Journal of Basic Microbiology
Environment · Health · Techniques

Research Paper

Mating type genes and genetic markers to decipher intraspecific variability among *Fusarium udum* isolates from pigeonpea

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To ascertain the variability in *Fusarium udum* (*Fu*) isolates associated with pigeonpea wilt is a difficult task, if based solely on morphological and cultural characters. In this respect, the robustness of five different genetic marker *viz.*, random amplified polymorphic DNA (RAPD), enterobacterial repetitive intergenic consensus (ERIC), BOX elements, mating type locus, and microsatellite markers were employed to decipher intra-specific variability in *Fu* isolates. All techniques yielded intra-specific polymorphism, but different levels of discrimination were obtained. RAPD-PCR was more discriminatory, enabling the detection of thirteen variants among twenty *Fu* isolates. By microsatellite, ERIC- and BOX-PCR fingerprinting, the isolates were categorized in seven, five, and two clusters, respectively. Cluster analysis of the combined data also showed that the *Fu* isolates were grouped into ten clusters, sharing 50–100% similarity. The occurrence of both mating types in *Fu* isolates is reported for the first time in this study. All examined isolates harbored one of the two mating-type idiomorphs, but never both, which suggests a heterothallic mating system of sexual reproduction among them. Information obtained from comparing results of different molecular marker systems should be useful to organize the genetic variability and ideally, will improve disease management practices by identifying sources of inoculum and isolate characteristics.

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords: Diversity / Mating type / Microsatellite / Rep-PCR / RAPD

Received: June 24, 2014; accepted: December 10, 2014

DOI 10.1002/jobm.201400483

Introduction

Pigeonpea (*Cajanus cajan* L. Millspaugh) wilt caused by *Fusarium udum* Butler (*Fu*) is one of the most important diseases and responsible for 16–47% yield loss under favorable environmental conditions [1]. In India, disease incidence varies from 5.3% to 22.6% [2] and losses only due to this disease are estimated to US $71 million. Use of resistant cultivars is the most effective and economic mean to manage the disease. However, high level of genetic variability among the *Fu* population and resistant cultivars' selective pressure has led to wide variation in virulence and aggressiveness of the *Fu* population in the field [3].

Pathogenic variability in *Fu* has been assessed traditionally through pathogenecity tests using host differentials containing different resistance genes. This is a laborious and time-consuming procedure requiring at least 40 days for the analysis [4]. *Fu* isolates have been identified by a range of cultural and morphological characteristics, such as shape and size of the macroconidia, presence or absence of microconidia and chlamydospores, fungicide sensitivity, colony color, growth rate, and differences in host range and pathogenicity [5, 6]. Although valuable, these criteria

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alone are not always sufficient as morphological features may vary under different environmental conditions [4]. Therefore, more effective genetic markers are needed to understand genetic variation in *Fu*.

Molecular techniques, combined with morphological studies have proven to be effective for characterization of *Fusarium* populations [6, 7]. Since past few years, several DNA-based molecular markers such as rDNA-ITS sequencing [8], RFLP [9], RAPD [6, 10], and SSR [11, 12] have been developed and widely implemented for more accurate characterization and discrimination between *Fusarium* species and within species. Although, the PCR-based DNA-fingerprinting is a fast, reliable, and comparatively low cost method to study genetic diversity of fungi, its effectiveness depends on primers chosen for analysis and quality of DNA. There are several highly conserved and repetitive DNA sequences present in the genome and that can be used to decipher genetic diversity of *Fusarium* employing PCR with different primers homologous to repetitive sequences. Three families of repetitive sequences (Rep) including repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC) sequences, and BOX element have been identified [13]. Godoy and colleagues showed that an enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and PCR restriction fragment length polymorphism (PCR-RFLP) were useful for genotyping *Fusarium* isolates [14]. Rep-PCR genomic fingerprinting with primers ERIC, BOX, and REP had the capacity to discriminate *Fusarium culmorum* isolates and revealed high level of genetic diversity among them [15].

Mating type characteristics of fungi are indirectly associated with genetic diversity as a determinant of recombination frequency in a population. Mating types are controlled by a locus with the idiomorphic allelles, MAT-1 and MAT-2 which contain conserved sequences of alpha box domain and high mobility group (HMG) box domain, respectively [16]. O'Donnell *et al*. [17] reported that MAT-1 and MAT-2 alleles have a contiguous arrangement on the nuclear genome consistent with nine phylogenetically distinct homothallic species of *F. graminearum*. *F. culmorum* carries either MAT-1 or MAT-2 alleles [18, 19]. This species has previously been reported to have heterothallic mating [20]. European isolates of *F. culmorum* dominantly carry MAT-2 alleles [18] while most of the Turkish isolates carry MAT-1 or MAT-2 alleles [19].

To date, several molecular methods have proven useful for analysis of intra-species genetic variation in *Fusarium* species [21]. But so far, no comparative assessment of various molecular marker systems to decipher genetic variability among *Fu* isolates has been reported. Thus, the objective of this work is to characterize the *Fu* isolates, using a range of molecular markers *viz*. mating type (MAT) primers, simple sequence repeats (SSRs), random amplified polymorphic DNA (RAPD), ERIC- and BOX- PCR analysis.

Materials and methods

Isolates

Twenty virulent isolates of *Fu* obtained from National Agriculturally Important Microbial Culture Collection (NAIMCC), National Bureau of Agriculturally Important Microorganisms (NBAIM), Mau, Uttar Pradesh, India, were used in the present study. The isolates were selected on the basis of host specificity, geographic origin and pathogenicity (Table 1). These isolates were maintained by regular sub-culturing on potato dextrose agar (PDA) at 28 ± 2 °C and stored as spore suspension in 15% glycerol at -80 °C.

Morphological and cultural characterization

Test isolate was plated on PDA at room temperature. Three 5-mm plugs were aseptically punched from actively sporulating areas near the growing edge of five-day-old culture of these isolates. Each plug was placed onto PDA Petri plates and incubated under the same conditions as starter cultures. After seven days, colony size, shape, margin, and color were recorded. Colony diameter of every culture was recorded daily for 7 days. Growth rate was calculated as the 7 day average of mean daily growth (mm per day). Three cultures of each isolate were investigated and experiments were conducted twice. For examination of conidial morphology, cultures were washed with sterile water and drops of the suspension were placed on microscope slides and mixed with lactophenol/cotton blue to stain the conidia. Length and width were measured for 30 conidia per isolate.

Genomic DNA isolation

Total genomic DNA from fungus was extracted with cetyltrimethylammonium bromide (CTAB) as described by Kumar *et al.* [22]. The purity of genomic DNA and quantification of template DNA for PCR was measured in duplicate using UV spectrophotometer (Shimazdu UV-160).

Genetic diversity analysis by mating type sequences

To identify the mating type of test isolates, MAT1–1 (5′-GTCGTCGATGGTGATGAAAGAAA-3′), MAT1-R (5′-CCGCACTGGAGCTCAAATGGT-3′), MAT2–2 (5′-GTTGCATCTCCGTCTGCGCCA-3′), MAT2-R (5′-GGCTGCAAGGATGACTGGCAT-3′) primers were used [23]. The PCR was performed in 25 μl reaction volume containing 10× PCR buffer, 25 mM

Table 1. Isolates of *Fusarium udum* used in the study.

Code no.	Accession no.	Culture	Biological origin	Geographical region	Virulence	Mating type*
*Fu*1	NAIMCC-F-02854	*F. udum*	*Cajanus cajan*	Hyderabad, Andhra Pradesh	+++	MAT1
*Fu*2	NAIMCC-F-02853	*F. udum*	*C. cajan*	Faridkot, Punjab	+	MAT1
*Fu*3	NAIMCC-F-02852	*F. udum*	*C. cajan*	Hissar, Haryana	+	MAT1
*Fu*4	NAIMCC-F-02860	*F. udum*	*C. cajan*	Latur, Maharashtra	+++	MAT1
*Fu*5	NAIMCC-F-02850	*F. udum*	*C. cajan*	Ranchi, Jharkhand	++	MAT1
*Fu*6	NAIMCC-F-02849	*F. udum*	*C. cajan*	Mujaffarpur, Bihar	+++	MAT2
*Fu*7	NAIMCC-F-02851	*F. udum*	*C. cajan*	Berhampur, West Bengal	++	MAT2
*Fu*8	NAIMCC-F-02844	*F. udum*	*C. cajan*	Aligarh, Utter Pradesh	+++	MAT1
*Fu*9	NAIMCC-F-02847	*F. udum*	*C. cajan*	Jabalpur, Madhya Pradesh	+	MAT2
*Fu*10	NAIMCC-F-02842	*F. udum*	*C. cajan*	IIPR Kanpur, Utter Pradesh	+++	MAT2
*Fu*11	NAIMCC-F-02855	*F. udum*	*C. cajan*	Guntur, Andhra Pradesh	+	MAT1
*Fu*12	NAIMCC-F-02848	*F. udum*	*C. cajan*	Sagar, Madhya Pradesh	++	MAT1
*Fu*13	NAIMCC-F-02845	*F. udum*	*C. cajan*	Bahraich, Utter Pradesh	+++	MAT2
*Fu*14	NAIMCC-F-02843	*F. udum*	*C. cajan*	Varanasi, Utter Pradesh	+++	MAT2
*Fu*15	NAIMCC -F-02861	*F. udum*	*C. cajan*	Badnapur, Maharashtra	+++	MAT1
*Fu*16	NAIMCC -F-02857	*F. udum*	*C. cajan*	Banglore, Karnatak	+	MAT2
*Fu*17	NAIMCC -F-02858	*F. udum*	*C. cajan*	Gulberga, Karnataka	++	MAT2
*Fu*18	NAIMCC -F-02859	*F. udum*	*C. cajan*	Aloka, Maharashtra	+++	MAT1
*Fu*19	NAIMCC -F-02856	*F. udum*	*C. cajan*	Krishnagiri, Tamil Nadu	+	MAT1
*Fu*20	NAIMCC -F-02846	*F. udum*	*C. cajan*	Allahabad, Utter Pradesh	+++	MAT1

+, less virulent; ++, moderately virulent; +++, highly virulent.
*Pathogenecity assay was done on fifteen days old seedling of susceptible cultivar (cv. TTB-7) by artificial inoculation of each isolate under glass-house. Virulence was measured by using a scale 1–9 [38], where those below 3 were considered as less virulent, 4–7 moderately virulent and above 7 highly virulent. Mating type was determined by PCR (see Fig. 1).

dNTP, 10 pM each of forward and reverse primers, one unit of *Taq* DNA polymerase (Bangalore Genei, India) and 25 ng of genomic DNA in PCR tubes. Thermal Cycler (G Storm GS4, Somerset, UK) was programmed as follows: initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 57.5/60.8 °C (MAT1/MAT2) for 30 s and elongation at 68 °C for 3 min and then a final extension at 68 °C for 15 min.

Genetic diversity analysis by ERIC and BOX elements

Genetic diversity among *Fu* isolates was analyzed by rep-PCR using the BOXA1R (5′-CTACGG CAAGGCGACGCT-GACG-3′), ERIC 1 R (5′-ATGTAAGCTCCTGGGGATTCA-3′) and ERIC 2 F (5′-AAGTAAGTGACTGGGGTGAGC-3′) primers [24]. All the PCR reactions were carried out in 25 μl reaction mixture containing 5× Gitschier buffer, 50 ng DNA template, 2 mM $MgCl_2$, 25 mM dNTP mixture, 50 pmol of each of primer, and one unit of *Taq* DNA polymerase (Bangalore Genie, India). Thermal Cycler (G Storm GS4, Somerset, UK) was programmed as an initial denaturation at 94 °C for 5 min, 40 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min and a final extension at 72 °C for 10 min.

Genetic diversity analysis by RAPD primer

Genetic diversity among *Fu* isolates was also assessed by RAPD primer using the 10 RAPD primer set (Bangalore Genei, India) (Supporting Information Table S1). All the PCR reactions were carried out in 25 μl reaction mixture containing 10× PCR buffer (Bangalore, Genei), 5 pmole of primer, dNTPs (25 mM), one unit of *Taq* DNA polymerase (Bangalore Genie, India) and 50 ng genomic DNA as a template in a thermal cycler. The thermal profile used was initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation step (94 °C for 1 min), annealing (47 °C, 1 min), extension (72 °C for 1.5 min), and a final extension step (72 °C for 7 min).

Genetic diversity analysis by microsatellites

Three EST-SSR primer sets (FOM-8, FOL-2, and FOL-4) developed by Kumar *et al.* [2] were used to analyze the genetic diversity in *Fu*. The PCR was performed in 10 μl reaction volume containing 1× PCR buffer (10 mM Tris HCl pH 9.0, 1.5 μM $MgCl_2$, 50 mM KCl, 0.01% gelatin), 25 mM dNTP (Bangalore Genei), 10 pM each of forward and reverse primers, one unit of *Taq* DNA polymerase (Bangalore Genei, India), and 25 ng of genomic DNA was used as template in PCR tubes. PCR program me and gel electrophoresis was as described by Kumar *et al.* [2]. 100 bp and 500 bp DNA ladder (MBI Fermentas) was used to estimate the fragment size.

Visualization, scoring of bands, and construction of dendrogram

Amplified products were resolved in 1.5% agarose gels using 1× TAE buffer on a gel electrophoresis apparatus. Ethidium bromide (0.25 mg ml^{-1}) was used as an

intercalating agent. The gel was run at 6 V cm^{-1} of the length of gel till the bands resolved. The amplified bands, after separation on the gel, were visualized and documented using a gel documentation imaging system (Bio-Rad, USA). The presence or absence of individual, distinct, and reproducible bands was scored as '1' for presence and '0' for absence. Binary data were used to calculate Jaccard similarity coefficient. Cluster analysis was performed using NTSYSpc version 2.0 [25] and dendrogram was constructed using the unweighted pair-group method with arithmetic average (UPGMA).

Statistical analysis

Experimental data for conidial morphology and growth rate were analyzed using standard analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). Standard errors were calculated for all mean values. All RAPD, ERIC, BOX, MAT, and microsatellite-PCR reactions were repeated to ensure validity of results. A combined dendrogram was constructed for the isolates on the basis of the different fingerprint patterns generated by each primer [26].

Results

Morphological and cultural characterization

Colony color, chlamydospore position, and sporulation pattern revealed some interesting trends and on the basis of these *Fu* isolates could be separated into eleven groups (Table 2; Supporting Information Fig. S1). Most of the isolates (*Fu*2, *Fu*3, *Fu*4, *Fu*8, *Fu*16, and *Fu*19) produced white, creeping growth with intercalary chlamydospores on aerial hyphae (Supporting Information Fig. S1). Isolate *Fu*5 and *Fu*18 showed yellowish cottony growth with profuse aerial hyphae and intercalary chlamydospores. Other isolates appeared as white to purple whitish cottony growth with profuse aerial hyphae having both terminal and intercalary chlamydospores (*Fu*1 and *Fu*10), creamy white creeping growth without aerial hyphae and intercalary chlamydospores (*Fu*6), pinkish fluffy and appressed growth with intercalary chlamydospores (*Fu*7), whitish fluffy and appressed with both type of chlamydospores (*Fu*9 and *Fu*12), pinkish, creeping growth with aerial hyphae and both type of chlamydospores (*Fu*11), pinkish fluffy and appressed with both type of chlamydospores (*Fu*13), whitish cottony growth with profuse aerial hyphae with both type of chlamydospores (*Fu*14), yellowish cottony growth-fluffy with intercalary chlamydospores (*Fu*15), pinkish or yellowish creeping growth without aerial hyphae and absence of chlamydospores (*Fu*17 and *Fu*20), respectively.

According to mycelia growth rate, the *Fu* isolates were divided into eight different groups. Isolates *Fu*12, *Fu*13, and *Fu*18 (11–11.14 mm day^{-1}) grew faster than other isolates. Least growth rate was recorded in case of *Fu*10 (5.02 mm day^{-1}) and *Fu*11 isolates (5.26 mm day^{-1}) (Table 2). A perusal of data indicated that there was a significant difference in growth rate among test isolates.

The hyphae of the cultured isolates were highly branched, slender, septate, and produced conidia and chlamydospores. Macro and microconidia were present in all the isolates, but the size varied between $31.0 \pm 2.37 \times 4.9 \pm 1.09$ and $13.9 \pm 1.15 \times 4.1 \pm 1.09$ μm (*Fu*3 isolate) to $13.8 \pm 1.54 \times 2.9 \pm 1.07$ and $4.9 \pm 1.17 \times 2.8 \pm 1.08$ μm (*Fu*5 isolate), respectively, with 1–8 septation in all isolates (Table 2). Distinct pattern of profuse sporulation of macro- and micro-conidia was observed among *Fu* isolates. Clustering of *Fu* isolates on the basis of sporulation showed a non-significant relationship in terms of macro- and microconidia production among *Fu* isolates. Isolate *Fu*2, *Fu*5, and *Fu*19 showed profuse sporulation of macrocondia, while six isolates (*Fu*1, *Fu*4, *Fu*6, *Fu*7, *Fu*12, and *Fu*16) indicated significant production of microcondia (Table 2).

Mating type genes

PCR assay was performed to assign mating types (MAT1 and MAT2) for 20 different isolates of *Fu* (Table 1). A single product was generated by PCR from each *Fu* isolates using primer pairs complementary to the alpha domain and HMG domain genes, respectively. A 320 bp portion of the alpha domain (MAT1) was obtained from twelve isolates. Similarly, a 650 bp portion of HMG domain (MAT2) was detected in eight isolates (Fig. 1).

RAPD-PCR analysis

Among the ten RAPD primers, three primers *viz.,* OPA-2 (TGCCGAGCTG), OPA-3 (AGTCAGCCAC), and OPA-11 (CAATCGCCGT) were chosen based on their capacity to reveal variability among isolates (Fig. 2). These primers produced a total of 258 fragments among all the 20 isolates. The size of RAPD fragments ranged 300–1600 bp. RAPD analysis of genomic DNA from the pathogenic isolates revealed the presence of 13 clusters at the arbitrary level of 50% similarity (Fig. 2). Maximum isolates were clustered in group I (*Fu*1, *Fu*2, *Fu*3, and *Fu*4) followed by group II (*Fu*19 and *Fu*20), III (*Fu*15 and *Fu*18), IV (*Fu*6 and *Fu*7), and IX (*Fu*13 and *Fu*16).

ERIC-PCR analysis

The genetic discrimination among the 20 *Fu* isolates was assessed using ERIC-PCR and a high level of variability in the banding pattern was obtained (Fig. 3). The number of

Table 2. Morphological characteristics of *F. udum* isolates used in the present study.

Isolate No.	Colony Type	Colony colour	Pigmentation	Growth (mm day^{-1})	Spore Size [μm]				Conidia Sporulation			
					Macroconidia		Microconidia					
					Length	Width	Length	Width	Macro	Micro	No. of septa	Clamydospore Position
*Fu*1	Cottony growth, profuse aerial mycelium	Purple white	Dark gray	*8.57 ± 0.49^{d}	28.5 ± 1.54^{bc}	4.2 ± 1.11^{a}	11.2 ± 1.12^{bc}	3.9 ± 1.19^{a}	+	+++	3–8	**Both
*Fu*2	Creeping growth without aerial mycelium	White	Blackish gray	8.74 ± 0.49^{d}	29.4 ± 1.49^{abc}	4.1 ± 1.14^{ab}	12.1 ± 1.12^{bc}	3.8 ± 1.08^{a}	+++	++	3–4	Intercalary
*Fu*3	Creeping growth without aerial mycelium	White	Light red	8.0 ± 0.44^{e}	31.0 ± 2.37^{a}	4.9 ± 1.09^{a}	13.9 ± 1.15^{a}	4.1 ± 1.09^{a}	+	++	3–4	Intercalary
*Fu*4	Creeping growth without aerial mycelium	White	Orange	6.89 ± 0.45^{f}	17.5 ± 1.48^{e}	3.4 ± 1.17^{b}	6.2 ± 1.12^{e}	3.0 ± 1.12^{ab}	++	+++	2–3	Intercalary
*Fu*5	Creeping growth without aerial mycelium	Yellowish	Orange	5.90 ± 0.29^{g}	13.8 ± 1.54^{g}	2.9 ± 1.07^{b}	4.9 ± 1.17^{f}	2.8 ± 1.08^{b}	+++	++	2–4	Intercalary
*Fu*6	Creeping growth without aerial mycelium	Creamy White	Blackish gray	8.63 ± 0.32^{d}	28.2 ± 2.49^{bc}	3.8 ± 1.04^{ab}	11.0 ± 1.07^{bc}	3.7 ± 1.06^{a}	++	+++	1–3	Intercalary
*Fu*7	Fluffy and appressed	Pinkish	Light orange	9.21 ± 0.48^{cd}	16.8 ± 2.36^{f}	2.8 ± 1.04^{b}	6.8 ± 1.18^{e}	3.2 ± 1.07^{ab}	++	+++	3–5	Intercalary
*Fu*8	Creeping growth without aerial mycelium	White	Light red	9.44 ± 0.46^{bc}	17.3 ± 2.44^{ef}	3.1 ± 1.08^{b}	5.2 ± 1.15^{f}	3.1 ± 1.07^{ab}	++	+	3–8	Intercalary
*Fu*9	Fluffy and appressed	White	Dark gray	9.90 ± 0.44^{b}	25.4 ± 2.28^{cd}	3.6 ± 1.15^{b}	9.1 ± 2.11^{c}	3.9 ± 1.09^{a}	+	+	2–4	Both
*Fu*10	cottony growth Profuse aerial mycelium	White	Blackish gray	5.04 ± 0.4^{h}	12.0 ± 1.33^{g}	2.8 ± 1.03^{b}	4.8 ± 1.30^{f}	2.1 ± 1.02^{b}	++	+	3–7	Both
*Fu*11	Creeping growth without aerial mycelium	Pinkish	Light orange	5.26 ± 0.35^{h}	27.3 ± 2.45^{bcd}	3.9 ± 1.21^{ab}	10.8 ± 2.15^{c}	3.1 ± 1.11^{ab}	++	+	1–3	Both
*Fu*12	Fluffy and appressed	White	Light Pink	11.03 ± 0.42^{a}	26.1 ± 2.48^{c}	3.7 ± 1.06^{b}	9.8 ± 1.64^{c}	3.6 ± 1.05^{a}	+	+++	2–4	Both
*Fu*13	Fluffy and appressed	Pinkish	Light pink	11.14 ± 0.61^{a}	24.6 ± 2.32^{d}	3.8 ± 1.10^{ab}	8.2 ± 1.14^{d}	3.2 ± 1. 03^{ab}	+	++	3–8	Absent
*Fu*14	Cottony growth profuse aerial mycelium	White	Yellowish	8.39 ± 0.3^{de}	18.4 ± 1.45^{e}	4.0 ± 1.02^{ab}	7.8 ± 1.28^{d}	2.8 ± 1.05^{b}	+	++	3–8	Both
*Fu*15	Cottony growth- fluffy	Yellowish	Light orange	6.63 ± 0.39^{f}	28.2 ± 2.12^{bc}	4.3 ± 1.08^{a}	11.4 ± 1.19^{b}	3.9 ± 1.13^{a}	++	++	1–3	Intercalary
*Fu*16	Creeping growth without aerial mycelium	White	Light orange	6.86 ± 0.52^{f}	18.2 ± 1.75^{e}	3.9 ± 1.12^{ab}	7.6 ± 2.14^{de}	2.2 ± 1.11^{b}	+	+++	3–5	Intercalary
*Fu*17	Creeping growth without aerial mycelium	Pinkish	Creamy blue	7.51 ± 0.44^{e}	15.8 ± 1.39^{f}	3.8 ± 1.09^{ab}	4.9 ± 1.12^{f}	2.2 ± 1.09^{b}	+	++	1–2	Absent
*Fu*18	Cottony growth profuse aerial mycelium	Yellowish	Blackish gray	11.0 ± 0.32^{a}	16.8 ± 1.35^{f}	3.4 ± 1.14^{b}	5.6 ± 2.05^{ef}	2.8 ± 1.12^{b}	++	+	3–7	Intercalary
*Fu*19	Creeping growth without aerial mycelium	White	Light pink	8.74 ± 0.55^{d}	25.1 ± 2.44^{d}	3.5 ± 1.12^{b}	8.6 ± 1.16^{d}	2.5 ± 0.02^{b}	+++	++	1–2	Intercalary
*Fu*20	Creeping growth without aerial mycelium	Yellowish	Light orange	9.74 ± 0.32^{b}	26.2 ± 2.49^{cd}	3.9 ± 1.13^{ab}	10.0 ± 1.32^{c}	3.8 ± 0.15^{a}	+	+	3–7	Absent

+, little sporulation; ++, moderate sporulation; +++, profuse sporulation.

*Values are mean ± standard error of 30 conidia per isolate. Means followed by same letter in superscript within a column are not significantly different ($p \leq 0.05$) according to Duncan's Multiple Range Test (DMRT).

**Both indicate the formation of thick-walled asexual spore by the rounding-up of a cell at intercalary and terminal position.

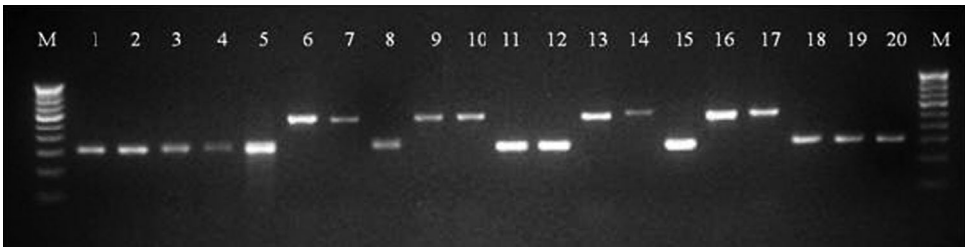

Figure 1. PCR amplification of MAT1 (320-bp) and MAT2 (650 bp) gene in *Fu* isolates representing distinct geographical lineages. Lanes 1–20 are different *Fu* isolates as mentioned in Table 1. M is a 100-bp DNA marker.

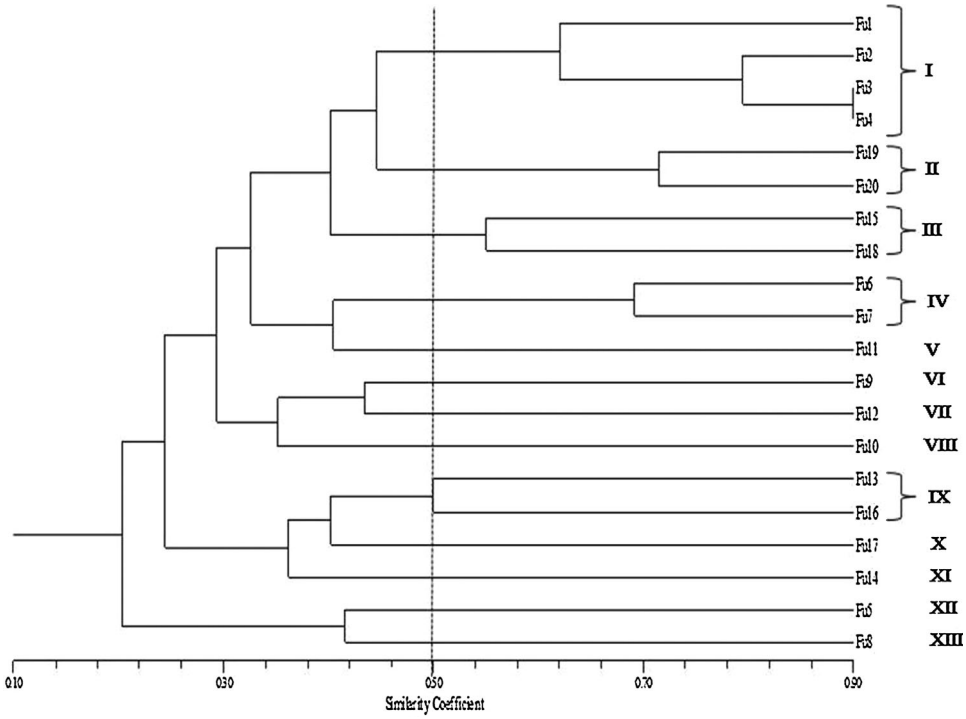

Figure 2. Dendrogram derived from RAPD PCR analysis obtained with OPA2, OPA3, and OPA11.

bands in the amplification profile was 190, and their size was found to vary from 150 bp to 3000 bp among these isolates (Fig. 3). Cluster analysis based on the Jaccard's similarity coefficient (50%) showed that the isolates were divided into seven groups and giving a ratio of eight bands/isolate.

BOX-PCR analysis

Analysis of BOX-PCR banding pattern (Fig. 4) showed that the *Fu* isolates were clustered into five clusters, sharing 50–100% similarity. The banding pattern showed a total of 246 fragments in the range of 200–4000 bp, giving a ratio of five polymporphic bands/isolate. A perusal of the dendrogram revealed that thirteen isolates (*Fu*1, *Fu*2, *Fu*3, *Fu*4, *Fu*5, *Fu*7, *Fu*9, *Fu*10, *Fu*13, *Fu*14, *Fu*15, *Fu*16, and *Fu*17) formed a major cluster (cluster I), while only three (*Fu*18, *Fu*19, and *Fu*20) and two (*Fu*11 and *Fu*12) isolates were grouped in third (cluster III) and fourth cluster (cluster IV), respectively.

Microsatellite analysis

Three SSR primers were used for amplification of microsatellite loci of 20 isolates of *Fu.* All isolates amplified polymporphic bands ranging 200–800 bp. A total 60 alleles were produced by test primers. The dendrogram based on Jaccards similarity coefficient depicted two major clusters I and II at 50% genetic dissimilarity (Fig. 5). Cluster I contained eleven isolates,

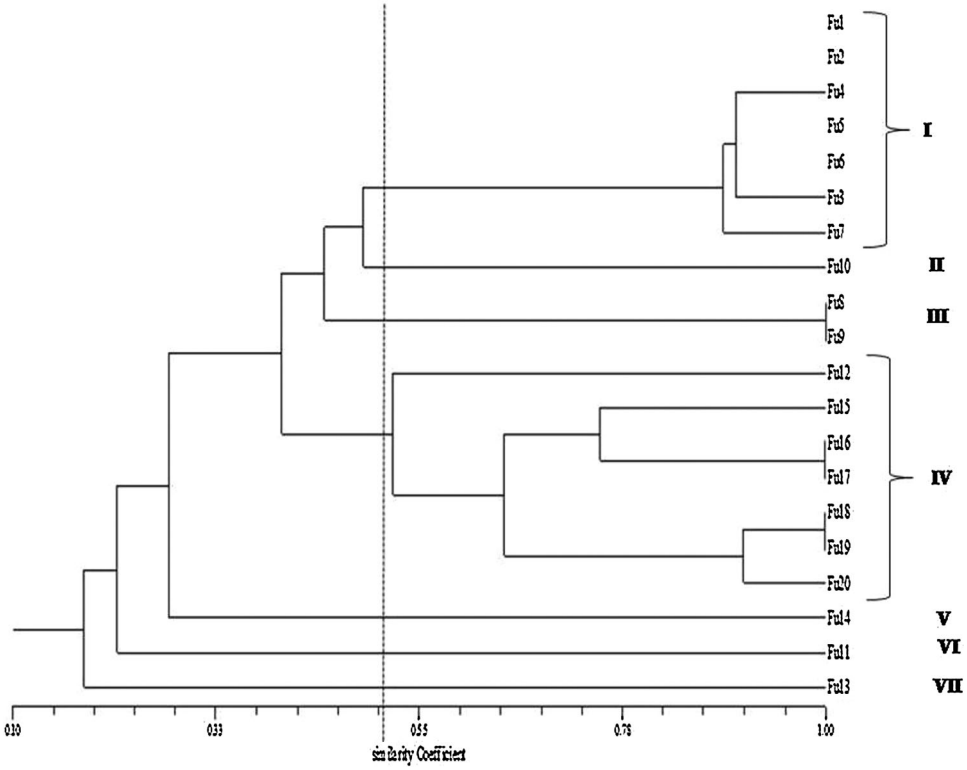

Figure 3. Dendrogram based on banding pattern obtained from ERIC-PCR fingerprints.

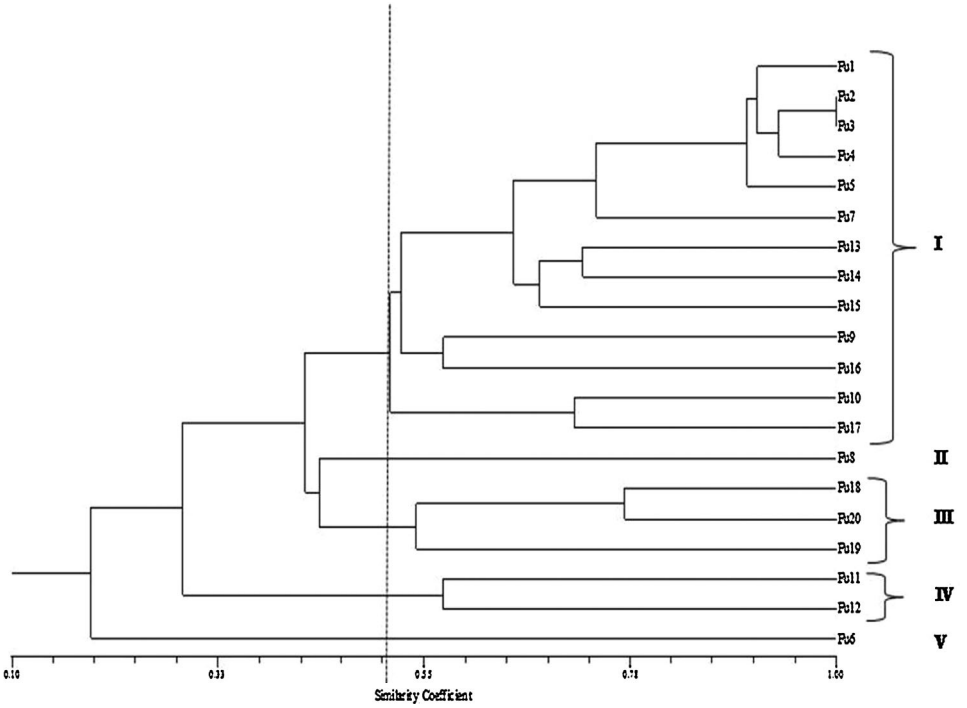

Figure 4. Dendrogram based on banding pattern obtained from BOX-PCR fingerprints.

and cluster II contained nine isolates. The highly diverse first cluster branched into four sub-clusters at 69% genetic diversity among *Fu* isolates. The first sub-cluster included seven isolates (*Fu*1–*Fu*7), and fourth sub-cluster included two isolates (*Fu*11 and *Fu*12). Isolates *Fu*5 and *Fu*10 were depicted in cluster I with less similarity value from rest of other isolates. The second major cluster branched into further two sub-clusters and showed 69% genetic diversity. The first sub-cluster included seven isolates (*Fu*8, *Fu*9, *Fu*13, *Fu*14, *Fu*18, *Fu*19, and *Fu*20), while *Fu*18 and *Fu*19 formed the second sub-cluster (Fig. 5).

Discussion

A detailed understanding of pathogen's phenotypic and genetic diversity is imperative to interpret their contribution to disease epidemiology and management. In this context, a polyphasic approach was undertaken with the aim of identifying and characterizing the *Fu* species responsible for causing wilt disease in piegonpea in India. *Fu* isolates were grouped into 11 distinct morphological types based on colony attributes. *Fu* isolates from the same site or diverse geographical origins have been shown to exhibit high variability in cultural characteristics and virulence [5, 27]. This may be due to the fact that the isolates are derived from genetically distinct clones. The exchange of contaminated seeds and cultures probably contributed to the existence of variable population of *Fu* in wider geographical areas. Moreover, non-stability of most prominent pigeonpea genotypes also supported the argument that the isolates of this fungus are not stable and parasexual recombination might have a role in the evolution of races. Thus, relying on morphology and ability to grow on selective culture media for the discrimination of *Fu* isolates is time consuming and laborious. In addition, considerable expertise is needed to clearly define intra-species differentiation [28].

The present study is the first report to determine the presence of mating types (MAT1 and MAT2) in *Fu* isolates of Indian origin. In present study, the amplification of the *MAT* idiomorph sequence revealed that 60% of the examined *Fu* isolates possess the *MAT-1* idiomorph and 40% possess the *MAT2* allele. No one isolate showed both amplicons, which would be distinctive of homothallic species. Similar results were reported for several species from *Fusarium* [29–34]. The obtained results may suggest that *Fu* has a potentially heterothallic origin. Such a conclusion is in agreement with the hypothesis proposed by Turgeon [32], that some *Fusaria* may exhibit a sporadic and cryptic sexual cycle. Taylor *et al.* [33] also suggested that all fungi with no known sexual stage are originally heterothallic, and most of them should display a sexual reproduction. The maximum effective reproductive strategy occurs when the mating type idiomorphs are present in a 1:1 ratio [34]. However, the absence of teleomorphs on the one hand and the source of high intra-species variability on the other, are still poorly

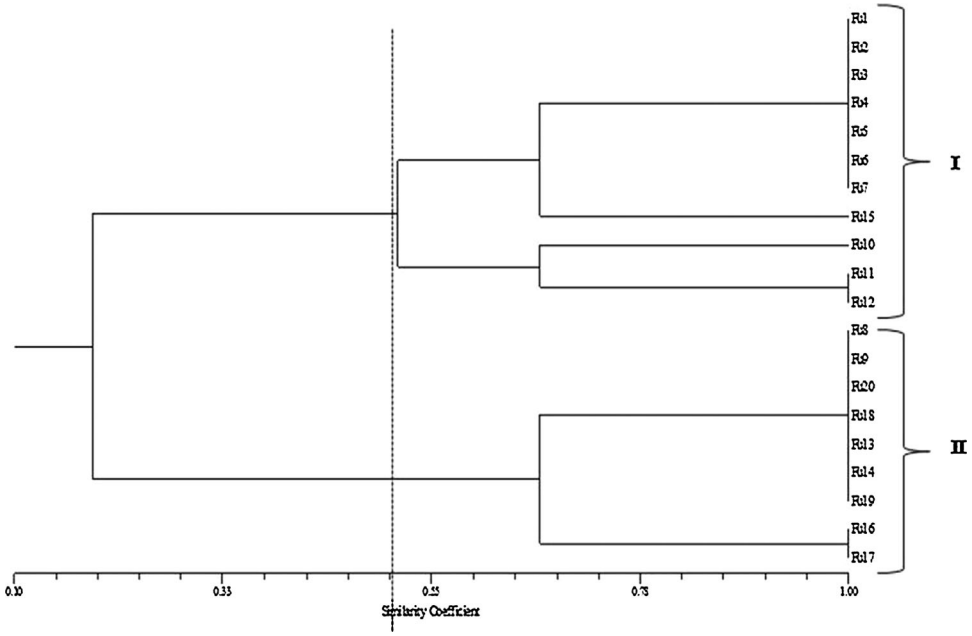

Figure 5. Dendrogram derived from microsatellite marker analysis obtained with FomSSR8, FolSSR-2, and FolSSR-4.

understood and extensive studies needed to assess a hypothetic genetic linkage between particular *MAT* allele and genes controlling virulence.

The present study also revealed the usefulness of DNA polymorphism techniques to detect genetic variation among *Fu* isolates. These techniques are especially valuable to enhance epidemiological knowledge because typing is the prime step in epidemiological studies [35]. The study of DNA polymorphisms involves the selection of a target sequence, and several approaches have been used to achieve this task. One approach involves the exploitation of ubiquitously conserved known genes that display sequence variation. In particular, comparative nucleotide sequencing of rDNA subunits, such as ITSs, has been used widely for distinguishing between fungal species, and to develop specific protocols for identifying fungal species [24]. Another approach involves the screening of random parts of the genome to identify distinctive nucleotide sequences by techniques, such as microsatellites, RAPD, ERIC- and BOX-PCR. The results indicated that BOX elements and ERIC-PCR are suitable for the rapid molecular characterization of *Fu* isolates. Some of the *Fu* isolates such as *Fu*8, *Fu*16, and *Fu*19 which were not differentiated by morphology can be discriminated by BOX and ERIC-PCR banding patterns. In general, both techniques were found to produce reproducible results especially with purified genomic DNA as a template, and when the primer concentration and composition of buffer were strictly controlled.

All the molecular marker systems have shown a high success rate in PCR amplification and detected a significant variation among *Fu* isolates. It has been observed that using the same set of isolates at a similarity coefficient of approximately 50%, RAPD showed higher level of variation compared to other molecular marker systems (microsatellite, ERIC- and BOX-PCR). This finding agrees with the observation that the DNA sequences are highly conserved among the species and potentially useful to distinguish between organisms [36]. Also, RAPD utilizes fragment amplification of the whole genome, therefore being well suited to detect differences between closely related organisms. It is also worth mentioning here that all the marker systems revealed considerable intra-species variability, although grouping on the basis of virulence, geographical origin and mating types was not detected. Considering the large areas and diverse eco-environments in India where pigeonpea wilt has occurred, results suggest that geographic isolation and ecological conditions may have had a significant effect on the distribution of *Fu.* Further, in order to improve the clarity of the dendrogram, single similarity matrices was generated by combining the banding patterns of RAPD, microsatellite, BOX- and ERIC-PCRs. In fact, it was found that the combined dendrogram (Supporting Information Table S2, Fig. S2) gave the most information among all the individual cluster analyse, although less robust than RAPD. These findings are contradictory with the study of Gurjar *et al*. [37], who advocated that the combined use of molecular methods was more powerful to detect fine differences among pathogen subpopulations. We also support the notion and postulated that a larger population of *Fu* originated from different geographic locations should be analysed before reaching a final conclusion.

In conclusion, RAPD, mating type, microsatellite, ERIC, and BOX-PCR based fingerprinting techniques have proved to be powerful molecular tools for examining intra-specific variation within *Fu* isolates. The present study also provides evidences regarding the presence of *MAT* idiomorphs within *Fu* isolates of Indian origin. Mating type genes are frequently used in population studies, as their presence, relative frequency and distribution within a population may facilitate the identification of the pathogen population in pigeonpea crop in order to improve genetic resistance as well as in epidemiological studies.

Acknowledgments

This study was made possible through the financial support from the Indian Council of Agriculture Research (ICAR) under the "Outreach project on *Phytophthora, Fusarium*, and *Ralstonia* disease in horticulture and field crops."

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