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Fusarium ananatum sp. nov. in the *Gibberella fujikuroi* species complex from pineapples with fruit rot in South Africa

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ABSTRACT

Pineapple (*Ananas comosus*) is native to South America and widely planted as a fruit crop in the tropics and sub-tropics. This plant is susceptible to a number of fungal diseases of which the most severe is fusariosis. The disease is caused by *Fusarium guttiforme* and occurs only in South and Central America. The occurrence of a similar disease on pineapples in South Africa has prompted a re-evaluation of the *Fusarium* sp. associated with pineapple fruit rot. Phylogenetic relationships of isolates from pineapples collected in Brazil and South Africa were assessed based on sequence data for the translation elongation factor-1-α, histone H3 and β-tubulin gene regions. Analyses showed that the South African isolates represent a species distinct from Brazilian isolates. The South African isolates are characterised by a concentration of aerial mycelium at the centres of the colonies, different to the Brazilian isolates that have an even distribution of aerial mycelium. Both phylogenetic and morphological data show that the disease on pineapple in South Africa is caused by a new *Fusarium* species described here as *F. ananatum* sp. nov.

Introduction

Pineapple [*Ananas comosus* (L.) Merr.] is native to South America and is the fourth most important crop planted in the tropics (Ploetz 2001). The first report of a serious disease caused by a *Fusarium* species and known as fusariosis of pineapple was from Argentina in 1954 (Rohrbach 1994). Ten years later, this disease was reported from Brazil, where pineapple is an economically important crop. Pineapple fusariosis was so serious at that time that Brazil lost its position as the world's leading pineapple producer (Ploetz 2001; Ventura *et al.* 1993a).

All pineapple plant parts can be infected with the causal agent of fusariosis, but symptoms are most conspicuous on the fruit. Symptoms include bent or dead stem apices, shortened stems, disrupted phyllotaxy, general stunting and chlorosis (Pires de Matos 1995; Ploetz 2001). Fruit symptoms include an initial discolouration of the infected area exemplified by light to dark brown fruitlet septa, which may extend into the fruitlet core. The diseased areas become sunken and profuse pink fungal sporulation and gum exudation is evident (Pires de Matos 1995; Rohrbach 1994). Infection can occur from the start of flowering through all stages of fruit development. However, it is most severe when plants are infected during the early stages of flowering (Ploetz 2001).

Previously, the fungus causing fusariosis in pineapple was identified as *F. subglutinans* (Wollenw. & Reinking) P.E. Nelson,

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Toussoun & Marasas (basionym: *F. moniliforme* J. Sheld. var. *subglutinans* Wollenw. & Reinking). The species is specific to pineapple, and causes fruitlet core rot and fusariosis (Ploetz 2001). Based on its host specificity, Ventura *et al.* (1993b) proposed a new *forma specialis*, *F. subglutinans* f. sp. *ananas* for the fungus. Nirenberg & O'Donnell (1998) described this fungus as *Fusarium guttiforme* Nirenberg & O'Donnell.

F. guttiforme forms part of the *G. fujikuroi* complex. Species in this complex can be distinguished from each other based on morphological characteristics and DNA sequence comparisons (Nirenberg & O'Donnell 1998; O'Donnell *et al.* 1998). The morphological characteristics used to distinguish between species in this group include conidiophore arrangement on the aerial mycelium, the number of conidiogenous openings on the polyphialides, the presence or absence of sterile coils or curved hyphae, and macroconidial morphology (Aoki *et al.* 2001; Nirenberg & O'Donnell 1998). DNA based characterisation most commonly considers sequence data for the (β-tubulin, translation elongation factor-1α and mitochondrial small subunit (mtSSU) genes (Aoki *et al.* 2001; O'Donnell *et al.* 1998).

Pineapple fusariosis has been reported only from South America (Ploetz 2001). In Cuba, a disease called "fusariose" caused by *F. subglutinans* has been reported (Borras *et al.* 2001; Hildalgo *et al.* 1999), but it is not clear whether it is the same disease as fusariosis in South America. A similar disease known as fruitlet core rot occurs in Hawaii (Rohrbach & Pfeiffer 1976), and the associated '*F. moniliforme*' strains have been incorrectly referred to as *F. guttiforme* (Nirenberg & O'Donnell 1998; Rohrbach & Schmitt 2003). Recently, a *Fusarium* species was isolated from pineapples with a fruit rot disease in South Africa and there was concern that this might reflect a first report of fusariosis in the country. Alternatively, the disease might have been the same as the one known as 'black spot' and associated with *Penicillium funiculosum* and '*F. moniliforme*' in South Africa (Edmonstone-Sammons 1958). The aim of this study was to compare South African isolates from diseased pineapples with those from Brazil, including the ex-type isolate of *F. guttiforme*. Isolates were compared based on DNA sequences for the translation elongation factor-1α, the partial β-tubulin gene, and the histone H3 gene as well as on their morphology.

Materials and methods

Symptoms and isolations

Diseased pineapple fruit were obtained from Hluhluwe, KwaZulu Natal. The symptoms on these fruit included an initial off-colour appearance, followed by the tissue becoming sunken with characteristic V-shaped lesions appearing on the outside of the fruit. This extends to the internal tissue and ultimately manifests itself as a core rot.

Primary isolations from the diseased material were done by placing small pieces (3 mm) of diseased tissue onto *Fusarium* selective medium (Nelson *et al.* 1983) in Petri dishes. These Petri dishes were incubated at 25 °C under cool-white fluorescent illumination. The plates were checked routinely and all the colonies with typical *Fusarium* morphology were transferred to half-strength potato dextrose agar (PDA) (Merck, Darmstadt, Germany). Single conidial cultures were stored using a cryopreservation method at −70 °C in 15 % glycerol aqueous solution.

Fungal isolates

All isolates used in this study (Table 1) are maintained in the *Fusarium* culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and in the Medical Research Council (MRC) Culture Collection, Tygerberg, Cape Town, South Africa. Representative isolates have also been deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

Morphological comparisons

Isolates were grown on synthetic low nutrient agar (SNA) (Nirenberg 1976) and carnation leaf agar (CLA) (Nelson *et al.* 1983) for 7 d at 25 °C, under near ultraviolet light. Fungal structures produced on these media were mounted on microscope slides in lactophenol with cotton blue and used in the morphological comparison of the South African and Brazilian groups of isolates. Colony colour was assigned using the colour charts of Rayner (1970) for isolates grown on PDA 7 d at 25 °C, under near ultraviolet light. Growth rates were determined by placing a single macroconidium (Nelson *et al.* 1983) on a PDA plate and calculating the average growth in mm over 5 d. The standard errors were determined for data representing each isolate at all temperatures. The presented measurements are the average of 50 measurements per morphological structure.

DNA extraction and amplification

Isolates were grown in complete medium (CM) (Correll *et al.* 1987) at 25 °C for 7 d. DNA was isolated using a modified version of the technique described by Raeder & Broda (1985). Mycelium was placed in Eppendorf tubes and ground with *ca.* 10 μg sterile, chemically treated sand in 500 μL of DNA extraction buffer [DEB: 200 mM Tris—HCl pH 8, 150 mM NaCl, 25 mM EDTA, 0.59 % SDS]. Thereafter, 500 μL of phenol and 300 μL chloroform were added, mixed and centrifuged for 30 min at 10 000 rpm. The phenol/chloroform step was repeated until the interface was clean. The supernatant was transferred to a new tube and double the volume of 100 % ethanol was added and mixed. The DNA was allowed to precipitate at 4 °C overnight and then pelleted by centrifugation for 30 min at 11 000 rpm. Pellets were washed with 300 μl 70 % ethanol, dried and resuspended in 50 μL sterile distilled water and 3 μL RNase (2.5 μM) (Roche Pharmaceuticals, Basel, Switzerland).

Extracted DNA was used as template in PCR reactions to amplify regions of the histone H3, β-tubulin (BT) and translation elongation factor-1α (TEF) genes. The histone H3 gene region was amplified using primer sets H3-1a (5′-ACTAAGCAGACCGCCCGCAG-3′) and H3-1b (5′-GCGGG CGAGCTGGATGTCCTT-3′) (Glass & Donaldson 1995). Part of the TEF was amplified using the primer set EF1 (5′-CGAAT CTTTGAACGCACATTG-3′) and EF2 (5′-CCGTGTTTCAA GACGGG-3′) (Carbone & Kohn 1999). The BT gene region was amplified using the primer set T1 (5′-AACATGCGTGAGATTG

Table 1 – List of the *Fusarium* strains included in the phylogenetic analyses, representing closely related species in the *Gibberella fujikuroi* species complex.

Strain number[a]	Species	Original identification	Origin	Collector	GenBank accession number			Reference for DNA sequences
					B-tubulin	Elongation factor 1-α	Histone H3	
MRC 8165/FCC 2986/CMW 18685/CBS 118516, ex-type	*F. ananatum*	*Fusarium* sp.	*Ananas comosus*, South Africa	PS Van Wyk	DQ282174	DQ282167	DQ282181	This study
MRC 8166/FCC 2988/CMW 18686/CBS 118517	*F. ananatum*	*Fusarium* sp.	*Ananas comosus*, South Africa	PS Van Wyk	DQ282178	DQ282171	DQ282182	This study
MRC 8167/FCC 2990/CMW 18687/CBS 118518	*F. ananatum*	*Fusarium* sp.	*Ananas comosus*, South Africa	PS Van Wyk	DQ282176	DQ282169	DQ282183	This study
MRC 8168/FCC 2991/CMW 18688/CBS 118519	*F. ananatum*	*Fusarium* sp.	*Ananas comosus*, South Africa	PS Van Wyk	DQ282175	DQ282168	DQ282180	This study
FCC 4251/CMW 28597	*F. ananatum*	*Fusarium* sp.	*Ananas comosus*, South Africa	A Jacobs	EU668309	EU668312		This study
FCC 4252/CMW 28598	*F. ananatum*	*Fusarium* sp.	*Ananas comosus*, South Africa	A Jacobs	EU668310	EU668313		This study
FCC 4253/CMW 28599	*F. ananatum*	*Fusarium* sp.	*Ananas comosus*, South Africa	A Jacobs	EU668311	EU668314		This study
NRRL 25300, ex-type	*F. begoniae*		*Begonia* hybrid, Germany	Unknown	AY329045	AY329036		O'Donnell *et al.* 1998
NRRL 13618, ex-type	*F. bulbicola*		*Nerine bowdenii*, Germany	Unknown	U61546	AF160294		O'Donnell *et al.* 1998
NRRL 25331, ex-type	*F. circinatum*		*Pinus radiata*, USA	Unknown	U61547	AF160295	AF150852	O'Donnell *et al.* 1998; Steenkamp *et al.* 1999
MRC 6213/KSU 10850	*F. circinatum (MAT H-2)*[b]		*Pinus* sp., South Africa	A Viljoen			AF150844	Steenkamp *et al.* 1999
MRC 7488/KSU 10847	*F. circinatum (MAT H-1)*		South Africa	A Viljoen			AF238478	Steenkamp *et al.* 1999
NRRL 25181, ex-type	*F. concentricum*		*Musa sapientum*, Costa Rica	Unknown	AF333951	AF333935		O'Donnell *et al.* 1998
MRC 6570/KSU 1993	*F. fujikuroi (MAT C-1)*		*Oryza sativa*, Taiwan	JF Leslie			AF150873	Steenkamp *et al.* 1999
MRC 6571/KSU 1995	*F. fujikuroi (MAT C-2)*		*Oryza sativa*, Taiwan	JF Leslie			AF150872	Steenkamp *et al.* 1999
MRC 6782/CMW 30032/CBS 124146	*F. guttiforme*	Published as *F subglutinans* f. sp. *ananas* in Steenkamp *et al.* 1999	*Ananas comosus*, Brazil	JA Ventura	DQ282177	DQ282170	AF150834	This study
MRC 6783/CMW 30033/CBS 124145	*F. guttiforme*	Published as *F subglutinans* f. sp. *ananas* in Steenkamp *et al.* 1999	*Ananas comosus*, Brazil	JA Ventura	DQ282173	DQ282166	AF150833	This study
MRC 7539, ex-type	*F. guttiforme*		*Ananas comosus*, Brazil	H Nirenberg	DQ282172	DQ282165	DQ282179	This study
NRRL 22945	*F. guttiforme*	Published as *F. guttiforme* in O'Donnell *et al.* 1998 but reidentified in this study as *F. ananatum*	*Ananas comosus*, England	Unknown	U34420	AF160297		O'Donnell *et al.* 1998
MRC 6784	*F. guttiforme*	Published as *F subglutinans* f. sp. *ananas* in Steenkamp *et al.* 1999	*Ananas comosus*, Brazil	JA Ventura			AF150836	Steenkamp *et al.* 1999

(*continued on next page*)

Table 1 – (*continued*)

Strain number[a]	Species	Original identification	Origin	Collector	GenBank accession number			Reference for DNA sequences
					B-tubulin	Elongation factor 1-α	Histone H3	
MRC 6785	*F. guttiforme*	Published as *F subglutinans* f. sp. *ananas* in Steenkamp *et al.* 1999	*Ananas comosus*, Brazil	JA Ventura			AF150835	Steenkamp *et al.* 1999
MRC 7559	*F. mangiferae*		*Mangifera indica*, South Africa	Unknown			AF236779	Steenkamp *et al.* 1999
MRC 3477	*F. mangiferae*		*Mangifera indica*, South Africa	Unknown			AF150868	Steenkamp *et al.* 1999
NRRL 13488, ex-type	*F. nygamai*		*Sorghum bicolor*, Australia	Unknown	U34481	AF160273		O'Donnell *et al.* 1998
MRC 7548/KSU 5111	*F. nygamai (MAT G-1)*		Lab cross	JF Leslie			AF150854	Steenkamp *et al.* 1999
MRC 7549/KSU 5112	*F. nygamai (MAT G-2)*		Lab cross	JF Leslie			AF150855	Steenkamp *et al.* 1999
MRC 6212	*F. oxysporum*		South Africa	A Viljoen			AF150832	Steenkamp *et al.* 1999
NRRL 26374	*F. oxysporum*		Unknown	Unknown	AF008518	AF008483		O'Donnell *et al.* 1998
NRRL 22944	*F. proliferatum*		*Cattleya* hybrid, Germany	Unknown	U34471	AF160280		O'Donnell *et al.* 1998
NRRL 31071	*F. proliferatum*		Unknown	Unknown			AF291059	Steenkamp *et al.* 1999
MRC 6568/KSU 4853	*F. proliferatum (MAT D-1)*		Lab cross	JF Leslie			AF150871	Steenkamp *et al.* 1999
MRC 6569/KSU 4854	*F. proliferatum (MAT D-2)*		Lab cross	JF Leslie			AF150870	Steenkamp *et al.* 1999
NRRL 22946, ex-type	*F. pseudocircinatum*		*Solanum* sp., Ghana	Unknown	U34482	AF160271		O'Donnell *et al.* 1998
NRRL 13999	*F. sacchari*		Unknown	Unknown	U34469	AF160278		O'Donnell *et al.* 1998
MRC 6524/KSU 3852	*F. sacchari (MAT B-1)*		Lab cross	JF Leslie			AF150861	Steenkamp *et al.* 1999
MRC 6525/KSU 7853	*F. sacchari (MAT B-2)*		Lab cross	JF Leslie			AF150860	Steenkamp *et al.* 1999
MRC 7873	*F. sterilihyphosum*		*Mangifera indica*, South Africa	Unknown			AF236774	Steenkamp *et al.* 1999
MRC 7605	*F. sterilihyphosum*		*Mangifera indica*, South Africa	Unknown			AF236773	Steenkamp *et al.* 1999
MRC 6512/KSU 2192	*F. subglutinans (MAT E-2)*		*Zea mays*, USA	JF Leslie	AF366552	AF160289	AF150844	O'Donnell *et al.* 1998; Steenkamp *et al.* 1999
MRC 6483/KSU 990	*F. subglutinans (MAT E-1)*		*Zea mays*, USA	JF Leslie			AF150845	Steenkamp *et al.* 1999
MRC 1077	*F. subglutinans*		*Zea mays*, South Africa	FC Wehner			AF150837	Steenkamp *et al.* 1999
NRRL 22045	*F. thapsinum*		Unknown	Unknown	U34473	AF160270		O'Donnell *et al.* 1998
MRC 6536/KSU 4094	*F. thapsinum (MAT F-1)*		Lab cross	JF Leslie			AF150857	Steenkamp *et al.* 1999
MRC 6537/KSU 4093	*F. thapsinum (MAT F-2)*		Lab cross	JF Leslie			AF150856	Steenkamp *et al.* 1999
NRRL 22172	*F. verticillioides*		Unknown	Unknown	U34468	AF160262		O'Donnell *et al.* 1998
MRC 6155/KSU 149	*F. verticillioides (MAT A-1)*		*Sorghum bicolor*, USA	JF Leslie			AF150858	Steenkamp *et al.* 1999
MRC 6191/KSU 999	*F. verticillioides (MAT A-2)*		*Zea mays*, USA	JF Leslie			AF150859	Steenkamp *et al.* 1999

a MRC: Culture collection of the Medical Research Council, Tygerberg, Cape Town, South Africa. NRRL: Agricultural Research Service Culture Collection at the National Centre for Agricultural Utilization Research, Peoria, IL, USA. FCC: The *Fusarium* culture collection of the Tree Protection Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. CMW: The culture collection of Mike Wingfield housed at TPCP, FABI, University of Pretoria. CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
b Mating type tester strains are indicated in brackets with their associated mating population represented by the letter and the number indicating the mating idiomorph.

TAAGT-3′) and T222 (5′-GACCGGGGAAACGGAGACAGG-3′) (O'Donnell *et al.* 2000). The PCR reaction consisted of 1× Roche *Taq* reaction buffer with $MgCl_2$, dNTPs (250 μM each), primers (0.2 μM each), template DNA (25 ng) and Roche *Taq* polymerase (0.5 U) (Roche Pharmaceuticals). The PCR reaction conditions, for the amplification of the histone H3 gene, were an initial denaturation at 92 °C for 1 min. This was followed by 30 cycles of denaturing at 92 °C for 1 min, annealing at 63 °C for 1 min and elongation at 72 °C for 1 min, followed by a final elongation step at 72 °C for 5 min. The TEF and BT gene regions were amplified by initial denaturation at 94 °C for 2 min. This was followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and elongation at 72 °C for 1 min, with a final elongation step at 72 °C for 5 min. The resulting PCR amplicons were purified using a QIAquick PCR Purification kit (QIAGEN, Hilden, Germany).

DNA sequencing and phylogenetic analyses

DNA sequences were determined from PCR amplicons using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, (Applied Biosystems, Warrington, UK) using the primers H3-1a, H3-1b, EF1, EF2, T1, and T222. Sequences generated in this study have been deposited in GenBank (Table 1).

DNA sequences were manually aligned by inserting gaps. Gaps were treated as new state in the subsequent analysis. Phylogenetic analysis was based on parsimony using PAUP 4.0* (Phylogenetic Analysis Using Parsimony* and Other Methods version 4; Swofford 2002). Heuristic searches were done with random addition of sequences (100 replicates), tree bisection-reconnection (TBR) branch swapping, and MULPAR effective and MaxTrees set to auto-increase. Phylogenetic signal in the data sets (*g*1) was assessed by evaluating tree length distributions over 100 randomly generated trees (Hillis & Huelsenbeck 1992). The consistency (CI) and retention (RI) indices were determined for all data sets. Phylogenetic trees were rooted with *F. oxysporum* as monophyletic sister outgroup to the rest of the taxa. Bootstrap analyses were performed to determine branching point confidence intervals (1000 replicates) for the most parsimonious trees generated for the TEF, BT and histone H3 data sets. The combinability of the TEF and BT data sets was tested using the partition homogeneity test in PAUP 4.0* (Farris *et al.* 1994).

Bayesian analyses utilized the Metropolis-coupled Markov Chain Monte Carlo search algorithm as implemented in the program MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001). All Bayesian analyses consisted of 1 000 000 generations running one cold and three heated chains, with Bayesian inference posterior probabilities (biPP) calculated after a burnin was determined. BInt analyses utilized the GTR + I substitution model with separate parameters for each gene (partition) and an eight-category gamma model. The data sets were deposited in Treebase SN 4077.

Mating studies and MAT genes

In order to determine the mating types of the seven isolates from pineapple in South Africa, the *MAT-1* and *MAT-2* loci were amplified using PCR, as described by Steenkamp *et al.* (2000). The *MAT* idiomorphs were amplified with the primer sets GFmat1a (5′-GTTCATCAAAGGGCAAGCG-3′), GFmat1b (5′-TAAGCGCCTC-TTAACGCCTTC-3′), GFmat2c (5′-AGCGT CATT-ATTCG-ATCAAG-3′) and GFmat2d (5′-CTACGTTGA GAGCTGTACAG-3′) (Steenkamp *et al.* 2000). The following PCR reaction mixture was used: 1× PCR buffer with $MgCl_2$, dNTP (250 μM) each, primers (0.1 μM of each), template DNA (25 ng) and 0.5 U Roche *Taq* polymerase (Roche Pharmaceuticals). The PCR reaction conditions were an initial denaturation at 92 °C for 1 min, followed by 35 cycles of denaturation at 92 °C for 30 s, annealing at 63 °C for 30 s and elongation at 72 °C for 30 s. A final elongation step was done at 72 °C for 5 min. The products were resolved on a 1 % agarose gel, containing ethidium bromide (0.2 μg/ml) and visualised under UV light. The presence of the *MAT* idiomorphs in the pineapple isolates was confirmed by amplification of the *MAT* loci.

Results

Symptoms and isolations

The external fruit symptoms on South African pineapples (Fig 1) were similar to but less severe than those reported for *F. guttiforme* infections on pineapples (Ploetz 2001; Rohrbach 1994). Isolations from these symptoms yielded cultures that resembled *F. guttiforme*. Seven of these isolates were used in this study.

Morphological comparisons

Sequence comparisons and phylogenetic analyses on the TEF, BT and histone H3 genes

The amplification of the TEF, BT and histone H3 gene regions resulted in products of 640, 520 and 540 bp, respectively.

Parsimony analysis of combined and separate data sets for the TEF and BT gene regions were done to determine the phylogenetic placement of *F. guttiforme* isolates from Brazil in relation to the South Africa pineapple isolates in the *Gibberella fujikuroi* species complex. The partition homogeneity test showed sufficient probability to accept the null hypothesis ($P < 0.5$) to combine the data sets for the TEF and BT gene regions. Alignment of combined data by inserting gaps resulted in a total of 1059 characters used in the comparison of the different species in the combined data sets. All parsimony-uninformative and constant characters were excluded, resulting in 127 parsimony-informative characters. Heuristic searches on the data set generated one most parsimonious tree (Fig 2). Alignment of separate TEF data set by inserting gaps resulted in a total of 528 characters used in the comparison of the different species. All parsimony-uninformative and constant characters were excluded, resulting in 53 parsimony-informative characters. Heuristic searches on the data set generated six most parsimonious trees (Fig 3). The separate BT data set consisted of 484 parsimony-uninformative and 46 parsimony-informative characters. Heuristic searches on the data set generated two most parsimonious trees (Fig 4).

In the combined data set, the Brazilian isolates from pineapple, grouped together with the ex-type isolate of *F. guttiforme* to form a distinct clade (Fig 2). The South African

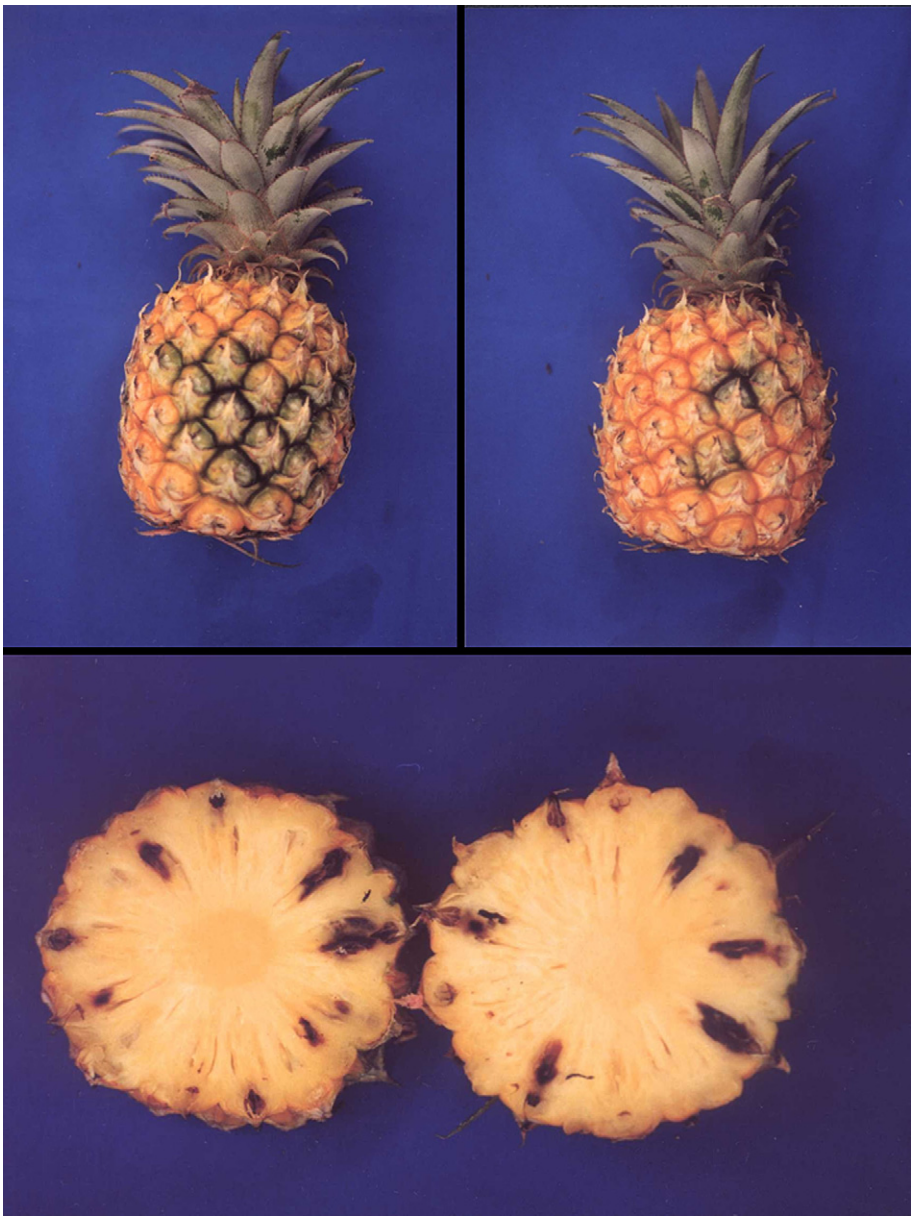

Fig 1 – Internal and external lesions formed on susceptible pineapple fruits.

isolates from diseased pineapple formed a separate clade, with 99 % bootstrap support. The grouping of all *Fusarium* isolates associated with pineapples from both Brazil and South Africa was supported by a bootstrap value of 96 %. Some differences were found in the sequences of the South African pineapple isolates, most likely indicating genetic variation in this population.

In the separate TEF data set (Fig 3), the Brazilian isolates from pineapple grouped together with the ex-type isolate of *F. guttiforme* to form a distinct clade, although not supported by a high bootstrap value. The South African isolates from diseased pineapple formed a separate clade, with 87 % bootstrap support. Some differences were found in the sequences of the South African pineapple isolates, most likely indicating genetic variation in this population. In the separate BT data set the Brazilian isolates from pineapple, grouped together with the ex-type isolate of *F. guttiforme* to form a distinct clade (Fig 4). The South African isolates from diseased pineapple formed a separate clade with 95 % bootstrap support.

Parsimony analysis on the histone H3 gene region was done separately to those of the other gene regions because data from representatives of the different mating populations of *G. fujikuroi* were included to test the grouping of four South African and Brazilian isolates from pineapple. All parsimony-uninformative and constant characters were excluded, resulting in 85 parsimony-informative characters. Heuristic searches on the data set generated one most parsimonious tree after reweighing of the characters based on the CI value (Fig 5). The topography of the tree was similar to that obtained for combined sequences of the TEF and BT gene regions. The Brazilian isolates grouped together with the ex-type culture of *F. guttiforme* (MRC 7539) and they represent a discrete taxon.

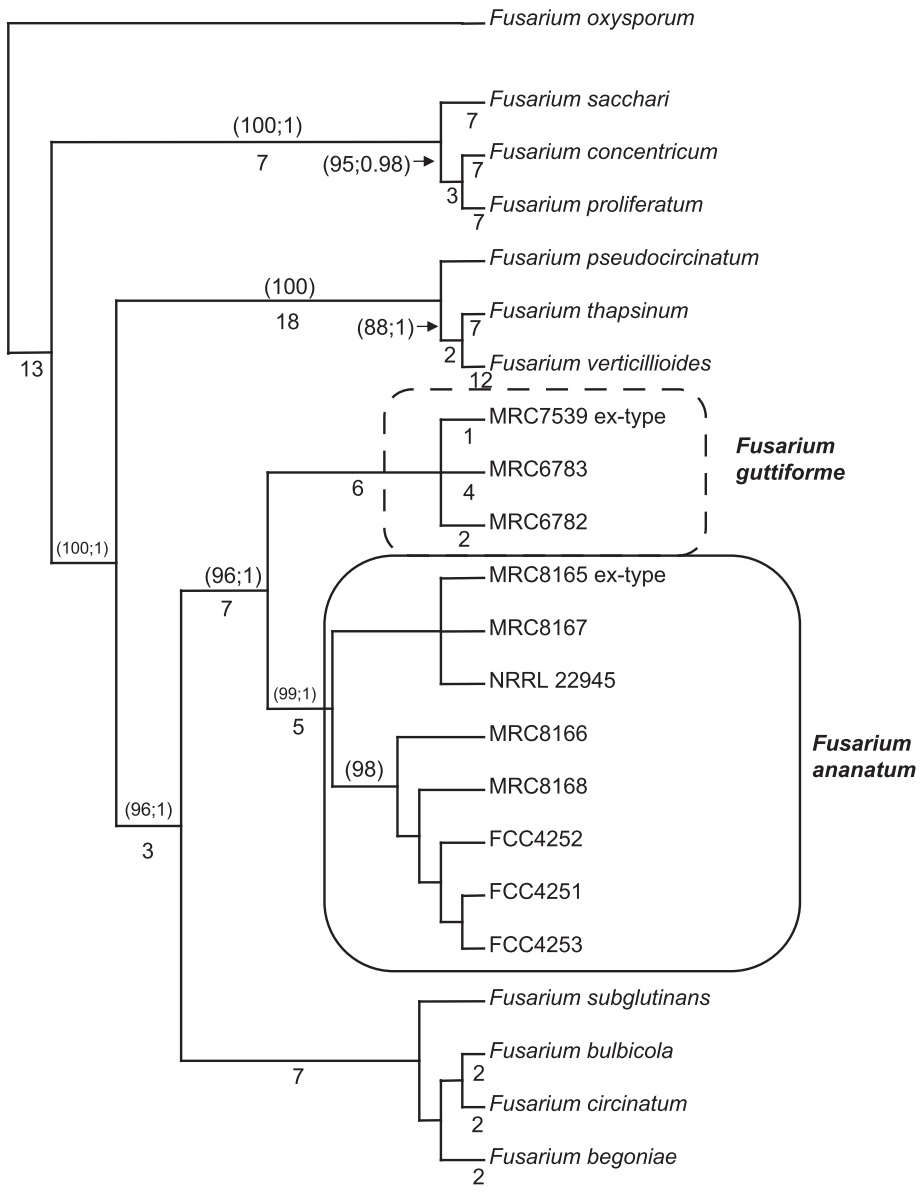

Fig 2 – Phylogenetic tree of *Fusarium ananatum* and related species produced using parsimony of the combined data of the translation elongation factor-1α and β-tubulin genes, with *F. oxysporum* as outgroup. Bootstrap values above 50 % (percentages of 1000 bootstrap replicates) and the Bayesian posterior probability values are indicated in brackets above the branches of the tree. Branch lengths are indicated below the branches. Parsimony-informative characters = 127; CI = 0.8593; RI = 0.9395; g1 = −0.860858.

The South African isolates from diseased pineapple resided in a distinct clade supported by a bootstrap value of 88 %.

Based on the results of the phylogenetic analyses, careful morphological comparisons were done. The South African isolates with the ex-type of *F. guttiforme* as well as other Brazilian isolates of the species showed that they could be distinguished based on a number of morphological characteristics. The Brazilian isolates were characterised by dark purple colonies on PDA but with dispersed aerial mycelium and where the conidiophores produced in these hyphae are prostrate. The South African isolates were characterised by saffron-coloured colonies on PDA (Fig 6). In older cultures, a dark purple colour appeared at the colony centres in the South African isolates. Furthermore, the aerial mycelium of these isolates was concentrated at the middle of the SNA plates.

Mating studies and MAT genes

No perithecia were observed in any of the crosses between or amongst the pineapple isolates from Brazil and South Africa. There were also no positive results for crosses between the South African pineapple isolates and the mating tester strains for eight of the biological species in *G. fujikuroi* species

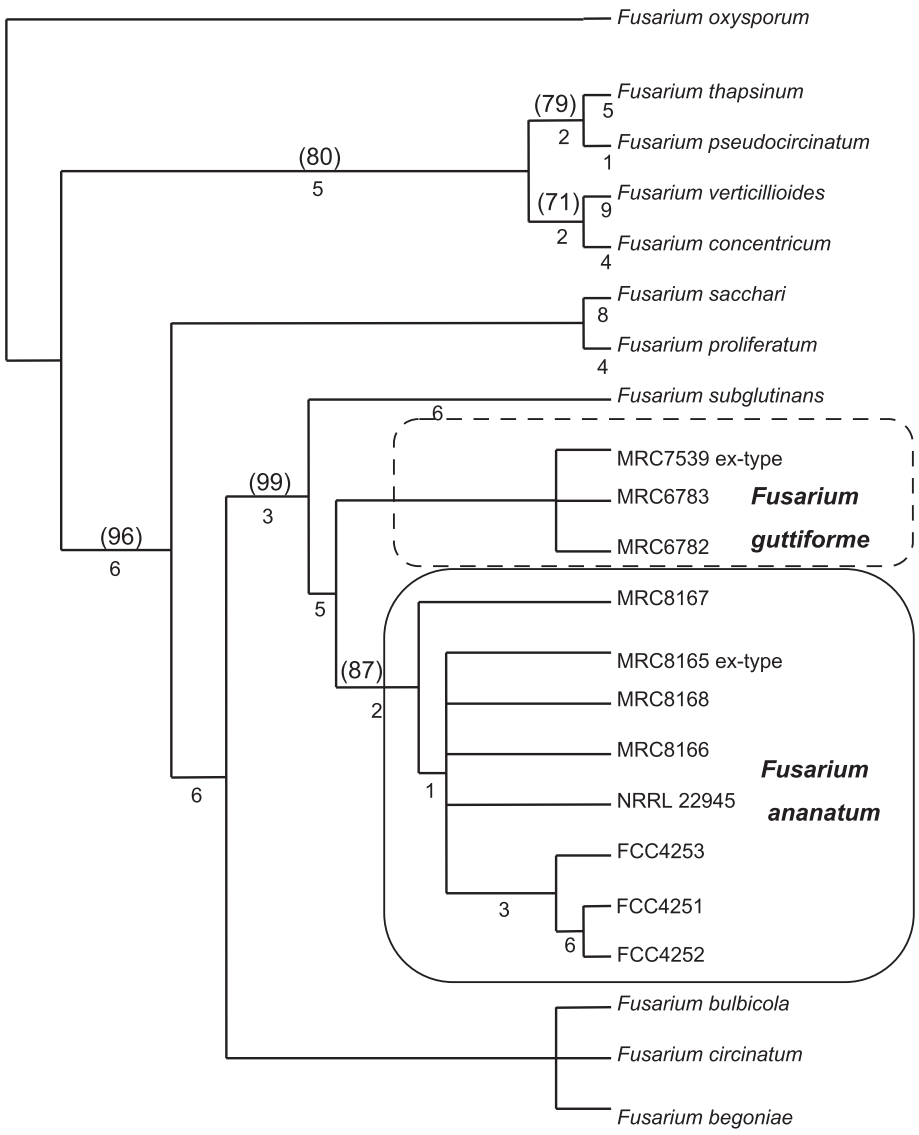

Fig 3 – Phylogenetic tree of *Fusarium ananatum* and related species produced using parsimony of the translation elongation factor-1α gene with *F. oxysporum* as outgroup. Bootstrap values above 50 % (percentages of 1000 bootstrap replicates) are indicated in brackets above the branches of the tree. Branch lengths are indicated below the branches. Parsimony-informative characters = 53; CI = 0.6737; RI = 0.8510; g1 = −0.476216.

complex. The control crosses for all the tester strains produced fertile progeny showing that conditions for these tests were appropriate to stimulate sexual recombination. All of the seven South African isolates had the *MAT-2* idiomorph and were thus of the same mating type.

Taxonomy

The *Fusarium* isolates from pineapple in South Africa could easily be separated from those of *F. guttiforme* from pineapple in Brazil based on DNA sequence comparisons for three different gene regions. These isolates were also phylogenetically distinct from those of all other species in the *G. fujikuroi* species complex. The isolates from pineapple in South Africa could also be distinguished from *F. guttiforme* based on distinct morphological characteristics such as the nature of the conidiophores and the colony colour. This species is, therefore, described as a new taxon as follows.

Fusarium ananatum A. Jacobs, Marasas & van Wyk, **sp. nov.** (Fig 7)

MycoBank no.: MB 511686

Etym.: The specific epithet refers to host, *Ananas comosus*, from which the species was isolated.

Margo coloniae integra. Coloniae crescunt circiter 3.2 mm/d in 25 °C in PDA. Mycelium aerium in medio coloniae congregatum; in PDA incrementum conformatione orbium concentricorum signatum. Mycelium aerium in PDA croceum, infra

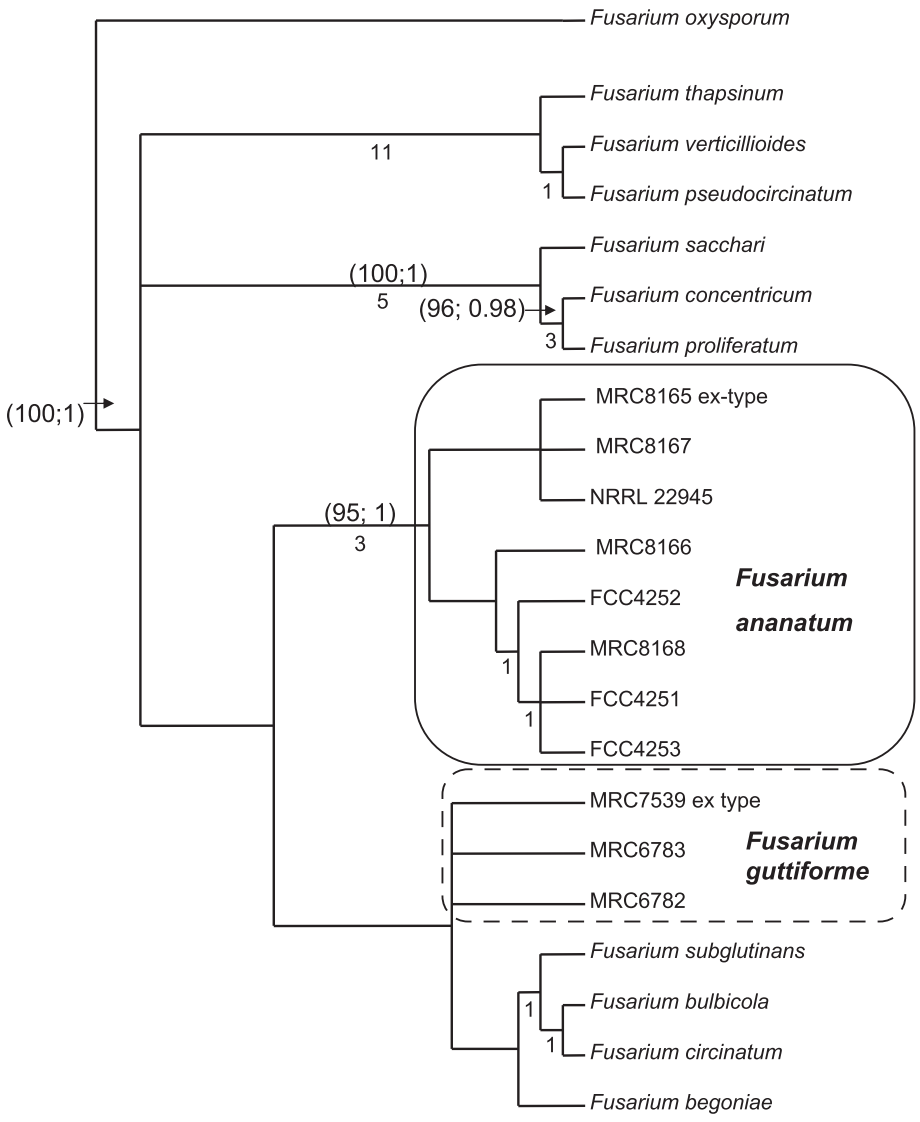

Fig 4 – Phylogenetic tree of *Fusarium ananatum* and related species produced using parsimony of the β-tubulin gene region with *F. oxysporum* as outgroup. Bootstrap values above 50 % (percentages of 1000 bootstrap replicates) and the Bayesian posterior probability values are indicated in brackets above the branches of the tree. Branch lengths are indicated below the branches. Parsimony-informative characters = 46; CI = 0.8593; RI = 0.9395; g1 = −0.860858.

bubalinum. Sclerotia, sporodochia chlamydosporaeque desunt. Hyphae in SNA (2–)3–5(–6) μm latae. Conidia in pseudocapitulis aggregata. Conidiophorae in mycelio aerio erectae e substrato orientes, ramosae vel non, centrum coloniae *versus* plurimae, (15–)22–40(–47) × (1–)2–3 μm. Conidiophorae sympodialiter ramosae, mono- et polyphialidibus instructae. Phialides mycelii aerii cylindricae, monophialidicae, 10–46(–56) × (1–)2–3 μm, ramis 3–17(–22) × (1–)2–3 μm, et polyphialidicae aperturis conidiogenis 2–4, 25–90(–115) × 1–2(–4) μm. Microconidia plerumque non septata raro uniseptata, (6–)8–16(–17) × 1–2 μm. Macroconidia in mycelio aerio facta subfalcata vel fere recta, superficiebus dorsalibus ventralibusque subparallelis, parietibus tenuibus, (14–)16–31(–32) × 3–6 μm.

Colony margin entire. Colonies on PDA with average growth rate of 3.2 mm/d at 25 °C. Aerial mycelium concentrated at the middle of the colony and growth on PDA characterised by the formation of concentric circles. Aerial mycelium on PDA saffron (7f) in colour, reverse of colony buff (19″f) in colour. Sclerotia, sporodochia and chlamydospores absent. Hyphae on SNA (2–)3–5(–6) μm wide. Conidia accumulating in false heads. Conidiophores on aerial mycelium originating erect from substrate, branched or unbranched, numerous towards centre of colony, (15–)22–40(–47) × (1–)2–3 μm. Conidiophores sympodially branched bearing mono- and polyphialides. Phialides of the aerial mycelium, cylindrical, monophialidic, 10–46(–56) × (1–)2–3 μm, with branches 3–17(–22) × (1–)2–3 μm, and polyphialidic, with 2–4 condiogenous openings, 25–90(–115) × 1–2(–4) μm. Microconidia mostly 0-septate with 1-septate conidia occurring less abundantly, (6–)8–16(–17) × 1–2 μm. Macroconidia produced only on aerial mycelium and not in sporodochia,

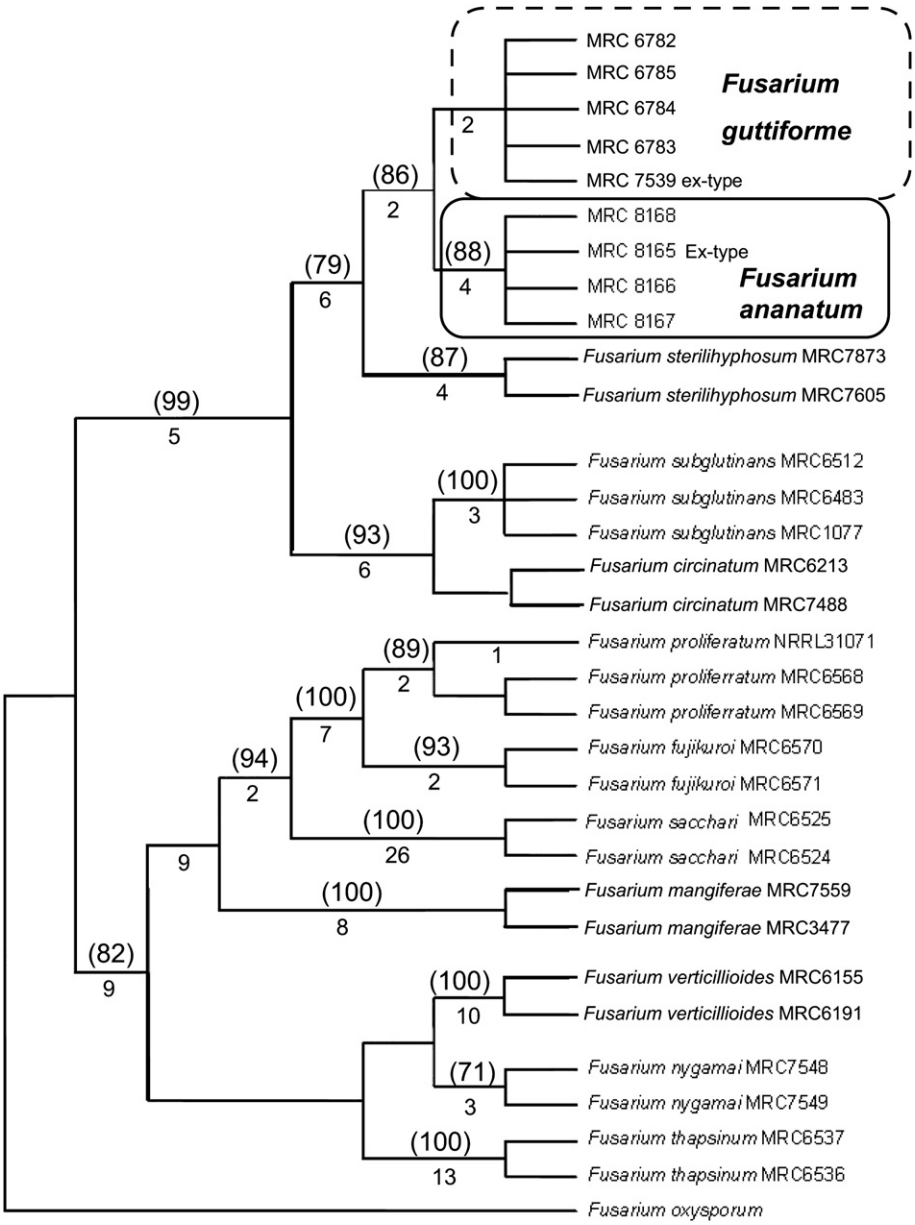

Fig 5 – Phylogenetic tree of *Fusarium ananatum* and related species produced using parsimony of the histone H3 gene with *F. oxysporum* as outgroup. Bootstrap values above 50 % (percentages of 1000 bootstrap replicates) are indicated in brackets above the branches of the tree. Branch lengths are indicated below the branches. Parsimony-informative characters = 53; CI = 0.6737; RI = 0.8510; g1 = −0.476216.

slightly sickle-shaped to almost straight, with the dorsal and ventral surfaces almost parallel with thin walls, 3–4 septate, (14–)16–31(–32) × 3–6 μm.

Materials examined: **South Africa**, *Hluhluwe*, Kwazulu Natal, *A. comosus*, November 2001, P.S. van Wyk, PREM 58713 (*holotype*; dried down culture of MRC 8165, FCC 2986, CMW 18685, CBS 118516). PREM 58714 (*paratype*; dried down culture of MRC 8166, FCC 2988, CMW 18686, CBS 118517); PREM 58715 (*paratype* dried down culture of MRC 8167, FCC 2990, CMW 18687, CBS 118518); PREM 58716 (*paratype* dried down culture of MRC 8168, FCC 2991, CMW 18688, CBS 118519).

Discussion

Results of this study have shown that *Fusarium* isolates from diseased pineapple in South Africa represent a new species described here as *F. ananatum*. This species forms part of the *G. fujikuroi* species complex and can be distinguished from all other species in this group based on DNA sequence comparisons and morphology. *F. ananatum* isolates were supported as monophyletic with high bootstrap support for all three gene phylogenies studied. By contrast, similar results were not observed for *F. guttiforme*.

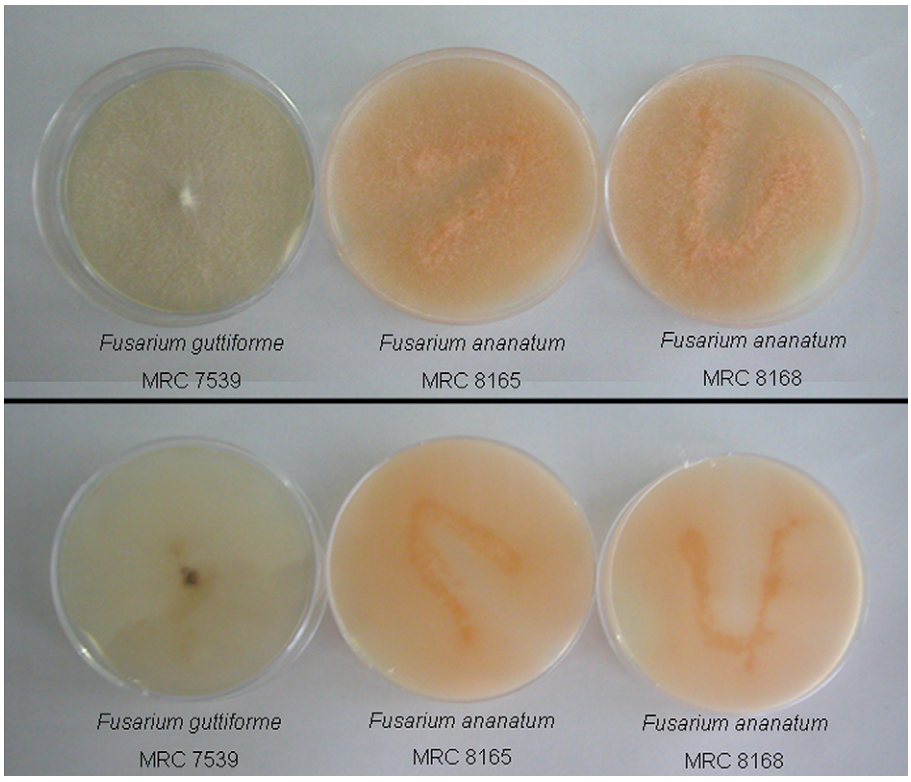

Fig 6 – Colony morphology of *Fusarium ananatum* and *F. guttiforme* on PDA at 25 °C after 5 d. First set of plates represent the top of the colonies and the second row of plates represent the reverse of the colonies.

Although DNA sequence comparisons form the most important basis for distinguishing *F. ananatum* from other related species, it is possible to distinguish the South African fungus based on morphology. The defining morphological characteristics include the nature of the conidiophores on the aerial mycelium and the distribution of the aerial mycelium on the surface of isolates. The original description of the *F. guttiforme* suggested that this species is characterised by erect and prostrate conidiophores (Nirenberg & O'Donnell 1998). After the re-evaluation of the ex-type material (MRC 7539) and the Brazilian isolates, we conclude that *F. guttiforme* has distinctly prostrate conidiophores. This is in contrast to *F. ananatum* that is characterised by erect conidiophores on the aerial mycelium. The colony morphology of these two groups on SNA is also distinct. *F. ananatum* is characterised by concentrations of aerial mycelium at the centres of plates. In contrast, *F. guttiforme* has aerial mycelium distributed evenly over the surface of the colonies. Concentric circles of mycelial growth were also observed in *F. ananatum* cultures while these are absent in *F. guttiforme*. Furthermore, the distinct saffron colony colour of *F. ananatum* on PDA distinguishes it from *F. guttiforme*, which has dark purple coloured colonies on PDA.

Despite numerous attempts to cross isolates, no perithecia were obtained between the tester strains for eight mating populations in the *G. fujikuroi* species complex (Desjardins 2003) and the South African isolates from pineapple. Crosses amongst isolates of *F. ananatum* also failed to produce perithecia. This clearly resulted from the fact that the seven isolates tested all had the same mating type idiomorph. It is thus possible that crosses amongst a larger number of isolates, assuming some have the *MAT-1* idiomorph, might produce a teleomorph.

Symptoms associated with both *F. guttiforme* and *F. ananatum* on fruit are similar but they are less severe in the case of the latter fungus. For *F. guttiforme*, these symptoms include a light to dark brown discolouration of the fruitlet septa that can extend to the fruitlet core (Rohrbach 1994). The infected fruit area initially appears off-colour, then becomes sunken with profuse pink sporulation and exudation of gum (Rohrbach 1994). Similar core discolouration occurs in fruit infected with *F. ananatum*. However, the infected fruit area is characterised by a V-shaped lesion that initially appears discoloured, and then becomes sunken. No exudation of gum was observed in the case of pineapples infected with *F. ananatum*, which appears to be a symptom typical of fusariosis.

In their re-evaluation of the *Gibberella fujikuroi* species complex, O'Donnell *et al.* (1998) included an isolate from *A. comosus* in England (NRRL 22945 = CBS 184.29 = IMI 375350 = DAOM 225144) as *F. guttiforme*. In the present study, this isolate grouped in the clade that accommodates the *F. ananatum* isolates (Figs 2–4). It is also the only isolate included in the morphological comparison by Nirenberg &

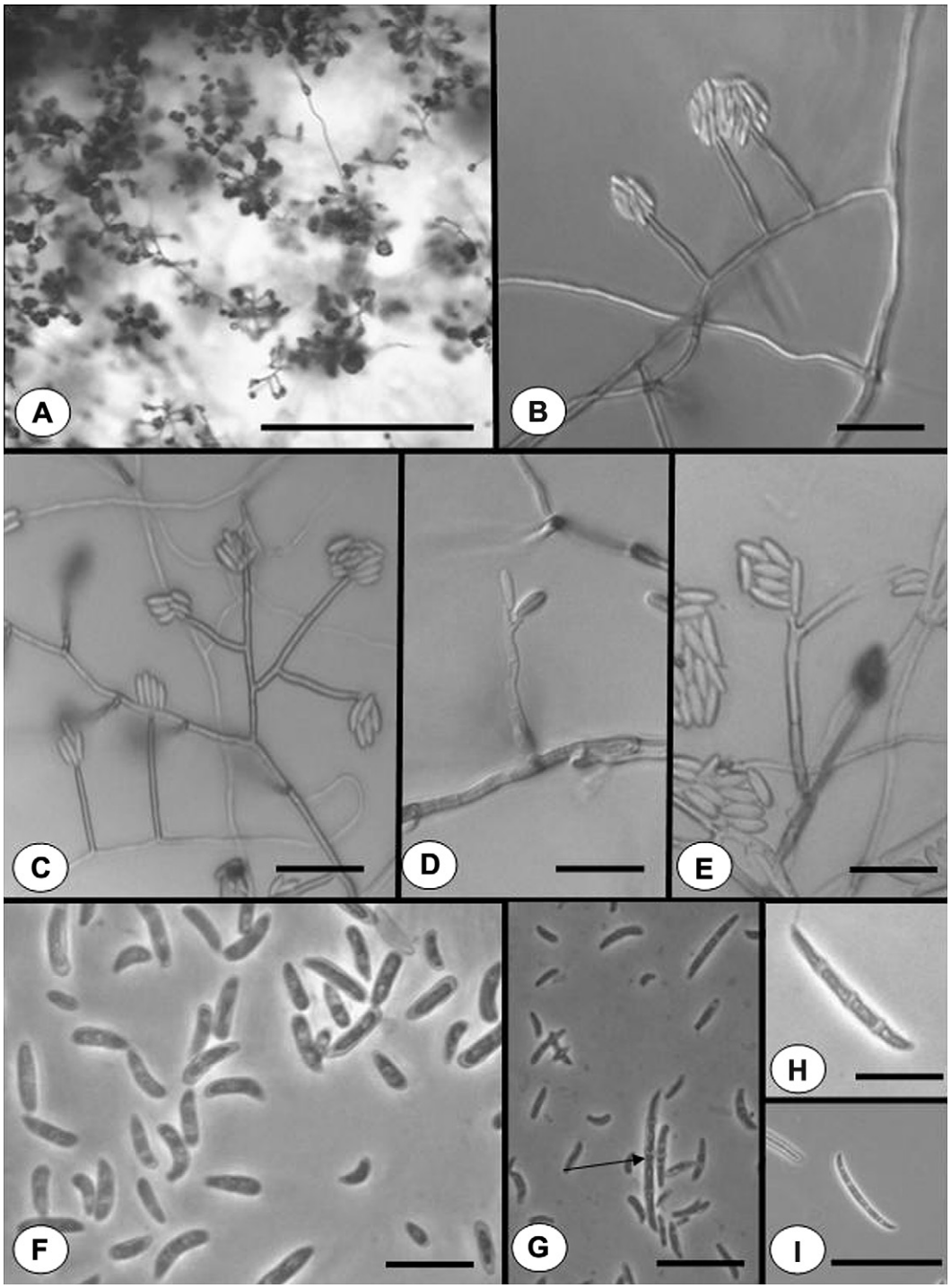

Fig 7 – Morphological characteristics of *Fusarium ananatum*. (A) Erect conidiophores on aerial mycelium on SNA. (B) False conidial heads on SNA. (C) Branched and unbranched monophialides on CLA. (D) Polyphialdes on SNA. (E) Polyphialides on CLA. (F) Microconidia on CLA. (G–I) Macroconidia on CLA. Bars (A) = 130 μm; (B, C, E–H) = 15 μm; (D) = 30 μm; (I) = 20 μm.

O'Donnell (1998) that originates from the United Kingdom and not a South American country. This unusual grouping is further supported by the fact that South Africa exports pineapples to the United Kingdom and, thus, may have inadvertently sent fruit infected with *F. ananatum* to that country. This emphasises the importance of basing phylogenetic relationships employing ex-type strains and not on other isolates that might not represent the fungus intended for comparison.

At present, nothing is known regarding the pathogenicity of *F. ananatum*. The fungus is closely associated with rot of fruit and it most likely is the cause of this disease. However, pathogenicity tests will be needed to determine its relative importance as a pathogen.

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REFERENCES

Aoki T, O'Donnell K, Ichikawa K, 2001. *Fusarium fractiflexum* sp. nov. and two other species within the *Gibberella fujikuroi* species complex recently discovered in Japan that form aerial conidia in false heads. *Mycoscience* **42**: 461–478.

Borras O, Santos R, Matos AP, Cabral RS, Arzola M, 2001. A first attempt to use a *Fusarium subglutinans* culture filtrate for the selection of pineapple cultivars resistant to fusariose disease. *Plant Breeding* **120**: 435–438.

Carbone I, Kohn LM, 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia* **91**: 553–556.

Correll JC, Klittich CJR, Leslie JF, 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* **77**: 1640–1646.

Desjardins AE, 2003. *Gibberella* from A(venaceae) to Z(eae). *Annual Review of Phytopathology* **41**: 177–198.

Edmonstone-Sammons C, 1958. Some aspects of black spot in pineapples. *South African Journal of Agricultural Science* **1**: 111–120.

Farris JS, Kallersj M, Kluge AG, Bult C, 1994. Testing significance of congruence. *Cladistics* **10**: 315–320.

Glass NL, Donaldson GC, 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**: 1323–1330.

Hillis DM, Huelsenbeck JP, 1992. Signal, noise, and reliability in molecular phylogenetic analyses. *Journal of Heredity* **83**: 189–195.

Hildalgo OB, Santos R, Tussel RT, Pires de Matos A, Cabrl RS, Avzola M, Perez MC, 1999. Phytotoxicity of *Fusarium subglutinans* culture filtrates *in vitro* plantlets and calli of resistant and susceptible pineapple (*Ananas comosus*). *Plant Pathology* **48**: 756–758.

Huelsenbeck JP, Ronquist F, 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754–755.

Nelson PE, Tousson TA, Marasas WFO, 1983. Fusarium *Species: an Illustrated Manual for Identification*. University Park, Pennsylvania State University Press.

Nirenberg HI, 1976. Untersuchungen über die morphologische und biologische Differenzierung in der *Fusarium*-Sektion *Liseola*. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft* **169**: 1–117.

Nirenberg HI, O'Donnell K, 1998. New *Fusarium* species and combinations within the *Giberella fujikuroi* species complex. *Mycologia* **90**: 434–458.

O'Donnell K, Cigelnik E, Nirenberg HL, 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* **90**: 465–493.

O'Donnell K, Nirenberg HI, Aoki T, Cigelnik E, 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. *Mycoscience* **41**: 61–78.

Pires de Matos A, 1995. Pathological aspects of the pineapple crop with emphasis on the fusariosis. *Revista de la Facultad de Agrarias (Maracay)* **21**: 179–197.

Ploetz RC, 2001. Significant diseases in the tropics that are caused by species of *Fusarium*. In: Summerell BA, Leslie JF, Backhouse D, Bryden WL, Burgess LW (eds), *Fusarium: Paul Nelson Memorial Symposium*. The American Phytopathological Society Press, St Paul, pp. 295–309.

Raeder U, Broda P, 1985. Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**: 17–20.

Rayner RW, 1970. *A Mycological Colour Chart*. Commonwealth Mycological Institute/British Mycological Society, London.

Rohrbach KG, Pfeiffer JB, 1976. Susceptibility of pineapple cultivars to fruit diseases incited by *Penicillium funiculosum* and *Fusarium moniliforme*. *Phytopathology* **66**: 1386–1390.

Rohrbach KG, 1994. Fusariosis. In: Ploetz RC, Zentmeyer GA, Nishijima WT, Rohrbach KG, Ohr HD (eds), *Compendium of Tropical Diseases*. The American Phytopathological Society Press, St. Paul, pp. 45–56.

Rohrbach KG, Schmitt D, 2003. Diseases of pineapple. In: Ploetz RC (ed), *Diseases of Tropical Fruit Crops*. CABI Publishing, Oxford, pp. 443–464.

Steenkamp ET, Wingfield BD, Coutinho TA, Wingfield MJ, Marasas WFO, 1999. Differentiation of *Fusarium subglutinans* f. sp. *pini* by histone gene sequence data. *Applied and Environmental Microbiology* **65**: 3401–3406.

Steenkamp ET, Wingfield BD, Coutinho TA, Zeller KA, Wingfield MJ, Marasas WFO, Leslie JF, 2000. PCR-based identification of *MAT-1* and *MAT-2* in the *Gibberella fujikuroi* species complex. *Applied and Environmental Microbiology* **66**: 4378–4382.

Swofford DL, 2002. *PAUP*: phylogenetic Analysis Using Parsimony (*and Other Methods)* Version 4. Sinauer Associates, Sunderland, MA.

Ventura JA, Zambolim L, Chaves GM, 1993a. Integrated management system for pineapple *Fusarium* disease control. *Acta Horticulturae* **334**: 439–453.

Ventura JA, Zambolim L, Gilbertson L, 1993b. Proposição de nova forma specialis em *Fusarium subglutinans* no abacaxizeiro. *Fitopatologia Brasileira Supplemento* **18**: 280.