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Population distribution and genetic relatedness in Indian *Fusarium udum* isolates based on ribosomal internal transcribe spacer and elongation factor

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Abstract

With the objective to study the geographical distribution pattern and pathotype classification, isolates from 12 major pigeonpea growing states of India were examined at morphological and molecular levels. Two DNA based internal transcribe spacer (ITS) region derived primers FDP 3 (ITS1/ITS2), FDP 25 (mRNA, LOC100383610) and two elongation factors FDP 4 (F98-BKR5) and FDP 29 (M9968PY) were employed to genetically differentiate the isolates. As a result, each marker system gave an average of 3 alleles/marker. The higher efficiency of ITS over EF-1α marker was revealed using detailed comparative analysis that included various parameters like gene diversity index, effective marker ratio, and marker index. Neighbour Joining tree analysis grouped the isolates into three major clusters and showed narrow existence of genetic divergence. Combination of genotyping data with pathological measurements indicates dominance of variant 1 in the Central zone, South zone and North East Plain Zone, while North East Plain Zone and North West Plain Zone were largely dominated by variants 2 and 1, with strong possibility of evolving other variants. The present study would help in identifying specific isolate and patterns of its distribution in various pigeonpea growing regions thereby enhancing the scope for precise resistance breeding for crop improvement.

Key words

Elongation factor, *Fusarium udum*, Genetic diversity, Internal transcribed spacer, Pathogenicity

Introduction

Fusarium sp. is one of the most devastating soil-borne pathogen worldwide for field crops (Roncero *et al.,* 2003). The pathogen invades a wide range of hosts causing severe loss to agricultural produce, extending up to 100% yield penalty in susceptible cultivars (Kiprop *et al.,* 2002). In legume crops, pigeonpea remains an important food legume in India, however its cultivation is subjected to vulnerability against number of root and foliar diseases, like *Fusarium* wilt, at different stages of crop growth (Mahesh *et al.,* 2010). The disease occurs almost at every pigeonpea growing areas in India, especially Maharashtra, Rajasthan, Madhya Pradesh, Uttar Pradesh and some parts in Southern India. Therefore, quick and accurate methods are required to identify various pathogenic isolates, based on morphological features and genetic analysis for appropriate disease management. Since the growth of isolates is influenced by a plethora of factors like environmental conditions, type of media, nutrients and cultural conditions however classification of these isolates exclusively on the morphological basis remain a difficult task. Therefore, DNA based fingerprinting technology will be the best tool to understand the underlying genetic basis of diversity and phylogenetic relationships among various *Fusarium* species.

So far, DNA-based marker system like random amplified polymorphic DNA (RAPD) in *Fusarium* sp. (Datta *et al.,* 2009), ITS in different *Fusarium* sp. (Datta *et al.,* 2011), restriction fragment length polymorphism (RFLP) marker system in *F. semitectum* (Avinash *et al.,* 2011), amplified fragment length polymorphism (AFLP) in *F. oxysporum* (Robert *et al.,* 2000) were employed effectively to differentiate fungal species. In the recent past, highly

conservative ITS regions in *F. oxysporum* f. sp *lycopercicon*, translation elongation factor (EF-1α) for genus *Fusarium* and *F. solani* (Arif *et al.,* 2012), intergenic spacer region (IGS) in *F. oxysporum* f.sp *cubense* (Dita *et al.,* 2010), vegetative compatibility group (VCG) in *F. oxysporum* f.sp *cubense* (Fourie *et al.,* 2009) and PCR detection of nuclear protein gene SGE 1 (secreted in xylem 1) for parasitic growth in *F. oxysporum* (Caroline *et al.,* 2009), matting type alleles (MAT1-1, MAT1-20) in *F.oxysporum* (Lidia *et al.,* 2012) and *F. oxysporum* f.sp *Phaseoli* (Bahar *et al.,* 2010) have widely been used in genetic diversity analysis. In certain ribosomal rRNA genes family: 26S, 28S, 18S, 5.8S, and 5S rRNA are arranged in head-to-tail tandem repeats fashion and separated by spacer region; *viz* internal transcribed spacer (ITS) and intergenic spacer region (IGS). These regions are mainly located in between 18S Small Sub Unit (SSU) and 28S Large Sub unit (LSU) of nuclear ribosomal DNA separated by 5.8S gene and source for variable sites. Therefore, the present study aimed at elucidating the population diversity of *F. udum* in different pigeonpea growing regions in India employing ITS and EF-derived markers, and establishing correlation between geographical distributions of isolates and their pathotypes.

Materials and Methods

A systematic collection comprising of 45 *F. udum* isolates that represent 12 states and four agro-ecological zones as North-Eastern Plains Zone (NEPZ), North Western Plain Zone (NWPZ), Central Zone (CZ) and Southern Zone (SZ) in India were used in the present study.

Mycelia growth estimation and pathogenicity test : A single conidium from individual isolates were inoculated in potato dextrose agar (PDA) plates for 4-5 days at 25°C to observe morphological character of mycelial growth, colour, type of septum and colony pattern as described by Sinha *et al.* (2008). The extent of pathogenicity was tested by purified isolates on wilt susceptible pigeonpea cultivar 'Bahar' under pot experiments in growth chamber. The isolates were further tested for host pathogen interaction on fifteen differential genotypes of pigeonpea to classify *F. udum* isolates into variants. Thus, sterilized seeds of pigeonpea in 0.5% $HgCl_2$ were put in sterilized soil inoculated with 5% inoculum ($1x10^7$conidia ml^{-1}). Disease symptoms were regularly monitored at 30 days after germination under day temperature of 37ºC and night temperature of 28ºC with relative humidity of 80%. The details relating to geographic origin and pathogenicity of various isolates are given in Table 1.

Genomic DNA extraction and quantification : Genomic DNA from 12-15 days old mycelia was extracted according to the Lee and Taylor procedure (1990). The quality and quantity of extracted DNA were estimated by nano spectrophotometer (Eppendorf) and gel-electrophoresis. DNA was normalized to working concentration of 20ng μl^{-1} for downstream PCR analysis.

PCR amplification of ITS and EF-1α loci : PCR amplification of four *Fusarium* specific primers ITS (2) and EF-1α (2) regions were performed in 45 isolates. The PCR reactions were performed in 20µl volume containing 1X Taq buffer, 0.2mM of dNTPs mix, 25 pmole each forward and reversed primer and 0.3 U of Taq polymerase (Merk Biosciences) with 25ng of template DNA. PCR conditions for primer pair were as follows; denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, annealing for 1 min (appropriate annealing temperature were used for each primers set), elongation Tm at 72°C for 2 min with final extension of 10 min at 72°C and finally amplified products were resolved in 3 % agarose gel, using 0.5X TBE buffer.

Data scoring and statistical analysis : Statistical analysis for distinct polymorphic fragments, detected by marker systems was converted to binary form to generate binary matrix. These binary data were then analysed using numerical taxonomy and multivariate analysis system (NTSYSpc 2.02i) software package (Rohlf, 2000) and Darwin (Version 5.0.156) software. The UnWeighted Neighbor-Joining tree constructed using Darwin (Version 5.0.156) software was used to establish the genetic relationship of the 45 *F. udum* isolates. The robustness of dendrogram was tested by generating cophenetic values for each dendrogram. Mantel matrix correspondence test was then used to compare cophenetic matrices (Mantel, 1967).

Demonstrating utility of the marker system : The utility of marker systems mental test was performed to compare the correlation coefficient between Jaccard's and cophenetic similarity matrix to analyse the 'goodness of fit' for dendrogram, generated by individual's marker system (ITS, EF-1α, ITS+EF-1α). This was further re-evaluated by genetic parameters like marker discriminatory power, polymorphism information content (PIC), marker index (MI) and effective marker ratio (EMR). The DI of primer is defined as $1- \sigma (pi)^2$ where pi is frequency of i^{th} allele, using Popgene software, version 1.31 (Yeh *et al.,* 1999), while EMR of primer was calculated as “product of fraction of polymorphic bands and number of polymorphic bands for an individual marker system” described by Milbourne *et al. (*1997). Marker index (MI) was used to evaluate the overall utility of each marker system by the following formula

$$MI = DI.\beta.n,$$

where DI is total gene diversity, β is percentage of polymorphic allele and n is the number of alleles detected per primer.

Results and Discussion

The pathogenicity of isolates was determined by inspecting the appearance of wilt symptoms and percentile mortality observed in susceptible pigeonpea cultivar “Bahar”. Based on disease severity scores, isolates were classified as high (80-100%), moderate (40 to below 80%) and weak (0-40%)

Table 1. Geographical distribution of *F. udum* isolates collected from different states in India and their pathogenicity index with morphological features of mycelial growth and colony growth pattern

Isolates	State	Pulses growing agroecological zone	Pathogenicity test			
			Pathogenicity	Mycelial growth rate	Growth pattern	Pathological reaction
MP-132	Madhaya Pradesh	CZ	NA	Slow	Fluffy	V3
KA-1	Karnataka	SZ	NA	NA	NA	NA
KA-8	Karnataka	SZ	NA	Medium	Intermediate	NA
KA-14	Karnataka	SZ	NA	Slow	Fluffy	V1
KA-15	Karnataka	SZ	NA	Slow	Fluffy	NA
AP-1	Andhra Pradesh	SZ	NA	Slow	Fluffy	NA
AP-5	Andhra Pradesh	SZ	NA	Slow	Appressed	NA
AP-7	Tamilnadu	SZ	NA	Slow	Fluffy	NA
MSF-4	Maharashtra	CZ	NA	Medium	Fluffy	V1
MSF-12	Maharashtra	CZ	NA	Medium	Fluffy	NA
MSF-14	Maharashtra	CZ	NA	Medium	NA	NA
AKOLA-1	Maharashtra	CZ	NA	Medium	Appressed	NA
MP-133	Madhaya Pradesh	CZ	NA	Slow	Fluffy	V1
MP-134	Madhaya Pradesh	CZ	NA	Slow	Appressed	NA
MP-137	Madhaya Pradesh	CZ	NA	NA	Intermediate	V2
MP-142	Madhaya Pradesh	CZ	NA	Slow	Fluffy	V1
H-1	Haryana	NWPZ	Weak	Slow	Appressed	V3
HF-1	Haryana	NWPZ	Moderately	Fast	Fluffy	NA
DF-3	Delhi	NWPZ	Moderately	Fast	NA	V2
RF-6	Rajasthan	NWPZ	Weak	Slow	Fluffy	V3
HF-23	Haryana	NWPZ	Moderately	Fast	Intermediate	V2
FU-12	Bihar	NWPZ	High	Fast	Fluffy	V2
FU-24	Bihar	NWPZ	Weak	Slow	Fluffy	NA
FU-37	Jharkhand	NWPZ	Moderately	Slow	Fluffy	V4 / V1
FU-43	Bihar	NWPZ	Moderately	Medium	Fluffy	V2 / V1
FU-61	West Bengal	NWPZ	Moderately	Medium	Fluffy	V4
FU-88	Jharkhand	NWPZ	Moderately	Slow	Appressed	V2
F-3	Uttar Pradesh	NWPZ	High	Medium	NA	NA
F-8	Uttar Pradesh	NWPZ	High	Fast	Fluffy	NA
F-17	Uttar Pradesh	NWPZ	High	Slow	NA	NA
I-3	Uttar Pradesh	NWPZ	High	Slow	NA	V1
I-8	Uttar Pradesh	NWPZ	High	Slow	Fluffy	V2
I-9	Uttar Pradesh	NWPZ	High	Slow	Intermediate	V1
ICRI-1	Andhra Pradesh	SZ	High	Fast	Fluffy	NA
NF-3	Uttar Pradesh	NEPZ	High	Slow	Intermediate	V2
NF-16	Uttar Pradesh	NEPZ	High	Slow	Intermediate	NA
NF-20	Uttar Pradesh	NEPZ	Weak	Fast	Intermediate	NA
NF-27	Uttar Pradesh	NEPZ	High	Slow	Fluffy	NA
NF-36	Uttar Pradesh	NEPZ	Weak	Slow	Fluffy	NA
NF-55	Uttar Pradesh	NEPZ	Weak	Slow	Intermediate	NA
NF-59	Uttar Pradesh	NEPZ	High	Medium	Fluffy	V2
NF-72	Uttar Pradesh	NEPZ	High	Fast	Appressed	NA
NF-81	Uttar Pradesh	NEPZ	High	Fast	Fluffy	V1
NF-92	Uttar Pradesh	NEPZ	High	Fast	Fluffy	V4
NF-117	Uttar Pradesh	NEPZ	High	Fast	Appressed	NA

pathogens. The differential behaviour of isolates in host-pathogen interaction was also observed and recorded. Accordingly, eleven isolates from NEPZ, eight from SZ, nine from CZ and seventeen from NWPZ were collectively designated as five variants *viz*. V1, V2, V3, V4 and V5 classes. In PCR analysis, ITS markers gave 90 reproducible and scorable products, with fragment sizes (average of 45 bands per primer) ranging from 690 bp to 850 bp (Fig 1a). However, EF-1α markers generated 101 polymorphic bands with product sizes lying within the range of 700-1000 bp (average of 50 bands per primer), with an average of

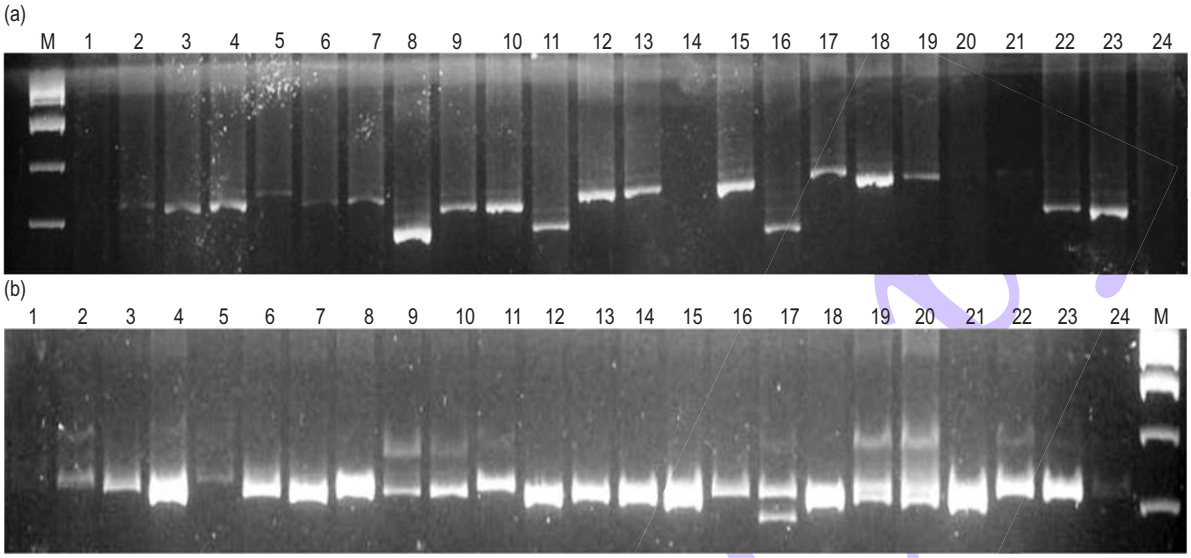

Fig. 1 : Gel photograph of *Fusarium udum* (a) ITS profile by primer pair FDP-2 (ITS1/ITS2) and (b) EF-1α profile by primer pair FDP-29 (EF-1α, M9968PY)

3 alleles per primer (Fig 1b). Concerning the PIC values of ITS markers, the values were 0.44 for FDP 3 (ITS1/ITS2) and 0.35 for FDP 25 (mRNA, LOC100383610), with an average of 0.39, whereas the PIC values for EF-1α were 0.27 for FDP 4 (EF-1α, F98-BKR5) and 0.37 for FDP 29 (EF-1α, M9968PY) with an average of 0.32. Furthermore, the usefulness of each marker system was evaluated by analyzing DI, EMR and MI, consequently these values for ITS were found to be higher *i.e.* 0.48, 6.0 and 1.44 respectively. A comparison among MI, DI and EMR values demonstrated higher effectiveness of ITS marker system as compared to EF-1α-derived markers (Table 2).

UnWeighted neighbour-Joining tree for both marker systems was constructed using Darwin 5 (Version 5.0.156) software. ITS marker analysis led to the grouping of 45 isolates into two clusters (Fig. 2). Cluster I included isolates belonged to eleven states and four agro ecological zones of which fourteen isolates (43%) were from Uttar Pradesh, namely 28, 30, 33, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44 and 45, where majority of the isolates representing V-1, V-2 and V-4, five (15%) isolates from Madhya Pradesh (1, 13, 14, 15 and 16) representing V-1, V-2 and V-3, three (9%) from Haryana (17, 18 and 21) representing V-2 and V-3, two each (6%) from Maharashtra (11, 12) and Bihar (22, 25) representing V-1 and V-2, one each (3%) isolates from Karnataka (4) representing V-2, Delhi (19) representing V-2, Rajasthan (20) representing V-3, Andhra Pradesh (6), West Bengal (26) representing V-4 and Tamilnadu (8). In this cluster, majority (55%) of isolates were from NEPZ. The second cluster comprised thirteen isolates from six different states and three agro ecological zone, in which three (23%) were from Uttar Pradesh namely 29, 31 and 32 representing V-1 and V-2, three (23%) isolate from Karnataka namely 2, 3 and 5, two each (15%) from Maharashtra (9, 10) representing V-1, Jharkhand (24, 27) representing V-1, V-2 and V-4, Andhra Pradesh (6, 7) and one (7%) isolates from Bihar (23). In case of Cluster II, majority (46%) of isolates were from NEPZ.

Marker analysis using EF-1α, grouped 45 isolates into three clusters (Fig. 3). Cluster first included fourteen isolates representing six states and four agro climatic zones, in which six isolates (42%) were from Uttar Pradesh namely 28, 37, 38, 40, 41 and 42, four (25%) from Madhya Pradesh (1, 12, 13, 14 and 15) representing V-1, V-2, V-3 and one each (6%) isolates from Haryana (18) and Jharkhand (27) representing V-2, Maharashtra (12) and Andhra Pradesh (7). Here majority (50%) of the isolates were from NEPZ. Second cluster consisted of total of twenty nine isolates from eleven different states, representing four zones, in

Table 2 : Effectiveness of marker system in detecting polymorphism of 45 *F.udum* isolates

	ITS marker	EF-1α
Total amplified bands	90	101
Total number of allele	6	6
Number of polymorphic allele	6	6
Percentage of polymorphic bands	100	100
Number of primers used	2	2
Number of polymorphic allele/primer	3	3
Range of PIC value	0.35-0.44	0.27-0.37
Average of PIC value	0.39	0.32
DI	0.48	0.41
EMR	6	6
MI	1.44	1.23

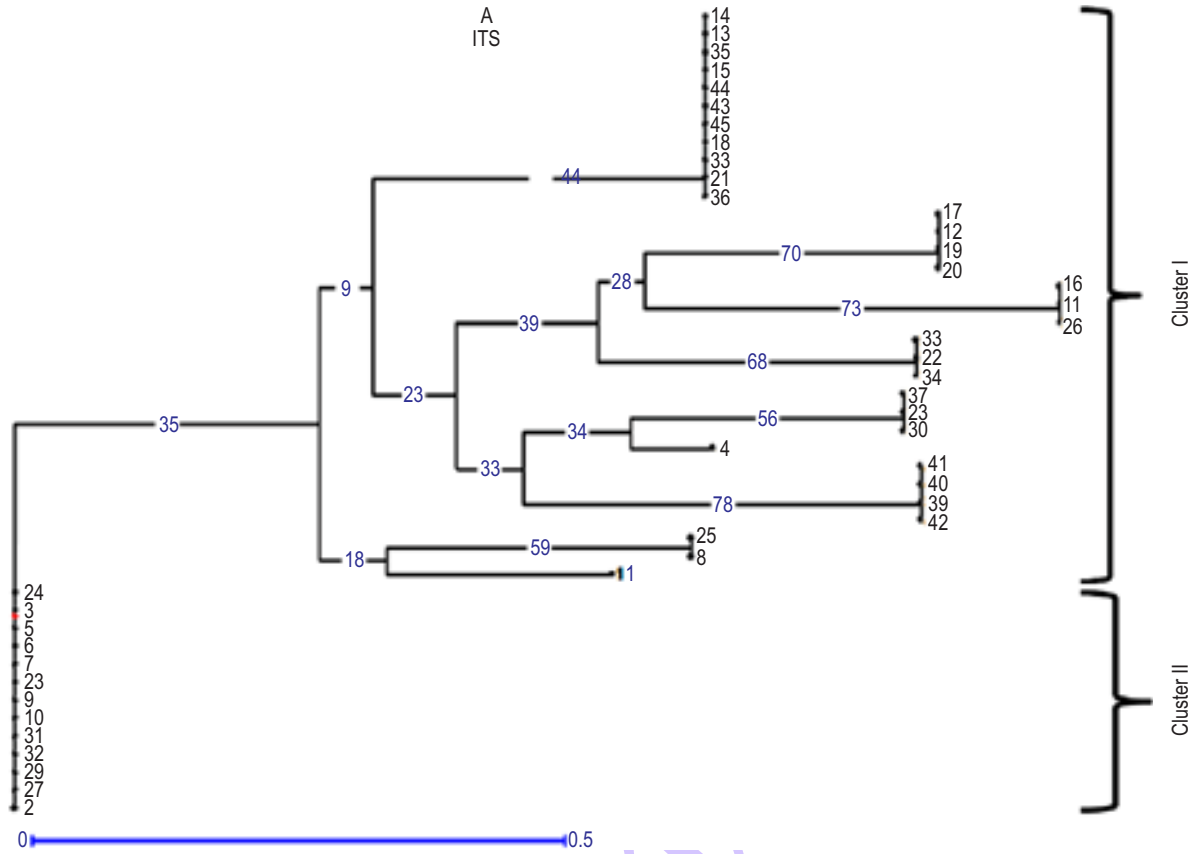

Fig. 2 : Dendrograms of 45 *Fusarium udum* isolates derived based on ITS marker system

which eleven isolates (37%) were from Uttar Pradesh namely 29, 30, 31, 32, 33, 35, 36, 39, 43, 44 and 45 representing V-1, V-2 and V-4, Four (13%) from Madhya Pradesh (3, 2, 5 and 16) representing V-1, three (10%) isolates from Bihar (22, 23 and 25), representing V-1/V-2 and Maharashtra (9, 10, 11) representing V-1 and one each (3%) isolates from Delhi (19) representing V-2, Rajasthan (20) representing V-3, West Bengal (26) representing V-4 and Tamilnadu (8), Haryana (17) representing V-3 and Jharkhand (24) representing V-1, V-4. Here majority (55%) the isolates were from NEPZ. Two isolates namely 4 and 21 from Madhya Pradesh (V-1) and Haryana (V-2) respectively, were placed in the third cluster.

In combined analysis (ITS+EF-1α), all the isolates were categorized into three main clusters (Fig. 4). First cluster had a total of twenty one isolates from nine different states representing four agro ecological zones. Of which, seven (33%) from Uttar Pradesh namely 29, 30, 31, 32, 33, 44 and 45 representing V-1, V-2 and V-4, three (14%) isolates from Maharashtra (9, 10, 11) representing V-1, Bihar (22, 23, 25) representing V-1 and V-2 and Karnataka (2, 3, 5), one-one (5%) isolates from Delhi (19) representing V-2, Rajasthan (20) representing V-3, Jharkhand (24) representing V-1/V-4, Andhra Pradesh (6) and Madhya Pradesh (16) representing V-1. Here majority (52%) of the isolates were from NEPZ. Second cluster consisted of total twenty one isolates from seven different states representing four zones, in which ten (47%) from Uttar Pradesh namely 28, 35, 36, 37, 38, 39, 40, 41, 42 and 43 representing V-1 and V-2, four (19%) isolates from Madhya Pradesh namely 1, 13, 14, 15 representing V-1, V-2 and V-3, three (14%) from Haryana namely 17, 18 and 21 representing V-2, V-3 and one each (5%) isolates from Jharkhand (27) representing V-2, Karnataka (4) representing V-1, Maharashtra (12) and Andhra Pradesh (7). Here also majority (52%) of the isolates were from NEPZ. Three isolates namely 8, 26 and 34 from Tamilnadu, West Bengal and Andhra Pradesh, respectively were placed in third cluster.

The values of correlation coefficients of similarity matrix between ITS and EF-1α were fairly low (0.01). The value of cophenetic correlation coefficient between dendrogram and distance matrix was found to be higher for ITS (0.97) than EF-1 alpha (0.96). These results from cophenetic correlation coefficient between dendrogram and original distance matrix reconfirmed for superiority of ITS marker over EF-1 to represent

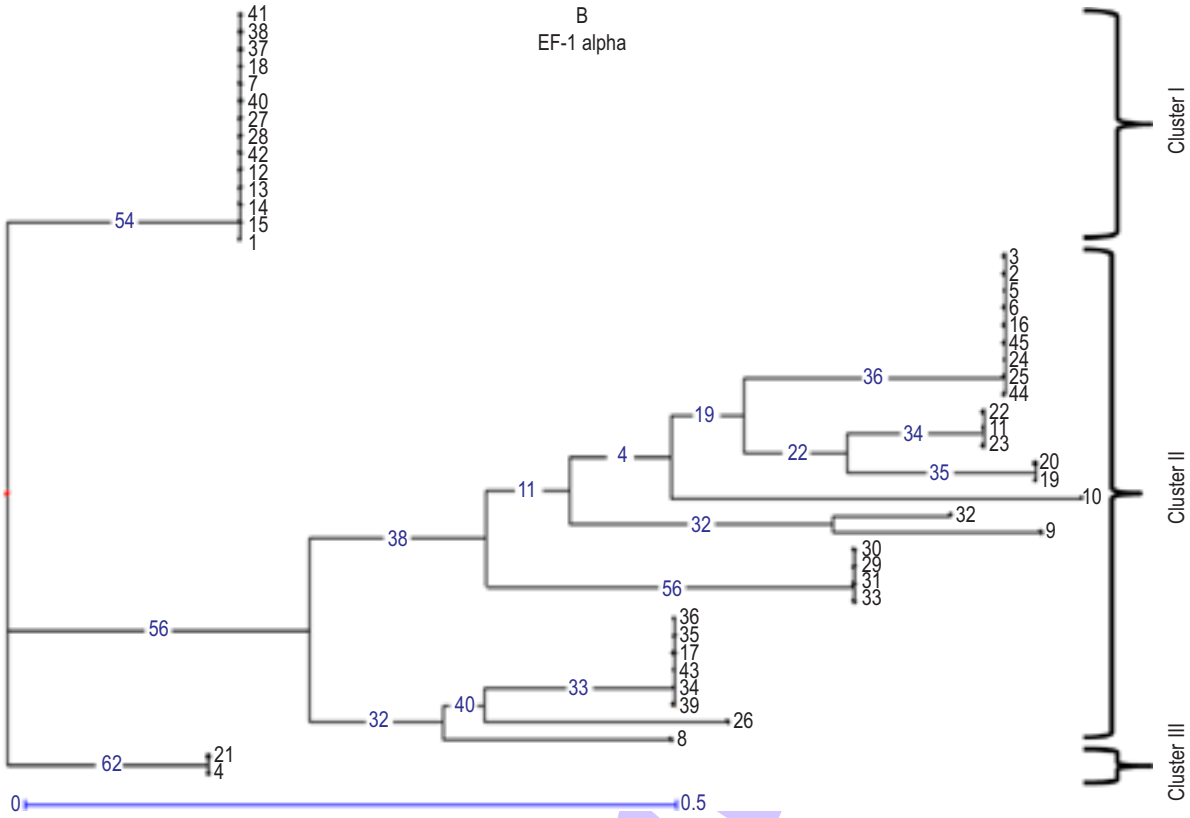

Fig. 3 : Dendrograms of 45 *Fusarium udum* isolates derived based on EF-1 alpha marker system

the relationship between isolates. Grouping the isolates based on colony growth pattern, mycelia growth rate and to make direct correlation among isolates into different pathotype is quite difficult (Kiprop *et al.,* 2002; Mishra and Vishwadhar, 2003; Baayen *et al.,* 2000; Bao *et al.,* 2002). Therefore, based on the host-plant reaction in fifteen differential sets of pigeonpea isolates were classified into five variants *viz*. V1, V2, V3, V4 and V5 (Tiwari and Vishwadhar 2011).

In order to analyse the population distribution of these isolates, information generated by combined analysis of two marker systems (ITS & EF-1α) was used. Several limitations associated with classical taxonomic method were overcome by using DNA marker systems, thereby offering a precise classification for the geographically distinct isolates. Beside isolates classifications, appropriate marker systems for *F. udum* differentiation were also applied to detect the level of polymorphism and discriminatory power efficiency. The values observed for EMR, DI and MI were significantly higher for ITS. The result obtained in this study revealed that ITS marker system was more powerful and effective for population discrimination in fungus than EF-1α (Nilsson *et al.,* 2008). The findings suggest that both marker systems were unique in origins, that amplified unique sequences from different regions. Earlier reports also suggested that ITS marker was more informative, ideal and more successful in reliable differentiation of *Fusarium* sp. isolates and species for many other eukaryotic taxa (Oechsler *et al.,* 2009). Moreover, in an effort to add more robustness to the present analysis, single dendrogram was constructed by combining genotyping data generated using two markers systems (ITS and EF-1α). The combined analysis (ITS+EF-1alpha) resulted in grouping of isolates into three major clusters (cluster I, cluster II & cluster III). In fact, cluster I represented isolates from CZ, SZ, NWPZ and NEPZ where it included variant 1, variant 2, variant 3 and variant 4 but the percentage occurrence of variant 1 was more prevalent. However, cluster II included isolates from CZ, SZ, NWPZ and NEPZ, where the percentage occurrence of variant 2 was more prevalent than the other. Likewise, in cluster III isolates representing the SZ and NEPZ belonged to variant 4. With this finding it can be interpreted that the occurrence of variant 1 and 4 was more prevalent in the agro climatic zone. The phylogenetic relationship, evident from bootstrap UnWeighted Neighbour-Joining analysis and statistical analysis, clearly indicated that Indian isolates of *F. udum* had variable ITS sequences and best choice for molecular analysis.

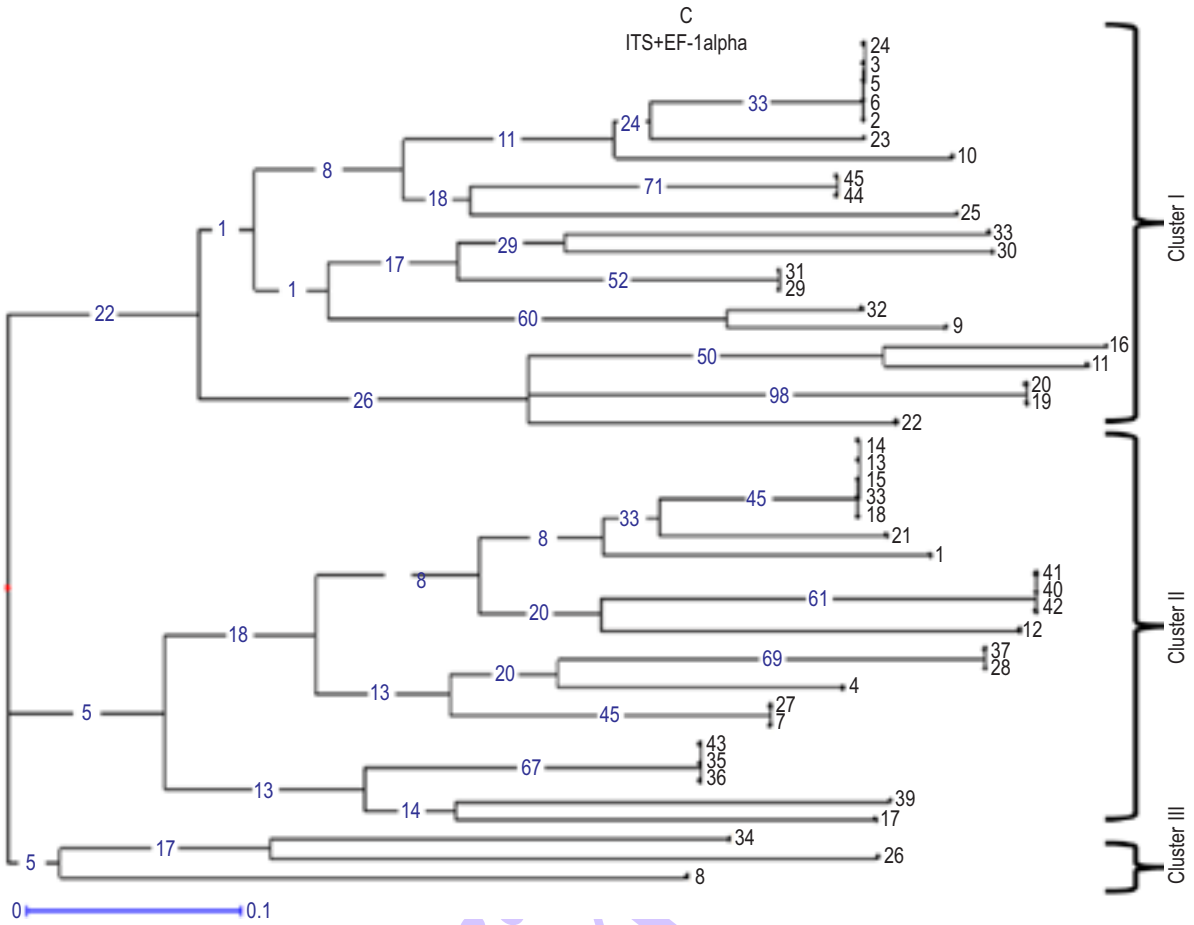

Fig. 4 : Consensus tree of 45 *Fusarium udum* isolates derived based on ITS+EF-1α marker system

In a similar manner, Wang *et al.* (2011) found that the ITS rDNA sequences were variable in 14 *Z. Nicaraguensis*. Gomes *et al.,* (2002) also reported sequence variations in 26 isolates of ectomycorrhizal fungi belonging to 8 genera and 19 species in the ITS of rDNA region using restriction fragment length polymorphism (RFLP). In addition, the study highlighted existence of genetic variation in pathogens at species level and revealed relative superiority of ITS-based marker system over EF-1α. Dissecting *F. udum* isolates genetically and establishing the evidence about their distribution across different pigeonpea growing states strongly support the localization of specific variants confined to specific geographical region. However, at the same time gradual genetic evolution of the novel variants alongside existing one cannot be ignored. Moreover, given the complexity in variants, precise classification is needed using virulence genes, which will be more meaningful in correlating the isolates with their geographical distribution and virulence status. In summary, this article highlights the growing demand for integrated resistance breeding schemes aiming at pyramiding multiple genes into good agronomic base against various variants. The variant-specific molecular tools would greatly assist the pigeonpea breeder to precisely manipulate the genetic architecture of *Fusarium* resistance, thereby speeding up the pace of development of wilt resistant publically accepted cultivars.

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