Austin Journal of Biotechnology & Bioengineering

Open Access

Austin Publishing Group

Research Article

Genetic Variations among Ten Isolates of *Fusarium equiseti* (Corda) Saccardo Isolated from Fruits and Vegetables

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Received: September 22, 2014; **Accepted:** November 01, 2014; **Published:** November 03, 2014

Abstract

We studied ten- isolates of *F. equiseti* isolated from different fruits and vegetables, which were identified on the basis of morphological characteristics and Random amplified polymorphic DNA (RAPD). Analysis of these isolates was made by using 25 different universal decamer primers to confirm the identity. UPGMA study differentiated of ten isolates into two main clusters on the basis of similarity. The aim of the present study was to assess genetic variations among ten- isolates of *F. equiseti* isolated from fruits and vegetables by RAPD. The dendrogram obtained from the data showed that hierarchical clustering separated the isolates into three groups according to their similarity coefficients. The similarity coefficients of all isolates ranged from 0.02 to 0.15.

Keywords: Dendrogram; *Fusarium equiseti*; Genetic diversity; RAPD; UPGMA

Introduction

Fusarium species are commonly associated with many economically important crop diseases, such as, vascular- wilt, root-rot, stem- rot, and fruit and vegetable decay. The disease can cause yield and economic losses, hence study of distribution and diversity of these species is very important [1,2]. Nelson *et al.* [3] illustrated the morphology of *F. equiseti*. The fungus was found to be associated with various diseases like cankers of sour cherry trees [4], rots of pumpkin [5] or cucurbit fruits in contact with soil [6]. It is resistant to antifungal agents like fluconazole and flucytosine [7].

Isolates of *F. equiseti* can be distinguished on the basis of growth rate and other morphological characters like shape and size of conidia on PDA [3]. However, molecular markers like random amplified polymorphic DNA (RAPD) can be used for confirmation of identity and differentiation among different isolates. RAPD assays have been used extensively to define fungal populations at species, intraspecific, race and strain levels. The use of molecular marker based on the polymerase chain reaction for species identification and as a diagnostic tool became very popular during the last decade [8] and usually RAPD-PCR technique is used for detecting genetic variability [9]. The morphological and molecular characterization of *Fusarium* species within the Liseola section isolated from corn grains were studied in different geographic regions of Brazil [10]. Moreover, phylogenetic analysis of *Fusarium*-wilt caused by *F. oxysporum* f.sp. *melonis* (FOM) was performed by random amplified polymorphic DNA (RAPD) profiling and by translation elongation factor-1α (TEF-1α) sequencing [11]. Katkar *et al.,* [12] reported that *F. oxysporum f.sp.ciceri* were screened by using 30 RAPD primers for evaluation of genetic diversity. The biodiversity of *Fusarium* sps. isolated from the roots of oil palm and date palm was studied using RAPD molecular marker [12].

The aim of the present study was to assess the genetic variation among ten- isolate of *F. equiseti* (Corda) Saccardo isolated from fruits and vegetables by using RAPD-PCR method.

Materials and Methods

Isolation and morphological identification

Infected fruits were collected from different locations of Amravati city of Maharashtra (Table 1).

Different isolates of *F. equiseti* were recovered from infected materials on potato dextrose agar (PDA) and incubated at 25 ± 2^{0}C for 3-4 days.

All the isolates of *F. equiseti* were identified on the basis of morphological and cultural characters [9].

DNA extraction

The total DNA was extracted using fungal genomic DNA isolation kit from Chromous Biotech Pvt. Ltd, Bangalore, India according to manufacturer's instructions.

Table 1: *Fusarium equiseti* isolated from different fruits and vegetables.

S.N.	Hosts	Botanical name	Place of collection
1	Brinjal (Fruit) (BB)	*Solanum melongena*	Amravati
2	Tomato (Fruit) (CC)	*Lycospersicum esculentum*	Amravati
3	Carrot (Stem) (DD)	*Daucus carota*	Amravati
4	Papaya (Fruit) (EE)	*Carica papaya*	Amravati
5	Papaya (Fruit) (FF)	*Carica papaya*	Amravati
6	Banana (Fruit) (GG)	*Musa paradisiaca*	Amravati
7	Beet (Vegetable) (HH)	*Beta vulgaris*	Amravati
8	Potato (Tuber) (II)	*Solanum tuberosum*	Amravati
9	Ladies finger (Vegetable) (JJ)	*Abelmoschus esculentus*	Amravati
10	Papaya (Fruit) (KK)	*Carica papaya*	Amravati

Citation: Bonde SR, Gade AK and Rai MK. Genetic Variations among Ten Isolates of *Fusarium equiseti* (Corda) Saccardo Isolated from Fruits and Vegetables. Austin J Biotechnol Bioeng. 2014;1(5): 5.

Table 2: Primer and their sequences used in RAPD-PCR test.

S. N.	Primer	Sequences 5'-3'	No. polymeric bands	No. of monomeric bands	Total bands	%of plymorphism
1	RFu 1	CCTGGGCCAG	0	0	0	0
2	RFu 2	CCTGGGCGAG	0	0	0	0
3	RFu 3	CCTGGGCTGG	0	0	0	0
4	RFu 4	CCTGGGCTAT	0	0	0	0
5	RFu 5	CCTGGGCTTG	**2**	**4**	**107**	**1.9**
6	RFu 6	CCTGGGCTAC	0	0	0	0
7	RFu 7	CCTGGGCTTA	0	0	0	0
8	RFu 8	CCTGGGTCGA	0	0	0	0
9	RFu 9	CCTGGGTGCA	0	0	0	0
10	RFu 10	CCTGGGTGAC	**2**	**2**	**90**	**2.2**
11	RFu 11	CCTGGCTTAC	0	0	0	0
12	RFu 12	CCTGGGTTAC	0	0	0	0
13	RFu 13	CGGGGGATGG	0	0	0	0
14	RFu 14	CTCCCTGACC	0	0	0	0
15	RFu 15	GAGCACCTGT	0	0	0	0
16	RFu 16	GAGCACGTCA	0	0	0	0
17	RFu 17	GAGCACGGCA	0	0	0	0
18	RFu 18	GAGCACGGAG	0	0	0	0
19	RFu 19	GAGCTCGCAT	0	0	0	0
20	RFu 20	GAGGGCATGT	0	0	0	0
21	RFu 21	CCGGCCCCAA	0	0	0	0
22	RFu 22	CCGGCCTTAA	0	0	0	0
23	RFu 23	CCGGCCATAC	**1**	**4**	**77**	**1.3**
24	RFu 24	CCGGCCTTCC	0	0	0	0
25	RFu 25	CCGGCTGGAA	**1**	**3**	**82**	**1.2**

RAPD analysis

The isolated DNA of ten different isolates of *F. equiseti* were screened using twenty- five random fungal primers (Table 2) from Random Fungal Primer Kit (RFu 'D'), Genie Pvt. Ltd, Bangalore, India, Amplifications were performed using PCR mixture in a total volume of 25 µL containing 12.5µl PCR master mix (2X) (Fermentas Life Sciences, Canada) 5 µl of template DNA (20 ng), 1.5 µl $MgCl_2$ (25 *mM*), 0.3 µl *Taq* DNA polymerase (Genexy, 5U/µl), 1 µl each primer and 4.7 µl nuclease free distilled water (supplied with Fermentas PCR master mix).PCR was carried out on gradient PCR machine (Palm-Cycler Corbett Research, Australia). The programme included an initial denaturation at 94^{0}C for 2 min, 35 cycles with denaturation at 94^{0}C for 30 sec, annealing 40 ^{0}C for 1 min, extension at 72^{0}C for 2 min and final extension at 72^{0}C for 5 min with holding temperature at 4^{0}C for 10 min. All experiments were repeated for three times. PCR products were electrophoresed on 1.5% agarose by using 1X TAE buffer (Fermentas Life Sciences, Canada), stained with ethidium bromide, visualized in a UV-transilluminator and the gels were photographed using Gel Doc (AlphaImager, Gel documentation system, USA), system.

Results and Discussion

Morphological and cultural studies

All the ten- isolates of *F. equiseti* grew rapidly with dense aerial mycelia on potato dextrose agar. The detailed morphological studies including macroscopic and microscopic characteristics have been given in Table 3.

Macroscopic characteristics: Ten different isolates of *F. equiseti* were recovered from infected fruits and vegetables (Table 1) on Potato Dextrose Agar (PDA) and identified on the basis of morphological and cultural characteristics. The mycelium of *F. equiseti* was initially yellow but became brown with increasing age. It produces pale-brown to dark-brown pigments on PDA plate (Figure 1A, B). All the isolates exhibited similar growth rate of about 7.1-9 cm after 6 days incubation at 25 ± 2^{0}C. Morphological characters of *F. equiseti* showed resemblance with morphological characters reported by many researchers in their study carried out in the past for *F. equiseti* [2,3].

Microscopic characteristics: Microscopic examinations showed the presence of septate, hyaline hyphae and absence of microconidia in majority of isolates. Macroconidia were long slender, abundant and borne on aerial mycelium, generally 5-7 septate and 23.55 to 58.45 µm in length (Figure 2A). The apical shape of macroconidia was tapered and elongated or even whip-like while the basal shape was prominent foot-shaped (Figure 2B). Bi-septate mesoconidia were found in a few isolates (Figure 2C). Two types of chlamydospores were found in the present study. Interestingly, in one case chlamydospores were found in the hyphae singly and sometimes in chains (Figure 2D), while in another case, single chlamydospores were present. (Figure 2E) which is a specific characteristic of *F. equiseti*. It provides the strength for the identification of all these isolates on the basis of microscopic characteristics. Aigbe *et al.*[2] reported the similar microscopic characters for *F. equiseti* isolated from cow pea in Nigeria.

RAPD analysis

RAPD-PCR analysis was carried out to screen genomic DNA isolated from ten isolates of *F. equiseti* using twenty five random decamer primers of Fungal RAPD Primer (RFu 'D') kit (Table 2). The culture of *F. equiseti* procured from IMTECH (MTCC 3731),

Table 3: Morphological characteristics of *F. equiseti* associated with different fruits and vegetables.

S.N.	Host	Colony Color		Type of Mycelium	Colony diameter* (cm)	Common Conidial septation	Length of macroconidia** (µm)	Meso-conidia	Chlamydo-spore
1	Brinjal (Fruit) (BB)	White	Cream	Aerial	6.1	4-7	58.42 ± 1.71	Present	Present
2	Tomato (Fruit) (CC)	White	Brown	Aerial	8.1	3-6	47.82± 0.52	Absent	Present
3	Carrot (Stem) (DD)	White	Brown	Aerial	8.6	3-4	26.27± 0.16	Present	Absent
4	Papaya (Fruit) (EE)	Cream	Cream	Aerial	9.1	3-7	42.06 ± 0.15	Absent	Present
5	Papaya (Fruit) (FF)	Cream	Brown	Aerial	8.2	3-5	43.52 ± 3.31	Absent	Present
6	Banana (Fruit) (GG)	White	Cream	Aerial	8.2	3-6	56.41 ± 0.24	Absent	Present
7	Beet (Vegetable) (HH)	Cream	Yellowish	Aerial	7.6	3-5	23.55 ± 0.13	Absent	Absent
8	Potato (Tuber)	White	Brown	Aerial	8.1	3-5	32.33 ± 1.40	Present	Present
9	Ladies finger (II) (Vegetable)	Cream	Brown	Aerial	8.2	3-4	34.2 ± 1.46	Present	Present
10	Papaya (JJ)	Yellow	Brown	Aerial	8.0	3-6	44.11 ± 0.44	Absent	Present

* Diameter of colony after 6 days of growth at 25 ± 2°C.

** Mean values of 10 random conidia ± standard deviation.

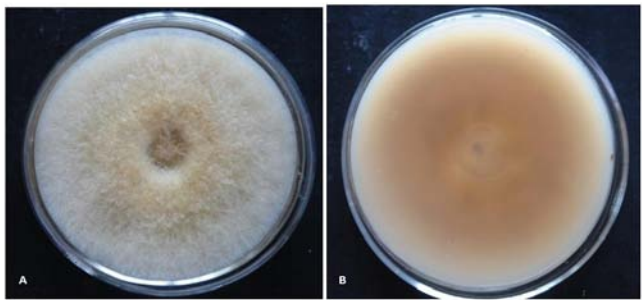

Figure 1: Growth of *F. equiseti* on PDA medium (A) Dorsal view (B) Ventral view isolated from Brinjal (*Solanum melongena*).

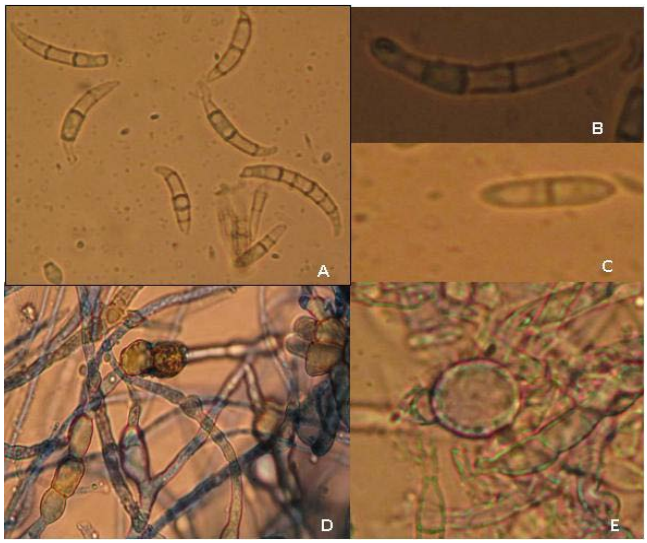

Figure 2: Microscopic characteristics of *F. equiseti* **(A)** Macroconidia **(B)** Single Macroconidium (6 septate) (tapering apical and notched foot shape) **(C)** Mesoconidia **(D)** Chlamydospores in chain in hyphae **(E)** Chlamydospore singly in conidia.

Chandigarh was taken as reference for RAPD study. In the preliminary experiments, twelve out of the twenty five primers tested produced distinct and reproducible band profile, and polymorphisms produced by ten primers. Four of twelve primers were used for comparative analysis of ten isolates of *F. equiseti*. The primers, including RFu 5 (5'-CCTGGGCTTG-3'), RFu 10 (5'–CCTGGGTGAC-3'), RFu 23 (5'-CCGGCCATAC-3'), and RFu 25 (5'-CCGGCTGGAA-3') generated polymorphic bands in all ten isolates of *F. equiseti* (Figure 3). Primers RFu 5, RFu 10, Rfu 23 and RFu 25 showed monomorpic and polymorphic bands (Table 2). The amplified fragments were ranged 0.5 kb to 3.0 kb, except a single band which ranged upto 5.0 kb. RAPD assays of all ten isolates with four above mentioned primers yielded 170 bands, which were found to be polymorphic. Above data showed that RAPD is a convenient method for distinguishing the isolates of *F. euiseti* and also reveal a significant genetic variation among these isolates. *F. oxysporum* also demonstrated the genetic variation and differentiation among its isolates. Assigbetse *et al.* [14] differentiated races of *F. oxysporum* f. sp. *vasinfectum* on cotton by using RAPD as a molecular tool. They reported the significant genetic variation in these isolates of F. *oxysporum*. In another study, Edel and colleagues [15] observed that the soil isolates of *F. oxysporum*

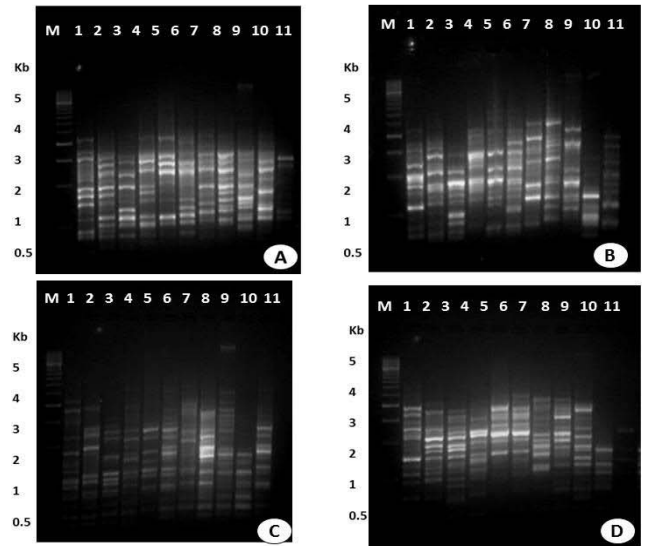

Figure 3: RAPD patterns on 1.5% agarose gel of amplified fragments generated from 10 isolates of *F. equiseti* with random primers **(A)** RF u-5 **(B)** RFu - 10 **(C)** RFu-23 (D) RFu-25. Lane M- DNA marker (100 bp), lane 1- mtcc-3731, lane 2- Brinjal *(Solanum melongena*), lane 3- Tomato *(Lycospersicum esculentum*), lane 4-Carrot (*Daucus carota*), lane 5-Papaya 1 *(Carica papaya*), lane 6-Papaya 2 *(Carica papaya*), lane 7-Banana (*Musa paradisiaca*), lane 8- Beet *(Beta vulgaris*), lane 9-Potato *(Solanum tuberosum*), lane 10-Ladies finger (*Abelmoschus esculentus*), lane 11-Papaya 3 (*Carica papaya*).

Table 4: Distance matrix by UPGMA Analysis.

Isolates	MTCC 3731	Brinjal	Tomato	Carrot	Papaya 1	Papaya-2	Banana	Beet	Potato	Ladies Finger	Papaya 3
MTCC 3731	0.000										
Brinjal	0.050	0.000									
Tomato	0.066	0.031	0.000								
Carrot	0.074	0.042	0.043	0.000							
Papaya 1	0.063	0.038	0.045	0.051	0.000						
Papaya 2	0.074	0.060	0.089	0.057	0.031	0.000					
Banana	0.051	0.036	0.060	0.031	0.034	0.019	0.000				
Beet	0.052	0.043	0.060	0.045	0.051	0.036	0.016	0.000			
Potato	0.040	0.031	0.051	0.074	0.052	0.063	0.041	0.029	0.000		
Ladies Finger	0.047	0.020	0.034	0.062	0.051	0.074	0.055	0.046	0.021	0.000	
Papaya 3	0.110	0.089	0.132	0.156	0.087	0.146	0.156	0.159	0.082	0.083	0.000

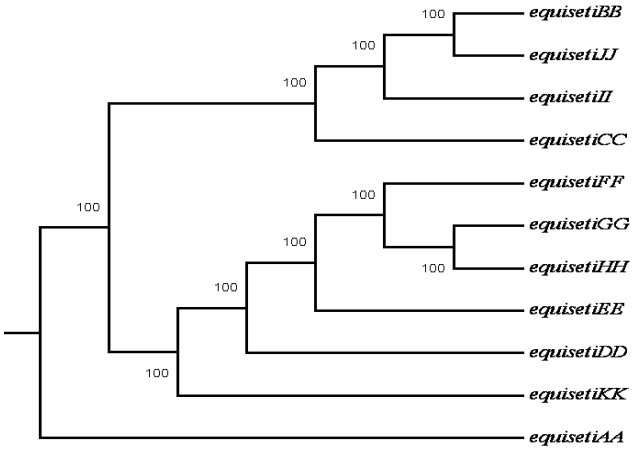

Figure 4: Dendrogram showing genetic relationship among the ten isolates of *F. equiseti*
AA) MTCC 3731, (BB) Brinjal *(Solanum melongena)*, (CC) Tomato *(Lycospersicum esculentum)*, (DD) Carrot (*Daucus carota*), (EE) Papaya 1 *(Carica papaya)*, (FF) Papaya 2 *(Carica papaya)*, (GG) Banana (*Musa paradisiaca*), (HH) Beet *(Beta vulgaris)*, (II) Potato *(Solanum tuberosum)*, (JJ) Ladies finger (*Abelmoschus esculentus*), (KK) Papaya 3 (*Carica papaya*).

in France showed genetic diversity. Our finding supports the work carried out by Leslie and co-workers [9]. They observed inter- and intra specific genetic variation in different *Fusarium* species. RAPD-PCR technique was used to rapid identification and differentiation of *Fusarium* species [16]. Moreover Costano [17] studied genetic diversity of *F. oxysporum* f. sp. *dianthi* in Southern Spain in 132 isolates collected from carnation wilted plants. RAPD marker used to estimate genetic variation among 12 isolates of the *F. solani isolates* causing dry root rot of sweet orange (*Citrus sinensis* osbeck) [18].

Unweighted Pair Group Method with Arithmetic Mean analysis (UPGMA) Cluster Analysis

F. equiseti isolates were analyzed using UPGMA clustering approach with bootstrap value of 1000. UPGMA, a multivariate statistics analysis of the RAPD data separated the *F. equiseti* in two clusters. UPGMA dendrogram showed MTCC (3731) culture of *F. equiseti* and other isolates reported from India in two different clades. *F. equiseti* isolated from brinjal (BB) (*Solanum melongena)*, tomato (CC) (*Lycospersicum esculentum*), potato (II) (*Solanum tuberosum*) and ladies finger (JJ) (*Abelmoschus esculentus*) were in one clade while other isolates were in different clades. Isolates of *Fusarium* recovered from brinjal, potato and ladies finger were similar but *F. equiseti* isolated from tomato showed genetic variation as it was present in different clade in dendrogram. UPGMA analysis thus carried out in the present study showed the genetic variation in these isolates of *F. euiseti.* A distance matrix on simple matching coefficients was calculated from the data based on the RAPD of all ten isolates of *F. equiseti*. The matrix was used to construct a dendrogram using distance tool with UPGMA method of PHYLIP for establishing to analyze the level of relatedness among the ten isolates. The dendrogram obtained from the data showed that hierarchical clustering separated the isolates into three groups according to their similarity coefficients. The similarity coefficients among the all isolates ranged from 0.02 to 0.15. Distance matrix of different isolates of *F. equiseti* was analyzed (Table 4).

UPGMA is a simple agglomerative or hierarchical clustering method used in bioinformatics for the phylogenetic analysis. The results obtained in the present study are noteworthy and showed the similarity with other researchers. In their studies on isolates of *F. semitectum*, *F. solani* and *F. oxysporum* respectively, they used data generated from RAPD banding pattern for the UPGMA analysis and found that there were genetic variations in different isolates of same *Fusarium* [19-21]. A modified mathematical model by Nei and Li [22] for the evolutionary change of restriction site was used. Although the ten isolates of *F. equiseti* were recovered from different infected fruits and vegetables showed the similar morphological characteristics. Interestingly, our results suggest a significant genetic variation among the isolates of *F. equiseti*. It seems that the fungal species showed the host dependent genetic variation. Similarly biodiversity of sixteen *Fusarium* isolates, isolated from the roots of oil palm and date palm in Nigeria was studied. In this RAPD was used to detect the phylogenetic similarity between them. The UPGMA dendrogram clearly separated these sixteen *Fusarium* isolates into five groups (clusters). The first at SC values of 100 grouped six Fusarium isolates of both oil and date palms [13].

Conclusion

Our results suggest a significant genetic variation among the isolates of *F. equiseti* by RAPD analysis, The genetic variation shown by fungal species is host dependent. We report RAPD analysis for genetic variation among different isolates of *F. equiseti* isolated from fruits and vegetables and we suggest that RAPD marker may be used as one of the reliable alternatives for the determination of genetic variation among the *Fusarium* species when a little variation in morphological characters exist.

Acknowledgement

The authors are grateful to Defense Research and Development Organization (DRDO), New Delhi for providing financial assistance for the present research.

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Austin J Biotechnol Bioeng - Volume 1 Issue 5 - 2014
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Citation: Bonde SR, Gade AK and Rai MK. Genetic Variations among Ten Isolates of *Fusarium equiseti* (Corda) Saccardo Isolated from Fruits and Vegetables. Austin J Biotechnol Bioeng. 2014;1(5): 5.