Analysis of Kenyan isolates of Fusarium solani f. sp. phaseoli from common bean using colony characteristics, pathogenicity and microsatellite DNA

Article *in* AFRICAN JOURNAL OF BIOTECHNOLOGY · July 2008

DOI: 10.5897/AJB08.847

CITATIONS
13

READS
118

5 authors, including:

Ezekiel Kiprop
University of Eldoret
16 PUBLICATIONS **112** CITATIONS

SEE PROFILE

Florence Olubayo
University of Nairobi
70 PUBLICATIONS **384** CITATIONS

SEE PROFILE

Joel W Ochieng
University of Nairobi
71 PUBLICATIONS **1,114** CITATIONS

SEE PROFILE

African Journal of Biotechnology Vol. 7 (11), pp. 1662-1671, 3 June, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Analysis of Kenyan isolates of *Fusarium solani* f. sp. *phaseoli* from common bean using colony characteristics, pathogenicity and microsatellite DNA

Agnes W. Mwang'ombe[1]*, Pixley K. Kipsumbai[1], Ezekiel K. Kiprop[2], Florence M. Olubayo[1] and Joel W. Ochieng[3,4]

[1]Department of Plant Science and Crop Protection, Faculty of Agriculture, University of Nairobi, P.O Box 30197-00100, Nairobi, Kenya.
[2]Department of Biological Sciences, School of Science, Moi University, P.O. Box 1125-30100, Eldoret, Kenya.
[3]Faculties of Agriculture and Veterinary Medicine, University of Nairobi, Kenya.
[4]Centre for Plant Conservation Genetics, Southern Cross University, P.O. Box 157 Lismore NSW, 2480 Australia.

Accepted 24 January, 2008

***Fusarium solani* (Mart) f.sp. *phaseoli* (Burk) Synd. and Hans., is a plant pathogenic fungus that causes root rot in garden bean (*Phaseolus vulgaris* L.). To evaluate methods used in classifying strains of this pathogen, 52 *Fusarium solani* f.sp. *phaseoli* isolates from infected bean plants grown on different farms in Taita hills of Coast province, Kenya, were cultured and characterized using morphology, pathogenicity and microsatellite DNA. All the isolates showed high variability in aerial mycelial growth, mycelia texture, pigmentation (mycelia colour) when cultured on potato dextrose agar medium, and conidial measurements on Spezieller Nahrstoffarmer agar medium. Colonies were grouped into luxuriant, moderately luxuriant and scanty on aerial mycelial growth; fluffy and fibrous based on mycelial texture; purple, pink and white based on mycelia colour; and long, medium and short macroconidial length. All the isolates were pathogenic on GLP-2 (Rosecoco), a susceptible bean variety commonly grown in Kenya. DNA analysis showed that the isolates carried a high genetic diversity (gene diversity = 0.686; mean number of alleles = 9). Neighbour-Joining phylogenetic clusters reconstructed using microsatellite variation showed three major clusters. However, the microsatellite groupings were independent of the altitude, colony characteristics and virulence of the isolates.**

Key words: *Fusarium solani, phaseoli*, fungus, microsatellite, genetic diversity, *Phaseolus vulgaris*.

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) farming characterises most peasant households in developing countries. They are cheaper to grow and require less land area as they can be intercropped with other food crops (Gethi et al., 1997). In Kenya, garden beans are the most widely grown legume and second most important food crop (Gethi et al., 1997). It is predominantly grown by small-scale farmers and often intercropped with Maize (*Zea mays*) and other food crops (Wortmann et al., 1998). Apart from providing families the cheapest source of protein, bean harvests lead to increased household incomes from sales. Bean root rot disease caused by a number of soil-borne pathogenic fungi including *Macrophomina phaseolina*, *Rhizoctonia solani*, *Pythium* spp. and *F. solani* f.sp. *phaseoli* are some of the major causes of low yields (Nderitu et al., 1997). Bean root rot caused by *F. solani* f.sp. *phaseoli* has been reported worldwide, including Mexico, Brazil, Colombia, Peru, Ecuador, Chile, Venezuela, Costa Rica and Malawi (Kraft et al., 1981). In

*Corresponding author. Email: mwangombe@kenyaweb.com. Tel: +254 20 2055126. Fax: +254 20 631255.

Kenya, the disease has been reported in many bean-growing areas including western Kenya (Otsyula and Ajanga 1994; Muriungi 1997), Embu and Taita hills (Nderitu et al., 1997), where yield losses are estimated to between 10 and 100% (Nderitu et al., 1997).

The high variability in cultural characteristics exhibited by *F. solani* f. sp*. phaseoli* isolates (Nelson et al., 1983; Nirenberg 1989; Kwasna et al., 1991) poses a challenge to efforts aimed at breeding for resistance to bean root rot disease. Moreover, host specificity (Li et al., 2000) as well as a ribosomal DNA nucleotide sequence (O'Donnel and Gray 1995; Suga et al., 2000) has shown that *phaseoli* is a very diverse form, almost indiscernible from other related forms such as *glycines*. High variability has generally been observed in the genus *Fusarium* using dominant AFLP markers and cultural characteristics (Kiprop et al., 2002). However, dominant markers and morphology may be under selection and can provide biased inferences of genetic relationships. Analysis using a neutrally evolving and co-dominant marker such as microsatellites could shed insights into the variability within *F. solani* f.sp. *phaseoli*, to aid breeding progr-ammes aimed at developing resistance to this fungus.

Microsatellites also referred to as simple sequence repeats (SSRs; Bruford and Wayne, 1993) have many advantages over DNA sequencing, including a greater representation of different genomic regions and faster evolution that may lead to more informative characters. SSRs are suitable for studies involving deep divergences as well as very closely related taxa (Avise, 1994). Specifically, the use of SSRs in fungal diversity studies is prosperous since these markers are fairly well characterised (Bergemann and Miller 2002; Fournier et al., 2002; Sirjusingh and Kohn 2002; Rehner and Buckley 2003; Welser et al., 2003; Kretzer et al., 2004) and their transferability across species (Sirjusingh and Kohn, 2002) and genera (Mwangombe et al., 2007) has been demonstrated. The present study tested the level of phenotypic and microsatellite DNA variation and relationships among *F. solani* f. sp*. phaseoli* isolates from Taita hills, a bean growing area of Kenya. We aimed to answer the following questions: (1) are isolates of *F. solani* f. sp*. phaseoli* in Taita Hills isolated by distance, i.e., does genetic similarity of isolates depend on distances separating bean farms (2) is there congruence between pathogenicity and cultural characteristics or SSR-based clustering of isolates.

MATERIALS AND METHODS

Sampling and fungal isolation

Samples analysed in this study were collected in Taita hills of Coast Province, Kenya, in the year 2002 during two common bean root rot surveys. The first survey was done in May 2002 during the long rains while the second was done in October 2002 during the short rains. The root or hypocotyl portions of bean plants showing symptoms of root rot were collected from 50 fields (sites). The same fields were visited during the two surveys. The plant samples were mainly from two major varieties Rosecoco (GLP-2) and Red haricots (GLP-585) widely grown in the area.

During laboratory fungal isolation, 52 single spore isolates of *F. solani* f.sp. *phaseoli* were obtained from the plant tissues, with an extra isolate each from sites T7 and T38 (Table 1). One centimetre portions of plant samples with root rot symptoms were cut and placed aseptically onto a plate containing agar and incubated at room temperature for 3 days. Hyphal tip transfer was done onto plates containing potato dextrose agar (PDA) and incubated at room temperature in a 12 h light/dark cycle until the formation of conidia. Conidial suspension from the cultures was prepared and streaked on plates with sterile water agar and single germinating conidia was transferred to fresh PDA after 18 - 24 h and incubated at room temperature, and these were maintained as single spore isolates. Identification of the pathogen was done using colony characteristics (Booth, 1978; Gerlach and Nirenberg, 1982), and pathogenicity tests using a susceptible Kenyan bean variety Rosecoco (GLP-2).

Colony characteristics

A small portion of each isolate was transferred onto a PDA plate and incubated at room temperature for 8 days. The cultures from these plates were used to determine the nature of aerial mycelial growth, mycelial texture and pigments (colour) produced on the mycelia. The nature of aerial mycelia growth and mycelial texture were determined by visual observation while pigmentation on the mycelium was determined with the help of a mycological colour chart (Rayner, 1970).

To study the conidial morphology, *F. solani* f.sp. *phaseoli* isolates were sub-cultured onto Spezieller Nahrstoffarmer agar (SNA) medium. The plates were incubated at room temperature in a 12 h light/dark cycle for 10 days. A block of 1 cm^2 of agar from SNA was cut and mounted directly on a slide with a drop of water and a cover slip. Morphology of macroconidia and microconidia was described, and measurements made in micrometres (µm). Fifty conidia per isolate were measured to determine the length, width and the number of septa, using a compound microscope with a micrometer. Determination of the width was by measuring the widest section of the conidia.

Pathogenicity tests

Pathogenicity tests for all isolates of *F. solani* f.sp. *phaseoli* were done on commonly cultivated susceptible bean variety Rosecoco (GLP-2), obtained from Hortitec Seed Company (Nairobi, Kenya). The inocula were obtained from cultures grown on PDA for one week. 5 ml of sterile distilled water was added to each PDA plate and scraped gently with the edge of a glass slide to obtain a spore suspension. The suspension was then sieved in four layers of cheesecloth to remove mycelial fragments. The spore concentration was determined with the help of haemocytometer. The planting media was composed of soil, manure (cow dung) and sand in the ratio 2:1:1 by volume (Isanda 1995). The planting media was steam sterilized at 80°C overnight, allowed to cool and kept in 20 cm diameter polythene sleeves and kept in a glasshouse with a prevailing temperature of 23 to 30°C with a 12 h photoperiod. A soil inoculation method of Abawi and Pastor-Corrales (1990) was adopted. A spore suspension of each isolate was mixed with steam-sterilized soil at a rate of $3x10^4$ conidia per g of soil. Four bean seeds were planted in pots filled with steam sterilized soil mixture and covered with approximately 3 cm of infected soil and watered daily. Non-inoculated soil planted with bean seeds were used as controls.

Table 1. Colony characteristics, pathogenicity, and phylogroups of 52 *Fusarium solani* f.sp. *phaseoli* isolates. [a]Site- Taita hills represented as T followed by the field number; [b]in metres above sea level; [c]percentage pathogenicity; [d]SSR clustering.

Isolate	Site[a]	Altitude (masl)[b]	Aerial mycelia	Mycelia texture	Mycelia colour	Mico-conidia (µm)	Macro-conidia (µm)	Path (%)[c]	Phylo group[d]
Fs 01	T1	1575	Moderately luxuriant	Fluffy	Pink	7x3.4	34x4.5	91.7	2
Fs 02	T2	1583	Luxuriant uniform	Fibrous	Pink	8x3.4	36x4.2	83.3	2
Fs 03	T3	1558	Scanty, suppressed	Fibrous	Purple	6x2.8	30x4.2	100	3
Fs 04	T4	1496	Luxuriant	Fluffy	Purple	8x3.0	37x4.7	100	3
Fs 05	T5	1630	Scanty, suppressed	Fibrous	Pink	6x3.2	48x4.2	100	2
Fs 06	T6	1400	Luxuriant suppressed	Fluffy	Pink	8x3.5	36x4.0	91.7	1
Fs 07	T7	1593	Scanty	Fibrous	Purple	9x3.8	38x4.0	66.7	1
Fs 08	T8	1628	Luxuriant uniform	Fluffy	Pink	10x3.5	42x4.2	66.7	2
Fs 09	T9	1480	Moderately luxuriant	Fluffy	Pink	12x3.5	48x4.4	91.7	2
Fs 10	T10	1477	Scanty, suppressed	Fibrous	Purple	7x3.0	28x3.7	58.7	2
Fs 11	T11	1622	Scanty	Fibrous	Purple	6x3.2	30x3.8	100	2
Fs 12	T12	1493	Moderately luxuriant	Fluffy	Pink	7x3.4	30x3.6	83.3	2
Fs 13	T13	1656	Scanty, Suppressed	Fibrous	Purple	8x3.4	30x3.8	66.7	2
Fs 14	T14	1485	Luxuriant uniform	Fibrous	Pink	8x3.5	30x3.8	58.3	2
Fs 15	T15	1501	Moderately luxuriant	Fibrous	Pink	8x3.2	30x4.0	83.3	1
Fs 16	T16	1656	Luxuriant	Fluffy	White	9x3.6	42x4.2	91.7	2
Fs 17	T17	1482	Luxuriant	Fluffy	Pink	10x3.5	45x4.0	100	1
Fs 18	T18	1462	Luxuriant suppressed	Fibrous	Purple	9x3.2	37x4.0	100	1
Fs 19	T19	1477	Moderately luxuriant	Fibrous	Pink	9x3.2	38x4.0	66.7	1
Fs 20	T20	1555	Scanty, uniform	Fluffy	Purple	11x3.6	39x4.2	100	2
Fs 21	T21	1599	Luxuriant	Fibrous	Purplish/Violet	10x3.4	42x4.0	100	2
Fs 22	T22	1501	Moderately luxuriant	Fluffy	Pink	9x3.0	43x4.2	75.0	1
Fs 23	T23	1465	Luxuriant uniform	Fibrous	Pink	7x2.8	28x3.6	50.0	2
Fs 24	T24	1534	Scanty uniform	Fluffy	Pink	8x3.2	30x3.6	66.7	2
Fs 25	T25	1476	Scanty uniform	Fibrous	Purple	11x3.2	4.2x3.7	100	2
Fs 26	T26	1642	Luxuriant	Fluffy	Purple	9x3.5	40x4.2	75.0	2
Fs 27	T27	1462	Scanty suppressed	Fibrous	Purple	7x2.9	28x4.0	75.0	2
Fs 28	T28	1486	Luxuriant uniform	Fluffy	Pink	12x3.6	45x4.1	100	2
Fs 29	T29	1673	Luxuriant	Fibrous	Pink	10x3.4	41x4.0	100	2
Fs 30	T30	1568	Scanty uniform	Fluffy	Pink	11x3.2	42x3.9	50.0	2
Fs 31	T31	1570	Scanty uniform	Fibrous	Pink	10x3.4	40x4.2	66.7	1
Fs 32	T32	1497	Moderately luxuriant	Fibrous	Purple	8x3.2	32x3.5	83.3	2
Fs 33	T33	1493	Luxuriant suppressed	Fluffy	Pink	7x2.8	34x3.8	58.3	2

Table 1. Contd.

Fs 34	T7	1593	Luxuriant	Fluffy	White	8x3.0	35x4.2	91.7	2
Fs 35	T34	1651	Luxuriant suppressed	Fibrous	Pink	9x3.2	38x4.2	58.3	2
Fs 36	T35	1590	Moderately luxuriant	Fluffy	Pink	9x3.0	38x4.0	91.7	2
Fs 37	T36	1486	Scanty uniform	Fluffy	Pink	8x2.8	28x4.0	100	2
Fs 38	T37	1599	Moderately luxuriant suppressed	Fibrous	Pink (mauve)	10x3.8	44x4.4	83.3	2
Fs 39	T38	1459	Moderately luxuriant	Fluffy	Pink	11x3.6	48x4.5	100	2
Fs 40	T39	1456	Scanty, suppressed	Fluffy	Purple	10x3.5	42x4.2	91.7	2
Fs 41	T40	1701	Moderately luxuriant uniform	Fluffy	Pink	7x3.0	28x3.8	66.7	2
Fs 42	T38	1459	Moderately luxuriant	Fluffy	White	8x3.0	28x3.8	83.3	2
Fs 43	T41	1456	Scanty uniform	Fluffy	Purple	9x3.4	34x4.2	100	2
Fs 44	T42	1623	Luxuriant uniform	Fibrous	Pink	10x3.2	36x4.2	50.0	3
Fs 45	T43	1500	Luxuriant	Fluffy	Pink	9x3.4	40x4.2	75.0	2
Fs 46	T44	1651	Scanty, uniform	Fibrous	Purple	8x3.2	28x3.8	100	2
Fs 47	T45	1485	Luxuriant uniform	Fluffy	Pink	7x3.2	30x3.8	83.3	2
Fs 48	T46	1684	Scanty, uniform	Fibrous	Purple	10x3.4	34x4.0	91.7	2
Fs 49	T47	1695	Luxuriant	Fluffy	Pink	9x3.4	30x4.0	100	2
Fs 50	T48	1480	Moderate luxuriant uniform	Fibrous	Pink	10x3.2	35x3.9	75.0	2
Fs 51	T49	1485	Luxuriant uniform	Fluffy	Pink	10x3.4	36x4.0	91.7	2
Fs 52	T50	1622	Scanty uniform	Fibrous	Purple	13x3.6	48x4.5	58.3	2
Mean						9 x 3.5	36x4.0	82.5	
LSD (P=0.05)								19.1	

Data on percentage plants showing root rot symptoms was taken 7 weeks after sowing. The experiment was laid in a complete randomized design with three replicates.

DNA extraction

DNA was extracted from 52 single spore isolates of *F. solani* f.sp. *phaseoli*. Erlenmeyer flasks (500 ml) containing 200 ml of Czapek Dox (Oxoid) liquid medium (Coddington and Gould, 1992) were inoculated with three 5 mm^2 agar blocks excised from margins of 7 day old cultures on PDA medium, placed on a rotary shaker at 120 rpm and incubated at 24 – 25^oC under a dark condition for 7 days. The mycelia were harvested by filtration through two layers of cheesecloth; freeze dried using liquid nitrogen and ground to a fine powder using a mortar and pestle. DNA extraction was done according to the CTAB (Cetyltrimethyl-Ammonium Bromide) method (Doyle and Doyle, 1990). The DNA pellets were dissolved in 500 µl of TE buffer and stored at 4^oC. The DNA was quantified by separating 10 µl of each sample, along with undigested λ DNA (Roche Diagnostics Gmbh, Mannheim, Germany) of known concentration (20 ng/µl) on a 1% agarose gel containing 0.01 µL/mL ethidium. The relative fluorescence of each sample and the reference standard was then compared in the presence of UV light.

DNA microsatellite analysis

Seven SSR marker (microsatellite) pairs adopted from other fungal taxa as indicated in Table 2 were synthesized at the Sequencing and Oligonucleotide Synthesis Unit of the International Livestock Research Institute (ILRI), Nairobi. For each primer pair, the forward primer was fluo-

Table 2. Microsatellites sequence and source, expected (H_E) and observed (H_O) heterozygosity, mean number of alleles (A) and inbreeding coefficient (F_{IS}) per locus obtained from genotyping 52 *Fusarium solani f.sp. phaseoli* isolates.

Marker[a]	Primer sequences (F + R)	Ta (°C)	Size Range bp	*A*	H_E	H_O	F_{IS}	*A* Focal[b]
RB23	CAG CCG TCT TTC TCT CTC C GCC TTG AAT CACTACCTCCA	55	182-194	6	0.64	0.60	0.07	4
RE14	TAC CCA TTG CCT TGT TTC C ACT CCG CGT TCT GCT AGA G	55	177-181	3	0.48	0.40	-0.05	3
RE102	GGA CTT GTC AGC GTC AAG TCA ACC ATC TCA AGG TAT GTC	58	133-153	10	0.85	0.80	0.11	6
BC5	CGT TTT CCA GCA TTT CAA GT CAT CTC ATA TTC GTT CCT CA	58	144-176	11	0.81	0.80	0.15	10
AY117125	GCT TCT ACC ATA GTG AC ACC ACG AGT GCT TCT ACC ATA CTC	55	252-300	7	0.47	0.40	0.10	5
AF513014	GGCATATTGAGTATGGTATGGATG CTC CCG AGA TCT TGT TCA	58	189-253	16	0.81	0.80	0.01	10
AY212027	GAG CTG TGC GCG AGT CTG TG ACT GCT CCT TCGA GTC GTC A	55	283-303	10	0.73	0.70	0.08	10
			Mean	9.0	0.68	0.63	0.05	

[a]Marker source: RB23, RE14 and RE102 from Bergemann and Miller (2002); BC5 from Fournier et al. (2002); AY117125 from Kretzer et al. (2004); AF513014 from Welser et al. (2003); and AY212027 from Rehner and Buckley (2003).
[b]*A* focal is the mean number of alleles in the focal taxa (taxa from which the SSR were isolated).

rescently labelled with a dye to enable fluorescent detection.

The Polymerase chain reactions (PCR) were carried out in a total volume of 10 ul containing 5 - 10 ng genomic DNA, 5 pMol/ul of each primer, 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 0.125 mM of each dNTP (Roche, Germany), 1X PCR buffer (10 mM Tris–HCl, pH 8.3) including 50 mM KCl, 0.001% gelatin (Sigma, St Louis, MO, USA), 0.25% nonidet P40 (BDH, Poole, UK) and 2 mM $MgCl_2$. The cycling profile included an initial denaturation step at 95°C for 10 min, followed by 35 cycles of 30 s at 94°C, 1 min at 55 - 58°C depending on the primer pair used (Table 2), 1 min at 72°C and a final step of 15 min at 72°C using a GeneAmp 9700 (Applied Biosystems) thermal cycler.

The PCR fragments were fractionated and sized under denaturing conditions on a 5% polyacrylamide gel using an automated ABI377 DNA sequencer and the internal size standard Genescan 350-TAMRA (Applied Biosystems). Data were collected with the ABI PRISM 377 (version 2.1) software (Applied Biosystems). Fluorescent DNA fragments were analysed using the GENESCAN (version 3.1) and the GENOTYPER (Version 2.0) softwares (Applied Biosystems). The third order least square was used for base calling. Allele size data from SSRs were exported to an Excel spreadsheet for statistical analyses.

Data analysis

The data on conidial measurements of *F. solani* f.sp. *phaseoli* isolates grown on SNA media, and percentage bean plants with root rot symptoms during pathogenicity tests were analysed using GENSTAT computer package (Lane and Payne, 1996). The means were separated using least significant difference (LSD) at P = 0.05. The disease assessment started from the third week after planting and inoculation to the seventh week. To determine the level of virulence of the isolates during the pathogenicity tests, a disease scale was used that was adopted from Reddy and Raju (1993), where 0 - 10% resistant (= avirulent), 11 - 20% moderately resistant (= moderately avirulent), 21 - 40% moderately susceptible (= low virulence), 41 - 60% susceptible (= virulent) and 61 - 100% highly susceptible (highly virulent).

Gene diversity (Nei, 1987), inbreeding coefficient F_{IS} (an estimate of departure from random mating expectations at each individual locus), and genetic diversity indices (observed heterozygosity, mean number of alleles) were calculated using FSTAT computer programme V 2.9 (Goudet, 2001). Two measures were employed to estimate the between-individual genetic distance: the average square distance (D1) of Goldstein et al. (1995), and Cavalli-Sforza and Edward's (1967) chord distance. Chord distance models short-term divergence due to drift alone (Nei, 1987) and is thus most suitable for comparing closely related groups, while the average square distance accounts for size homoplasy, and is suitable for reconstructing trees that include more distantly related taxa. Both distances were computed using the MICROSAT programme available from the Human Population Genetics Laboratory (HPGL), Stanford University. Genetic distances based on allele size variation are modelled on the premise that when a mutation occurs, the new mutant is related to the allele from which it was derived. In this case, the difference in length between alleles contains phylogenetic information (Goldstein et al., 1995). The allele sizes analysed were nucleotide counts rather than repeat scores, using the option that allows for repeat lengths = 2. Duration of linearity was calculated for each locus and averaged over loci. The primer error (size of the region flanking the SSR) was entered and corrected for, by assuming a default of no error (that is, 0 nucleotides). Genetic distance matrices were imported into the computer programme PHYLIP (Felsenstein 1995) for phylogenetic tree reconstruction. Neighbour-Joining (NJ) trees were drawn using NEIGHBOUR assuming no outgroup information. All phylogenetic trees were displayed using TREEVIEW Version 1.5 available from the Department of Zoology, University of Glasgow. To determine if phylogenetic groupings correlated with geographical location, altitude or pathogenicity, a correlation analysis was done using the computer

programme XLSTAT version 7 (Addinsoft, New York).

RESULTS

Colony characteristics

The *F. solani* f.sp. *phaseoli* isolates exhibited high variability in colony characteristics on PDA and/or SNA media (Table 1). The growth of aerial mycelia was luxuriant, moderately luxuriant and scanty with 40.4, 25.0 and 34.6% isolates, respectively. Mycelial texture was either fluffy (51.9% isolates) or fibrous (48.1%). The mycelial colours observed were purple, pink and white with 59.6, 34.6 and 5.8% isolates, respectively.

The hyphae of the cultured isolates were highly branched, slender, septate and produced conidia and chlamydospores. The microconidia observed on the isolates were 0 - 1 septate, and their length ranged from 6.0 to 13.0 µm, while the width ranged from 2.8 to 3.6 µm (Table 1). The mean microconidial length x width was 9 x 3.5 µm. The most commonly observed macroconidia from all the isolates ranged from 3 to 7 septate. The 3-septate macroconidia ranged in length from 28 to 48 µm, while the macroconidial width was 3.6 to 4.5 µm. The mean macroconidial length x width was 36 x 4.0 µm. For comparison purposes, the 3-septate macroconidial length were grouped into three: long (≥ 40 µm), medium (30-39 µm) and short (< 30 µm) with 34.6, 51.9 and 13.5% isolates, respectively.

The variation in aerial mycelial growth, mycelial texture, mycelia colour and macroconidial length were observed in both higher (≥ 1,500 m.a.s.l) and lower (<1,500 m.a.s.l) altitudes of Taita hills. A high proportion (68.0%) of the isolates with fibrous texture was either scanty or moderately luxuriant in growth, while most (77.8%) isolates with fluffy texture were either luxuriant or moderately luxuriant in growth. Isolates with luxuriant growth were either fibrous or fluffy in texture, and with three types of pigments. Isolates with moderately luxu-riant texture are either fibrous or fluffy, and majority (84.6%) of them were pink. Isolates with scanty growth are either fibrous or fluffy, although most (72.2%) had a purple colour.

Pathogenicity

All the 52 isolates of *F. solani* f.sp. *phaseoli* were found to incite disease to varying levels of virulence. Symptoms observed on the infected plants were reddish streaks on the taproot and hypocotyls. These streaks covered the entire root on uprooting forming brown corky appearance. On the surface, symptoms included pale to yellow leaves, which were affected progressively starting with the older leaves, leading to defoliation. There were no symptoms observed on the un-inoculated bean controls grown under the same conditions.

The mean disease incidence was 85.5% with significant differences (p = 0.05) among the 52 isolates (Table 1). The lowest disease incidence was 50.0% while the highest was 100%. The isolates were grouped into two, based on their virulence to the susceptible bean variety. The highest virulence was observed for isolate 44 (84.6%) while the lowest was from isolate 8 (15.4%). Both levels of virulence (low and high) were represented in low and high altitudes of Taita hills, as well as groupings based on colony characteristics.

DNA microsatellite results

A total of 63 different alleles were detected when the 52 individual isolates of *F. solani* f.sp. *phaseoli* isolates were typed at seven microsatellite loci (Table 2). The allele sizes and their frequencies are given in Table 3. Genetic diversity at the seven SSRs was high (mean number of alleles = 9; Table 2). Observed heterozygosity (H_O) and the value expected under Hardy-Weinberg (H_E), and the mean numbers of alleles (MNA) are given in Table 2. H_O was not significantly lower than H_E. Locus-specific F_{IS} values ranged from – (negative) 0.05 to 0.15, while multilocus F_{IS} was 0.05 (Table 2). It should be noted that multilocus F_{IS} is not necessarily the average of single locus F_{IS} values.

SSR-based Neighbour-Joining phylogram clustered using the matrix of Cavalli-Sforza and Edward's (1967) chord distance is shown in Figure 1. The phylogram supported three major clusters: cluster 1 comprising 15.4% of the isolates, cluster 2 comprising 78.8%, while the rest (5.8%) are supported on the third cluster. There was no correlation between phylogenetic groupings and geographic location of isolate (r = 0.2), or altitude (r = -0.06). Similarly, there was no relationship between the SSR-clusters with the aerial mycelial growth, mycelial texcture, pigmentation, and macroconidial length. However, there was a positive correlation between SSR-based phylogenetic clusters and pathogenicity (r = 0.92). Generally, both highly virulent and virulent isolates were in clusters 2 and 3, while cluster 1 isolates are all highly virulent. Specifically, 90% of isolates in clade 1 incited early disease symptoms (pathogenicity group A; Table 1) on cv-Rosecoco, a susceptible host plant. Phylogenetic trees in this study were not bootstrapped because this procedure is not necessary in individual-specific Neighbour-joining clustering. As stated by Ochieng et al. (2007), bootstrapping provides a confidence interval that contains the phylogeny that would be estimated from repeated sampling of many characters from the underlying set of all characters, not the true phylogeny. Bootstrapping is thus a measure of repeatability, rather than measures of accuracy (Ochieng et al., 2007).

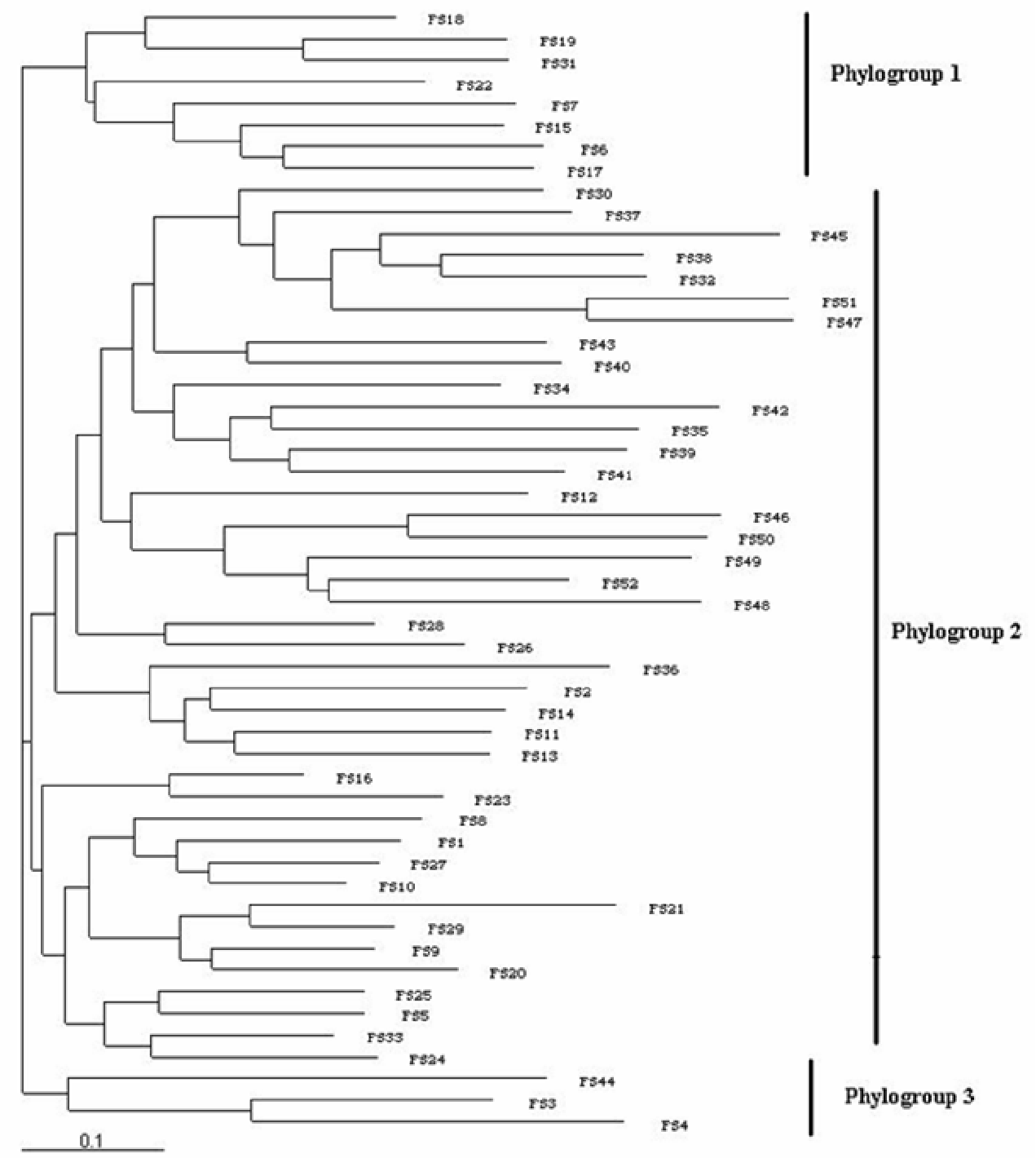

Figure 1. Dendrogram showing the genetic relationships among 52 *Fusarium solani* f.sp. *phaseoli* isolates obtained from Taita hills, Kenya, based on length variation at seven microsatellite loci.

DISCUSSION

Single spore isolates of *F. solani* f.sp. *phaseoli* collected from Taita hills showed variation in colony characteristics. Inoculation conditions have been shown to affect the forms of isolates (Nelson et al., 1983), perhaps explaining why different forms of luxuriant and scanty growths were observed in this study. Previous studies have shown re-

Table 3. Size and frequency of each allele obtained per locus obtained from genotyping 52 *Fusarium solani f.sp.* phaseoli isolates. The most frequent allele is marked in boldface.

RB23	freq	AY212027	freq	RE14	freq	AY117125	freq	RE102	freq	BC5	freq	AF513014	freq
182	0.03	283	0.04	177	0.02	252	0.02	133	0.12	144	0.08	189	0.11
184	0.52	285	0.02	179	0.34	278	0.01	135	0.06	146	0.06	191	0.01
186	0.28	287	0.02	**181**	0.64	290	0.04	**137**	0.23	148	0.02	211	0.05
188	0.01	289	0.02			**292**	0.72	139	0.11	152	0.03	**213**	0.38
190	0.06	291	0.15			298	0.06	141	0.02	**156**	0.33	215	0.08
194	0.09	**293**	0.46			299	0.01	143	0.17	158	0.05	223	0.01
		295	0.11			300	0.14	145	0.20	166	0.01	227	0.03
		297	0.12					147	0.02	168	0.24	229	0.02
		301	0.01					149	0.04	170	0.11	231	0.04
		303	0.04					153	0.01	172	0.04	235	0.11
										176	0.03	237	0.01
												239	0.03
												243	0.03
												247	0.02
												251	0.01
												253	0.07

sults similar to the ones observed here: two mycelia textures on isolates cultured on PDA medium- the fluffy and fibrous textures (Gerlach and Nirenberg, 1982); three mycelia colours- white, purple and pink observed on PDA medium (Gerlach and Nirenberg, 1982; Nelson et al., 1983; Kwasna et al., 1991). The conidia of *F. solani* f.sp. *phaseoli* cultures revealed the abundance of microconida, macroconida and chlamydospores, while pathogenicity tests revealed two virulence levels for *F. solani* f.sp. *phaseoli* on a bean variety susceptible to root rot. The highly virulent group is more abundant than the virulent one, similar to those reported in western Kenya in an earlier study (Muriungi, 1997). Microsatellite based phylogenetic clustering revealed three distinct clusters in basal topology. The clusters were significantly correlated with pathogenicity (duration from inoculation to symptoms expression on the susceptible bean plant); however, these clusters appeared to be independent of colony characteristics. Cultural characteristics have also been shown to be incongruent with AFLP based phylogenetic clustering of a related Fussarium species, *F. udum* (Kiprop et al., 2002). The most parsimonious explanation for this observation could be that morphological diversification in this pathogen is not selectively neutral.

An average of nine (9) alleles per locus (total of 63 different alleles at seven loci), were detected from 52 individual isolates of *F. solani* f.sp. *phaseoli*. The number of alleles per locus ranged from three (3) to 11. At each locus, the number of alleles was either equal to or greater than that detected in the focal taxa (Table 2). This is inconsistent with the principle of ascertainment bias. Ascertainment bias describes the observation that when the size distribution of microsatellite alleles across different species is compared, the absolute allele sizes in the species from which the microsatellite was derived are often greater than those found in closely related species (Ellegren et al., 1995; Rubinstein et al. 1995). When clones for sequencing and primer development are biased (Ellegren et al., 1995), or evolution within species occur in a directional manner (Rubinstein et al., 1995), ascertainment bias may result. The allele sizes are usually correlated with the number of alleles (Ochieng et al., 2007); accordingly, the fungus studied here was expected to show a lower diversity than that observed in the taxa from which the microsatellite primers were developed. The observation of a higher diversity may mean a very rapid evolutionary diversification in this group following divergence from their common ancestor. Multiple sources of molecular data suggest to an evolutionary diversification and loss of host specificity within member forms of *F. solani*. In a molecular differ-entiation study comparing the various forms of *F. solani* on pathogennicity, mitochondrial and nuclear gene sequences, Li et al. (2000) demonstrated that *phaseoli* caused the sudden death syndrome (SDS) on soybean foliage, a disease and symptom thought to be caused specifically by *glycines*. The study by Li et al. (2000) also showed that only *glycines* and *phaseoli* formed macroconidia, whereas all the other forms of *F. solani* only formed microconodia. Li et al. (2000) demonstrated that *phaseoli* isolates were phylogenetically paraphyletic or polyphyletic: some clustered with non-SDS causing *F. solani*, others showed a closer phylogenetic affinity with pea or tomato and alfalfa pathogenic fungi (Li et al., 2000). This

would suggest to an evolutionary diversification and subsequent loss of host specificity in *phaseoli*. In another study (Suga et al., 2000) involving the nuclear ribosomal internally transcribed spacers (nrITS) of the various forms of *F. solani*, phylogenetic clusters comprised strains belonging to the same special form (monophyletic), except *eumartii* and *phaseoli.* Indeed, these two forms were separated from the polytomic cluster composed of all other special forms (Suga et al., 2000), suggesting that the *phesoli* clade was attracted (Long branch attraction; Felsenstein, 1978) to the outgroup, because it evolved faster than the other forms. Under a Long Branch Attraction scenario, rapidly evolving lineages can be mistakenly inferred to be closely related even when they are evolutionarily not. Other hypotheses that may explain the high diversity and incongruence between cultural characteristics and SSR clustering are microevolution on ascomycetes due to combination of haploid-based genetic systems (Brasier, 1995), and complex ancestry of filamentous fungi such as *Fusarium* spp. because of their numerous nuclei within cells (Kristler et al., 1995; O'Donnell and Cigelnik, 1997).

Taxonomic misidentification or existence of biological species complexes can lead to erroneous conclusions in genetic analyses. Samples for this study were concluded to be *F. solani* f.sp. *phaseoli*, based on an assumption of host specificity. In practice, however, this pathogen can well exist in a symbiosis relationship with a closely related species or *forma specialis* without notice. Alternatively, the set of isolates could comprise different forms of *Fusarium solani*. The latter case can result into exceedingly large phenotypic and genomic variation as reported here. Although the genus *Fusarium* comprises Ascomyceteous fungi that have lost the need for or ability to produce their sexual spores (ascospores), a possibility that some members could have "regained" their sexual reproduction has not been verified. *Fusarium solani* are known to reproduce asexually through a haploid mycelium or conidia. The present data suggest that reproduction primarily occurs by spores as opposed to mycelial expansion, accounting for the observed diversity. However, sexual pathogenic Ascomycetes can exist as mycelia, reproduce and cause most of infections with the asexual stage (conidia), then produce the sexual stage at the end of the growing season when the food supply is diminishing (Agrios, 1997). The exceedingly high genetic diversity recorded in this study and in previous studies provides a compelling case to review the reproductive strategy in this pathogen.

Small-scale agricultural systems rely heavily on local crop varieties for seeds, thereby restricting the introduction of new genotypes of the fungus via infected seeds. Theoretically for sexual reproducing forms, exchange of seeds among farmers (gene flow) would lead to a homogenizing effect, leading to similarity in genotypes. In this study, we report a high genetic diversity in *Fusarium solani* f. sp. *phaseoli*, based on the present study and this agrees with the results of previously published data. Moreover, there was phylogeographic structure from either cultural characteristics or SSR in this study. Exchange of bean seeds among farmers is therefore unlikely to account for this observation.

In conclusion, this study has demonstrated that microsatellites can be used to classify strains of Kenyan *F. solani* f.sp. *phaseoli* fungus and to predict pathogennicity. The lack of correlation between cultural characteristics and pathogenicity indicates that morphology is an unreliable predictor of the pathogenicity for *phaseoli*. The high genetic variation for this fungus, both in phenotype and genotype suggest that the lineage could be diversifying. *F. solani* f. sp. *phaseoli* is known to be very persistent in soil and capable of surviving in infested fields almost indefinitely, and some strains may become pathogenic or non-pathogenic. This study was based on a limited number of SSRs (7), within a narrow geographical range. A more expansive study using more SSR makers, and covering diverse agro-ecological zones and regions, to include both pathogenic and non-pathogenic isolates and other related *Fusarium* species, is needed to better understand the diversification in this fungus. Although covering a small administrative area, this is the first study on the genetic diversity of *F. solani* f.sp. *phaseoli* in Kenya using microsatellite analysis

ACKNOWLEDGEMENTS

This research was funded by The Rockefeller Foundation under FORUM for Agriculture Resource Husbandry. We thank the University of Nairobi, The Kenya Agricultural Research Institute's Biotechnology Laboratory at Katumani, and The International Livestock Research Institute for facilities and technical support in DNA extraction and microsatellite genotyping. We are grateful to all the bean farmers of Taita Hills for facilitating access and sampling on their farms.

REFERENCES

Abawi GS, Pastor-Corrales MA (1990). Root rots of beans in Latin America and Africa: Diagnosis, research methodologies and management strategies. CIAT, Cali. Colombia, p. 114.

Agrios GN (1997). Plant Pathology (4th Edition) Department of Plant Pathology, University of Florida. Academic Press.

Bergemann SE, Miller SL (2002). Size distribution and persistence of genes in local populations of the late stage Ectomycorrhizal basidiomycetes, Rusula brevipes, University of Wyoming, Botany Department, Laramie WY 82071- 3165.

Booth E (1978). *The genus Fusarium*. Commonwealth Mycological Institute, Kew, Survey, England.

Brasier CM (1995). Episodic selection as a force in fungal microevolution, with special reference to clonal speciation and hybrid introgression. Can J. Bot. 73: 1213-1221.

Cavalli-Sforza LL, Edwards WF (1967). Phylogenetic analysis: models and estimation procedures. Evolution 32: 550-570.

Coddington A, Gould DS (1992). Use of RFLPs to identify races of fungal pathogens. In: Techniques for rapid detection of plant pathogens, 162-176 (Eds Duncan JM, Torrance L), Blackwell Scientific Publications.
Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. FOCUS 12: 13-15.
Ellegren H, Primmer CR, Sheldon BC (1995). Microsatellite evolution: directionality or bias in locus selection. Nat. Genet. 11: 360-362.
Felsenstein J (1995). PHYLIP: phylogeny inference package. University of Washington, Seattle.
Felsenstein J (1978). Cases in which parsimony or compatibility methods will be positively misleading. Syst. Zool. 27: 401-410.
Fournier E, Giraud T, Loiseau A, Vautrin D, Estoup SA, Solignac M, Cornuet JM, Brygoo Y (2002). Characterization of nine polymorphic microsatellite loci in the fungus Botrytis cinerea. Mol. Mycol. Notes 2: 253-255.
Gethi M, Mwithi FM, Macharia N, Njoroge K (1997). Maize bean intercropping system in medium potential area of Kenya. Farmer Practice and Research Challenges, Afr. Crop Sci. Conf. Proc. 3: 756-770.
Gerlach W, Nirenberg H (1982). The genus Fusarium, a pictorial atlas. Berlin and Hamburg: Kommissionnsverlag Paul Parey.
Goldstein DB, Ruiz LA, Cavalli-Sforza LL, Feldman MW (1995). An evaluation of genetic distances for use with microsatellite loci. Genetics 139: 463-471.
Goudet J (2001). A computer program to calculate F-statistics. Heredity 86: 485-456.
Isanda GO (1995). Assessment of the level of bean (*Phaseolus vulgaris* L.) infection by Colletotrichum lindemuthianum (Sacc. Magn.) Brit. and CAV. It's implication on disease incidence and severity. Msc. Thesis. University of Nairobi.
Kiprop EK, Baudoin JP, Mwang'ombe AW, Kimani PM, Mergeai G, Maquet A (2002). Characterization of Kenyan isolates of *Fusarium udum* from pigeonpea [*Cajanus cajan* (L.) Millsp.] by cultural characteristics, aggressiveness and AFLP analysis. J. Phytopathol. 150: 517-525.
Kraft JM, Burke DW, Hagland WA (1981). Fusarium diseases of beans, peas and lentils. Pennysvania State University Press, University Park, PA. USA.
Kretzer AM, Molina R, Spatafora JW (2004). Microsatellite markers for the Ectomycorrhizal basidiomycetes Rhizopogon vinicolor. Mol. Ecol. 9: 1171-1193.
Kristler HC, Benny U, Boehm EWA, Katan T (1995). Genetic duplication in Fusarium oxysporum. Curr Genet. 28: 173-176.
Kwasna H, Chelkowski J, Zajkowski P (1991). Fusarium seria mycota vol. 22. Polish Academic of Sciences , Institute of Botany, Krakow.
Lane PW, Payne RW (1996). Genstat^{R} for Windows^{TM}. An Introductory Course, 2nd Edition, Lawes Agricultural Trust.
Li S, Tam YK, Hartman GL (2000). Molecular differentiation of Fusarium solani f.sp. glycines from other Fusarium solani based on mitochondrial small subunit rDNA sequences. Ecol. Popul. Biol. 90: 491-497.
Muriungi SJ (1997). Bean root rot complex, it's management by microbial agents and plant resistance, MSc. Thesis, University of Nairobi.
Nderitu JH, Buruchara RA, Ampofo KO (1997). Integrated pest management of beans. African Highland Initiative.
Nei M (1987). Molecular evolutionary genetics. Columbia University Press, New York.
Nelson PE, Toussoun TA, Cook RJ (1983). Fusarium diseases, biology and taxanomy. Pennsylvania State University Press.
Nirenberg HI (1989). Identification of Fusarium occurring in Europe on cereals and potatoes. In: *Fusarium Mycotoxins,* Taxonomy and Pathogenicity, 179-193 (Ed J Chelkowski), Elsevier, Amsterdam.
Ochieng JW, Muigai AWT, Ude GN (2007). Phylogenetics in plant biotechnology: principles, obstacles and opportunities for the resource poor. Afr. J. Biotechnol. 6: 639-649.
Ochieng JW, Steane DA, Ladiges PY, Baverstock PR, Henry RJ, Shepherd M (2007). Microsatellites retain phylogenetic signals across genera in eucalypts (Myrtaceae). Genet. Mol. Biol. 30: 1125-1134.
O'Donnell K, Cigelnik E (1997). Two divergent intagenomic rDNA ITS2 types within a monophyletic lineage of the fungus Fusarium. Mol. Phylogenet. Evol. 7: 103-116.
Otsyula RM, Ajanga SI (1994). Control strategy for the root rot in western Kenya. Proceedings of the fourth KARI Scientific Conference, Nairobi, Kenya.
Rayner RW (1970). A mycological colour chart. Commonwealth Agricultural Bureaux.
Reddy MV, Raju TN (1993). Pathogenic variability in pigeonpea wilt-pathogen Fusarium udum. In: Plant Disease Problems in Central India, 32-34 (Murahdharan K, Reddy CS). Indian Phytopathological Society, Directorate of Rice Research, Hyderabad.
Rubinstein DC, Amos W, Leggo J, Goodburn S, Jain S, Li SH, Margolis RL, Ross CA, Ferguson-Smith MA (1995). Microsatellite evolution - evidence for directionality and variation in rate between species. Nat. Genet. 10: 337-343.
Sirjusingh C, Kohn L M (2002). Characterization of microsatellite in the fungal plant pathogen Sclerotinia sclerotionum. Mol. Ecol. Notes 1: 267-269.
Suga H, Hasegawa T, Mitsui H, Kageyama K, Hyakumachi M (2000). Phylogenetic analysis of the phytopathogenic fungus Fusarium solani based on the rDNA-ITS region. Mycol. Res. 104: 1175-1183.
Wortmann CS, Kirby RA, Eledi CA, Allen DJ (1998). Atlas of common bean (Phaseolus vulgaris) production in Africa. CIAT, Cali, Colombia.