# 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

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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-µL PCR reaction mix was prepared that contained 0.4 mM of each deoxynucleoside triphosphate (dNTPs), 1 × 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 ºC for 2 min, followed by 35 cycles of denaturation at 92 ºC for 30 s, primer annealing at 62 ºC (*MAT* 1) or 54 ºC (*MAT* 2) for 40 s, elongation at 72 ºC for 2 min, and a final extension at 72 ºC 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-µL PCR reaction mixture for TEF was prepared as described above and reaction conditions set as follows: denaturation at 95 ºC for 2 min, followed by 30 cycles of 30 s at 95 ºC, 40 s at 60 ºC,[26] 1 min at 72 ºC, and a final extension of 7 min at 72 ºC. 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, Straße, 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.4 [34] and maximum likelihood methods were employed by using PhyML version 2.4.3 [35] 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

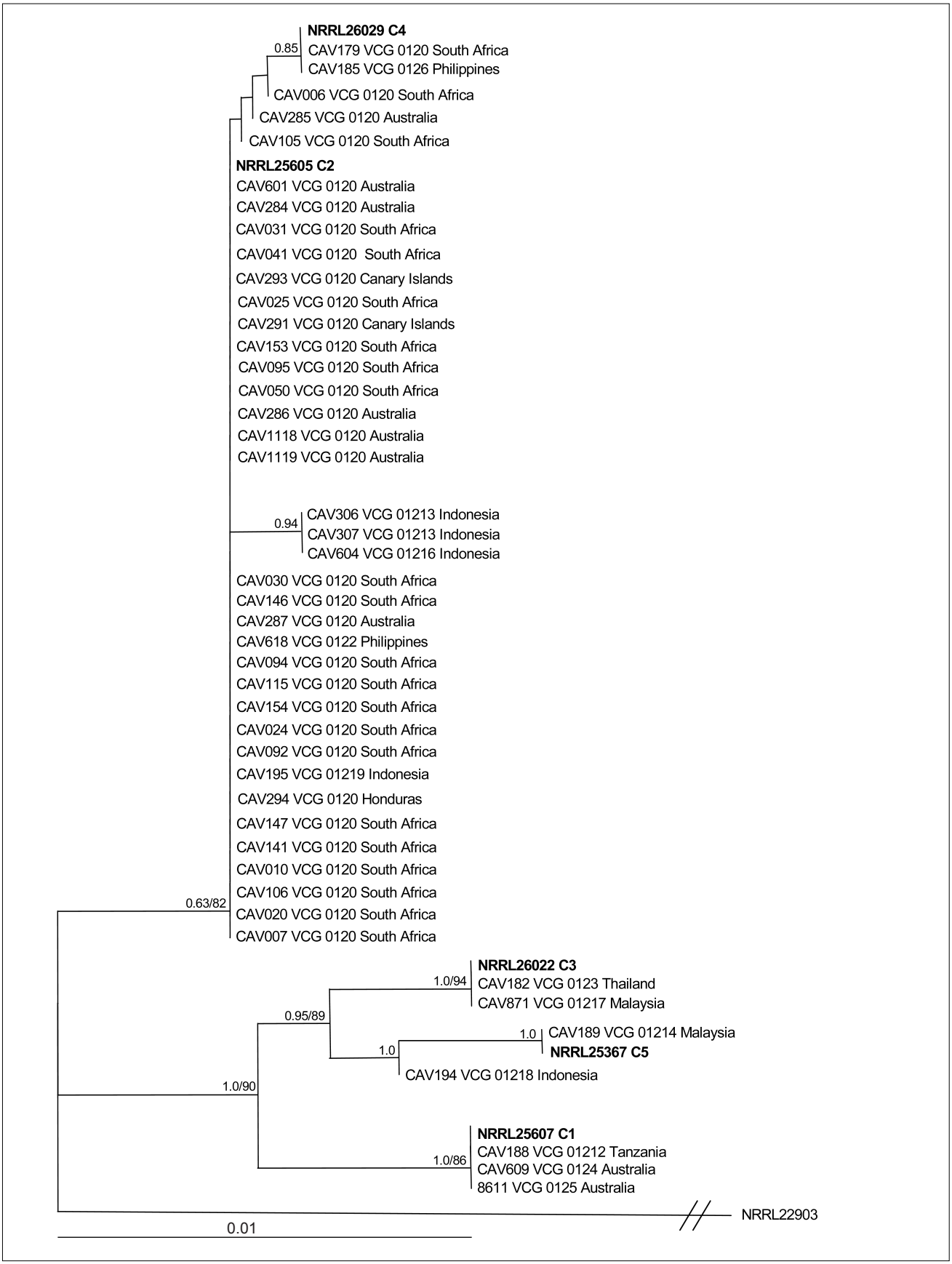
| CAV number* | *Foc* number | Other name† | Geographic origin | Host origin | Race | VCG‡ | MAT | Donor or collector | TEF |
|---|---|---|---|---|---|---|---|---|---|
| CAV 050 | *Foc* 001 | | Burgershall, South Africa | Williams | 4 | 120 | II | A. Viljoen | |
| CAV 105 | *Foc* 008 | | Kiepersol, South 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 *nit*-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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