Chapter 5

Development of microsatellite markers for the banana wilt pathogen *Fusarium oxysporum* f. sp. *cubense* (*Foc*).

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ABSTRACT

Fusarium oxysporum f. sp. *cubense* (*Foc*) is a serious fungal pathogen causing great losses to the banana industry worldwide. We describe the development of primers that target eight microsatellite markers to be used for population genetics studies on *Foc*. In this study the ISSR-PCR technique was used to develop the markers. The eight microsatellite loci amplified consistently well and were polymorphic among *Foc* isolates from different geographical origins. These polymorphic markers will be useful in large-scale studies to investigate the population structure, variation and movement of *Foc*.

INTRODUCTION

Fusarium oxysporum f. sp. *cubense* (*Foc*), the causal agent of Panama disease or Fusarium wilt, is one of the most serious problems affecting banana production in the world. In South Africa and other areas, the major commercial cultivar is Cavendish. This cultivar is highly susceptible to *Foc* race 4. The presence of this race in South Africa is therefore a serious threat to banana cultivation in the country (Stover 1962, Ploetz 1990).

The best defense against *Foc* infection at present is to prevent spread of the pathogen through education of farmers on appropriate sanitation practices (Robinson 1996). These include the use of disease-free planting material and cleaning of farming equipment to remove inoculum. Where Fusarium wilt occurs, the most effective and economical method for control is to grow genetically resistant cultivars. Because genetic resistance can be compromised by changes in the pathogen population, it is necessary to understand the genetic variability of the pathogen. One way to achieve this is to characterize isolates using polymorphic genetic markers such as microsatellites. To this end, the aim of this study was to develop microsatellite markers for *Foc* and to confirm their utility by examining *Foc* isolates representative of different geographical regions.

MATERIALS AND METHODS

Genomic DNA from the South African isolate *Foc* 135 (Table 1) was used in PCR reactions containing internal short sequence repeats (ISSR) primers: 5' NDV$(CT)_8$, DBB$(CAA)_5$, 5' DHB$(CGA)_5$, 5' YHY$(GT)_5$G, DBD$(CAC)_3$, 5'NDB$(CA)_7$C, 5' HVH$(GTG)_5$, 5' DDB$(CAT)_5$ and 5' HBDB$(GACA)_4$ (Lieckfeldt, Meyer & Borner 1993, Meyer & Mitchell 1995, Buscot *et al*. 1996). Each PCR reaction contained 0.4 mM of each dNTP, 1x PCR buffer containing

15mM $MgCl_2$ (Roche Molecular Biochemicals, Alameda, CA), 1.0 pmole of each primer (in combination), 0.25 units Expand High Fidelity *Taq* polymerase (Roche Molecular Biochemicals), 2 ng genomic DNA and sterile, deionised water to a final volume of 25 µl. PCR reactions were performed on a Hybaid TouchDown PCR machine (Hybaid Limited, UK).

Reaction conditions were: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 30 s, primer annealing at 48°C for 45 s and elongation at 72°C for 2 min. A final extension at 72°C for 10 min was included to complete the reactions. Amplified products were resolved on a 1.5% or 2% (w/v) agarose gel in 1x Tris-Acetate-EDTA (TAE) buffer (Sambrook, Fritsch & Maniatis 1989), stained with ethidium bromide and visualised under UV illumination. Product sizes were estimated using a molecular weight standard (100 bp ladder Promega, Corp., Madison, WI). PCR products were purified using the Magic PCR Preps Purification System (Promega), according the manufacturer's instructions.

The PCR products from the ISSR primer combinations were cloned using the pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions and propagated in *E. coli* JM109 cells. White bacterial colonies containing recombinant plasmids were selected and grown in 2 ml Luria-Bertani (LB) broth (Sambrook *et al.* 1989) supplemented with 100 µg/ml ampicillin (Sigma Chemicals Co, USA). Plasmid DNA containing cloned PCR amplicons was extracted using alkaline lysis (Sambrook *et al.* 1989). The DNA was treated with RNAse H (10 mg/ml, Roche Molecular Biochemicals) and digested with *Eco*RI (Roche Molecular Biochemicals) to excise inserts for sizing.

Plasmids containing inserts between 150-1500 base pairs (bp) in size were sequenced with universal M13 plasmid primers using the BigDye terminator cycle sequencing kit (Perkin-

Elmer Applied Biosystems) and ABI PRISM™ 377 and 3100 sequencer (Perkin-Elmer Applied Biosystems, Inc., Forster City, Calif). Sequences were analysed using the Sequence Navigator™ 1.0.1 computer program (Perkin-Elmer Applied Biosystems). Primer pairs were designed from sequences flanking microsatellite regions using Vector NTI (3.1). These primers were designed to have annealing temperatures between 56˚C and 68˚C and amplify different sized (120-480 bp) fragments. Inserts that contained microsatellite regions near the 3' or 5' ends required genome walking. This was performed as described by Siebert *et al.* (2001).

The primers were used in PCR reactions to test for amplification of the expected fragment. Genomic DNA of five *Foc* isolates originating from three different geographic regions (Table 1) was selected to test the primers. Each PCR reaction contained 0.4 mM of each dNTP, 1x PCR buffer containing 15 mM $MgCl_2$, (Roche Molecular Biochemicals) 1.0 pmole of each primer, 0.25 units Expand High Fidelity *Taq* polymerase (Roche Molecular Biochemicals), 2 ng genomic DNA and sterile double distilled water to a final volume of 25 µl. Reaction conditions were: initial denaturation at 96°C for 2 min, 10 cycles of denaturation at 94°C for 20 s, specific primer annealing temperature for 45 s, elongation at 72°C for 45 s. Another 25 cycles were carried out with a 5 s extension step after each cycle with the annealing time of to 40 s. A final extension step of 10 min at 72°C was included. Amplified products were electrophoresed, stained and visualized as before.

PCR products were purified using the Magic PCR Preps Purification System (Promega). Only primer pairs, which resulted in the amplification of single amplicons for all five isolates, were tested further. These amplicons were cycle sequenced on an ABI PRISM™ 3100 Autosequencer (Perkin-Elmer Applied Biosystems) using the BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems). Sequences of the five

isolates were aligned manually using Sequence Navigator (Perkin-Elmer Applied Biosystems) and checked for polymorphisms. Where primer pairs yielded polymorphic products, one primer from a primer pair was 5' end-labelled with a fluorescent dye (Applied Biosystems) and used for subsequent analysis.

The polymorphic primer pairs were used to amplify PCR products from an additional nine (Table 1) geographically diverse *Foc* isolates. These reactions were performed in 15 µl volumes containing 1 x PCR buffer, 50 µM of each dNTP, 0.2 µM of each labeled and unlabeled primer, 0.25 units of *Taq* polymerase and 2 ng of DNA. The same cycling conditions were used as above. The annealing temperature for all primers was 58°C since all primers annealed sufficiently at this temperature. Fluorescently labelled PCR products were fractionated on an ABI Prism 3100 DNA sequencer. The sizes of the DNA fragments were determined using the Genescan® 2.1 and Genotyper® 3.0 analysis software packages (Perkin Elmer Corp.) with LIZ™ as an internal size standard (Applied Biosystems). A UPGMA (Unweighted Pair-Group Method with Arithmetic mean) dendrogram obtained using Nei's (1972) genetic distance matrix was constructed in POPGENE version 1.31. This was done to evaluate genetic distance between all the isolates from the three geographic origins.

RESULTS

From a total of fourteen primer pairs that were tested, eight primer pairs amplified polymorphic regions. Genescan analysis identified a total of 36 alleles across 8 polymorphic loci (Table 2). No null alleles were detected. The smallest number of alleles per locus was two and the largest was eight. Allele sizes for all loci combined, ranged from 148 to 467 bp in length.

DISCUSSION

The number of alleles detected in our sample was high despite the small sample size. *Foc* isolates from South Africa and Australia were more similar to each other than they were to the Asian isolates. The isolates from Asia displayed the highest size variability, reflecting the presence of many unique alleles. This finding is consistent with the view that *Foc* probably originated in Southeast Asia (Fig 1) (Ploetz & Pegg 1999).

The polymorphic microsatellite markers developed in this study will enable researchers to study bigger populations of *Foc* to gain more insights into the structure and diversity of geographically distinct populations. Better characterization of population structure may help to identify patterns of disease spread and the origin of new pathotypes.

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Table 1: Isolates of *Foc* used for the development and testing of microsatellite markers.

Isolate no[1]	Country of origin	VCG designation[2]	Donor
Foc 1	South Africa	VCG 0120	E. Grimbeek
Foc 135	..	..	..
Foc 145	..	..	..
Foc 147	..	..	A. Viljoen
Foc 46	Australia	..	N. Moore
Foc 49	..	VCG 0124	..
Foc 50	..	VCG 0125	..
Foc 52	..	VCG 0129	..
Foc 54	Philippines	VCG 0122	R. Ploetz
Foc 57	Malawi	VCG 01214	..
Foc 58	..	VCG 01216	N. Moore
Foc 60	Indonesia	VCG 01218	..
Foc 61	..	VCG 01219	..
Foc 229	Indonesia	VCG 01213/01216	..

[1]All isolates are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[2]Vegetative compatibility groups (VCGs) are a phenotypic character used to group fungal isolates based on heterokayon formation (Puhalla 1985).

Table 2: PCR primer information and the core microsatellites regions amplified.

SSR primer	Primer sequence	Tm (°C)[1]	Ta[2] (°C)[3]	Polymorphic fingerprints	[3]Core sequence	Fluorescent label	Allelic range
G1	F 5′CTC GTC CTT TGC GAA TGA CC	59.4	58	Yes	*GA_{19}*	6-FAM™	444,454,455,457,460,467
MV15/1	R 5′ GAC CAC CTC GGT GAT GGT GAG ACG G	69.5	58				
G2	F 5′ GAG CTG CTG GTC TCG ATG TGG	63.7	58	Yes			
G2/3	R 5′ GCA GCA TGT ACG TTA CTC AAT CTG GCG GC	69.5	58		*TGG_7*	PET™	342,343,344,345,346,347,348,353
G4	F 5′ CGT CCT CAA GAG CAG CGA C	61.0	58	Yes	*GAT_6*	NED™	386,387,389,390
G4/5	R 5′ GCG CCT GGC GTT ACT GGC AGT TTG G	69.5	58				
G16	F 5′ GAG AAC TGG GCG TAT TTG TTA GAT CAC	63.4	58	Yes	*$GGC*_4$ *GAC_3*	VIC™	276,277,280,281,282,285
G16/8	R 5′ GCT TAG GCC GAG GGA GGC AGA G	67.7	58				
G20	F 5′ GTG GAG CAA CAG AAT GTG GCC GAG CC	69.5	58	Yes			
G20/21	R 5′ GCG GCT CCA GGA GCT GCT CTG AAG TG	71.1	58		*$GTT_7GAT_2GTT_2GA_2$*	6-FAM™	284,285
G40	F 5′ CCA CGG CTT GTC CGA GCT AGT GGA G	74.3	58	Yes			
G40/41	R 5′ CAA GCC GCT CTC CAC GGC GAA GGC G	69.5	58		*$GGT_1GT_1GGT_7$*	NED™	210,211,215,219,222
G42	F 5′ GGA GGT AAT GTT ACG CAA GAG G	63.3	58	Yes	*CA_8*	PET™	203,205
G42/43	R 5′ CGA CAC TCA AGC AAG AGT GTG C	62.4	58				
M42	F 5′ CGC GTC TCC AAT CAA GCC TCA ACC	66.1	58	Yes	*$CAACAG_2CAA*_4$	VIC™	149,150,153
M43	R 5′ GGC GGT TCG GTT GAT AGG GCT CCC AG	71.1	58				

[1] calculated melting temperature
[2] actual optimum annealing temperatures for PCR primers
[3] * varying lengths of sequence on either side of the core sequence

Fig. 1 UPGMA dendrogram of *Foc* from South Africa, Australia and Asia obtained using Nei's (1973) genetic distance based on allele frequency. South Africa and Australia have the least genetic distance compared with Asia.