Molecular characterization of *Fusarium globosum* strains from South African maize and Japanese wheat

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Abstract

The fungus *Fusarium globosum* was first isolated from maize in South Africa and subsequently from wheat in Japan. Here, multiple analyses revealed that, despite morphological similarities, South African maize and Japanese wheat isolates of the fungus exhibit multiple differences. An amplified fragment length polymorphism-based similarity index for the two groups of isolates was only 45%. Most maize isolates produced relatively high levels of fumonisins, whereas wheat isolates produced little or no fumonisins. The fumonisin biosynthetic gene *FUM1* was detected in maize isolates by Southern blot analysis but not in the wheat isolates. In addition, most of the maize isolates produced sclerotia, and all of them produced large orange to dark purple sporodochia in carrot agar culture, whereas wheat isolates did not produce either structure. In contrast, individual isolates from both maize and wheat carried markers for both mating type idiomorphs, which indicates that the fungus may be homothallic. However, a sexual stage of *F. globosum* was not formed under standard self-fertilization conditions developed for other homothallic species of *Fusarium*. The inability to produce the sexual stage is consistent with the high similarity of 87–100% and G_{ST} index of 1.72 for the maize isolates, which suggests that these isolates are undergoing asexual but not sexual reproduction. Together, the results suggest that the South African maize and Japanese wheat isolates of *F. globosum* are distinct populations and could be different species.

Introduction

Fusarium globosum Rheeder, Marasas and Nelson was first isolated from maize (*Zea mays* L.) samples harvested in the former Transkei region of South Africa [33] and subsequently from wheat (*Triticum aestivum* L. em. Thell.) culms in subtropical Japan [1]. This species is characterized by abundant globose microconidia produced singly or in botryose clusters on monophialides and polyphialides in aerial mycelium. Clavate to ellipsoidal microconidia also are produced on both types of phialides, as well as in chains and in false heads. Like other species of *Fusarium* in Section Liseola [47], *F. globosum* does not produce chlamydospores.

South African maize isolates of *F. globosum* can produce B-series analogues of fumonisin (FB) mycotoxins [43]. Fumonisins were first implicated in various animal diseases such as equine leukoencephalomalacia [24] and subsequently demonstrated to cause liver cancer in rodents [14,

17] and pulmonary edema syndrome in pigs [35]. These mycotoxins also have been linked epidemiologically to esophageal cancer in both South Africa and China [10, 41]. More recently, it has been proposed that fumonisins be considered a potential risk factor for birth defects, including human neural tube defects (NTD) in populations consuming fumonisin-contaminated maize. This is due to the interference of this mycotoxin with folic acid utilization via depletion of cellular sphingolipids required for normal functioning of the folate receptor α [25, 26].

The fumonisin biosynthetic (*FUM*) gene cluster consists of 17 genes that are localized to a 42.5 kb region of chromosome 1 in *F. verticillioides* [6, 7, 30]. The roles of most of these genes have been confirmed by gene deletion analysis. Deletion of *FUM1*, *FUM6* or *FUM8* blocks accumulation of all known fumonisins, indicating that these genes are indispensable for fumonisin production [29, 30, 37]. Also, strains lacking *FUM2* produced only fumonisin analogues (FB2 and FB4) that lack the C-10 hydroxyl group [32], and *FUM3* deletion mutants produce only analogues (FB3 and FB4) that lack the C-5 hydroxyl group [8]. The individual deletions of the remaining *FUM* genes resulted in various effects and influences on fumonisin production [6–9, 30, 48], including no effects on fumonisin accumulation with the deletions of *FUM15*, *FUM16*, *FUM17* or *FUM18* [9, 30, 32].

The teleomorph of *F. globosum* has not been identified, and it is not known if this *Fusarium* is capable of sexual reproduction. The ability to reproduce sexually is an important feature as meiotic recombination can generate and maintain genotypic variation that could result in the re-assortment of genes that direct traits such as virulence or toxin production. In *Fusarium* and other filamentous ascomycetes, sexual reproduction is controlled by the mating type (*MAT*) locus [13, 45]. In heterothallic species, there are two functional *MAT* alleles, which are designated *MAT1-1* and *MAT1-2* in *Fusarium* [49]. These alleles are idiomorphic as they exhibit no significant sequence similarity with regard to DNA or encoded proteins but map to the same chromosomal position. In heterothallic *Fusarium* species, two haploid individuals must have different *MAT* alleles in order to be sexually compatible and to complete the sexual cycle. In homothallic species, on the other hand, individual haploid strains have both *MAT-1* and *MAT-2* idiomorphs, which can be closely linked or fused [44]. These individuals are self-fertile and can complete the sexual cycle with or without a mate.

There is little sequence data available for *F. globosum*. The GenBank database contains only 19 nucleotide sequences corresponding to 8 different genes from various *F. globosum* strains. Nine sequences are for the translation elongation factor-1α gene (*TEF-1α*) and exhibit a 99–100% identity to one another. However, close scrutiny of these sequences and accession information indicates that six are not from *F. globosum*. GenBank accessions AF160285, AY337440 and AY337441 were derived from maize isolates of *F. globosum* from South Africa. Accession AM404137 was derived from strain ITEM 6013, which is listed as a *F. globosum* isolate from Kansas. However, ITEM 6013 is in fact a maize isolate from South Africa, i.e. ITEM 6013 = KSU 11555 = MRC 6647 ex holotype of *F. globosum* (Logrieco and Leslie, personal communication). Similarly, GenBank accessions AM404135 and AM404136 are sequences that were derived from strains ITEM 1590 and ITEM 1874, respectively. However, according to the ITEM database, these strains are *Fusarium proliferatum*, rather than *F. globosum*.

Finally, accessions DQ854859, DQ85460 and DQ854461 were derived from strains attributed to Spanish isolates of *F. globosum* [23]. However, these three sequences are 100% homologous to the TEF-1α sequences for *F. proliferatum* strains ITEM 1590 and ITEM 1874, which strongly suggests that these three Spanish strains are probably *F. proliferatum* and not *F. globosum*. Databases containing incorrect data could result in much confusion with the incorrect identification of newly isolated strains.

The main objectives of this study were to determine (1) the mating type and the sexual reproductive capability of the *F. globosum* isolates; (2) the genetic variation among these isolates, especially between the *F. globosum* strains isolated from the two host plants in the two geographical locations; and (3) the levels of fumonisin B analogues produced by *F. globosum* strains.

Materials and methods

Fungal isolates

Resuspended lyophilized culture material of 18 *F. globosum* strains, 15 isolated from maize in South Africa and 3 strains isolated from wheat in Japan, was used for the phylogenetic analysis, fumonisin quantitation and *MAT*-typing (Table 1). These were the only known *F. globosum* strains identified from the specific hosts in the respective geographical locations. All isolates are preserved in the culture collection of the PROMEC Unit, Medical Research Council (MRC), South Africa. Five *F. globosum* strains were used in a Southern Blot analysis and included two maize isolates ex-holotype MRC 6647 (sclerotial-producing) and MRC 6657 (non-sclerotial), and the three wheat isolates MRC 7883, MRC 7884 and MRC 7885.

Table 1. Fumonisin B analogues produced by *Fusarium globosum* strains isolated from maize in South Africa and wheat in Japan.

Fusarium globosum strains		Maximum fumonisin levels (mg kg^{-1}) for			
MRC no.	Other no.	FB_1	FB_2	FB_3	Total FB
Maize Isolates, South Africa					
6646		50	ND[b]	3.9	54
6647[a]	CBS 428.97	32	ND[b]	1.9	33
6648	CBS 429.97	57	ND[b]	2.7	60
6649		121	0.6	5.9	128
6650		2	ND[b]	ND[b]	2.1
6651		117	0.6	5.7	123
6652		52	5.0	6.6	64
6654		10	0.5	1.3	12
6655		126	0.7	5.4	132
6656		159	0.9	6.8	167
6657	CBS 430.97	121	0.6	6.8	128
6659		83	0.4	4.1	88
6660	CBS 431.97	114	0.6	5.6	120
6661		6	0.7	ND	6.7
6662		5	0.4	ND	5.4
Wheat Isolates, Japan					
7883	BBA 69017	12	ND[b]	ND[b]	12
7884	BBA 69018	ND[b]	ND[b]	ND[b]	ND
7885	BBA 69019	2.1	ND[b]	ND[b]	2.1

[a] MRC6647 = *ex* holotype

[b] ND = Not detected (level of detection < 0.01 μg kg^{-1})

DNA isolation

Isolates used in the phylogenetic and *MAT*-typing studies were cultured by inoculating approximately 1 ml of a spore suspension (typically 106–107 conidia) in 100 ml of Malt Extract Broth (Difco) and incubated at 25°C for 14 days in the dark. The mycelia were harvested by filtration through Whatman No. 1 filter paper and stored at −20°C until DNA extraction. Mycelia

were ground to a fine powder in liquid nitrogen, and total genomic DNA was extracted with the NucleoSpin Plant L Extraction Kit (Macherey–Nagel, Germany) following the manufacturer's instructions. The five strains used in the Southern Blot analysis were grown in liquid GYEP medium (2% glucose, 0.3% yeast extract, and 1% peptone) [37], incubated for 3 days at 25°C, and the mycelia were harvested by vacuum filtration. Genomic DNA was isolated from lyophilized harvested mycelium using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's specifications.

PCR amplification of *MAT* idiomorphs

Mating-type idiomorphs *MAT-1* and *MAT-2* for the *F. globosum* isolates were identified with PCR-based assays using Gfmat1 and Gfmat2 primer sets, respectively, as described in Steenkamp *et al*. [39]. Positive PCR controls used were *F. sacchari* mating type tester strains MRC 6525 and MRC 6524 for the *MAT-1* and *MAT-2* amplification reactions, respectively. PCR products were electrophoresed to estimate size of fragments in a 1% agarose gel along with a 100-bp marker (Promega), purified by using the Wizard SV Gel and PCR Clean-up System (Promega), and subsequently submitted for sequencing. The resulting DNA sequences were compared using the BLAST algorithm against *MAT* sequences present in the GenBank database.

Self-fertilization

The method as described by Bowden and Leslie [5] was used for inducing self-fertilization to determine the perithecial producing ability of potentially homothallic strains. Briefly, cultures were grown on carrot agar in 6-cm Petri dishes at 25°C in the dark. After 7 days, 1.5 ml of 2.5% Tween 60 (Sigma) was applied to the culture and spread across the surface with a glass rod, with subsequent incubation of up to 8 weeks at 18°C as well as at 22°C with 12 h photoperiod.

A sclerotia-producing maize isolate (MRC 6647) was also crossed with a non-sclerotial maize isolate (MRC 6657) according to the method described by Klittich and Leslie [18], with the non-sclerotial cultures used as the male strain. The wheat isolates were crossed with each other and used as both male and female strains. All strains used as male parents were cultured on complete media slants and incubated for 7 days at 25°C under light. The conidia of each male culture were suspended in 3 ml 2.5% Tween 60; 1.5 ml aliquot was transferred to the female carrot agar cultures where it was spread across the surface with a glass rod and incubated under similar conditions as described with self-fertilization.

Amplified Fragment Length Polymorphisms

Amplified fragment length polymorphisms (AFLPs) were prepared according to the protocol of Vos *et al*. [46] with modifications as described by Zeller *et al*. [50]. *Fusarium* isolates *F. subglutinans* MRC 7828 and *F. circinatum* MRC 7870 were used as reference strains [12]. Four selective amplification reactions generated AFLP fingerprints by using combinations of IRDye™700-labeled *Eco*RI primers with two base selection (Biolegio, Malden, The Netherlands) and unlabeled *Mse*I primers with two base selection. The two base selective primer combinations for the 4 amplification reactions were (1) *Eco*RI+AA and *Mse*I+AA, (2) *Eco*RI+AC and *Mse*I+AA, (3) *Eco*RI+ and *Mse*I+AC, and (4) *Eco*RI+AA and *Mse*I+GA. PCR conditions were as follows: 13 cycles of 10 s at 94°C, 30 s at 65°C (decreased 0.7°C per cycle) and 1 min at 72°C, 23 cycles of 10 s at 94°C, 30 s at 56°C and 1 min (increase of 1 s per cycle) at 72°C, and 1 cycle of 1 min at 72°C. AFLP electrophoresis was performed on a model 4200 LI-COR® automated DNA sequencer as described by Myburg *et al*. [27]. Digital gel images obtained from the LI-COR® system were analyzed with the AFLP-Quanta Software according to the manufacturer's user manual. Polymorphic bands were scored as '−' indicating absence, '+' indicating presence of bands and '?' indicating

missing/unknown data.

Similarity analysis

The Dice Coefficients, determined using the combined AFLP data sets, were conducted with NTSYSpc software program to determine genetic distance among the isolates.

The genotypic diversity of the isolates was calculated using the Stoddart and Taylor [40] formula.

The maximum value for G_{ST} is the number of individuals in the population, which occurs when each individual in the sample has a different genotype. The phylogram was generated using distance analysis using the AFLP scores.

Southern Blot analysis

Oligonuclueotide primers used to generate probes for genes *FUM1*, *FUM2*, *FUM8*, *FUM14*, *FUM19* and *FUM21* are presented in Table 2. The PCR products used as templates to generate the probes were amplified from the isolated genomic DNA of *F. globosum* MRC 6647 (ex-holotype strain) following the protocol described by Proctor *et al*. [31]. The 32P-labeled hybridization probes were prepared with the Ready-to-Go kit (Amersham Biosciences).

Table 2. Oligonucleotides used to generate probes for Southern Blot analysis.

FUM gene	Oligonucleotide number	Nucleotide Sequences (5′–3′)	Predicted product size (bp)
FUM1	rp32	ACAAGTGTCCTTGGGGTCCAGG	700
	rp33	GATGCTCTTGGAAGTGGCCTACG	
FUM2	rp2056	CAGACGTCTCTTCTTGCCTGCT	1,000
	rp2057	GCYGCRCATTGTGGTATCGTAAGTC	
FUM8	rp679	CGTAGTAGGAATGAGAAGGATG	800
	rp680	GCAAGCTTTGTGGCTGATTGTC	
FUM14	rp2059	CARTCATSATAGAGGACGACACTG	1,250
	rp2061	CTCTTGAACTCATACTGCTGCAA	
FUM19	rp1935	GTCTCCCAACGCCCTGCCTATCT	900
	rp1937	GACAGCAGAACTAGGCTCATCGAGT	
FUM21	rp1944	GTAATGGCWCAAACCCTTGCAATCA	900
	rp1949	GTCTGGRCGCAAAMGGCKGCATC	

The genomic DNA was digested with the restriction endonuclease *Hin*dIII, subjected to electrophoresis and transferred to a nylon membrane. Southern Blot analysis was carried using the protocol described by Proctor *et al*. [31].

Fumonisin analysis

All isolates were cultured on maize patties (30 patties per strain) consisting of 30 g of ground yellow maize kernels mixed with 30 ml sterile dH_2O placed in 90-mm glass petri dishes and autoclaved at 121°C for 1 h on two consecutive days. Autoclaved patties were inoculated with 1 ml of a spore suspension prepared from lyophilized cultures diluted 1:100 with dH_2O. Cultures were incubated in the dark at 25°C for 21 days, and then dried at 50°C for 24 h, ground to a meal and stored at 4°C until analysis.

Sample extracts from the cultures were prepared by the method of Sydenham *et al*. [42]. In brief, 100 ml CH_3OH/H_2O (3:1 v/v) was added to 5 g culture material from each strain and homogenized for 3 min using a Polytron homogenizer (Polytron PT300, Kinematika). Extracts were centrifuged at 4,000×g for 10 min at 4°C, filtered (Whatman No. 4), and the filtrate adjusted to pH 6.0 with 1 M NaOH. Aliquots (5 ml) of the resultant filtrates were purified on solid-phase extraction cartridges containing silica-based strong anion-exchange (SAX) media (Varian, Harbor City, CA). The fumonisins were selectively eluted with a 1% solution of CH_3COOH in CH_3OH. The eluate was evaporated to dryness, in the form of a fine powdery residue around the vials, at 60°C under nitrogen. The residue was redissolved in CH_3OH prior to injection.

Levels of fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) were determined by using reverse-phase high pressure liquid chromatography (HPLC) coupled with fluorescence detection, as their o-phthaldialdehyde (OPA) derivatives. The HPLC was performed at a flow rate of 1 ml/min on a Luna C18 column (Phenomenex, Torrance, CA), and the mobile phase consisted of mixtures of CH_3OH/0.1 M $Na_2B_4O_7 \cdot 10H_2O$ (76:24 v/v). Fumonisin analogues were detected and quantified based on comparisons of retention times and peak area with standards [34].

Results

Mating type analysis

In PCR experiments, primer pair Gfmat1 amplified an approximately 250-bp fragment from the *F. sacchari MAT1-1* tester strain MRC 6525, but not the *MAT1-2* tester strain, and from all isolates of *F. globosum* analyzed. Likewise, primer pair Gfmat2 amplified a fragment of approximately 800 bp from the *F. sacchari MAT1-2* tester strain MRC 6524, but not the *MAT1-1* tester strain, and from all isolates of *F. globosum* examined. As expected, sequence analysis revealed that the Gfmat1 PCR product from all strains corresponded to the α-domain of the *MAT1-1-1* gene and that the Gfmat2 product included the HMG-domain of the *MAT1-2-1* gene. Amplification of fragments of both the *MAT1-1* and *MAT1-2* idiomorphs from individual isolates of *F. globosum* indicates that this species has at least a portion of both idiomorphs and that it maybe homothallic.

Self-fertilization

Based on the results of the molecular mating type analysis, we attempted to induce the sexual cycle by self-fertilization of all *F. globosum* isolates using the carrot agar assay that has been used to induce self-fertilization in *F. graminearum*/*Gibberella zeae*, a well-characterized homothallic species of *Fusarium*. Carrot agar cultures were incubated at 18 and at 22°C for up to 8 week cycles, but none of the 18 *F. globosum* isolates analyzed produced perithecia. However, 9 of the 15 maize isolates produced sclerotia (Fig. 1a–d), and all produced large masses of sporodochia (Fig. 1e, f) under these conditions. In contrast, none of the wheat isolates produced sclerotia or sporodochial masses in the carrot agar cultures.

Similarity analysis of AFLP data sets

AFLP bands (Fig. 2) were scored from amplification with four selective primer combination reactions. Dice Coefficients of the combined AFLP data sets used to determine genetic distance among the isolates resulted in the identification of two subgroups within *F. globosum* with a similarity of 45% (Fig. 3). A high Dice Coefficient of 87–100% was observed among the maize isolates. The G_{ST} statistic was calculated to estimate the degree of genotypic diversity of the individual clades representing the different host origin. The G_{ST} values obtained for the isolates of the individual maize and wheat clusters were 1.72 and 1, respectively. The low variability observed for the South African maize isolates suggests a clonal population. The results from the Japanese

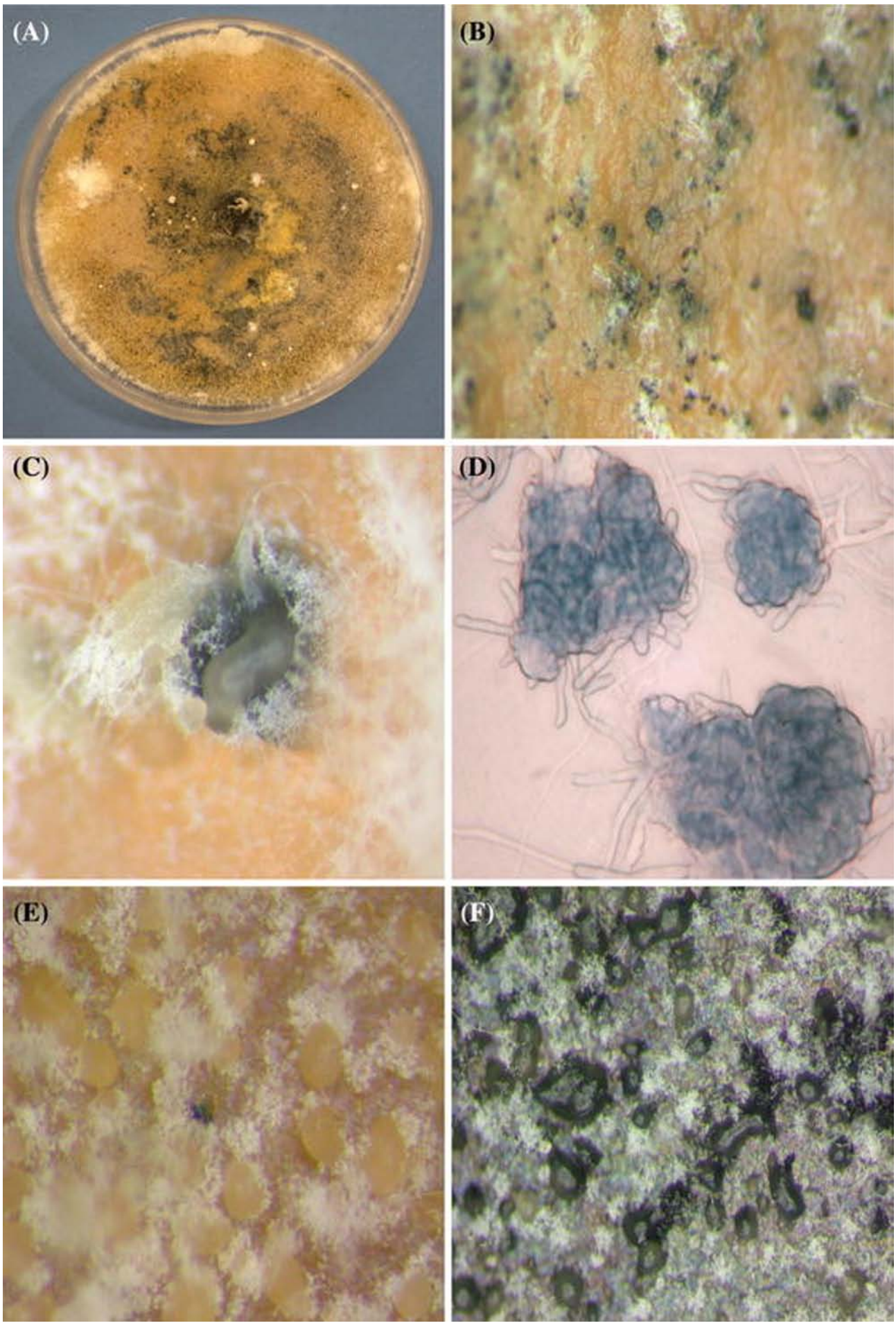
(A)
(B)
(C)
(D)
(E)
(F)

Figure 1. Sclerotia produced by maize isolates grown on carrot agar. **a** *Fusarium globosum* MRC 6647 (ex-holotype) at 4 weeks of incubation; **b** MRC 6647 culture with sclerotia produced on surface of carrot agar; **c** sclerotia with sporodochia produced by MRC 6649; **d** sclerotia under light microscope, 40× magnification. *F. globosum* cultures (4×) showing large **e**–**f** sporodochia on carrot agar after 4–8 weeks incubation. The dark sporodochia were observed on older cultures that produced more pigment with time

wheat isolates should be interpreted with caution as the isolate number is very low.

Southern Blot analysis

Southern Blot analysis was employed to determine the presence or absence of fumonisin biosynthetic genes *FUM1*, *FUM2*, *FUM8*, *FUM14*, *FUM19* and *FUM21* in the genomes of *F. globosum* strains from Japanese wheat and South African maize. These genes are distributed across the entire length of the fumonisin biosynthetic gene cluster and are required for fumonisin production in *F. verticillioides* [29, 30, 37]. Genomic DNA from five *F. globosum* strains was digested with *Hin*dIII, blotted and then hybridized to 32P-labeled probes for each of the six FUM genes. Hybridization signals were detected for *FUM2*, *FUM8*, *FUM14*, *FUM19* and *FUM21* in the South African maize and Japanese wheat isolates examined (Fig. 4). However, the hybridization signal for *FUM1* was detected only in the maize isolates (Fig. 4). In addition, there was differentiation in hybridization signal of *FUM21* between the maize and wheat isolates.

Fumonisin profiles

The type and levels of the fumonisin B analogues produced by the *F. globosum* strains are summarized in Table 1. FB_1 was detected in 17 of the 18 isolates, with the exception being the wheat isolate MRC 7884. The maize isolates were on average higher producers of FB_1 with relatively low levels detected in the culture material of the wheat isolates. The maize isolates also produced moderate levels of FB_2 and FB_3, with FB_3 on average being produced at higher levels. Nine of these strains produced both FB_2 and FB_3, three strains produced FB_3 but not FB_2, and two strains produced FB_2 but were negative for FB_3. MRC 6650 was the only maize isolate negative for both of these mycotoxins. None of the Japanese wheat isolates produced FB_2 and FB_3 in culture.

Discussion

Amplification of fragments of the *MAT1-1* and *MAT1-2* idiomorphs from *F. globosum* indicates that markers for both idiomorphs may be present in individual strains of this species, which suggest that both idiomorphs are present. If this is the case, *F. globosum* would be potentially homothallic. Homothallic species of *Fusarium*, such as *F. graminearum*/*G. zeae*, can produce ascospore-bearing perithecia in carrot agar cultures. However, multiple attempts to induce perithecial production in *F. globosum* under these conditions were not successful. Although the *MAT* locus controls mating in ascomycetes, other factors (e.g. temperature) may affect mating. In fact, strains of *Fusarium oxysporum* have *MAT* genes that can be transcribed [49], but sexual reproduction has not yet been observed in this species.

Genetic factors other than the *MAT* idiomorphs also can play a role in the ability of the fungus to reproduce sexually, and therefore account for the genetic variation among strains. A study by Shim *et al*. [38] demonstrated that *FSR1* has a role in fungal virulence and sexual fertility in *F. verticillioides* and *F. graminearum*. *FSR1* encodes an 823-codon open reading frame interrupted by two introns, and the translated Fsr1 protein shares 60% sequence homology with the Sordaria macrospora Pro11, a multimodular protein with a regulatory role in cell differentiation and ascocarp development. Fsr1 mutants of *F. verticillioides* retained their male fertility but cannot function as a

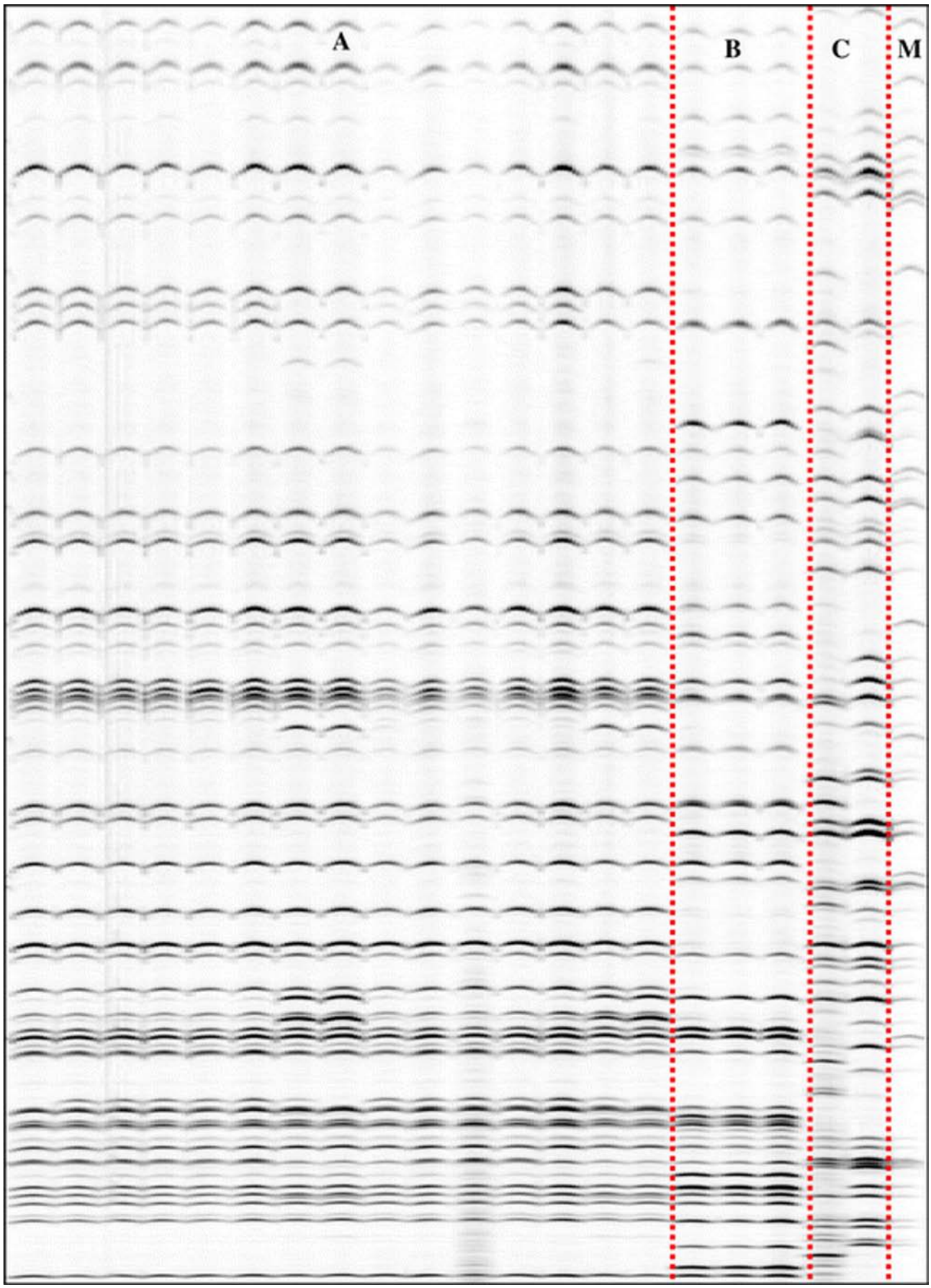

Figure 2. Li-Cor AFLP gel electrophoresis of selective amplification reactions using primer pair *Eco*RI+AC and *Mse*I+AC. (A) 15 maize isolates; (B) 3 wheat isolates, (C) reference strains *F. subglutinans* MRC 7828 and *F. circinatum* MRC 7870, and (M) Marker.

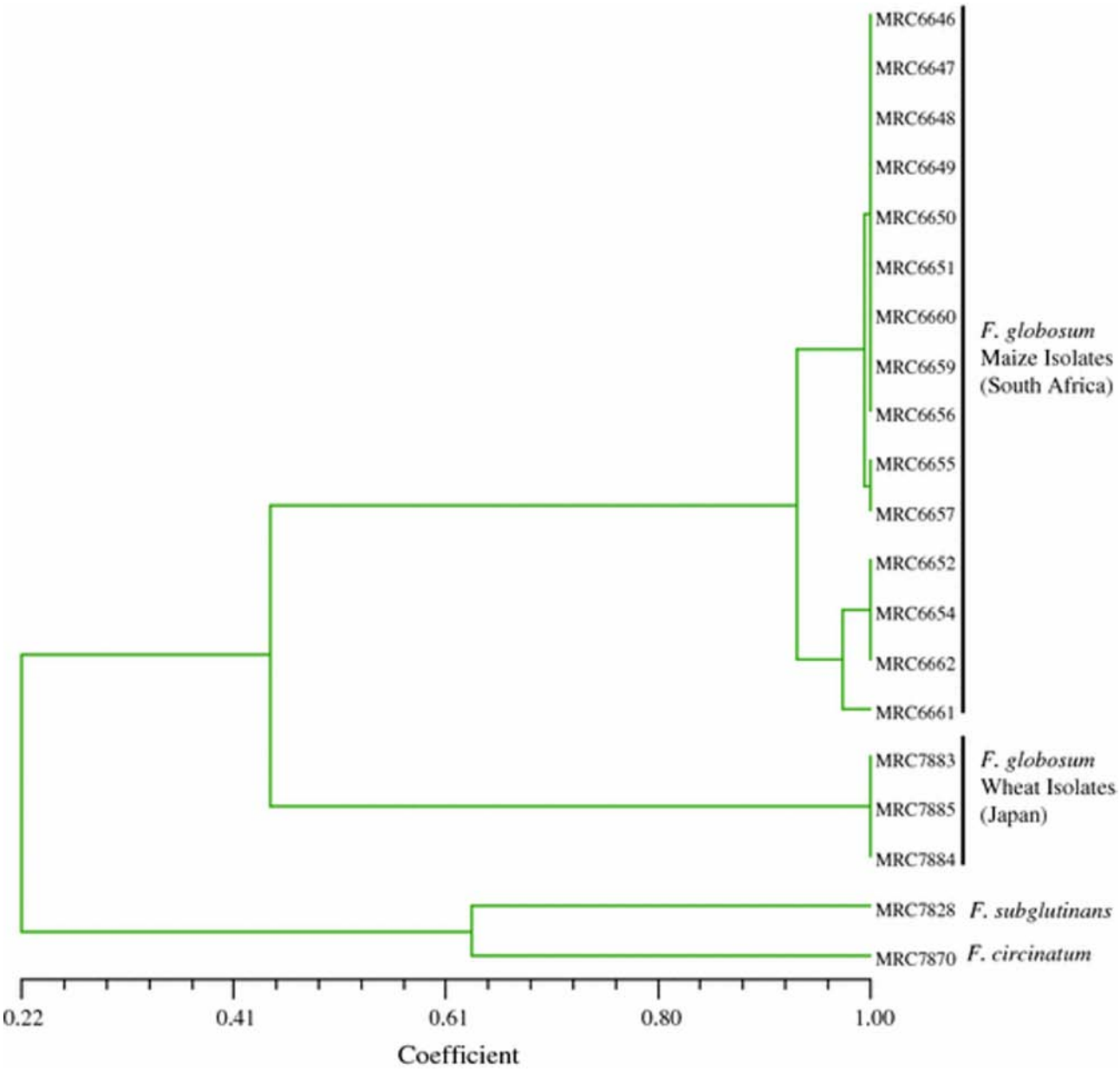

Figure 3. Genetic distance analyses of AFLP data matrix using the Dice Coefficient of Similarity for *F. globosum* strains isolated from maize and wheat with *F. subglutinans* MRC 7828 and *F. circinatum* MRC 7870 as reference strains.

female parent. Similarly, the disruption of *FSR1* in the homothallic fungus, *F. graminearum*, resulted in a loss of self-fertility and perithecial development. These results suggested that Fsr1 protein appears to be critical in the mating of filamentous fungi by specifically controlling the development of female fertility.

Although *F. globosum* isolates did not form perithecia in carrot agar culture, most maize isolates of the fungus produced, sclerotia, compact masses of hardened mycelia. In ascomycetes, sclerotia serve as resting structures that allow fungi to survive adverse conditions such as drought, cold or heat. Sclerotia can be an indicator of female fertility in *Fusarium*. However, the production of such structures can vary widely within a species [21]. Sclerotia-like structures are frequently observed in cultures of sexually reproducing species such as *F. verticillioides*, *F. proliferatum* and *F. sacchari*, as well as with *Fusarium* species where no sexual stage has been identified, such as *F. oxysporum* and *F. miscanthi*. In carrot agar, maize isolates of *F. globosum* also formed large orange sporodochia that darkened with age. Such sporodochia were not reported in the original description of *F. globosum* [33]. However, this may reflect the fact that the original description of *F. globosum* utilized

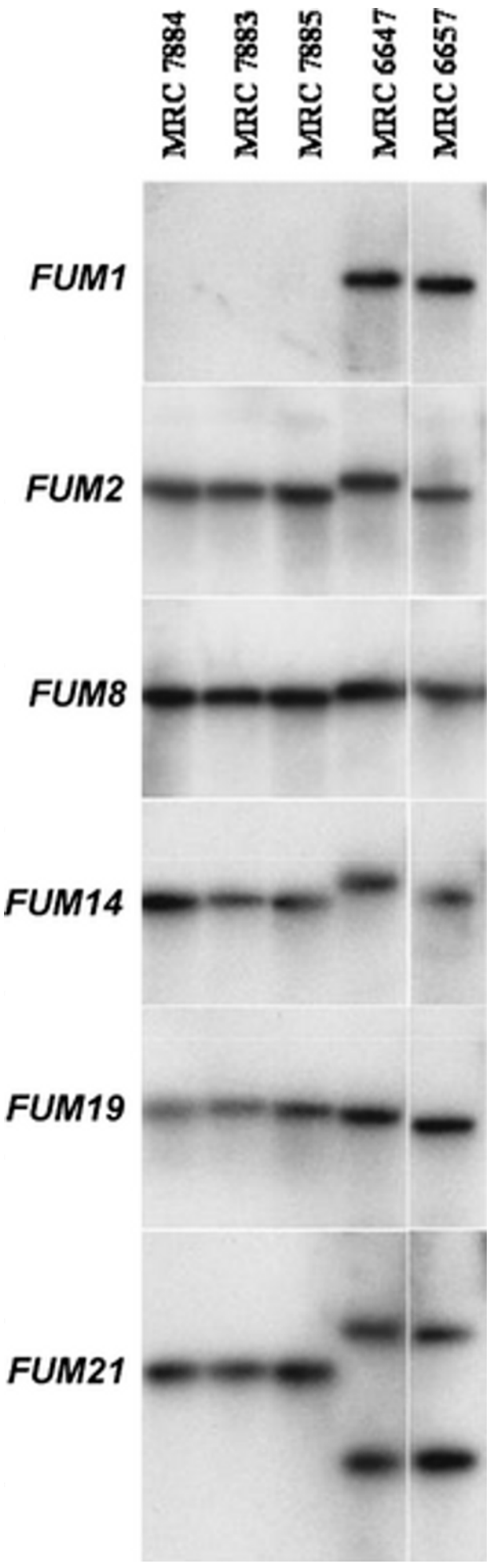
MRC 7884
MRC 7883
MRC 7885
MRC 6647
MRC 6657
FUM1
FUM2
FUM8
FUM14
FUM19
FUM21

Figure 4. The Southern Blot analysis was employed to determine the presence or absence of fumonisin biosynthetic genes *FUM1*, *FUM2*, *FUM8*, *FUM14*, *FUM19* and *FUM21* in the genomes of *F. globosum* strains from Japanese wheat (MRC 7883, MRC 7884, MRC 7885) and South African maize (MRC 6647, MRC6657). Hybridization signal was detected for *FUM2*, *FUM8*, *FUM14*, *FUM19* and *FUM21* in both the South African maize and Japanese wheat isolates examined, whereas the hybridization signal for *FUM1* was only detected in the maize isolates

carnation leaf agar (CLA) and potato dextrose agar (PDA), but not carrot agar.

AFLP analysis provided the opportunity to compare the genetic variability among the wheat and maize isolates, which exhibited morphological differences on carrot agar. The AFLP similarity analysis resolved the *F. globosum* isolates into two distinct clades; one clade corresponded to the Japanese wheat isolates and the other to the South African maize isolates. The Dice Coefficient of 45% observed between the two *F. globosum* strongly suggests that the wheat isolates from Japan are not *F. globosum*. It is possible that differences in the two populations of *F. globosum* may be related to their association with different hosts, but it is also possible that the differences between the populations resulted from their different geographical origins. However, because there are only three Japanese wheat isolates available, definitive statements about host and geographical specialization do not seem warranted when based on so few isolates.

O'Donnell *et al*. [28] investigated the phylogenetic relationships of the *Gibberella fujikuroi* complex, including *F. globosum*, using parsimony analyses performed on datasets of the 28S rDNA, mtSSU rDNA and β-tubulin gene sequences. All three Japanese wheat isolates and four South African maize isolates (viz. MRC 6647, MRC 6648, MRC 6657 and MRC 6660) were listed as strains included in the study. The analysis resolved *F. globosum* and other species such as *F. fujikuroi*, *F. proliferatum* and *F. sacchari* into a clade that O'Donnell *et al*. [28] designated as the Asian clade. However, sequences from multiple isolates were not reported in the analysis; instead, results from only one sequence per species (i.e. exemplar sequences) were reported. Thus, the analysis of O'Donnell *et al*. [28] did not reveal sequence variation within *F. globosum*. In the strict consensus trees based on the internal transcribed spacer (ITS) sequences (GenBank accession number U61687), only the Japanese wheat (*Triticum*) isolates were included in the analysis by O'Donnell *et al*. [28]. Also, the nucleotide sequences of 28S rDNA (U61661), mtSSU rDNA (U61609) and β-tubulin genes (U61583, U61557 and U61635) deposited in the GenBank database only lists a Japanese wheat isolate, viz MRC 7884 (NRRL 25190), as the organism used for sequencing. It is not clear whether different strains or a representative strain for each of the *Fusarium* species was used for analyses. The sequencing of a Japanese wheat isolate rather than the ex-holotype isolate from maize in South Africa, which is MRC 6647, as a representative strain could be the reason why *F. globosum* was grouped in the Asian clade. Alternatively, if both South African maize and Japanese wheat isolates were included in the analyses, it is possible that DNA sequence data provided insufficient resolution to distinguish between the closely related South African maize and Japanese wheat isolates. It was suggested that AFLPs be the method of choice for phylogenetic studies of closely related organisms when other characters (e.g. morphological characters) are identical [3, 16]. An advantage of the AFLP procedure is the fact that the whole genome is used as the source of information for the banding patterns. This technique was first applied by Leissner *et al*. [19] to discriminate between strains of *F. graminearum*. AFLP has since proven to be a powerful tool for evaluating genetic variation in populations and has resolved many other questions concerning mating type and *formae speciales* in the genus *Fusarium* [2, 4, 11, 21, 22, 36, 51].

South African maize isolates and Japanese wheat isolates can also differ in conidial germination as Glenn [15] reported that conidial germ tubes of maize isolates MRC 6647 and MRC 6660 penetrated the surface of an agar medium, whereas germ tubes of Japanese wheat isolate MRC 7884

(NRRL 25190) did not. These results further support the hypothesis that the maize and wheat isolates represent distinct populations.

The results obtained from the Southern Blot analysis also demonstrated dissimilarity between the South African maize isolates and the Japanese wheat isolates. The hybridization signal for *FUM2*, *FUM8*, *FUM14*, *FUM19* and *FUM21* were detected in the genomes of all five *F. globosum* strains examined, which included two maize and three wheat isolates. In contrast, *FUM1* was detected only in the maize isolates MRC 6647 (ex-holotype) and MRC 6657. A 290-bp *FUM1* fragment was amplified from genomic DNA of all maize isolates, including the strains used in the Southern Blot analysis (data not shown). These *FUM1* fragments were 100% homologous and showed 72% homology to the *FUM1* locus AF15573 (GenBank) of *Gibberella moniliformis*. These results indicate that in the Japanese wheat isolates, *FUM1* is either absent or diverged markedly from the *FUM1* orthologue in the South African maize isolate. This would be an obvious explanation for the dissimilarity in fumonisin profiles between the South African maize and the Japanese wheat isolates.

Most maize isolates produced FB_1 FB_2 and FB_3 analogues of fumonisins; a few isolates did not produce detectable levels of either FB_2 or FB_3, and MRC 6650 produced only FB_1 at very low levels, similar to the wheat isolates, and very low levels of FB also were observed in MRC 6661 and MRC 6662. Generally, strains that produce fumonisins at 0 to ~10 mg/kg are thought to have the same production profile, which would most likely be no or trace production. Genetic factors, specifically the *FUM* genes, could result in the different fumonisin profiles observed between the different host isolates. Inactivation of *FUM1*, *FUM6* or *FUM8* blocks fumonisin production in *F. verticillioides*, which indicates these three genes are essential for fumonisin production [29, 37]. It is possible that one of these genes could have been inactivated or deleted in the fumonisin non-producing wheat isolate MRC 7884. Similarly, strains lacking FB_2 and/or FB_3 could be lacking the activity of the *FUM2* and/or *FUM3* proteins, as *FUM2* and *FUM3* deletion mutants were shown to only produce FB_2 and FB_4, and FB_3 and FB_4, respectively [8, 32]. The fumonisin B profiles where at least two different profiles were observed, support the other results in this study that the maize and wheat isolates are not the same species.

The AFLP analyses shed some insight into the reproductive mode of *F. globosum*. The Dice similarity index observed among the maize isolates is very high, which suggests that the maize isolates of *F. globosum* are clonal and reproduce by asexual rather than sexual cycle [20]. This is supported by the low index of genetic diversity (G_{ST}) among the maize isolates. This low variability within these strains, and the association of each group with one host, would also be indicative of clonality, and therefore the lack of meiotic recombination which is the major source of variability. However, the possibility of sexual reproduction should not be ruled out, because sexual spores of a homothallic haploid fungus are identical to the parent and therefore would result in clonality. In order to devise effective strategies to control pathogen growth, to diminish fumonisin production in host plants, and thereby reduce the impact on human and animal health, knowledge of the genetic variability of mycotoxin-producing fungi is essential to be able to identify populations that are potentially toxigenic or shows differential host preferences.

In conclusion, isolates of *F. globosum* from South African maize and Japanese wheat differ in multiple traits. AFLP analysis indicates that the two groups of isolates represent genetically distinct populations that may be different species. Further research is needed to clarify this. The absence of perithecia coupled by the low genetic variability observed among the maize *F. globosum* strains implies that this *Fusarium* species is indeed an asexually reproducing fungus.

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