

CHARACTERISATION OF SOUTH AFRICAN ISOLATES OF *FUSARIUM OXYSPORUM* F.SP. *CUBENSE* FROM CAVENDISH BANANAS

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ABSTRACT

Fusarium wilt, caused by the soil-borne fungus *Fusarium oxysporum* f.sp. *cubense* (*Foc*), is a serious vascular disease of bananas in most subtropical and tropical regions of the world. Twenty-four vegetative compatibility groups (VCGs) and three pathogenic races have been identified in *Foc*, reflecting a relatively high genetic diversity for an asexual fungus. To characterise a South African population of *Foc*, a collection of 128 isolates from diverse geographic origins were isolated from diseased Cavendish bananas and subjected to VCG analysis and sequencing of the translation elongation factor 1- α (TEF) gene region. The presence of mating type genes was also determined using *MAT*-1 and *MAT*-2 specific primers. VCG 0120 was established as the only VCG of *Foc* present in the South African population studied. Only the *MAT*-2 idiomorph was present in all the local isolates of *Foc*. A phylogenetic analysis of DNA sequences of the TEF gene region revealed that the South African isolates grouped closely with VCG 0120 isolates from Australia and Asia. These results suggest that the South African population of *Foc* was most likely introduced in a limited number of events and that it had spread with infected planting material within the country. The presence of only one mating type and the limited diversity in this pathogen render it unlikely to rapidly overcome disease management strategies involving host resistance.

INTRODUCTION

Fusarium wilt of bananas is caused by the fungus *Fusarium oxysporum* f.sp. *cubense* (E.F. Smith) Snyder and Hansen (*Foc*), a pathogen that is generally considered to be one of the most destructive *formae speciales* of *F. oxysporum*.^{1,2} The disease seriously hampers banana production once *Foc* is introduced into fields and it is difficult to manage. Because the pathogen persists in infested soils for long periods,³ control strategies involve the use of tissue culture-derived plantlets to prevent the introduction of *Foc* into disease-free fields, as well as the implementation of sanitation practices to prevent spread.^{4,5} The most effective means of controlling *Fusarium* wilt, however, is the replacement of susceptible banana cultivars with resistant ones.

Fusarium wilt became notorious because it almost destroyed the Gros Michel-based banana export industry in Central America during the mid-1900s. The disease was eventually managed by replacing Gros Michel bananas with highly resistant Cavendish cultivars.¹ Since then, Cavendish bananas have been found to succumb to a new race of *Foc*, called *Foc* race 4, in other banana-producing areas of the world.⁶ Cavendish cultivars are the only banana varieties produced commercially in South Africa. Since the 1970s, *Fusarium* wilt has destroyed almost 40% of all Cavendish bananas grown in the Kiepersol and southern KwaZulu-Natal areas of the country.⁵ The disease has also been discovered in two further production areas since the turn of the century.⁷ New replacement cultivars for Cavendish bananas have not been readily accepted by producers and markets, primarily because of a slight difference in taste. Owing to the parthenocarpic nature of Cavendish bananas, unconventional improvement, rather than classical breeding, now offers the most feasible option to develop *Fusarium*-wilt-resistant Cavendish banana cultivars.

Knowledge of the genetic diversity of fungal populations and their mode of reproduction is important for implementing management strategies to reduce disease impact.^{8,9} *Foc* has a relatively diverse population structure for an apparently asexual fungus that consists of three races^{3,4,10} and 24 vegetative compatibility groups (VCGs).^{6,11,12} A teleomorph for *F. oxysporum* has never been observed and the pathogen appears to rely on mutations and parasexuality as the main basis for genetic variation.^{13,14,15,16} Polymerase chain reaction (PCR) amplification experiments have demonstrated the presence of both *MAT* idiomorphs in at least two *formae speciales* of *F. oxysporum*,^{17,18,19} but *MAT* idiomorphs in *Foc* have not yet been reported.

Studies to determine diversity in *Foc* have included both phenotypic and genotypic markers. The phenotypic characters most commonly used are pathogenic race and vegetative compatibility.^{20,21,22} *Foc* races 1, 2 and 4 are distinguished from one another based on their virulence to a defined group of banana cultivars under field conditions.^{3,4,23} *Foc* race 1 attacks Gros Michel, Silk, Apple, Lady Finger and Latundan cultivars, while race 2 attacks Bluggoe bananas and race 4 attacks all cultivars susceptible to *Foc* races 1 and 2, as well as Cavendish bananas. Although predisposing factors, such as cold temperatures, are associated with damage caused by *Foc* race 4 in the subtropics,⁵ these factors are not involved in *Fusarium* wilt of Cavendish bananas in the tropics. To recognise the effect of environmental factors and differences that exist between populations of *Foc* causing disease to Cavendish bananas in the subtropics and tropics, the pathogens are referred to as 'subtropical' and 'tropical' race 4, respectively.

Vegetative compatibility is a useful means of subdividing *Foc* into genetically isolated groups, but does not measure genetic relatedness among isolates. In addition, VCGs are phenotypic markers that may be subjected to selection pressures.^{24,25} Therefore, neutral DNA-based techniques would be more suitable for analysing genetic variation within and between *Foc* populations. DNA sequence analyses of several *formae speciales* of *F. oxysporum*, including isolates of *Foc*, have shown that the *Fusarium* wilt fungus represents

two genetically distinct lineages.²⁶ Concordant evidence from the gene genealogies further revealed that *Foc* harbours at least five clonal lineages.²⁶

Foc is believed to have spread worldwide through infected planting material.^{1,2,10} The route of entry of the pathogen into South Africa is unknown due to incomplete records of banana production in the country. It is thought that Indian labourers, who worked on sugar cane plantations in KwaZulu-Natal during colonial times, could have introduced infected rhizomes into South Africa.²⁷ In this study, the identity of *Foc* VCGs in a collection of isolates from South Africa was determined. The phylogenetic relationship of the South African isolates in relation to those from other banana-producing countries was assessed by DNA sequence data for the translation elongation factor 1- α (TEF) gene regions. We further considered whether both mating type genes were present in the South African *Foc* population to determine if sexual reproduction might occur in this fungus.

METHODS

Fungal isolates

Foc isolates ($n = 152$) from different banana genotypes and geographic origins were selected for this study. These included 128 isolates collected from Kiepersol and Komatipoort (Mpumalanga Province), Tzaneen (Limpopo Province) and the south-coast region of the KwaZulu-Natal Province in South Africa, and 24 VCG tester isolates from the collections of Dr N. Moore of the Queensland Department of Primary Industries (Australia), Dr S. Bentley of the University of Queensland (Australia) and Dr R. Ploetz of the University of Florida (USA). The VCG tester isolates represented *Foc* races 1 and 2, as well as *Foc* 'tropical' and 'subtropical' race 4. All cultures were single-spored and were maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

Generation of nitrate non-utilising mutants and VCG testing

Vegetative compatibility of the 128 South African *Foc* isolates was determined using the technique described by Puhalla.²⁸ In this technique, isolates are assigned to VCGs based on heterokaryon formation between complementary nitrate non-utilising (*nit*) mutants produced on media supplemented with chlorate. *Nit* mutants were produced for all South African isolates as well as for the known VCG tester strains. The *nit-1* and *nit-3* mutants were then paired with each of the *nit-M* tester strains on minimal medium (MM) at least twice.²⁹ *Nit-M*, *nit-3* and *nit-1* mutants of the same isolate were also paired to test for self-compatibility. Complementary *nit* mutants that formed dense, wild-type growth on MM were assigned to the same VCG. Vegetatively incompatible isolates were detected by their inability to form a heterokaryon when paired on MM.

DNA extraction

After VCG identification, 45 *Foc* isolates were selected for sequence analysis and mating type identification (Table 1). These isolates consisted of 21 *Foc* isolates representative of the different geographic areas, the Cavendish cultivars grown in South Africa and the 24 VCG tester isolates. For mating type identification, only the 21 South African *Foc* isolates were considered. All 45 *Foc* isolates were grown in 100 mL potato dextrose broth (Biolab Diagnostics, Wadeville, South Africa) in 250-mL flasks, without shaking, at room temperature for 7–10 days, after which the mycelium was harvested and freeze dried. For extraction of total DNA, freeze-dried mycelia were ground to a fine powder in liquid nitrogen and added to DNA extraction buffer (200 mM Tris-HCl, pH 8; 150 mM NaCl; 25 mM EDTA, pH 8; 0.5% SDS),³⁰ followed by phenol-chloroform extraction.³¹ The DNA concentration was estimated by comparing the intensity of ethidium bromide fluorescence of the DNA samples to known concentrations of lambda DNA marker (marker III) (Roche

Molecular Biochemicals, Mannheim, Germany) following gel electrophoresis.

Identification of mating type genes

To determine if *MAT-1* or *MAT-2* idiomorphs were present in the South African population of *Foc*, DNA of the 21 representative *Foc* isolates were subjected to PCR analysis with primers designed by Steenkamp et al.³² Additional primers were designed for *MAT-1* (FO-MAT-1-For 5'ACC GCC AGC CGT CGT GCA GTG 3' and FO-MAT-1-Rev 5'CTT GCG GGG GTA TGA GAA CGC 3') based on the *MAT-1* idiomorph sequences in GenBank, while a *MAT-2* reverse primer was designed specifically for the high mobility group (HMG) box (FF1 *Foc* 5' GTA TCT TCT GTC CAC CAC AG 3') and used with the forward primer Gfmat2c.³²

For each isolate, a 25- μ L PCR reaction mix was prepared that contained 0.4 mM of each deoxynucleoside triphosphate (dNTPs), 1 \times PCR buffer, 1.0 pmol of each primer, 0.25 units Expand High Fidelity *Taq* polymerase (Roche Molecular Biochemicals, Germany), 2 ng DNA, and sterile deionised water. PCR reactions were performed on a Hybaid TouchDown PCR machine (Hybaid Limited, Middlesex, United Kingdom) and reaction conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 92 °C for 30 s, primer annealing at 62 °C (*MAT 1*) or 54 °C (*MAT 2*) for 40 s, elongation at 72 °C for 2 min, and a final extension at 72 °C for 7 min. The amplified product was resolved by electrophoresis in a 1.5% (w/v) agarose gel in TBE buffer (Tris boric acid EDTA; pH 8.0), stained with ethidium bromide and visualised under UV illumination.³¹ Size estimates of the PCR fragments were determined using a molecular weight standard (100-bp ladder; Promega, Madison, Wisconsin, USA).

DNA sequence analyses

A standard 25- μ L PCR reaction mixture for TEF was prepared as described above and reaction conditions set as follows: denaturation at 95 °C for 2 min, followed by 30 cycles of 30 s at 95 °C, 40 s at 60 °C,²⁶ 1 min at 72 °C, and a final extension of 7 min at 72 °C. The amplified products were verified using electrophoresis in 1.5% agarose gels in Tris borate EDTA (TBE, pH 8.0) buffer. The resulting PCR amplicons were purified using a QIAquick PCR Purification kit (QIAGEN, StraÙe, Germany), according to the specifications of the manufacturer.

Purified PCR products were sequenced in both directions using the PCR primers listed above. DNA sequences were determined using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA) and an ABI PRISM™ 377 automated sequencer. For comparative purposes, five TEF sequences,²⁶ which represent the two clades and clonal lineages, were obtained from GenBank and included in the analyses. NRRL 22903, previously used by O'Donnell et al.²⁶ as an out group, was also included in the current study as an out group. All sequences from the current study were deposited in GenBank (<http://www.ncbi.nlm.nih.gov/>) (Table1).

Datasets were aligned with MAFFT (<http://mafft.cbrc.jp/alignment/software/>) software.³³ Bayesian inference was accomplished using MrBayes version 3.b.4³⁴ and maximum likelihood methods were employed by using PhyML version 2.4.3³⁵ Bootstrap confidence levels were assessed by 1000 parsimony replications.

RESULTS

Generation of *nit* mutants and VCG testing

Nit mutants were successfully generated for all the South African isolates and the known VCG testers of *Foc*. Almost all isolates produced at least one *nit-M* mutant and several *nit-1* and/or *nit-3* mutants. Pairings of *nit-M* mutants with *nit-1* or *nit-3* mutants of the same isolate produced a zone of wild-type growth where the two *nit*-mutants formed a heterokaryon. When paired with the different VCG tester isolates, *nit-1* mutants from the South

TABLE 1
Geographic origin and sequence information of *Fusarium oxysporum* f.sp. *cubense* (Foc) isolates used for the sequence of the translation elongation factor 1- α (TEF) region

CAV number [*]	Foc number	Other name [†]	Geographic origin	Host origin	Race	VCG [‡]	MAT	Donor or collector	TEF
CAV 050	Foc 001		Burgershall, South Africa	Williams	4	120	II	A. Viljoen	
CAV 105	Foc 008		Kiepersol, South Africa	Cavendish	4	120	II	A. Viljoen	
CAV 106	Foc 009		Kiepersol, South Africa	Williams	4	120	II	A. Viljoen	AY217170
CAV 094	Foc 010		Kiepersol, South Africa	Williams	4	120	II	A. Viljoen	
CAV 092	Foc 011		Kiepersol, South Africa	Grand Naine	4	120	II	A. Viljoen	
CAV 095			Kiepersol, South Africa	Williams	4	120	II	A. Viljoen	
CAV 115			Kiepersol, South Africa	Williams	4	120	II	A. Viljoen	
CAV 006	Foc 006		Ramsgate, South Africa	Williams	4	120	II	A. Viljoen	
CAV 007	Foc 019		Ramsgate, South Africa	Williams	4	120	II	A. Viljoen	AY217172
CAV 010			Ramsgate, South Africa	Williams	4	120	II	A. Viljoen	AY217189
CAV 031	Foc 018		Munster, South Africa	Grand Naine	4	120	II	A. Viljoen	AY217171
CAV 030			Munster, South Africa	Williams	4	120	II	A. Viljoen	AY217186
CAV 020			Port Edward, South Africa	Williams	4	120	II	A. Viljoen	AY217187
CAV 141			Port Edward, South Africa	Grand Naine	4	120	II	A. Viljoen	AY217188
CAV 041	Foc 043		Port Edward, South Africa	Cavendish	4	120	II	A. Viljoen	
CAV 025	Foc 020		Umzumbi, South Africa	Williams	4	120	II	A. Viljoen	AY217173
CAV 024	Foc 023		Umzumbi, South Africa	Williams	4	120	II	A. Viljoen	
CAV 146	Foc 147		Tzaneen, South Africa	Grand Naine	4	120	II	A. Viljoen	AY217190
CAV 147	Foc 148		Tzaneen, South Africa	Grand Naine	4	120	II	A. Viljoen	AY217192
CAV 153			Tzaneen, South Africa	Grand Naine	4	120	II	A. Viljoen	
CAV 154			Tzaneen, South Africa	Grand Naine	4	120	II	A. Viljoen	
CAV 179	Foc 046	23486	Australia	Cavendish	4	120		N. Moore	AY217174
CAV 293	Foc 231	IC-1	Canary Islands	Dwarf Cavendish	4	120		R. Ploetz	AY217195
CAV 286	Foc 235	22424	Australia	Ladyfinger	4	120		K. Pegg, N. Moore	
CAV 601	Foc 237	23599	Australia	Cavendish	4	120		K. Pegg, N. Moore	
CAV 1118	Foc 240	W91307	Australia	Cavendish	4	120		K. Pegg, N. Moore	
CAV 1119	Foc 241	W91345	Australia	Ladyfinger	4	120		K. Pegg, N. Moore	
CAV 285	Foc 242	22410	Queensland, Australia	Cavendish	4	120		K. Pegg, N. Moore	AY217198
CAV 287	Foc 244	22615	Byron Bay, Australia	Lady finger	4	120		K. Pegg, N. Moore	AY217199
CAV 284	Foc 245	O-1220	Australia?	Mons		120		R. Ploetz	
CAV 294	Foc 246	34661	Honduras	Highgate	1	120		?	AY217200
CAV 291	Foc 247	C1	Canary Islands	Cavendish	4?	120		?	AY217201
CAV 618		Phil 10	Philippines		4	122		R. Ploetz	AY217180
CAV 182	Foc 048	Thai1-2	Thailand	Kluai Namwa	1	123		N. Moore	AY217176
CAV 609		23538	Australia		2	124		N. Moore	AY217177
		8611	Australia		2	125		N. Moore	AY217178
CAV 185	Foc 051	Phil 6	Philippines	Latundan	1	126		N. Moore	AY217179
CAV 188	Foc 047	STNP4	Tanzania	Ney Poovan	?	1212		R. Ploetz	AY217175
CAV 307	Foc 229	II 5	Sulawesi, Indonesia	Pisang Manurung	4	1213		R. Ploetz	AY217193
CAV 306	Foc 232	DMI 8	Sulawesi, Indonesia	Pisang Capatu	4	1213		R. Ploetz	AY217196
CAV 189	Foc 057	RPMW 40	Malawi	Bluggoe	2	1214		R. Ploetz	AY217181
CAV 604		Indo 50	Indonesia		4	1216		R. Ploetz	AY217182
CAV 871	Foc 059	Mal 7	Malaysia		?	1217		R. Ploetz	AY217183
CAV 194	Foc 060	Indo 5	Indonesia	Pisang Siem	?	1218		R. Ploetz	AY217184
CAV 195	Foc 061	Indo 25	Indonesia	Pisang Ambon	?	1219		R. Ploetz	AY217185

^{*}Isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

[†]Names as used in other culture collections from donor.

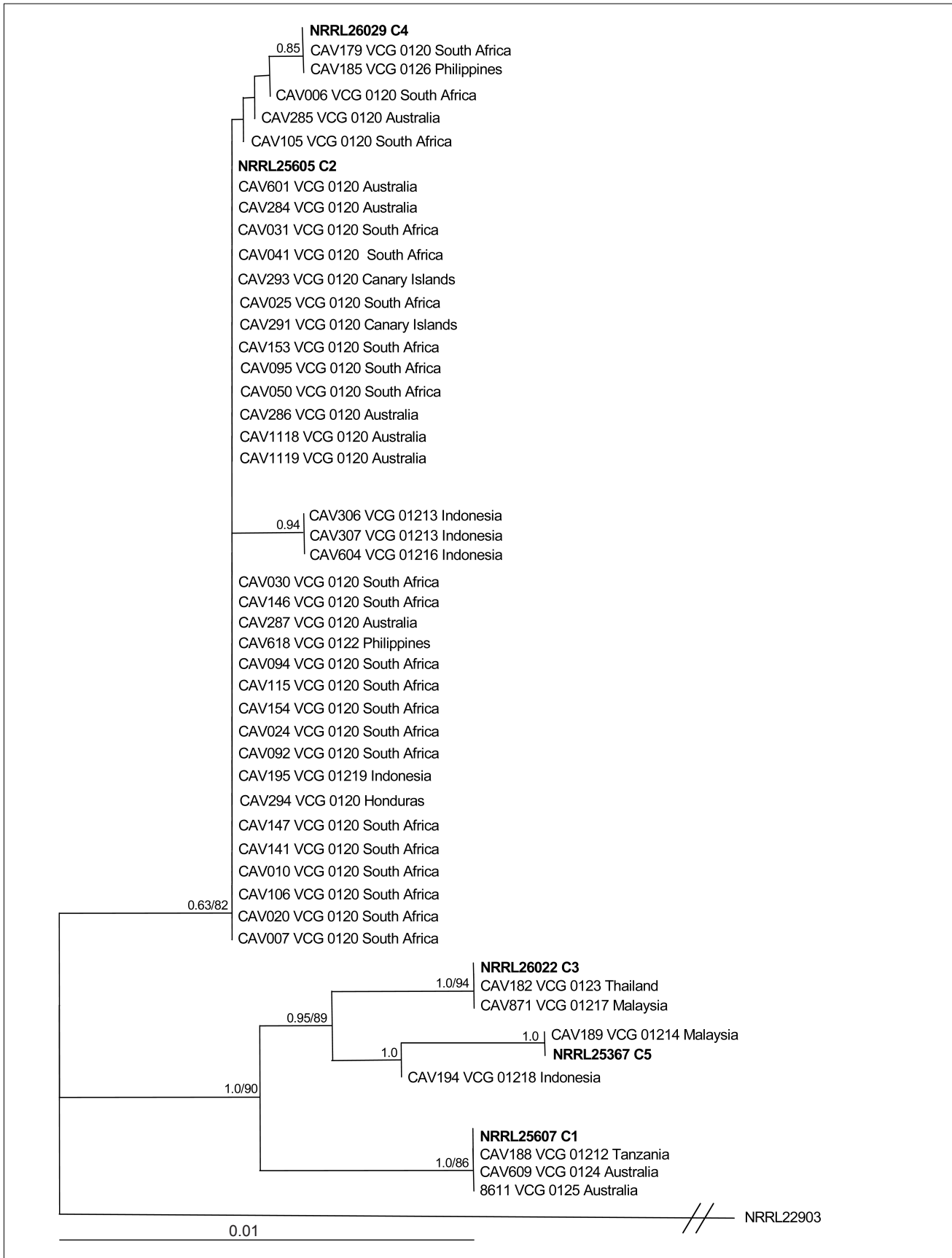
[‡]Vegetative compatibility groups (VCGs) are a phenotypic marker used to characterise fungal isolates based on heterokaryon formation.²⁸

African *Foc* population formed heterokaryons only with the *mit-M* tester isolate of VCG 0120 (Table 1). No complementary reactions resulted from pairings of the South African *Foc* isolates with testers representing any other VCG.

Identification of mating type genes

Amplification of genomic DNA with *MAT-2* specific primers

(*Gfmat2c* and FF1 *Foc*; *Gfmat2c* and *Gfmat2d*) produced an amplicon for all the South African *Foc* isolates tested (Table 1). A PCR reaction with the primer pair *Gfmat2c* and FF1 amplified a 700-bp fragment and the primer pair *Gfmat2c* and *Gfmat2d* resulted in a 200-bp PCR product. PCR using the *MAT-1* primers with genomic DNA as a template consistently failed to produce an amplicon.



NRRL numbers indicate sequences obtained from GenBank and represent the five clonal lineages of *Foc* previously reported by O'Donnell et al.²⁶ Bootstrap and Bayesian values are indicated above the branches. Geographic origin is listed beside isolate codes and vegetative compatibility group (VCG) designation.

FIGURE 1
Phylogenetic analysis of 45 *Fusarium oxysporum* f.sp. *cubense* (*Foc*) isolates based on the translation elongation factor 1- α (TEF) region

DNA sequence analysis

Amplification of the TEF region yielded a fragment of 700 bp. Alignment of the DNA sequences resulted in a data set of 598 characters. When aligned by TEF sequences, *Foc* isolates in the current study were broadly separated into two clades (Clades A and B; Figure 1). The South African population of *Foc* VCG 0120 'subtropical' race 4 grouped within Clade A with *Foc* isolates representing VCGs 0122, 0126, 0120/01215, 01212, 01213, 01216 and 01218. This grouping, as well as the relationships within it, however, was not supported by high bootstrap values. Isolates from the Indo-Malaysian region representing VCGs 01213 and 01216 appeared to form a subclade within Clade A. Clade B included isolates representing VCGs 0123, 0124, 0125, 01214, 01217 and 01219.

DISCUSSION

All isolates of *Foc* from commercial banana plantations in South Africa tested in this study belong to VCG 0120. This VCG is best known for its ability to cause disease of Cavendish bananas following incidents of environmental stress^{2,5} and has been reported from many banana-producing areas worldwide.^{2,6} The occurrence of a single VCG in South Africa indicates that the genetic diversity within the South African population is low and reconfirms the idea that the *Fusarium* wilt fungus was introduced into the country, most likely in a single or only a few events.

The phylogenetic tree generated in this study shows that the South African population of *Foc* harbours isolates that are closely related to *Foc* isolates from Australia, the Canary Islands and Central America. It seems likely, therefore, that VCG 0120 was originally introduced into subtropical and tropical Cavendish-producing areas – such as South Africa, Australia, the Canary Islands and Central America – with infected banana planting material from Southeast Asia.^{21,22} Planting material has also been moved between southern KwaZulu-Natal and Mpumalanga, which could have contributed to the movement of the *Fusarium* wilt pathogen between production areas.

Isolates of *Foc* globally are clearly heterogeneous. Sequencing results of this study showed that *Foc* can be divided into two phylogenetic clades with potentially separate evolutionary origins and five genetically distinct clonal lineages, as described by Koenig et al.³⁶ and O'Donnell et al.²⁶ Clade A can be divided into at least two lineages and Clade B into three lineages. The first clonal lineage in Clade A consisted almost entirely of isolates representing VCG 0120, while the second clonal lineage included isolates representing *Foc* 'tropical' race 4 (VCGs 1213 and 1216). Clade B consisted of three clonal lineages, all made up of isolates belonging to *Foc* races 1 and 2. Since the different pathogenic lineages may be capable of causing disease to different host genotypes,³⁷ banana improvement programmes must consider different pathogen lineages when developing plants with *Fusarium* wilt resistance. The race structure in *Foc* is not well defined and the genotypic groups defined above can, in future, be used to redefine pathotypes in *Foc*.

The occurrence of only the MAT-2 idiomorph in a representative population of isolates of *Foc* from South Africa provides strong evidence that sexual reproduction is absent in this fungus in the country. This finding is of great importance to the development of future management strategies for *Fusarium* wilt of bananas, since phytopathogenic fungi with an ability to reproduce sexually may overcome disease resistance in plants more rapidly than asexual forms. This has been true in bananas where the sexually reproducing fungus responsible for black Sigatoka, *Mycosphaerella fijiensis* Morelet, rapidly developed resistance to fungicides.³⁸ Both mating types have previously been reported for *F. oxysporum*^{17,18} but, to date, no studies have revealed clear evidence of sexual reproduction in contemporary populations.¹⁹ The stability of resistance to *Fusarium* wilt in Cavendish bananas in Central America may be further testimony to the absence of sexual reproduction and the consequent inability of the pathogen to generate new pathotypes.

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