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APPLICATION OF MOLECULAR MARKERS FOR GENETIC DISCRIMINATION OF *Fusarium* WILT PATHOGEN RACES AFFECTING CHICKPEA AND PIGEONPEA IN MAJOR REGIONS OF INDIA

J. DATTA AND N. LAL✉

Department of Life Sciences, C.S.J.M. University, Kanpur 208 024, India

Abstract

Genetic diversity in wilt pathogen has been characterized using 14 isolates of each of *Fusarium oxysporum* f. sp. *ciceri* (*foc*) and *Fusarium udum* (*Fud*) collected from major pulse growing regions of India. Out of 247 bands produced by 24 Randomly Amplified Polymorphic DNA (RAPD) primers in *Foc* isolates, 210 (85%) were polymorphic. A maximum of 14 amplicons were generated by primer OPF 05 whereas minimum 7 amplicons were generated by primer K7. A total of 24 alleles were produced by twelve Simple Sequence Repeats (SSR) primers with an average of two alleles per marker in *foc* isolates. The maximum number of 4 alleles was obtained with primer SSR 12. SSR amplicon size ranged from 100 to 400 bp. The Unweighted Pair Group Method with Arithmetic average (UPGMA) cluster analysis based on RAPD and SSR profiles grouped the fourteen *foc* isolates into four major clusters. The universal Inter Transcribed Spacer (ITS) primer pair amplified 630 bp bands in all fourteen *foc* isolates while significant length polymorphism was obtained only when analysed by restriction digestion with *Eco*RI and *Msp*I enzymes. The cluster analysis of ITS-RFLP grouped all 14 *Foc* isolates into three major clusters. Twenty four RAPD primers generated a total of 226 bands (ranging 0.3 to 3.0 kb) in *Fusarium udum* with an average of 9.4 bands per primer and a total of 27 alleles were produced by twelve SSR primers with an average of 2.25 alleles per marker. All isolates amplified a single band ranging from 100 to 450 bp. The universal ITS primer pair amplified 650 bp bands in all fourteen *fud* isolates while significant length polymorphism was obtained only when analysed by restriction digestion with *Eco*RI and *Hind* III enzymes. The cluster analysis of ITS-RFLP grouped all 14 *Fud* isolates into three major clusters. The cluster analysis using various markers show the grouping of *Fusarium* isolates strictly according to their cultural characteristics and degree of pathogenicity and not the geographical origin. This information will be helpful for pathologists and plant breeders to design effective resistance breeding programs in chickpea and pigeonpea taking into account the diversity in wilt pathogen.

Key words: Chickpea, *Fusarium*, Genetic diversity, ITS, Pigeonpea, Polymorphism, RAPD, rDNA, SSR.

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✉ **Corresponding author**
Tel: +919839342316
Fax: +91-512-2574720
E-mail: nl_pr@yahoo.co.in

INTRODUCTION

Chickpea and pigeonpea are among the world's most important pulse crops which suffer heavy yield losses up to 90% due to vascular wilt caused by *Fusarium*. The disease is prevalent in almost all pulses growing areas of the world, including the Indian subcontinent, Iran, Peru, Syria, Ethiopia, Mexico, Spain, Tunisia, Turkey, and the United States (31). Although much progress has been made in developing chickpea and pigeonpea lines with resistance to biotic constraints and tolerance to abiotic stresses, yield loss in these crops is very high due to the high incidence of diseases and insect-pests (32).

Fusarium wilt of chickpea and pigeonpea are caused by *Fusarium oxysporum* f. sp. *ciceri* and *F. udum,* respectively. Pathogenic isolates of *F. oxysporum* often display a high degree of host specificity and may be subdivided into *formae speciales* based on the plant species affected and into races based on the host cultivars attacked (1, 2). Eight races of the pathogen have been reported, of which six (1A, 2, 3, 4, 5, and 6) cause wilting syndrome and are economically more important when compared to races 0 and 1B/C that cause yellowing syndrome (17). The wilt pathogen is both soil and seed borne and difficult to eradicate as fungal chlamydospores survive in soil upto six years even in the absence of host plant (18). It's near ubiquity in soil worldwide and its ecological activities indicate a much more diverse role in nature. Biochemistry and physiology of the *Fusarium*-plant interaction have been characterized extensively (19), but definitive enquiry into identification of individual molecules essential for *Fusarium* pathogenesis to plants did not begin until molecular genetic technology became available for filamentous fungi (5, 12, 42). To develop effective strategy for management of wilt diseases, understanding of the molecular basis of pathogenesis and resistance mechanism is essentially required.

For integrated management of wilt, identification of isolates/races and developing strategy to incorporate resistance is very important. Infallible identification of races is critical to any resistance-breeding program as exact picture about the existence of number of physiological races in *Fusarium oxysporum* f. sp. *ciceri* and *Fusarium udum* is still not clear. Identification of any isolate/race by molecular tools like DNA fingerprinting is considered to be the most reliable method. Furthermore identification and classification of the race specific donors will help in pyramiding of resistance genes for developing varieties resistant against multiple races of pathogen.

A comparison at DNA sequences level provides accurate classification of fungal species to elucidate the evolutionary and ecological relationships among diverse species. In recent years, numerous DNA based methods have been increasingly used to study variability in pathogenic *Fusarium* population (41). DNA fingerprinting has been successfully used for *Fusarium* in characterization of individual isolates and grouping them into standard racial

classes. For breeding of resistant crop varieties, knowledge about the pathogen races in that particular crop area is very important especially to pyramid several resistance genes in an elite genotype. So far, eight races of the pathogen (race 0, 1A, 1B/C, 2, 3, 4, 5 and 6) have been identified by reaction on a set of differential chickpea cultivars (22). Races 1 to 4 have been reported from India, whereas 0, 1B/C, 5 and 6 are found in the Mediterranean region and USA. Randomly Amplified Polymorphic DNA offers several advantages and reduces the time needed for race identification in disease plants, provides genetic information on isolates, allowing for their fingerprinting. Gherbawy et al. (14) used RAPD technique for identifying *Fusarium subglutinans*, *F. proliferatum* and *F. verticillioides* strains isolated from maize in Austria. Pasquali et al. (34) characterized isolates of *Fusarium oxysporum* pathogenic on *Argyranthemum frutescens* L. using RAPD technique. Genetic similarity between isolates of *F. oxysporum* f. sp. *ciceri* was studied using 40 RAPD and 2 IGS primers and results indicate that there was little genetic variability among the isolates collected from the different locations in India (40). Cramer et al. (11) reported specific RAPD bandings which distinguish among races *F. oxysporum* f. sp. *phaseoli* and *F. oxysporum* f. sp. *betae*. Identification of pathogenic races 0, 1B/C, 5 and 6 of *Fusarium oxysporum* f. sp. *ciceri* has been reported using 40 RAPD primers (25). Similarly, simple sequence repeats (SSRs) provide a powerful tool for taxonomic and population genetic studies (7). Simple sequence repeats have been used as genetic markers in numerous DNA-fingerprinting and PCR fingerprinting experiments for strain typing of a variety of filamentous fungi and yeasts without prior knowledge of their abundance and distribution in the investigated fungal genomes (29). SSR markers distinguished the four races of *Fusarium oxysporum ciceri* causing varied levels of wilting with differential host cultivars (4). Bogale et al. (6) has shown that the polymorphism revealed with 8 SSR markers was sufficient for study of genetic diversity in *Fusarium oxysporum* complex.

The cluster of ribosomal DNA consists of tandem repeat of three coding (18S, 5.8S and the 28S), and two noncoding ITS and Intergenic spacer region (IGS). Designing primers from the rDNA region has far superior reliability compared to the use of random non-defined probes or primers. These markers occur in multiple copies with up to 200 copies per haploid genome arranged in tandem repeats and are most effective in detecting polymorphism (45). Inter Transcribed Spacer regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (8). Taxon-selective ITS amplification has already been used for detection of the fungal pathogens such as *Fusarium* and *Verticillium* spp. (30). O'Donnell (33) found a surprising level of divergence for ITS sequences within the species of *F. sambucinum*. Chakrabarti et al. (9) have shown that digestion of amplified IGS region with EcoR1 produced similar bands for both race1 and race 4 of *F. oxysporum* f. sp. *ciceri* but individual and distinctive banding patterns were observed for race 2 and race 3. Genetic diversity studies could reveal the adaptive potential of pathogenic populations (21) and sometimes RAPD patterns could reflect the variability of formae speciales (10). The aim of this study was to analyze the diversity of an isolate collection of *F. oxysporum* and *F. udum* recovered from diseased plants. The diversity of isolates was assessed by genetic analysis using RAPD, SSR markers and the restriction polymorphism analysis of Inter transcribed sequence (ITS) of the nuclear ribosomal DNA.

MATERIALS AND METHODS

Fusarium isolates

The *Fusarium oxysporum* f. sp. *ciceri* (*Foc*) and *Fusarium udum* (*Fud*) isolates collected from wilt affected fields of chickpea and pigeonpea from seven major pulse growing states of India were used as experimental material in the present study (Table 1). The isolates were selected based on the variability in their cultural, morphological and pathogenic characters. For facilitating easy identification of the isolates, the original isolate numbers have been used throughout the text.

Molecular markers

A total of 40 RAPD primers and 12 SSR markers were used in the present study. The RAPD primers from Operon series and SSR primers based on reported primer sequences were got synthesized from Operon Technologies, USA. Tables 2 and 3 show the Tm, source and sequence details of RAPD and SSR primers. The nuclear rDNA ITS region, including ITS2 and the 5.8S ribosomal gene, was amplified using primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTTATTGATATG3').

Collection and maintenance of the fungal isolates

The pathogens were isolated from fourth-node stem sections taken from wilted chickpea and pigeonpea plants collected from different agro-climatic regions of India according to the procedure described by Tullu et al. (43). The re-isolated pathogens were colonized on filter paper, dried in the transfer hood, and aseptically cut into small pieces. The colonized filter paper pieces were placed in potato-dextrose broth and incubated to produce liquid cultures of the pathogen. The liquid cultures were filtered through cheese cloth to remove mycelia. The spore suspension was pelleted by centrifugation. After discarding the supernatant, the conidia were washed with sterile water to adjust the spore suspension to 1 x 10^6 spores ml^{-1} with a haemocytometer. These isolates were further characterized at the laboratories of Department of Life Sciences, and Department of Biochemistry, I.B.S.B.T, C.S.J.M. University, Kanpur. Single spore culture of fungus was obtained by serial dilution method.

Isolation and purification of genomic DNA of Fusarium isolates

Single spore culture of each isolates was grown on PDA medium at 28°C and stored at 4°C until used. Mycelia for genomic DNA extraction were grown in 250 ml of PDB at 28°C for 5 days. After vacuum filtration, the mycelia were lyophilized and stored at -20°C. Genomic DNA for PCR was extracted using a modified method of Kim et al. (27). Prepared mycelium (approximately 0.5 g) was suspended in extraction buffer (4 M NaCl, 1 M Tris-HCl (pH 8.0), 0.5 M EDTA, 2% PVP) and extracted with phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform/ isoamyl alcohol [24:1]. DNA was then precipitated by adding 2.5 volumes of absolute ethanol and pelleted by centrifugation for 10 min at 10,000 rpm (Sorvall SS 34 rotor). The pellet

Table 1. *Fusarium oxysporum* f. sp. *ciceri* and *Fusarium udum* isolates used in the present study.

Isolates	Place of collection	Characteristics	Pathogenicity
Foc 17	Jhansi (Uttar Pradesh)	Long size/medium growth	Moderate
Foc 42	Mau (Uttar Pradesh)	Small size/slow growth	Moderate
Foc 92	Kanpur (Uttar Pradesh)	Medium size/medium growth	High
Foc 66	Lam Guntur (Andhra Pradesh)	Medium size/medium growth	High
Foc 65	Anantpur (Andhra Pradesh)	Medium size/medium growth	High
Foc 80	Bangalore (Karnataka)	Small size/slow growth	High
Foc 73	Gulberga (Karnataka)	Large size/slow growth	High
Foc 6	Narsinghpur (Madhya Pradesh)	Large size/fast growth	Moderate
Foc 56	Raisen (Madhya Pradesh)	Medium size/fast growth	High
Foc 91	Bharuch (Gujarat)	Medium size/slow growth	High
Foc 60	Porbandar (Gujarat)	Large size/fast growth	High
Foc 58	Bilaspur (Chattisgarh)	Large size/fast growth	High
Foc 79	Raipur (Chhattisgarh)	Medium size/fast growth	High
Foc 88	Amravati (Maharastra)	Medium size/ medium growth	Moderate
Fud 1	Uttar Pradesh	Long size/medium growth	Moderate
Fud 4	Uttar Pradesh	Medium size/medium growth	High
Fud 6	Bihar	Medium size/medium growth	High
Fud 7	Bihar	Small size/slow growth	High
Fud 9	Andhra Pradesh	Medium size/medium growth	High
Fud 11	Andhra Pradesh	Medium size/medium growth	High
Fud 14	Karnataka	Small size/slow growth	High
Fud 17	Karnataka	Small size/slow growth	High
Fud 18	Maharashtra	Large size/slow growth	High
Fud 19	Maharashtra	Medium size/medium growth	High
Fud 20	Madhya Pradesh	Small size/slow growth	High
Fud 22	Madhya Pradesh	Medium size/medium growth	High
Fud 23	Tamilnadu	Small size/slow growth	High
Fud 27	Tamilnadu	Medium size/medium growth	High

Table 2. RAPD primers used in the fingerprinting of *Fusarium* isolates.

Primer	Sequence 5'→3'	Primer	Sequence 5'→3'
K 1	5' TGCGTGCTTG 3'	P 19	5' GCGGCATTGT 3'
K 2	5'ACTTCGCCAC 3'	P21	5' CCAGACAAGC 3'
K 3	5' GGCTCATGTG 3'	OPD 11	5'AGCGCCATTG 3'
K 4	5' CAAACGTGGG 3'	OPD 13	5'GGGGTGACGA 3'
K 5	5'CGAGGTCGACG3'	OPD 16	5' AGGGCGTAAG 3'
K 6	5'CACCGCCCCAA 3'	OPA 3	5' AGTCAGCCAC 3'
K 7	5'GTCCTCAGTCCC 3'	OPA 4	5' AATCGGGCTG 3'
P1	5' CGTTGGATGC 3'	OPA 7	5' GAAACGGGTG 3'
P2	5' TACGGCTGGC 3'	OPA 11	5' CAATCGCCGT 3'
P3	5' GCGGCATTGT 3'	OPA 12	5' TCGGCGATAG 3'
P 8	5' CAGGCCCTTC 3'	OPF 01	5'ACGGATCCTG3'
P 17	5' TACGGCTGGC 3'	OPF 05	5'CCGAATTCCC 3'

was washed with 70 % ethanol, air dried and resuspended in 1 mM TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)). DNA concentration and purity were measured using a spectrophotometer (BioRad SmartSpec plus) at 260 nm and 280 nm. RNA was degraded with the treatment of RNase A (50 µg/ml) for 30 min at 37°C. Proteins were removed by phenol-chloroform extraction. Equal volume of phenol: chloroform: iso-amyl alcohol (25:24:1) was added, and mixed and the tubes were spun at 10,000 rpm for 5 minutes at room temperature. Aqueous phase extracted twice with chloroform: iso-amyl alcohol (24:1) was collected after centrifugation. DNA was precipitated by

Table 3. SSR primers used in the fingerprinting of *Fusarium* isolates.

Primer	Sequence 5'→3'	Tm (°C)	References
SSR 1	**F:** TGCTGTGTATGGATGGATGG **R:** CATGGTCGATAGCT	57	Bogale *et al.,* 2005
SSR 2	**F:** ACTTGGAGAAATGGGCTTC **R:** GGATGGAGTTTAATAAATCTGG	54	Bogale *et al.,* 2005
SSR 3	**F:** TGGCTGGGATACTGTGTAATTG **R:** TTAGCTTCAGCCCTTTGG	51	Bogale *et al.,* 2005
SSR 4	**F:** TATCGAGTCCGGCTTCCAGAAC **R:** TTGCAATTACCTCCGATCCAC	48	Bogale *et al.,* 2005
SSR 5	**F:** GTGGACGAACACCTGCATC **R:** AGATCCTCCACCTCCACCTC	68	Bogale *et al.,* 2005
SSR 6	**F:** GGAGGATGAGCTCGATGAAG **R:** CTAAGCCTGCTACACCCTCG	68	Bogale *et al.,* 2005
SSR 7	**F:** CGTCTCTGAACCACCTTCATC **R:** TTCCTCCGTCCATCCTGAC	57	Bogale *et al.,* 2005
SSR 8	**F:** ACTGATTCACCGATCCTTGG **R:** GCTGGCCTGACTTGTTATTCG	57	Bogale *et al.,* 2005
SSR 9	**F:** GGTAGGAAATGACGAAGCTGAC **R:** TGAGCACTCTAGCACTCCAAAC	57	Bogale *et al.,* 2005
SSR 10	**F:** CGAGCTAATGGTGGCAGGAT **R:** AACAACAAAACGGCTCATCG	50	Giraud *et al*., 2002
SSR 11	**F:** TATTTCGTGCAAGGACTTGG **R:** CTTGGTCCCTGGATATGGA	51	Giraud *et al*., 2002
SSR 12	**F:** AAGCGCCAACAGAGATGACGA **R:** GACTGCCGAAACACCGAAA	55	Giraud *et al*., 2002

adding two volumes of chilled absolute alcohol, pelleted and dissolved in $T_{10}E_1$ buffer. Genomic DNA samples were purified and quantified to 25 ng /µl to be used as template.

DNA fingerprinting using RAPD and SSR markers

Polymerase chain reaction (PCR) was performed in a 25 µl volume containing, *Taq* polymerase assay buffer (10 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$), 0.2 mM of each dNTP, 0.6 units of *Taq* polymerase (Bangalore Genei, Bengaluru, India), 20 pmoles of primer and 25 ng of DNA. The PCR regime comprised of an initial denaturation at 94°C for 3 mins followed by 40 cycles of denaturation at 94°C for 1 min, annealing of primers at suitable temperature for 1 min, extension at 72°C for 2 mins. Final extension was given at 72°C for 7 mins and reaction was held at 4°C. Amplification was performed using Biometra Thermal Cycler gradient (USA). Amplified products were resolved on 1.5 and 2% agarose gel at 45 V using 1X TBE buffer. PCR amplification with each primer was repeated twice before scoring for presence or absence of bands.

DNA fingerprinting with ITS-RFLP markers

The nuclear rDNA ITS region, including ITS2 and the 5.8S ribosomal gene, was amplified using primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTTATTGATATG3') (44). The amplification was performed in 50 µl reaction volume with 0.1 mM of each dNTP and 0.5 µM of both forward and reverse primer. Biometra thermal cycler was programmed for initial denaturation at 94°C for 4 min, and 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, the amplification was completed with a final extension at 72°C for 5 min. Electrophoresis and visualization of amplified bands was done as described above. The restriction enzyme digestion analyses were performed using 15 µl of the amplified PCR product. The enzymes *Eco*RI and *Msp*I were used as per the manufacturers' specifications (New England Biolabs). The restriction fragments were size separated by electrophoresis on 2.5% agarose gel. The gels were stained with ethidium bromide (0.5 µg/ml) and photographed under UV light. Unambiguous polymorphic DNA band were scored as "1" for presence and "0" for absence. UPGMA dendrogram was generated using software programme NTSYS-PC (version 2.02), based on Jaccards similarity coefficient (37).

RESULTS

RAPD amplification of Fusarium oxysporum f. sp. ciceri isolates

Out of 40 primers screened for amplification of DNA of *Fusarium* isolates, 16 resulted in either sub-optimal or non distinct amplification products. Therefore these were discarded and remaining 24 primers which generated reproducible RAPD patterns were used for subsequent analysis. A total of 247 bands were produced by 24 primers, out of which 85% (210) were polymorphic and 15% (37) were monomorphic. A maximum of fourteen amplicons were amplified by primer OPF05, whereas minimum seven amplicons were amplified by primer K7. Randomly Amplified Polymorphic DNA profile obtained with primer P8 is shown in Fig. 1a. The UPGMA cluster analysis based on RAPD profiles separated the fourteen isolates into four major clusters namely cluster I, II III and IV (Fig. 3a). Isolates *Foc* 17, *Foc* 42 and *Foc* 92 grouped together in cluster I. Cluster II comprised of *Foc* 66, *Foc* 65, *Foc* 80 and *Foc* 73. *Foc* 6, *Foc* 91, *Foc* 79 and *Foc* 88 fell in cluster III while *Foc* 60, *Foc* 58 and *Foc* 56 grouped in cluster IV. The maximum genetic similarity of 65% was observed between *Foc* 79 (Chhattisgarh) and *Foc* 88 (Maharashtra)

while a minimum of 13% similarity was found between *Foc* 17 and *Foc* 88. Isolate *Foc* 17 belonging to U.P. fell separate and totally demarcated from rest of the isolates.

SSR amplification of Fusarium oxysporum f. sp. ciceri isolates

Twelve SSR primers were used to generate amplification patterns in 14 *Foc* isolates. A total of 24 alleles were produced with an average of two alleles per marker. The maximum number of four alleles was obtained with primer SSR12 (Table 4). The SSR amplicon size ranged from 100-400 bp. Simple Sequence Repeats profile obtained with primer SSR1 is shown in Fig. 1b. The cluster analysis grouped 14 *Foc* isolates into four major clusters demarcating cluster IV completely (Fig. 3b). Cluster I comprised of *Foc* 17, *Foc* 42, *Foc* 92 and *Foc* 66. *Foc* 42 and *Foc* 92 clustered close to each other with similarity of 83% while *Foc* 66 shared 80% of genetic similarity with *Foc* 92. Cluster II consisted of *Foc* 65, *Foc* 80 and shared 82 % genetic similarity between them. *Foc* 73, *Foc* 6, *Foc* 56, *Foc* 91 fell in cluster III where *Foc* 6 and *Foc* 56 grouped closer with 84 % of similarity. Cluster IV contained closely grouping *Foc* 60, *Foc* 58 and *Foc* 79, *Foc* 88 with similarity values of 92 % and 88 % respectively.

ITS-RFLP amplification of Fusarium oxysporum f. sp. ciceri isolates

The universal ITS primer pair amplified 630 bp bands

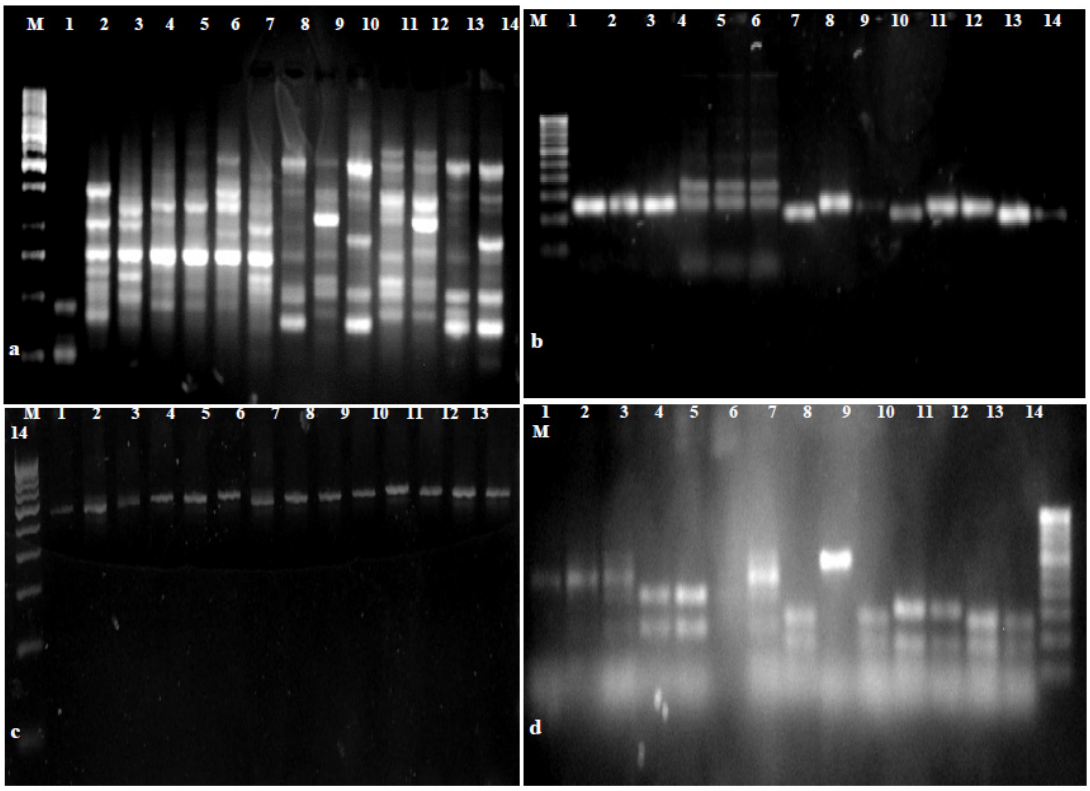

Figure 1. Amplification profile obtained with the different molecular markers in *Foc* isolates
a: RAPD profile obtained with the primer P-8, b: SSR profile obtained with SSR 1 primer pair, c: Amplification profile of ITS region with ITS-1and ITS-4 primers pairs, d: ITS-RFLP profile of rDNA with *Msp*I restriction endonuclease. Lanes M: 100 bp DNA ladder, 1: *Foc* 17, 2: *Foc* 42, 3: *Foc* 92, 4: *Foc* 66, 5: *Foc* 65, 6: *Foc* 80, 7: *Foc* 73, 8: *Foc* 6, 9: *Foc* 56, 10: *Foc* 91, 11: *Foc* 60, 12: *Foc* 58, 13: *Foc* 79, 14: Foc 8.

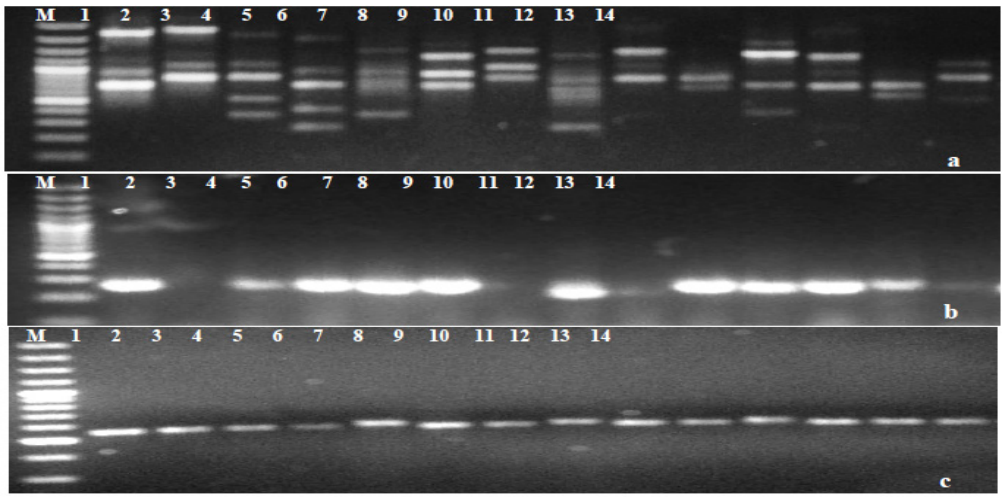

Figure 2. Amplification profile of *Fusarium udum* isolates obtained with different molecular markers.
a: Amplification profile of *Fusarium udum* isolates obtained with RAPD marker OPA11, b: SSR profile of *Fusarium udum* isolates obtained with SSR 1, c: Amplification profile of ITS region with ITS-1and ITS-4 primers pairs. Lanes M: 100 bp DNA ladder, 1: Fud 1, 2: Fud 4, 3: Fud 6, 4: Fud 7, 5: Fud 9, 6: Fud 11, 7: Fud 14, 8: Fud 17, 9: Fud 18, 10: Fud 19, 11: Fud 20, 12: Fud 22, 13: Fud 23, 14: Fud 27.

Table 4. Repeat motifs, no. of alleles and allele size from the SSR markers used in the fingerprinting of *Fusarium* isolates.

Marker name	Repeat motifs	Tm	Allele size (bp)	No. of alleles
SSR 1	$(GT)_{11}(GA)_6$	57	225bp, 250, 300 bp	3
SSR 2	$(TG)_9$	54	100 bp	1
SSR 3	$(CA)_9$	51	100 bp	1
SSR 4	$(AAC)_6$	48	200 bp	1
SSR 5	$(GGC)_7$	68	150 bp, 400 bp	2
SSR 6	$(CTTGGAAGTGGTAGCGG)_{14}$	68	100 bp, 200 bp, 300 bp	3
SSR 7	$(CCA)_5$	57	150 bp, 300 bp	2
SSR 8	$(CA)_{21}$	57	250 bp	1
SSR 9	$(CAACA)_6$	57	300 bp, 350 bp	3
SSR 10	$(AC)_{13}$	50	300 bp	1
SSR 11	$(AC)_{15}$	51	200 bp, 300 bp	2
SSR 12	$(AAG)_{28}$	55	100 bp, 200 bp, 300 bp, 400 bp,	4

from all fourteen isolates (Fig. 1c). Significant length polymorphism was not obtained unless analysed by digestion with restriction enzymes. Length polymorphism obtained after digestion of amplified ITS varied depending on the restriction enzyme and isolate used. Digestion of the amplified fragments by *Eco*RI and *Msp* I restriction enzymes revealed extensive polymorphism (Fig. 1d). The two enzyme which had restriction sites in the ITS region revealed maximum polymorphism in three isolates. *Msp* I restriction enzymes could detect more variation in the restriction sites in all isolates. Isolates from U.P. (*Foc* 14, *Foc* 42, *Foc* 92) and two other isolates *Foc* 73 (Karnataka) and *Foc* 56 (M.P.) were not restricted by *Msp* I restriction enzyme. The cluster analysis of ITS-RFLP grouped all 14 *Foc* isolates into three major clusters (Fig. 3c). Cluster I comprised of five isolates including three isolates from Uttar Pradesh (*Foc* 17, *Foc* 42 and *Foc* 92), one isolate from Karnataka (*Foc* 73) and one isolate from Madhya Pradesh (*Foc* 56). Cluster II was the largest cluster with seven *Foc* isolates. Within this cluster, two isolates from Andhra Pradesh, *Foc* 65 and *Foc* 66 shared close genetic similarities among themselves. Two isolates from Chhatisgarh (*Foc* 58 and *Foc* 79) also fell into cluster II. The other isolates within this cluster were *Foc* 60 (Gujarat), *Foc* 80 (Karnataka) and *Foc* 88 (Maharastra). The third cluster comprised of one isolate from Madhya Pradesh (*Foc* 6) and another isolate from Gujarat (*Foc* 91).

RAPD amplification of Fusarium udum isolates

Twenty four RAPD primers used for diversity analysis of 14 *Fusarium udum* isolates generated a total of 226 bands with an average of 9.4 bands per primer. The RAPD amplicon size ranged from 0.3 kb to 3.0 kb. RAPD profile obtained with primer OPA 11 is shown in Fig. 2a.

A dendrogram based on UPGMA analysis depicted the grouping of isolates into three major clusters namely cluster I, II and III (Fig. 4a). The major cluster I further divided into two sub-clusters IA and IB. Sub cluster IA comprised of two isolates *Fud* 1 and *Fud* 4 from Uttar Pradesh which share common characteristic of medium mycelium growth rate. Both shared 60% genetic similarity value and bootstrap replication obtained between the two was 45.1. Cluster IB further sub-divided into two clusters, IB1 and IB2. The sub-cluster IB1 comprised of two isolates *Fud* 6 and *Fud* 14 which shared 64% of genetic similarity and 40.7 bootstrap values. The maximum similarity of 69% was found between *Fud* 7 (Bihar) and *Fud* 20 (MP) with 66.3 bootstrap replication value. The isolate *Fud* 9 showed distinctness from all other isolates of cluster I and fell separately. The average bootstrap replication value among *Fud* 7, *Fud* 20, *Fud* 18 and *Fud* 9 was 94.0. Cluster II comprised of four isolates *viz.* *Fud* 22, *Fud* 23 and *Fud* 27 and *Fud* 17. *Fud* 22 (Madhya Pradesh) and *Fud* 23 (Tamilnadu) were in same cluster with second highest similarity (68%) and highest bootstrap replication 99.8. The isolate *Fud*27 (Tamilnadu) clustered separately and showed 57.3% average similarity and 94.0 bootstrap value with *Fud* 22 and *Fud* 23. *Fud* 17 and *Fud* 11 were most diverse from other 12 isolates and did not fall in any particular distinct cluster.

SSR amplification of Fusarium udum isolates

Twelve SSR primers were used for amplification of loci of 14 isolates of *Fusarium udum*. A total of 27 alleles were produced by SSR primers with an average of 2.25 alleles per marker. All isolates amplified a single band ranging from 100 to 450 bp. Maximum number of five alleles were amplified by primer SSR 9. SSR amplification pattern of SSR 1 is shown in Fig. 2b. The dendrogram based on Jaccards similarity coefficient depicted two major clusters I and II (Fig. 4b). In cluster I, the maximum similarity

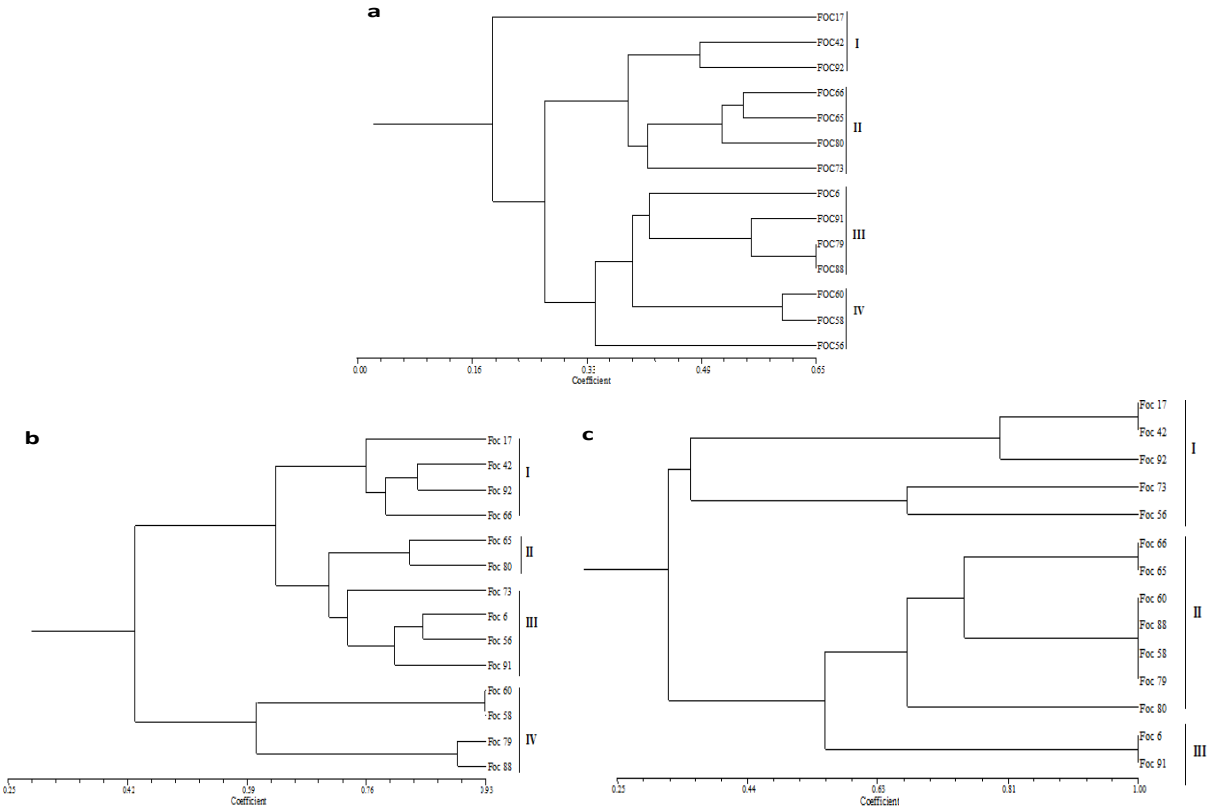

Figure 3. UPGMA cluster analysis showing relationship between *Fusarium oxysporum* f. sp. *ciceri* isolates using different molecular markers RAPD (a), SSR (b) and ITS-RFLP (c).

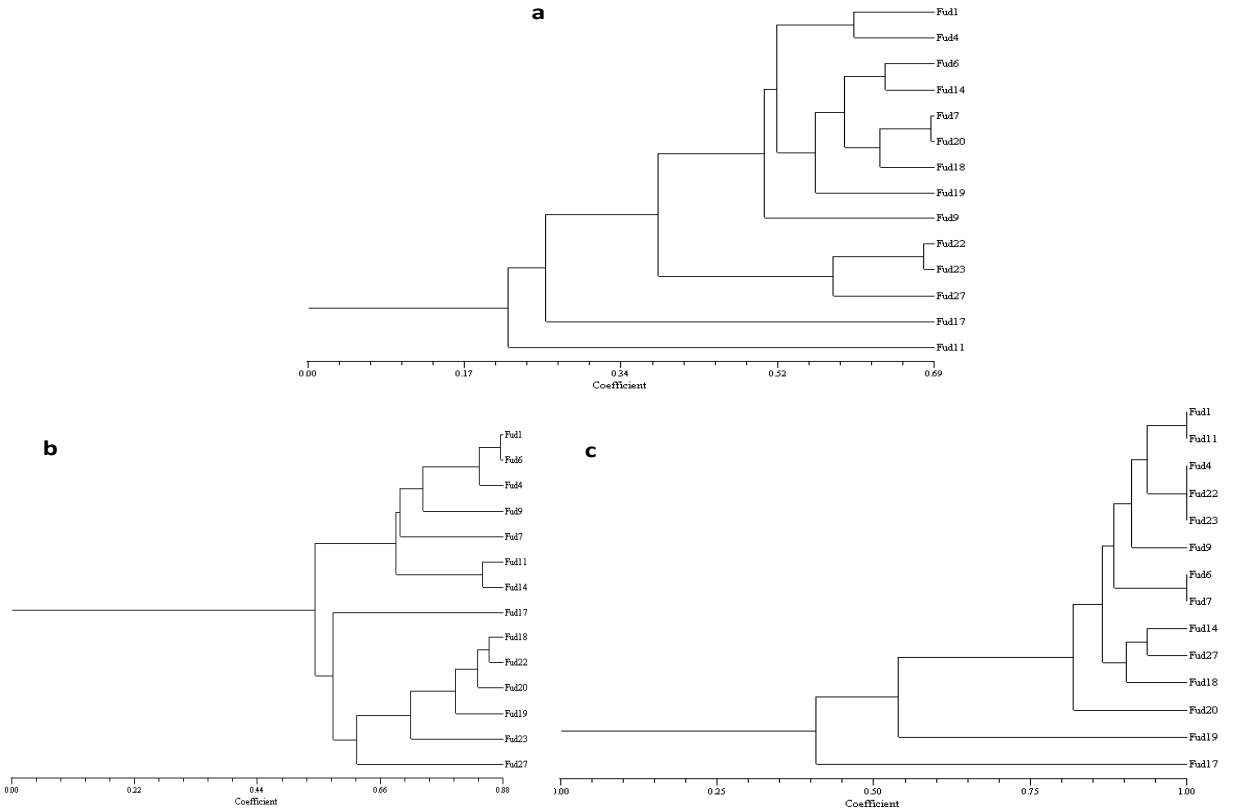

Figure 4. UPGMA cluster analysis showing relationship between *Fusarium udum* isolates using different molecular markers RAPD (a), SSR (b) and ITS-RFLP (c).

(88.0%) was found between *Fud* 1 (Uttar Pradesh) and *Fud* 6 (Bihar) with the bootstrap replication of 65.0 followed by *Fud* 4 (Uttar Pradesh) and *Fud* 6 (Bihar). Isolate *Fud* 11 (Andhra Pradesh) and *Fud* 14 (Karnataka) grouped close together and shared second highest similarity value (84.0%) due to their high pathogenesis. The cluster II comprised of 7 isolates *Fud* 17, *Fud* 18, *Fud* 19, *Fud* 20, *Fud* 22, *Fud* 23 and *Fud* 27. Isolates *Fud* 18 (Maharashtra) and *Fud* 22 (Madhya Pradesh) clustered together and showed 85% similarity value with 38.8 bootstrap replication due to their high pathogenicity. Isolate *Fud* 17 fell distinctly from all other isolates with bootstrap value of 48.9. The

isolates *Fud* 23 and *Fud* 27 were depicted in cluster II with less similarity value from rest of all other isolates of group.

ITS-RFLP amplification of Fusarium udum isolates

The universal primers ITS 1 and ITS 4 were used to amplify a region of the 5.8S rRNA gene. All the isolates amplified single amplicons of 650 bp (Fig. 2c). The amplified ITS was restricted with restriction enzymes for extensive polymorphism in rRNA regions. The ITS regions were restricted with five different hexa cutter restriction enzymes (*Eco* RI, *Eco*RV, *Sma* I, *Pst* I and *Hind* III. Out of five restriction enzymes, two enzyme (*Eco* RI and *Hin*d III) restricted the ITS region. The two enzymes which had restriction sites in the ITS region revealed the extensive polymorphism in the isolates. The UPGMA cluster analysis based on ITS-RFLP profiles clustered 14 isolates into three major clusters (Fig. 4c). Eight isolates *Fud* 1, *Fud* 11, *Fud* 4, *Fud* 22, *Fud* 23, *Fud* 9, *Fud* 6 and *Fud* 7 grouped closely in cluster I while three isolates *Fud* 14, *Fud* 27 and *Fud* 18 fell in cluster II. *Fud* 14 and *Fud* 27 fell closer to each other with 67 % of genetic similarity value. Three isolates *Fud* 20, *Fud* 19 and *Fud* 17 clustered separately from rest of the isolates in cluster III.

DISCUSSION

High level of genetic variability obtained in the study suggests that the isolates of *F. oxysporum* and *F. udum* are derived from genetically distinct clones. The exchange of contaminated seeds and cultures probably contributed to existence of variable population of *F. oxysporum* f. sp. *ciceri and F. udum* in wider geographical areas. Non-stability of most prominent genotypes of chickpea and pigeonpea further supported the view that the pathotypes of this pathogen are not stable and parasexual recombination plays a major role in the evolution of races. In the present study, the genetic variability of 14 Indian isolates of *F. oxysporum* f. sp. *ciceri* and *Fusarium udum* was determined through RAPD, SSR and ITS-RFLP. It appeared that the fingerprinting-based grouping was different from groups generated on the basis of virulence or geographical origin. Variation in symptom types (pathotypes) and pathogenic races have been reported to correlate to different geographical regions and to polymorphisms in molecular markers.

The present study generated significant information in terms of genetic variability of *F. oxysporum* f. sp. *ciceri*, which could be used further for development of area-specific resistant varieties of chickpea. The study also highlights the facts that both pathogenic virulence analysis and molecular markers are useful tools for analyzing the structure of the pathogen population, but further studies are needed to make them complementary to each other. Seventy-two isolates of *F. oxysporum* f. sp. *ciceri* were grouped into two distinct clusters using RAPD–PCR, one for wilting and another yellowing syndrome causing isolates (26). Ninety-nine isolates of *F. oxysporum* f. sp. *ciceris* were characterized by the RAPD marker and cluster analysis showed three groups of isolates. Races 0 and 1B/C grouped into two different clusters, whereas races 1–3, 5 and 6 were grouped into another cluster (23). One isolate from U.P. *Foc* 17 could not be grouped with other isolates and this result is in agreement with observations of Honnareddy and Dubey (20). The SCAR primer for identification of race of the pathogen was developed, because RAPD analysis was not able to distinguish all the races separately (24). Generalized race-specific patterns were not found through RAPD study (16).

Fusarium udum isolates from the same site or diverse geographical origins have been shown to exhibit high variability in cultural characteristics (36) and virulence or pathogenicity on pigeonpea genotypes (13, 35). Two isolates *Fud* 1 and *Fud* 4 from Uttar Pradesh grouped together in same cluster and they share common characteristic of medium mycelium growth rate. Studies of genetic diversity using isozyme markers have revealed low variation in *F. udum* isolates (39). There was no correlation between molecular diversity and geographical origin of the isolates. *F. udum* is a Deuteromycete and therefore natural populations of the pathogen may consist of clonal lineages produced by asexual reproduction. Using Amplified Fragment Length Polymorphism (AFLP) analysis, Gonzalez et al. (15) have classified *C. Lindemuthianum* isolates from Mexico into two major groups according to the type of common bean cultivar or system of cultivation from which they originated. The smaller subgroups generally associated with the geographical location from which they were obtained. Koenig et al. (28) identified 10 clonal lineages of *F. oxysporum* f. sp. *cubense* using RFLP analysis and the two largest lineages had pantropical distribution, while the minor lineages were found only in limited geographical regions. Amplified Fragment Length Polymorphisms based grouping of the isolates appeared independent of cultural and virulence traits. The genome analysis of *F. udum* isolates by RAPD, SSR and ITS-RFLP in this study has provided evidence that this pathogenic fungus varies genetically. This variability should be taken into consideration in pigeonpea improvement programmes aimed at breeding for wilt disease resistance. In order to determine the extent of genetic variation of this economically important fungus and relationships with cultural and pathogenic traits, more isolates from other countries and or geographical origins should be assayed using DNA based molecular techniques.

The traditional pathogenicity-based classification of isolates in *Fusarium* has several limitations. The use of vegetative compatibility tests, and various protein and DNA based techniques also has not solved the problem associated with taxonomy of isolates of *Fusarium*. Simple sequence repeat markers are advantageous in that they are hypervariable, abundantly found in eukaryotic genomes, and co-dominant. These characters make SSRs very useful in taxonomic and population genetic studies. The high degree of polymorphism revealed using the SSR markers used in this study should be sufficient for studies focussed in understanding the genetic diversity amongst isolates of *Fusarium.* The SSR primers should be particularly useful because the fungus is one of the very common plant pathogens. Their application should also enhance understanding relatedness of *formae speciales* in the genus *Fusarium.*

The entire three marker techniques used in this study effectively separated the *Fusarium* isolates into distinct clades. The primers and restriction enzyme combinations used here resulted in extensive polymorphism due to lack and/or alteration in primer binding and restriction sites. None of the three techniques correlated geographical origin based grouping or based on pathogenicity. This suggests that phylogenetic groups do not necessarily correlate with pathogenic or geographic groups. Other studies on

DNA fingerprinting of *Fusarium* wilt pathogens have also reported similar findings (3, 38). This result is perhaps not surprising as *formae speciales* are based on phenotypic characters which are influenced by a range of factors and linked to necessarily variations at DNA level.

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Other articles in this theme issue include references (46-73).

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