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Molecular characterization of *Fusarium* spp. Isolates by using RAPD technique

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Abstract
Genetic diversity of ten *Fusarium* spp. isolates was studied by using RAPD technique. As *Fusarium* species were commonly occurred on all five oilseeds in almost all varieties especially showing abnormalities. Out of seven primers used three primers viz., OPAD-4, OPAD-7 and OPAD-18 gave amplification and produced twenty seven bands. The diversity index in dendrogram clearly indicate the distinct features which gives clear idea about the relation between *Fusarium* species isolates and seed content.

Keywords: Genetic diversity, *Fusarium* spp. Isolates, RAPD technique and diversity index.

INTRODUCTION

Fusarium is one of the most ubiquitous, abundant, and important genera of seed-borne. The genus contains many species of environmental, agricultural and human health importance [1 and 2]. RAPD analysis is a fast, PCR-based method of genetic typing based on genomic polymorphisms. The technique is highly sensitive to nucleotide differences and can assay single nucleotide differences [3 and 4]. Molecular techniques based on the polymerase chain reaction (PCR) have been used as a tool in genetic mapping, molecular taxonomy, evolutionary studies and diagnosis of several fungal species [5, 6 and 7]. The random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique has been useful to distinguish species within genera, including *Fusarium* spp. [8]. RAPD technique was used to characterize *Fusarium* strains [9 and10]. Similarly, RAPD markers were used to study inter and intra-specific variation of twelve *Fusarium* species isolated from cotton-growing areas in Egypt [11]. The current study was undertaken to elucidate the genetic complexity of isolates of *Fusarium* spp. in Marathwada region of Maharashtra state based on RAPD analysis and to provide insight into its geographic origin.

MATERIALS AND METHODS

a) RAPD (Random Amplified Polymorphic DNA)

Ten isolates of *Fusarium* used in the present study were isolated from abnormal oilseeds collected from different market places of Marathwada region of Maharashtra state. The isolate were isolated and purified on GNA agar medium.

DNA Extraction method

The total genomic DNA of *Fusarium* was isolated from dry mycelia. Isolates were incubated at 28 ^{0}C for eight days on GNA media. Mycelia were harvested by scrapping with the help of sterile spatula and used for isolation of the DNA.

DNA isolation was done by CTAB method. Small quantity of dry mycelium mat was taken in a sterile eppendorf tube and crushed to very fine powder. Then prewarm CTAB buffer (600µl) was added. Further it was kept for lysis more than 2-3 hrs in water bath at 50^{0}C. Tubes were removed and centrifuged @10,000 rpm for 20 min. at room temperature. The supernatant was transfered to other sterile eppendorf tube. Further, 24:1 chloroform-isoamyl alcohol was added to it (600µl) and vortexed and centrifuged at 10,000 rpm for 20 min at room temperature. After that 0.5 µl of 3 M sodium acetate (pH-5.2) to each tube was added, mixed and chilled on ice for 15 min. and centrifuged for 30 min at 10,000 rpm at 4^0 C. The supernatant was removed and transferred to new sterile eppendorf tube. Then ice cooled isopropanol (600µl) was added and kept in -20^0 C for 15 min and centrifuged @ 10,000 rpm for 10 min at 4^0 C. Further the isopropanol was removed and drained out. Then 70% ethanol (600µl) was added and kept for 15 min followed by centrifugation @ 10,000 rpm for 5 min. The pellets were dried overnight and 100 µl ultrapure water was added for better dissolve of DNA and further used for electrophoresis.

RAPD Primers

Three decamer RAPD primers were used for initial screening. Following primers gave the amplification. The primer sequences are as follows.
Primer OPAD-4 – GTAGGCCTCA
Primer OPAD-7 - CCCTACTGGT
Primer OPAD-18 – ACGAGAGGCA

PCR amplification of DNA with RAPD primers

PCR technique has promoted the development of a range of molecular assay systems which detect polymorphism at molecular level. In this study we used the most widely adopted PCR based RAPD marker technology for characterizing the natural variation amongst the *Fusarium* spp. isolates. PCR reactions were carried out in a thermal cycler.

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Master mixture for PCR

The master mix for PCR was prepared by mixing the following components in the given proportion as follows:

Taq buffer (10X)	- 2.5µl
$MgCl_2$ (25mM)	- 2.5 µl
dNTP's (2.5mM)	- 2 µl
Taq polyerase (5Unit/ul)	- 0.2µl
MQ water	-14.8µl
Primer (10pm/uL)	- 2.0µl
Template DNA	-1.0µl (100ng/uL)
Total reaction volume	-25 µl

PCR thermal cycler

The polymerase chain reaction, usually called PCR, is an extremely powerful procedure that allows one to amplify a selected DNA sequence. The PCR procedure involves using synthetic oligonucleotides complementary to the known sequences spanning the region of interest to prime enzymatic amplification of this segment of DNA in the test tubes. DNA was amplified by Applied biosystem, Veriti 96 well Thermal Cycler programme. The PCR procedure involves three steps, each repeated many times to produce cycles of amplification.

First denaturation step is for 3 min at 95°C followed by 35 cycles of 30 sec at 95°C. Annealing temperature 35°C for 30 sec followed by renaturing temperature 68°C for 1min and final extension of 68°C for 1min. After completing 35 cycles, the samples were loaded on 1X TBE gel.

The products of first cycle of replication were then denatured, annealed to oligonuleotide primers and replicated again with DNA polymerase. The cycle is repeated many times until the desired level of amplification was achieved.

Post PCR processes

PCR products were resolved by horizontal electrophoresis using agarose gel (1.5%) with TBE buffer (1%) containing ethidium bromide

Electrophoresis

To prepare 1.5 % of agarose gel, 1.5 gm of agarose was dissolve in 100ml of 1X TBE buffer. It is then kept for cooling. Meanwhile, the gel tray was sealed at both the sides with tape. The comb was inserted in such a way that 1mm gap was created between the teeth and surface of tray could be made. Agarose solution was then poured into the tray to get the thickness of gel of about 4-5 mm and kept for about 30-40 min without disturbing. After solidifying the gel, the comb was removed gently. The gel tray was transferred into electrophoresis tank and TBE buffer was poured into the tank. DNA samples were loaded carefully into slots of submerged gel. The electric lead was connected in such a way that a negative terminal should be at the end where sample had been loaded. Electrophoresis was ran at 75 V until loading dye bromophenol blue migrate to the other end of the gel. Turn the button off and disconnect the electric leads after 1 hr. Agarose gel was taken out from the electrophoresis tank.

Band scoring and data analysis

The banding patterns generated through RAPD assay in the present study were used to differentiate between three *Fusarium* spp. isolates from different locations to deduce genetic diversity among them. A total of 10 samples were analyzed using three arbitrarily selected decamer primers revealed varying degree of polymorphism. For each fragment that was amplified using RAPD primers was treated as a unit rearrangement in genome. The gel pictures were taken by BioRad molecular imager GelDoc™ XR and documented to computer.

RESULTS AND DISCUSSION

The RAPD banding profiles were computed and analysed based on presence or absence of bands. Molecular weights of amplified bands were estimated by comparing with known molecular weight marker (100 bp DNA ladder, Promega). The DNA profiles generated for all samples were compared within and between the ten isolates of *Fusarium* species. Comparisons were carried out between samples amplified by the same primer in a pair-wise manner. Nei's genetic similarity coefficient between the isolates of *Fusarium* spp. was calculated from band sharing data and used for constructing a unweighted pair group methods with arithmetic (UPGMA). A total of 10 samples were analyzed using three arbitrarily selected decamer primers. Three primers generated 27 bands, ranging in size between 250 and 3700 bp. Three primers showed eight monomorphic bands of 250bp, 350bp, 800bp, 2400 bp, 3000 bp, 3200 bp, 3500 bp and 3700 bp generated by F. ver, F. equ1, F. oxy2, F. oxy1, F. oxy2, F. equ1, F. oxy2 and F. equ1respectively. Of the three primers used for RAPD, Primer OPAD18 showed maximum number of bands. On the other hand, Primer OPAD 7 produced the lowest number of fragments. Specific bands were produced by different primers at different places. Primer 4 gave five specific bands at 300, 600, 700, 900 and 1000 bases for isolate F.ver, F. sol, F.ver, F. equ1 and F. sol respectively as monomorphic bands. The Primer OPAD 7 gave five monomorphic bands at 400, 750, 800, 900 and1500 bp for isolate F. ros, F. equ2, F. oxy2, F. sol and F. oxy1 respectively as monomorphic bands. The Primer OPAD 18 gave bands at 250, 350, 600, 700, 750, 1500, 2400, 2500, 3000, 3200, 3500 and 3700 of isolate F. ver, F. equ1, F. oxy2, F. oxy1, F. ver, F. oxy2, F. oxy1, F. oxy2, F. oxy2, F. equ1, F. oxy2, F. equ1 respectively as monomorphic bands. The Primer OPAD 4 gave three, Primer OPAD 7 gave two and Primer OPAD 18 produced three polymorphic bands (Fig. 1). Similarity matrix was obtained by using PAST Ver. 1.83 software. Genetic similarity and distance indices were obtained based on pairwise comparison (Fig. 2). From similarity and distance index it is clear that 0.1 is the lowest similarity and 0.46 is the highest similarity among the *Fusarium* isolates. It was observed that, F. cul1 and F. cul2 was not amplified in PCR reaction, therefore, similarity indices were zero which was not produced scorable band. Isolates from similar species, similar host and same location had more similarity and hence less genetic distance was evidenced. The Neighbour Joining tree showed two Clads. Clad I having isolates F. ver, F.ros, F. sol. and F. dim where isolates F. sol. and F. dim. fall in Clad I with least genetic difference (0.461) and were isolated from sample of Osmanabad. It confirms and support for their less genetic variation. Isolate F. ros. and F. ver. partially claded with F. sol. and F. dim. The genetic distance among F. ros. and F. ver. is 0.315. This may be due to their host specificity and environmental adaptation. In

Clad II two separate clusters were observed. Out of that one with F. oxy1 and F. oxy2 which belongs to same host but their collection sites are different i.e. Latur and Nanded. Second cluster of this clad having two separate subgroups. One subgroup formed of F. equ1 and F. equ2 which were belongs to same location. Another subgroup formed of F. cul1 and F. cul2 which were isolated from same host.

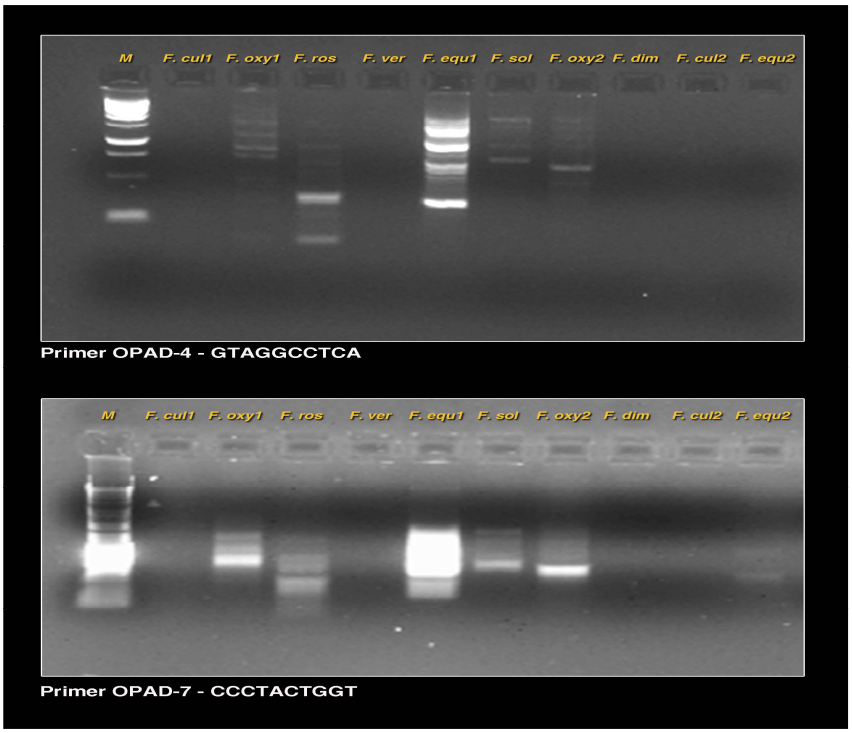

Fig 1. RAPD Fingerprints of ten *Fusarium* spp. isolates

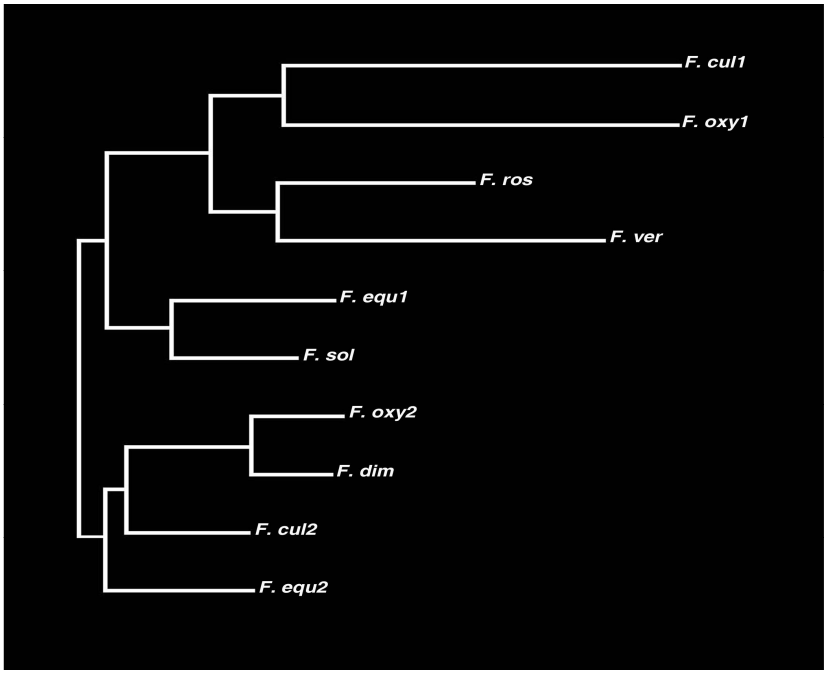

Fig 2. Dendrogram analysis of *Fusarium* spp. isolates

RAPD can be an efficient tool to differentiate geographically and genetically isolated populations, and has been used to verify the existence of locally adapted populations within a species that may have arisen either through genetic selection under different environmental conditions or as a result of genetic drift [12]. In our study we got genetic variation according to hosts and locations of isolates collected. This information may be useful in primary investigations to trace the genetic variability and adaptations among

fungal species of stored grains and their management. From clustering pattern it can be concluded that RAPD can give clear idea about genetic diversity among various *Fusarium* spp. Genetic distances could be due to their adoptability with various environmental conditions and hosts. Similar results were reported by several scientists [13, 14, 15, 16 and 17].

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