown rot	Asparagus (var. BI), stem	Sungai Ara, Penang
30	P1720A	Crown rot	Asparagus (var. California), stem	Sungai Ara, Penang
31	B1848A	Crown rot	Asparagus var. Jainan, stem	MARDI, Kelang, Selangor
32	A2059W	Root rot	Watermelon, root	Kampung Sungai Terap Dalam, Taiping, Perak
33	K3240U	Pokkah boeng	Sugarcane, young leaf	Padang Terap, Kedah
34	K3244U	Pokkah boeng	Sugarcane, young leaf	Padang Terap, Kedah
35	K3245U	Pokkah boeng	Sugarcane, young leaf	Padang Terap, Kedah
36	P4079π	Fruit rot	Dragon fruit, fruit	Tasik Gelugor, Penang
37	B4357DS	Fruit rot	Yam, fruit	Tanjung Karang, Selangor
38	A4714π	Stem rot	Dragon fruit, stem	Setiawan, Perak
39	S4882O		Corn grain	Ranau, Sabah
40	S4895O		Corn grain	Ranau, Sabah
41	K5011XL		Torch ginger, rhizome	Sungai Jed, Empangan Pedu, Kuala Nerang, Kedah
42	KL5058XL	Leaf spot	Torch ginger, leaf	Sungai Ayon, Empangan Pedu, Kuala Nerang, Kedah
43	C5187AR		Outdoor air	R&R, Gambang, Pahang
44	C5195AR		Outdoor air	Genting Highland, Pahang
45	A5199AR		Outdoor air	Gua Tempurung, Perak
46	Q5272O		Corn grain	Sri Aman, Sarawak
47	S5273O		Corn grain	Ranau, Sabah
48	Q5310O		Corn grain	Serian, Sarawak
49	Q5361O		Corn grain	Serian, Sarawak
50	C5417=	Infected flower	*Heliconia latispatha*	Gambang, Pahang
51	C5421=	Infected flower	*Heliconia latispatha*	Gambang, Pahang
52	C5426=	Infected flower	*Heliconia latispatha*	Gambang, Pahang
53	C5427=	Infected flower	*Heliconia latispatha*	Gambang, Pahang
54	C5431=	Infected flower	*Heliconia latispatha*	Gambang, Pahang
55	C5432=	Infected flower	*Heliconia latispatha*	Gambang, Pahang
56	C5433=	Infected flower	*Heliconia latispatha*	Gambang, Pahang
57	C5436=	Infected flower	*Heliconia latispatha*	Gambang, Pahang
58	C5438=	Infected flower	*Tagetes erecta*	Gambang, Pahang
59	C5439=	Infected flower	*Tagetes erecta*	Gambang, Pahang
60	C5446=	Infected flower	*Tagetes erecta*	Gambang, Pahang
61	C5496=	Infected flower	*Tagetes erecta*	Gambang, Pahang
62	P5791W		Root rot of watermelon	Kampung Sungai Terap Dalam, Taiping, Perak
63	Q5942O		Corn grain	Serian, Sarawak
64	Q5978O		Corn grain	Serian, Sarawak
65	T6106π	Stem rot	Dragon fruit	Kampung Jambu Bongkok, Marang, Terengganu
66	T6316π	Stem rot	Dragon fruit	Kampung Jambu Bongkok, Marang, Terengganu
67	C6662&		Grass	Cameron Highland, Pahang
68	C6663&		Grass	Cameron Highland, Pahang
69	P7184X	Tuber rot	Potato	Penang
70	P7420X	Tuber rot	Potato	Penang
71	S7579S		Debri	Poring, Sabah
72	P8112X	Tuber rot	Potato	Penang
73	P8114X	Tuber rot	Potato	Penang
74	P8115X	Tuber rot	Potato	Penang

(5'-CTGACCGCCTCTAAGTCAG-3') and CNS1 (5'-GAGACAAGCATATGACTACTG-3') (Appel & Gordon, 1995), 1.25 U/ of 5 Unit GoTaq®DNA polymerase (Promega), and 8 ng genomic DNA.

PCR amplification was carried out as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 35 sec, annealing at 59°C for 55 sec and extension at 72°C for 2 min and final extension at 72°C for 7 min. The amplified IGS region was subjected to 1.5% agarose gel, and run at 80 V, 400 mA for 90 min. The bands were visualized under UV light using Bio-Rad Molecular Imager® Gel Doc™ XR System and estimated based on 1 kb DNA marker using Quantity One® 1-D Analysis Software version 4.6.5.

IGS-RFLP analysis

The PCR products (amplified IGS region) were digested separately with five restriction enzymes: *Alu*1, *BsuR*1, *Bsu*151, *EcoR*1 and *Msp*1 (Thermo Fisher Scientific, USA). The reaction mixture consisted of 10 µL of the PCR products, digested with 1 µL of 10 U restriction enzyme, 1 µL of 10× restriction's buffer, and 1 µL distilled water. Each reaction mixture was incubated according to the manufacturer's instruction. Digestions of *Msp*1, *Alu*1, *BsuR*1, and *Bsu*151 were incubated at 37°C while digestion with *EcoR*1 was incubated at 65°C. Agarose gel 2.5% was used to separate the digested PCR products and run at 80 V, and 400 mA for 180 min. The estimated size of the restriction bands was based on a 100 bp DNA marker (Thermo Fisher Scientific, USA).

Cluster analysis and UPGMA dendrogram

The restriction bands were scored on a basis of presence (1) or absence (0). A data matrix was generated from the scoring and converted to a similarity matrix. The similarity matrix was then subjected to a cluster analysis using the unweighted pair group method with arithmetical averages (UPGMA). The UPGMA cluster analysis was based on simple matching coefficient (SMC) (Romesburg, 1984):

$$\text{Simple matching coefficient (SMC)} = \frac{a + d}{a + b + c + d}$$

a= number of bands present in two isolates
b= total number of bands unique in isolates 1
c= total number of bands unique in isolates 2
d= number of bands absent in two isolates

The data was analyzed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc, version 2.1) (Rohlf, 2000). *Fusarium solani* was chosen as an out-group.

Mating study

PCR amplification of mating type alleles

The isolates of *F. proliferatum* were crossed with known tester strains to evaluate its sexual compatibility. The mating-type alleles (*MAT-1* & *MAT-2*) carried by each isolate was determined by PCR amplification, to reduce the number of crosses performed. The isolates were only crossed with the tester strain of the opposite mating type (Leslie *et al*., 2004).

The isolates were amplified to determine the *MAT-1* and *MAT-2* alleles carried. Amplification reactions were carried out in a total volume 20 µL reactions containing 2 µL 25 mM $MgCl_2$, 2 µL 10 mM dNTP mix (Promega), 2 µL 10× buffer, 0.5 U/µL GoTaq® DNA polymerase (Promega), 2 µL of each primer: GFmat1a (5'-GTTCATCAAAGGGCAAGCG-3') and GFmat1b (5'-TAAGCGCCCTCTTAACGC TTC-3') for amplification of *MAT1* allele or GFmat2c (5'-AGCGTCATTATTCGATCAAG-3') and GFmat2d (5'-CTACGTTGAGAG-CTGTACAG-3') for *MAT-2* allele (Steenkamp *et al*., 2000) and 60 ng genomic DNA.

PCR amplification for both *MAT*-alleles was carried out as follows: initial denaturation at 94ºC for 1 min, denaturation at 94°C for 30 sec, annealing at 65°C for *MAT-1* allele and 53°C for *MAT-2* for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 5 min.

The PCR products were subjected to 1.5% agarose gel electrophoresis, carried out at 80 V, 400 mA for 90 min. The bands were estimated based on 1 kb DNA marker (Thermo Fisher Scientific, USA) using Quantity One® 1-D Analysis Software version 4.6.5.

Sexual crosses

Sexual crosses or mating study was carried out as described by Klittich and Leslie (1988) and Klaasen and Nelson (1996). Isolates of *F. proliferatum* were crossed with tester strains of mating population C (MP-C) with *Gibberella fujikuroi* as teleomorph and mating population D (MP-D), teleomorph *Gibberella intermedia*. Crosses with the tester strains of MP-C were also conducted as *F. proliferatum* were sibling species with *F. fujikuroi*.

Isolates that carried the *MAT-1* allele were crossed with the tester strains of *MAT2-C* and *MAT2-D* while isolates that carried the *MAT-2* allele were crossed with the tester strains of *MAT1-C* and *MAT1-D*. The tester strains were obtained from the Department of Plant Pathology, Throckmorton Plant Sciences Centre, Kansas State University, Manhattan, USA. To determine cross-fertility for *F. proliferatum*, tester strains used were *MATD-1* (KSU04854) and *MATD-2* (KSU04853) while tester

strains for *F. fujikuroi* were *MATC-1* (KSU01993) and *MATC-2* (KSU01995).

Production of blue or black perithecia with asci and ascospores are an indication of successful crosses between *F. proliferatum* isolates and the tester strains. From the crosses, cirrhus of ascospores can be observed emerging from the perithecia after 2–5 weeks of incubation. For observation of the ascospores in the asci, the perithecia were mounted on a slide and observed under a light microscope (Olympus CX-41).

RESULTS

Molecular identification and phylogenetic analysis

Amplification of the TEF-1α gene produced 750 bp band for all *F. proliferatum* isolates. A BLAST search of TEF-1α sequences produced 97–100% similarity with *F. proliferatum* in both Fusarium-ID and GenBank databases.

Maximum Likelihood (ML) phylogenetic tree generated with 1,000 replicates is shown in Figure 1, and the grouping of the isolates from various hosts and substrates can be divided into nine clades, A – I. Generally, the isolates did not cluster according to the hosts or substrates except isolates in Clades D, F and G. Clade D consisted of isolates from sugarcane with pokkah boeng symptoms and Clade F, isolates recovered from rot lesion of potato tuber. Two isolates from rice stem with bakanae disease symptoms were grouped in Clade G. The reference strain, *F. proliferatum* NRRL22944 was grouped in Clade F with five isolates recovered from rot lesion of potato tuber. The other clades (A, B, C, E, H and I) consisted of isolates from various hosts and substrates. Based on the grouping of the isolates, genetic variation was observed among the *F. proliferatum* isolates from various hosts and substrates. Other *Fusarium* species, *F. globosum*, *F. fujikuroi*, *F. inflexum* and *F. oxysporum* form separate clades.

RFLP-IGS and cluster analysis

Amplification of IGS region of *F. proliferatum* isolates produced a single band of 2600 bp. Table 2 shows the RFLP groups, IGS haplotypes and restriction patterns of *F. proliferatum* isolates. Depending on the restriction enzymes, 6 to 10 restriction patterns were resolved among 74 isolates of *F. proliferatum.* Isolates that produce the same restriction patterns for the restriction enzymes used were scored as the same IGS haplotype. A total of 67 IGS haplotypes were designated among the isolates, demonstrated that the restriction patterns produced were highly variable.

A dendrogram was generated using UPGMA cluster analysis to ascertain the genetic relationships of the *F. proliferatum* isolates from various hosts and substrates (Figure 2). The isolates were divided into three RFLP groups indicated that the isolates of *F. proliferatum* showed intraspecific variations. RFLP group I consisted of 37 isolates from different hosts/substrates, namely asparagus, banana, dragon fruit, debris, flowers, sorghum, grass, mango, outdoor air, potato, yam, rice grains, and watermelon. The similarity of the isolates in RFLP group I ranged from 84–100%. RFLP group II comprised 24 isolates from banana, corn, dragon fruit, flower, mango, onion, sorghum, sugarcane and torch ginger, with similarity ranged from 82–100%. Isolates in RFLP group III have similarity from 80–100% and consist of 13 isolates from corn, wheat, flowers, sorghum and torch ginger.

Mating study

From PCR amplification of *MAT* alleles, 40 isolates carried *MAT-1* while 34 isolates carried *MAT-2*. However, only 37 isolates of *F. proliferatum* that carry *MAT-1* allele crossed fertile with tester strain of *MATD-2*, and 33 isolates that carry *MAT-2* allele crossed fertile with tester strain of *MATD-1*. Four isolates (K680R, P902O, Q1054Q, and S4882O) did not cross fertile with any of the tester strain and regarded as infertile. None of the isolates was cross-fertile with MP-C tester strains.

Seventy isolates formed perithecia with asci and viable ascospores while four isolates (K680R, P902O, Q1054Q, and S4882O) did not produce any fertile progeny. After two months of incubation, black perithecia with ooze were visible (Figure 3A). In the perithecia, eight ascospores in an ascus were observed (Figures 3B & 3C). The ascospores were oval with 1-septate (Figure 3D). Isolates *F. proliferatum* cross-fertile with MP-D tester strains indicated that the isolates were able to form the teleomorph stage.

DISCUSSION

Species identity of morphologically identified *F. proliferatum* isolates in stock culture collection were confirmed based on TEF-1α sequences. The phylogenetic tree generated using TEF-1α sequences indicated a high intraspecific variability of the isolates, which was shown by the number of clades formed in the phylogenetic tree. Intraspecific variability of *F. proliferatum* from different hosts and origin based on phylogenetic analysis of TEF-1α sequences was also reported by Jurado *et al*. (2010). The study by Jurado *et al*. (2010) demonstrated a high level of genetic variation among the isolates of *F. proliferatum* of which the isolates were clustered into four groups and three sub-groups. The variability within *F. proliferatum*

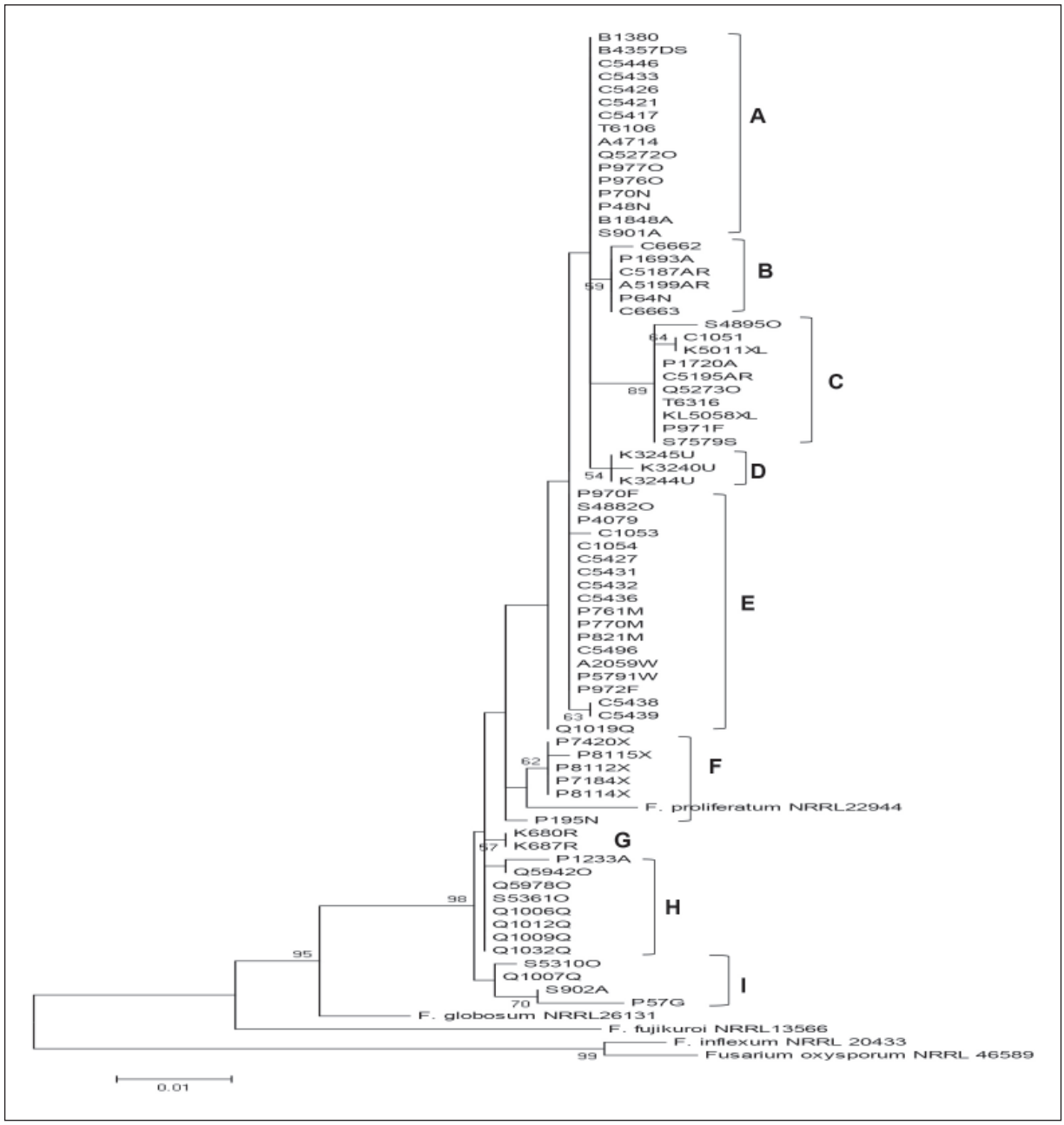

Fig. 1. Maximum likelihood phylogenetic tree of *F. proliferatum* isolates from various hosts/substrates based on TEF-1α sequences. The grouping of the isolates can be divided into nine clades (A – I). *Fusarium inflexum* and *F. oxysporum* are the out-groups.

isolates might be due to the species causing various types of diseases and differences in the hosts infected or substrates occupied (Leslie, 1995) as well as their ability to adapt and evolve in various environmental conditions (Medina *et al*., 2017). *Fusarium proliferatum* has also been reported to cause diseases on various crops including cereal grains (Mielniczuk & Skwarylo-Bednarz, 2020), corn (Scarpino *et al*., 2015; Wang *et al*., 2021), asparagus (Stepien *et al*., 2016; Djalali Farahani-Kofoet *et al*., 2020), dragon-fruits (Masratul Hawa *et al*., 2013), pineapple (Nurul Faziha *et al*., 2016), garlic (Mondali *et al*., 2021), mango (Omar *et al*., 2018) and even infected humans (Herbrecht *et al*., 2004; Sun *et al*., 2018).

Phylogenetic analysis showed that there was no relatedness between the grouping of the *F. proliferatum* isolates with the hosts. The results were similar with studies by Proctor *et al*. (2010) and Jurado *et al*. (2010) of which the phylogenetic analysis based on TEF-1α sequences of *F. proliferatum* isolates from diverse hosts and locations did not cluster according to the hosts and the locations. The results suggested that isolates of

Table 2. RFLP groups, IGS haplotypes, and restriction patterns of 74 isolates of *F. proliferatum* from various hosts /substrates

Isolates	RFLP Groups	IGS haplotype	Restriction band patterns				
			*Msp*1	*Eco*R1	*Bsu*R1	*Alu*1	*Bsu*151
P48N	I	1	A	C	B	C	A
P64N	I	1	A	C	B	C	A
Q1032Q	I	2	A	B	B	C	B
P7420X	I	3	H	C	D	C	A
P821M	I	4	A	C	B	C	B
C6662&	I	4	A	C	B	C	B
C5187AR	I	5	A	D	B	C	B
S901A	I	6	A	C	B	C	C
Q1009Q	I	7	A	D	B	C	C
A5199AR	I	7	A	D	B	C	C
B4357DS	I	8	A	B	B	C	C
C5195AR	I	9	A	D	B	C	D
P4079π	I	10	B	C	D	C	B
P8112X	I	11	B	C	D	C	F
P8114X	I	11	B	C	D	C	F
P8115X	I	12	B	E	D	C	F
S7579S	I	13	I	F	G	C	F
C1051=	I	14	A	D	B	C	F
C1054=	I	15	A	C	B	F	A
C1053=	I	16	B	C	B	F	A
P70N	I	17	B	D	B	C	C
B1380=	I	18	B	B	B	C	C
K687R	I	19	B	F	B	C	C
K680R	I	20	B	C	B	C	F
P1233A	I	20	B	C	B	C	F
C5426=	I	21	B	E	B	C	F
Q1006Q	I	22	B	C	B	C	B
S4882O	I	23	B	C	B	C	A
B1848A	I	24	H	C	B	C	F
P761M	I	25	H	C	B	C	A
P1720A	I	26	H	A	B	C	A
P2059W	I	27	G	C	B	C	A
S4895Q	I	28	B	C	B	C	A
P5791W	I	28	B	C	B	C	A
S902A	I	29	E	C	B	C	A
P7184X	I	30	E	C	B	C	B
P1693A	II	31	A	E	A	C	B
P57G	II	32	B	C	B	A	B
T6316π	II	33	B	C	A	A	B
P195N	II	33	B	C	A	A	B
P976O	II	34	B	C	B	G	B
Q1007Q	II	35	I	C	B	E	B
P770M	II	36	B	C	A	G	B
Q5272O	II	37	E	C	A	E	B
S5273O	II	38	B	C	A	C	B
C5446=	II	39	C	F	A	C	B
Q1019Q	II	40	E	C	A	A	B
K5011XL	II	41	B	E	A	E	B
C5438=	II	42	C	C	A	C	B
C5439=	II	43	E	C	A	E	B
C5427=	II	44	H	B	A	A	B
A4714π	II	45	D	I	E	D	A
K3240U	II	46	D	H	F	C	B
K3245U	II	47	I	H	G	E	B
K3244U	II	48	D	H	F	C	B
C5417=	II	49	D	H	F	C	C
C5421=	II	50	F	H	F	C	C
C5432=	II	51	D	H	C	C	B
T6106π	II	52	D	H	C	C	E
C5436=	II	53	D	H	C	C	B
C5431=	II	54	D	H	C	C	B
P970F	III	55	E	G	G	C	E
P971F	III	56	J	G	E	C	E
P972F	III	57	J	G	E	G	E
P977O	III	58	C	B	B	A	C
Q1012Q	III	59	A	B	B	A	C
C5433=	III	60	B	B	B	A	C
C5496=	III	61	A	B	A	A	C
C6663&	III	62	A	B	A	E	D
K5058XL	III	63	C	D	B	B	F
Q5310O	III	64	C	E	B	B	F
Q5978O	III	65	C	B	A	B	F
Q5361O	III	66	E	C	B	B	D
C5942O	III	67	B	E	A	B	D
		Total	**10**	**9**	**7**	**7**	**6**

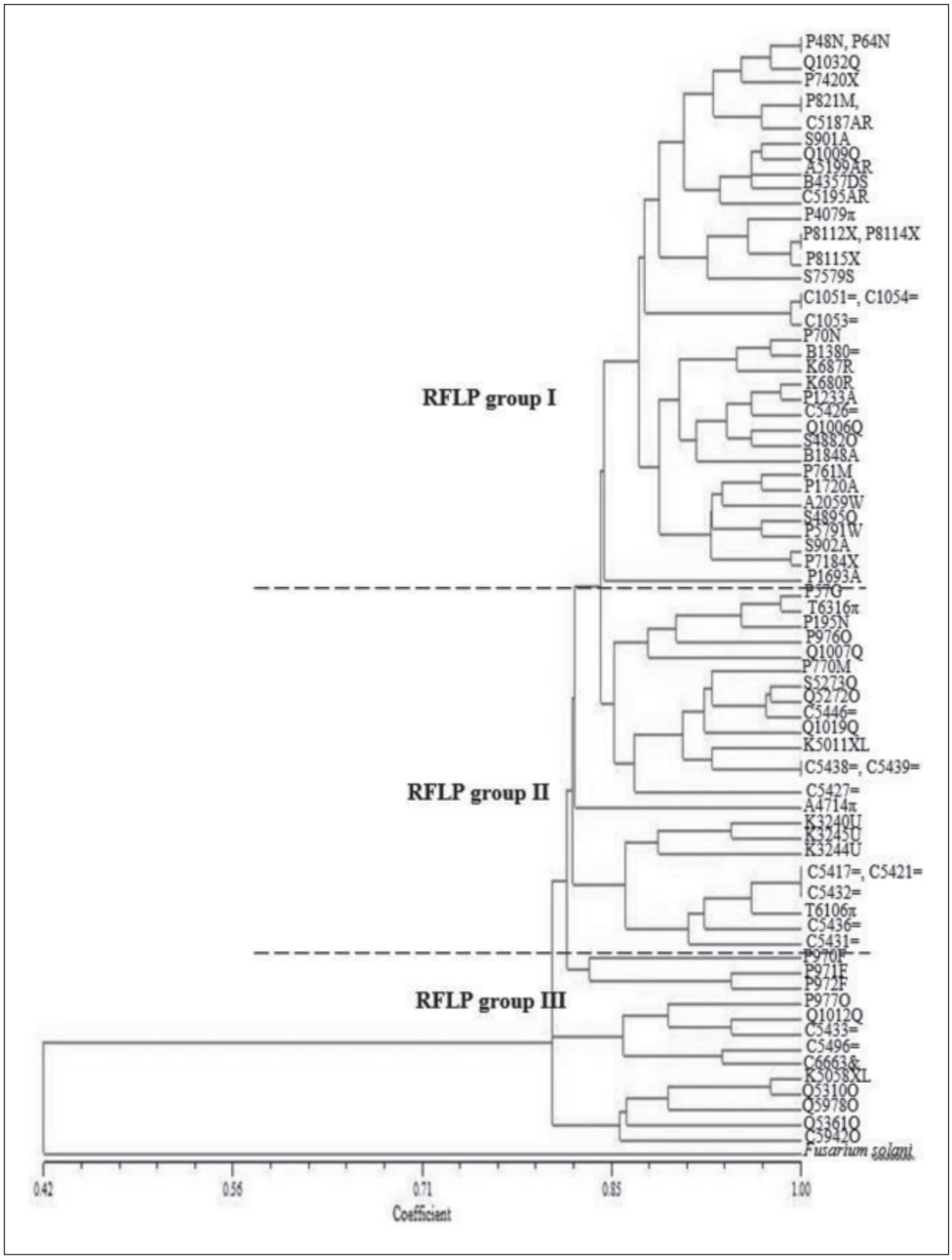

Fig. 2. Dendrogram constructed using UPGMA cluster analysis of *F. proliferatum* isolates based on RFLP-IGS analysis. *Fusarium proliferatum* isolates from various hosts/substrates are clustered in three RFLP groups, I, II, and III. *Fusarium solani* is the out-group.

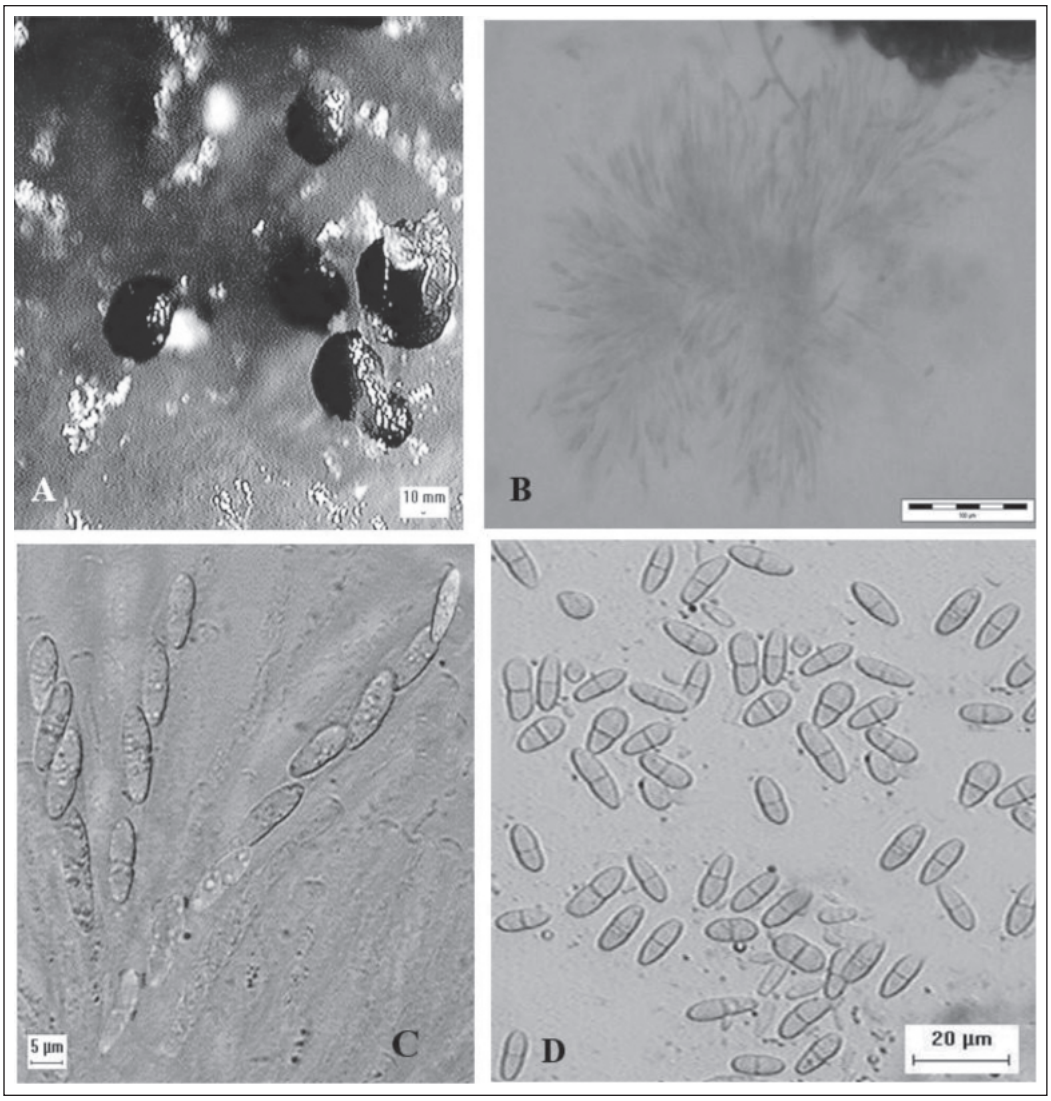

Fig. 3. Mating study of *F. proliferatum* with formation of perithecia and ascospores. **(A)** Black perithecia and ooze from the perithecia; **(B)** Asci and ascospores; **(C)** Ascospores in asci; **(D)** Ascospores.

F. proliferatum can infect various hosts when the conditions permitted. Other factors might be related to the evolution of the host and the pathogen due to environmental conditions as well as the pathogen introductions in an area or location due to the movement of infected planting materials and agricultural products (Almiman, 2017).

Restriction patterns produced by *F. proliferatum* isolates also indicated intraspecific variation in the IGS region. The variations in the IGS region resulted from minor alterations or variations in the nucleotide composition leading to different restriction patterns from the isolates of the same species (Apple & Gordon, 1995; Konstantinova & Yli-Mattila, 2004). The variations in the IGS region are also caused by insertions or deletions in the sub-repeats units within the region and unequal crossed over, which demonstrated that this region might be evolving intensively (Hillis & Dixon, 1991).

High levels of genetic variation were also shown by 67 haplotypes produced among isolates of *F. proliferatum* from various hosts and substrates. The results are similar to a study by Láday *et al*. (2008) of which 16 haplotypes were detected among 184 isolates of *F. proliferatum* using RFLP of mtDNA. Heng *et al*. (2012) also reported 53 haplotypes were detected among 74 isolates of *Fusarium* species from maize, sugarcane and rice using RFLP-IGS analysis. High levels of genetic variation of *F. proliferatum* are possibly due to rapid acclimatisation of the isolates to different ecological conditions for survival (McDonald & McDermott, 1993). This situation leads to modification of genetic structure which resulted in a high number of haplotypes from various hosts and substrates.

Four isolates of *F. proliferatum* did not produce perithecia and therefore these isolates were considered infertile. The results of the mating

study are an indication of biological species which showed that morphologically identified as *F. proliferatum* were certainly *F. proliferatum*. A high number of successful crosses was also reported by Armengol *et al*. (2005) where 34 from 36 isolates of *F. proliferatum* isolated from ornamental palms were categorized as MP-D. Several studies also showed similar results where a high number of MP-D crosses has been reported on several host plants such as asparagus (Bargen *et al*., 2009), date palm (Sharafaddin *et al*., 2019), onion and garlic (Stankovic *et al*., 2007; Salvalaggio & Ridao, 2013), rice (Matić *et al*., 2013) and corn kernels (Li *et al*., 2019).

All isolates of *F. proliferatum* were not crossed-fertile with tester strain of MP-C indicated interfertility did not occur even though *F. fujikuroi* was reported to be sibling species of *F. proliferatum* (Desjardins *et al*., 1997). A study by Heng *et al*. (2011) showed similar results where *F. proliferatum* isolates from maize, sugarcane and rice did not cross-fertile with MP-C tester strain.

In this study, both mating-type alleles (*MAT-1* & *MAT-2*) were detected and most of the *F. proliferatum* isolates were able to produce fertile progeny. Both mating types were also detected from isolates originated from the same host, namely banana (*MATD-1* - three isolates & *MATD-2* - one isolate), and *Heliconia latispatha* (*MATD-1* - seven isolates and *MATD-2* - one isolate). The results suggested a potential of genetic recombination among the isolates particularly isolates in the same field. A study by Armengol *et al*. (2005) on *F. proliferatum* as infected ornamental palms suggested the potential of genetic recombination in the field as *MATD-1* and *MATD-2* alleles were detected from the same host. Similar results were also reported by Abdalla *et al*. (2000) on *F. proliferatum* from date palm of which both mating-type alleles (*MATD-1* & *MATD-2*) were also detected and crossable isolates were able to produce fertile perithecia. The findings also suggested a potential for genetic recombination among isolates in the field. According to Armengol *et al*. (2005), a high frequency of genetic recombination in the field could improve the genetic pool available for *F. proliferatum* population.

Fusarium proliferatum is a well-known plant pathogen and mycotoxin producer, thus correct species identification is important as this will assist in developing attainable plant disease management, predicting the pathogen-host range as the species has a wide host range and predicting the types of mycotoxins produced particularly on cereal grains as well as other susceptible crops. In addition, the genetic variability of *Fusarium* spp. particularly *F. proliferatum* is essential as this species could survive and adapt to an environment that changes drastically. This capability can lead to the appearance of new strains that are resistant to fungicide and able to overcome host resistance (Klittich & Leslie, 1988).

In the present study, morphologically identified *F. proliferatum* isolates obtained from stock culture collection were re-identified and the species identity was confirmed using TEF-1α sequences and mating study. Phylogenetic analysis of TEF-1α sequences and RFLP-IGS indicated that the *F. proliferatum* isolates from various hosts and substrates were highly variable.

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