Molecular Diversity of Seed-borne *Fusarium* Species Associated with Maize in India

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Abstract: A total of 106 maize seed samples were collected from different agro-climatic regions of India. Sixty-two *Fusarium* isolates were recovered, 90% of which were identified as *Fusarium verticillioides* based on morphological and molecular characters. Use of the *tef*-1α gene corrected/refined the morphological species identifications of 11 isolates, and confirmed those of the remaining isolates. Genetic diversity among the *Fusarium* isolates involved multilocus fingerprinting profiles by Inter Simple Sequence Repeats (ISSR) UP-GMA and *tef*-1α gene genetic analyses; for which, we observed no significant differences among the isolates based on geographic origin or fumonisin production; most of the subdivision related to species. Genotyping was performed on the *F. verticillioides* isolates, using 12 primer sets from the fumonisin pathway, to elucidate the molecular basis of fumonisin production or non-production. One fumonisin-negative isolate, UOMMF-16, was unable to amplify nine of the 12 fumonisin cluster genes tested. We also used the CD-ELISA method to confirm fumonisin production for our 62 *Fusarium* isolates. Only 15 isolates were found to be fumonisin-negative. Interestingly, genotypic characterization revealed six isolates with various gene deletion patterns that also tested positive for the production of fumonisins via CD-ELISA. Our findings confirm the importance of molecular studies for species delimitation, and for observing genetic and phenotypic diversity, among the *Fusaria*.

Keywords: Maize, *Fusarium*, *tef*-1α gene, Inter simple sequence repeats, Fumonisin gene cluster, CD-ELISA.

1. INTRODUCTION

Maize is one of the most important food crops grown all over the world, and is the most susceptible to fungal contamination which can occur during pre- and post-harvest [1, 2]. The seed-borne fungi colonizing maize kernels often include mycotoxigenic species [3]. Among mycotoxigenic fungal pathogens, *Fusarium* species are common to maize and can cause disease at any time from the seedling stage through post-harvest storage. *Fusarium verticillioides* and *Fusarium proliferatum* belong to *Fusarium* section Liseola which contains other closely-related species that have the potential to produce fumonisins [4, 5]. There are at least 28 different forms of fumonisins that occur naturally; of which, Fumonisin B₁ (FB₁) is the most dominant form, followed by FB₂ and FB₃ [6]. Consumption of fumonisin-contaminated maize reportedly leads to disruption of sphingolipid metabolism, associated with human esophageal cancer, and increases risk for neural tube defects in children [7-9]. The regulatory limit for fumonisins in maize and maize products is set between 4000 to 200 μg/kg by European Union and Food and Drug Authority to prevent exposure of individuals to these fungal toxins [10].

*Fusarium verticillioides* is widely distributed throughout the world and is most often associated with infections of maize [11]. The genus *Fusarium* lacks many distinctive morphological characters that can be used to easily delimit species and often leads to inconsistent identification of species [12]. DNA-based comparisons (i.e. nucleotide sequences) have been increasingly used to distinguish between closely-related *Fusarium* species [13, 14]. The internal transcribed spacer (ITS) region is often used as a species delimiter, but Fusaria within the Gibberella teleomorph clade possess non-orthologous copies of the ITS2, which has lead to incorrect phylogenetic inferences [15]. The translation elongation factor-1α (*tef*-1α) gene occurs consistently as a single copy in *Fusarium*, and shows a high level of sequence diversity among closely-related species, thus making it a sequence-based marker of choice for delimitation of *Fusarium* species [12]. Isolates within each species are known to exhibit variations at the biochemical, physiological and molecular levels, which affect metabolite production, patho-

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2. MATERIALS AND METHODS

2.1. Identification of Seed-borne Fusarium Species From Maize Seeds

A total of 106 maize seed samples were collected from different agro-climatic regions throughout India and were subjected to the standard blotter method for isolation of Fusarium species [38]. A total of 62 Fusarium strains were isolated and putatively identified based on morphological characters [39]. For molecular confirmation of species identity we first inoculated 100 ml Erlenmeyer flasks containing 50 ml of potato dextrose broth with three mycelia plugs from each Fusarium strain. Mycelial mats were separated and Genomic DNA was extracted using a Hi PurA™ Plant Genomic DNA Miniprep Purification Spin Kit (Himedia, India), according to the manufacturer’s instructions. The concentration and purity of extracted DNA samples were determined using a Nano Drop spectrophotometer (Thermo Scientific, Nano drop-2000C, Germany). PCR assay for the specific detection of F. verticillioides was carried out using VERT-1 and VERT-2 primers [40], while the tef-1α gene, using the primer pairs EF1/ EF2 [41], offered intra-genus species delimitation. The PCR reactions were performed by following the protocol of Geiser et al. [12], with slight modifications. PCR-amplified products were separated on 1.2% agarose gel and the fragments were purified using a Qiagen PCR Purification Kit (Qiagen, Valencia, California, USA) agarose gel and the fragments were purified using a Qiagen PCR Purification Kit (Qiagen, Valencia, California, USA) and then queried by BLASTn search to confirm/identify species before being accessioned in GenBank.

2.2. Analysis of Molecular Diversity Among Fusarium Isolates

2.2.1. Phylogenetic Analysis

Phylogenetic relationships among our 62 Fusarium isolates were inferred using their respective tef-1α gene sequences mentioned above. A tef-1α sequence for F. redolens was downloaded from NCBI (AY714109) as the out-group taxa, and a multiple sequence alignment was generated using the Clustal W version 1.7 (MSA) program [42]. The phylogenetic tree was inferred by maximum likelihood method using the Mega 5.0 program [43] and the genetic distances were calculated using the Kimura 2-parameter genetic distance model [44].

2.2.2. ISSR Analysis

For ISSR analysis, a total of 30 primers were first synthesized at Sigma (India) Co., Ltd., and microsatellite regions were amplified [45, 46]. Twenty primers producing clear and reproducible amplicons were selected based on good amplification characteristics and their capacity to detect polymorphisms among the Fusarium isolates. Amplifications for all 62 isolates were performed twice using each primer, each with a primer-specific annealing temperature according to Divakara et al. [23]. Gels were visualized using Quantity One image analysis software (Bio-Rad, Germany). Unambiguous ISSR fragments were scored for the presence (1) or absence (0) of repeating patterns in each of the 62 isolates. Those isolates exhibiting similar banding patterns
were referred to as monomorphic, whereas those that exhibited a unique pattern were referred to as polymorphic. Cluster analysis of the isolates was performed using the UPGMA functionality in NTSYSpc 2.10 [47].

2.2.3. FUM Gene Deletion Analysis

The fumonisin gene cluster consists of 17 transcriptionally co-regulated genes which have various functions such as polyketide synthases, two fatty acid synthases, and numerous modifying enzymes like monoxygenases, dehydrogenases, aminotransferases and dioxygenases [48]. To evaluate the presence or absence of fumonisin biosynthesis genes in 56 Fusarium isolates, PCR (Eppendorf, Germany) was done for six fumonisin genes using 12 pairs of PCR primers. Most of the primer pairs were designed for the present study, based on the sequences of fumonisin biosynthesis genes in F. verticillioides (GenBank accession No. AF155773.5), but three were designed and used in previous studies [23, 48, 49]. Our nine PCR primers were designed using primer-BLAST [50] and verified for syntenic to other Fusarium species. The primers were synthesized at Sigma (India) Co., Ltd. Details of the genes, gene sequences, annealing temperature and length in base pairs are shown in (Table 1). A total of 35 PCR cycles were performed with the following conditions: initial denaturation at 94 °C for 5 min; repeated denaturation at 94 °C for 1 min, annealing for 45 sec (temperature used was primer specific; Table 1), and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 5 min. Amplified products were separated on 1.2% agarose gel and visualized in quantity one image analysis software (Bio-Rad, Germany).

2.3. Analysis of Total Fumonisin Production By CD-ELISA

Petri dishes containing corn meal agar (CMA) media were inoculated with each of the Fusarium isolates and incubated at 24±2 °C for 9 days. Small plugs of CMA cultures were transferred to 500 ml conical flasks containing 100 g of autoclaved corn kernels to which 25 ml distilled water was added. Corn cultures were shaken daily during the first week to ensure even distribution of the inoculum and aeration. The flasks were incubated four weeks at 25 °C for complete Fusarium colonization, at which point the maize grains were removed from the flasks and ground to powder using a blender, and 20 g was used for fumonisin extraction. Each 20 g sample was extracted with 100 ml of methanol:water (70:30) solvent and filtered using Whatman No. 1 filter paper. The supernatant was kept at 4 °C until further use [35]. Total fumonisins were quantified using commercially available CD-ELISA kits (Neogen Corp., Lansing, MI, USA) according to the manufacturer’s instructions.

3. RESULTS

3.1. Identification of Fusarium Species From Maize Seeds

A total of 62 Fusarium spp. were identified morphologically based on their white powdery appearance, as well as the presence of microconidia and macroconidia produced on false heads. Among these 62 isolates, 56 (90%) were identified as F. verticillioides based on the presence of the VERTF gene, while morphologically only 48 F. verticillioides isolates could be identified (Table 2). The tef-1a gene sequences from each of the 62 Fusarium isolates were compared with those from various known species of Fusarium in the NCBI database. The results confirmed that 56 isolates were F. verticillioides, two were F. andiyazi (UOMMF-2, UOMMF-18), and the remaining isolates identified as F. cf. incarnatum-equiseti complex (UOMMF-59), F. proliferatum (UOMMF-46), F. sacchari (UOMMF-20), and F. solani (UOMMF-7). Sequences were deposited at GenBank, and their accession numbers are indicated with their species identifications in (Table 2).

3.2. Analysis of Molecular Diversity Among Fusarium Isolates

3.2.1. Phylogenetic Analysis

Based on their tef-1a sequences, the 62 Fusarium isolates associated based more on species than any other ecological or phenotypic character (Fig. 1). Isolates UOMMF-7 (F. solani) and UOMMF-59 (F. incarnatum) were basal to the out-group species (F. redolens), and both appeared as distinct lineages with no cladal association. Strong bootstrap support (value = 95) associated F. redolens with the remaining 60 isolates examined. These 60 isolates were subdivided into two clades sharing strong bootstrap support (value = 89). Clade I had a very strong bootstrap support (value = 97) and was composed of five haplotype individuals, consisting of all 56 F. verticillioides isolates; of which, 45 had identical sequence and therefore shared a single haplotype. Another six F. verticillioides isolates shared a second haplotype, followed by three sharing a third haplotype, and two other isolates (UOMMF-29 and UOMMF-43) segregated into their own haplotypes. Isolate UOMMF-29 exhibited noticeable sequence diversity from the other F. verticillioides, including those from the same geographic origin (Andhra Pradesh). Clade II included the two F. andiyazi isolates (UOMMF-2, UOMMF-18) which had a very strong bootstrap support (value = 99), as well as the F. sacchari (UOMMF-20) and F. proliferatum (UOMMF-46) isolates, which shared marginal bootstrap support (value = 73).

3.2.2. ISSR Analysis

From the 20 ISSR primers screened, a total of 242 unambiguous and reproducible bands were scored (Table 3). The number of amplified fragments produced per primer pair ranged from three (ISSR9A9) to 18 (ISSR02), and the sizes of those fragments ranged from 150 to 2000 bp. Of the 242 amplified bands, 237 were polymorphic, with an average of 11.85 polymorphic fragments per primer pair. Based on the UPGMA analysis (Fig. 2) eight isolates, including three F. verticillioides isolates and five isolates of other Fusarium species, were separated from the rest of the isolates at the similarity level of 60 %. The rest of the isolates formed clade I including 53 F. verticillioides isolates and one F. andiyazi isolate. At the similarity level of about 82 % cluster I could be divided into seven separate isolates, cluster 1c of six isolates, another cluster of two isolates and to the cluster of the rest of F. verticillioides isolates. At the similarity level of about 88 % clusters 1a (12 isolates including one F. andiyazi isolate) and 1b (16 isolates) could be separated from the rest of F. verticillioides isolates.
Table 1. List of FUM gene cluster primers used in the study and their related functions.

| Primer   | Sequence                      | $T_a$ (°C) | Amplicon (bp) | Predicated Function                  | Mutant Phenotype | References |
|----------|-------------------------------|------------|---------------|--------------------------------------|-----------------|------------|
| Fum 1-F  | ATTGGTAAAGGGAGCAAGAC          | 63         | 798           | polyketide synthase                   | None            | [31, 65]   |
| Fum 1-R  | ACGCAAGCTGTCGTCAGGA           |            |               |                                      |                 |            |
| Fum 2-F  | CAAGAACCTCTGCTGTTCAAGT        | 57         | 770           | cytochrome P450 monooxygenase         | FB2 and FB4     | [31]       |
| Fum 2-R  | GAGCAATGATGTAATGTTGTTG        |            |               |                                      |                 |            |
| Fum 3-F  | AGCCGGAATTTGCTGTCAGTC         | 59         | 550           | deoxygenase                          | FB2 and FB4     | [31]       |
| Fum 3-R  | GGCTACACCTCTGAGCAAGA          |            |               |                                      |                 |            |
| Fum 6-F  | GTATCAGAACCACCCACCGTAT        | 63         | 904           | cytochrome P450 monooxygenase & rereductase | None            | [31]       |
| Fum 6-R  | TATCTTCGCTGACACACTGTT         |            |               |                                      |                 |            |
| Fum 7-F  | CTGGCTGCAAACAATGTCAC          | 59         | 739           | dehydrogenase                        | Tetrahydro-FB1 and -FB3 | [31]       |
| Fum 7-R  | CGATATCTGAGGTGCAAT            |            |               |                                      |                 |            |
| Fum 8-F  | CGTAGTAGAATGAGAAGAGAT         | 63         | 920           | α-oxoamine synthase                   | None            | [50, 63]   |
| Fum 8-R  | GCAAGCCTTGGGCGTGAGTTG         |            |               |                                      |                 |            |
| Fum 10-F | GTCCAGCACACTTCATCACCTTC       | 55         | 372           | acyl-CoA synthetase/acyl- protein synthetase | Hydrolyzed FB3 and FB4 | [31]       |
| Fum 10-R | AACACGAGTCTTGTGGTCA           |            |               |                                      |                 |            |
| Fum 11-F | GTCCAGTGGCTGCGTGCTGAGAAT      | 61         | 191           | tricarboxylic acid transporter        | FB1, FB2, FB3, and FB4,⁴ | [31]       |
| Fum 11-R | TCTTTCAAGGGATTCTCGGCTAC       |            |               |                                      |                 |            |
| Fum 13-F | ACATCTCCTGATTGCTCCTG          | 61         | 241           | short-chain dehydrogenase/reductase   | 3-keto FB3 & FB4 | [31]       |
| Fum 13-R | GATTCATTTTCCACACCTCTC         |            |               |                                      |                 |            |
| Fum 14-F | CTTCCAGTGGCTGCTCCTATC         | 59         | 632           | nonribosomal peptide synthase         | Hydrolyzed FB3 and FB4 | [31]       |
| Fum 14-R | CAAAGCCATCCTGCTCCTTAT        |            |               |                                      |                 |            |
| Fum 19-F | AGTAAGTCTCCACCTCCTCC         | 59         | 811           | ABC transporter                       | Increased ratio FB1:FB3 | [31]       |
| Fum 19-R | CCTCTACGCTGTTGATTTGAGTG       |            |               |                                      |                 |            |
| Fum 21-F | GCACATACAACGGGGAGTTG          | 63         | 598           | Cys-6 transcription factor            | None            | [23, 31]   |
| Fum 21-R | GGTGGGGAATAAGGTCAGTT          |            |               |                                      |                 |            |

a Annealing temperature for primer pair.

b Amplification product size of PCR products.

c With regard to fumonisins production.

d Half hydrolyzed and half keto-hydrolyzed forms.

Table 2. Fusarium isolates sampled in the present study with their ecological, molecular and phenotypic data.

| Isolate Code | GPS Coordinates     | Morphological Identification | Molecular Identification | NCBI Accession No | Fumonisins (µg/g) |
|--------------|---------------------|------------------------------|--------------------------|-------------------|-------------------|
|              |                     |                              |                          |                   |                   |
| KARNATAKA    |                     |                              |                          |                   |                   |
| UOMMF-1      | 13°10´N&76°18´E     | F. verticillioides           | F. verticillioides       | JX974610          | 10.2              |
| UOMMF-2      | 14°32´N&75°49´E     | F. oxysporum                 | F. andiyazi              | JX915765          | ND                |
| UOMMF-3      | 15°13´N&75°33´E     | F. verticillioides           | F. verticillioides       | JX915766          | ND                |
| UOMMF-4      | 14°21´N&76°36´E     | F. verticillioides           | F. verticillioides       | JX915767          | ND                |
### Table 2 contd....

| Isolate Code | GPS Coordinates     | Morphological Identification | Molecular Identification | NCBI Accession No | Fumonisins (µg/g) |
|--------------|--------------------|------------------------------|--------------------------|------------------|------------------|
| UOMMF-5      | 14°30’N & 75°51’E  | *F. verticillioides*         | *F. verticillioides*     | JX915768         | 0.5              |
| UOMMF-6      | 14°21’N & 76°36’E  | *Fusarium spp.*             | *F. verticillioides*     | JX915769         | 0.2              |
| UOMMF-7      | 14°28’N & 76°05’E  | *F. solani*                 | *F. solani*              | JX915770         | ND               |
| UOMMF-8      | 15°13’N & 75°34’E  | *F. verticillioides*         | *F. verticillioides*     | JX949660         | 12.1             |
| UOMMF-9      | 13°10’N & 76°18’E  | *F. verticillioides*         | *F. verticillioides*     | JX915771         | 10.0             |
| UOMMF-10     | 14°58’N & 75°19’E  | *Fusarium spp.*             | *F. verticillioides*     | JX915772         | 211.2            |
| UOMMF-11     | 12°19’N & 76°33’E  | *F. verticillioides*         | *F. verticillioides*     | JX915773         | ND               |
| UOMMF-12     | 12°38’N & 76°02’E  | *F. verticillioides*         | *F. verticillioides*     | JX915774         | 5.0              |
| UOMMF-13     | 12°29’N & 76°54’E  | *F. verticillioides*         | *F. verticillioides*     | JX915775         | ND               |
| UOMMF-14     | 12°38’N & 76°02’E  | *Fusarium spp.*             | *F. verticillioides*     | JX915776         | 0.4              |
| UOMMF-15     | 12°19’N & 76°34’E  | *F. verticillioides*         | *F. verticillioides*     | JX915777         | 23.2             |
| UOMMF-16     | 11°48’N & 76°42’E  | *F. verticillioides*         | *F. verticillioides*     | JX915778         | ND               |
| UOMMF-17     | 13°04’N & 77°35’E  | *F. verticillioides*         | *F. verticillioides*     | JX915779         | 2.1              |
| UOMMF-18     | 15°27’N & 75°01’E  | *Fusarium spp.*             | *F. andiyazi*            | JX974611         | ND               |
| UOMMF-24     | 13°04’N & 77°35’E  | *F. verticillioides*         | *F. verticillioides*     | JX974616         | 6.3              |
| UOMMF-26     | 13°10’N & 76°18’E  | *F. verticillioides*         | *F. verticillioides*     | JX974624         | 5.0              |
| UOMMF-47     | 14°32’N & 75°49’E  | *Fusarium spp.*             | *F. verticillioides*     | JX974639         | 144.1            |
| UOMMF-48     | 14°32’N & 75°49’E  | *F. verticillioides*         | *F. verticillioides*     | JX974622         | 30.2             |
| UOMMF-50     | 15°13’N & 75°33’E  | *F. verticillioides*         | *F. verticillioides*     | JX974642         | 362.1            |
| UOMMF-51     | 13°10’N & 76°18’E  | *F. verticillioides*         | *F. verticillioides*     | JX974623         | ND               |
| UOMMF-52     | 14°32’N & 75°49’E  | *F. verticillioides*         | *F. verticillioides*     | JX974643         | ND               |
| UOMMF-53     | 14°32’N & 75°49’E  | *F. verticillioides*         | *F. verticillioides*     | JX974644         | 30.1             |
| UOMMF-60     | 15°13’N & 75°33’E  | *F. verticillioides*         | *F. verticillioides*     | JX974651         | 1.2              |

#### TAMIL NADU

| Isolate Code | GPS Coordinates     | Morphological Identification | Molecular Identification | NCBI Accession No | Fumonisins (µg/g) |
|--------------|--------------------|------------------------------|--------------------------|------------------|------------------|
| UOMMF-19     | 10°28’N & 79°16’E  | *F. verticillioides*         | *F. verticillioides*     | Not submitted    | 0.4              |
| UOMMF-20     | 10°28’N & 79°16’E  | *Fusarium spp.*             | *F. sacharii*            | JX974612         | ND               |
| UOMMF-21     | 11°24’N & 78°31’E  | *Fusarium spp.*             | *F. verticillioides*     | JX974613         | 234.2            |
| UOMMF-22     | 11°24’N & 78°31’E  | *F. verticillioides*         | *F. verticillioides*     | JX974614         | 69.2             |
| UOMMF-23     | 10°59’N & 76°56’E  | *F. verticillioides*         | *F. verticillioides*     | JX974615         | 4.5              |
| UOMMF-25     | 10°59’N & 76°56’E  | *F. verticillioides*         | *F. verticillioides*     | JX974617         | 528.3            |

#### ANDHRA PRADESH

| Isolate Code | GPS Coordinates     | Morphological Identification | Molecular Identification | NCBI Accession No | Fumonisins (µg/g) |
|--------------|--------------------|------------------------------|--------------------------|------------------|------------------|
| UOMMF-27     | 16°18’N & 80°24’E  | *F. verticillioides*         | *F. verticillioides*     | JX974625         | 6.3              |
| UOMMF-28     | 15°40’N & 78°02’E  | *F. verticillioides*         | *F. verticillioides*     | JX974626         | 3.2              |
| UOMMF-29     | 15°40’N & 78°02’E  | *F. verticillioides*         | *F. verticillioides*     | JX974627         | 1.2              |
| UOMMF-30     | 16°59’N & 81°50’E  | *F. verticillioides*         | *F. verticillioides*     | JX974628         | 11.2             |
| Isolate Code | GPS Coordinates     | Morphological Identification | Molecular Identification | NCBI Accession No | Fumonisins (µg/g) |
|-------------|---------------------|------------------------------|--------------------------|------------------|------------------|
| UOMMF-31    | 17°11’N & 78°23’E   | *F. verticillioides*         | *F. verticillioides*     | JX974618         | 56.8             |
| UOMMF-32    | 17°11’N & 78°23’E   | *F. verticillioides*         | *F. verticillioides*     | Not submitted    | 3.6              |
| UOMMF-33    | 18°00’N & 79°33’E   | *F. verticillioides*         | *F. verticillioides*     | JX974619         | 0.2              |
| UOMMF-34    | 18°00’N & 79°33’E   | *F. verticillioides*         | *F. verticillioides*     | JX974620         | ND               |
| UOMMF-35    | 16°18’N & 80°24’E   | *Fusarium spp.*              | *F. verticillioides*     | JX974629         | 3.2              |
| UOMMF-37    | 16°59’N & 81°50’E   | *F. verticillioides*         | *F. verticillioides*     | JX974630         | 2.3              |
| UOMMF-38    | 16°59’N & 81°50’E   | *F. verticillioides*         | *F. verticillioides*     | JX974631         | 535.1            |
| UOMMF-39    | 16°18’N & 80°24’E   | *F. verticillioides*         | *F. verticillioides*     | JX974632         | 762.2            |
| UOMMF-62    | 15°40’N & 78°02’E   | *F. verticillioides*         | *F. verticillioides*     | Not submitted    | 1.5              |

**ANDHRA PRADESH**

| UOMMF-55    | 18°30’N & 73°50’E   | *F. verticillioides*         | *F. verticillioides*     | JX974646         | 26.3             |

**MAHARASHTRA**

| UOMMF-56    | 22°32’N & 88°21’E   | *F. verticillioides*         | *F. verticillioides*     | JX974622         | ND               |

**WEST BENGAL**

| UOMMF-37    | 18°18’N & 80°24’E   | *Fusarium spp.*              | *F. verticillioides*     | JX974658         | 0.2              |

**RAJASTHAN**

| UOMMF-40    | 24°39’N & 74°01’E   | *F. verticillioides*         | *F. verticillioides*     | JX974633         | 13.1             |
| UOMMF-41    | 24°39’N & 74°01’E   | *Fusarium.spp.*              | *F. verticillioides*     | JX974634         | 152.3            |
| UOMMF-42    | 24°39’N & 74°01’E   | *F. verticillioides*         | *F. verticillioides*     | JX974635         | 4.1              |
| UOMMF-43    | 24°39’N & 74°01’E   | *F. verticillioides*         | *F. verticillioides*     | JX974636         | 19.3             |
| UOMMF-44    | 28°07’N & 73°02’E   | *F. verticillioides*         | *F. verticillioides*     | JX974637         | 2.1              |
| UOMMF-49    | 26°54’N & 75°48’E   | *F. verticillioides*         | *F. verticillioides*     | JX974641         | ND               |
| UOMMF-54    | 28°07’N & 73°02’E   | *F. verticillioides*         | *F. verticillioides*     | JX974645         | 4.3              |
| UOMMF-56    | 24°39’N & 74°01’E   | *F. verticillioides*         | *F. verticillioides*     | JX974647         | 300.0            |
| UOMMF-61    | 28°07’N & 73°02’E   | *F. verticillioides*         | *F. verticillioides*     | JX974652         | 16.5             |

**UTTARAKHAND**

| UOMMF-58    | 28°38’N & 77°09’E   | *F. verticillioides*         | *F. verticillioides*     | JX974649         | 12.3             |

**HIMACHAL PRADESH**

| UOMMF-59    | 31°02’N & 76°41’E   | *F. verticillioides*         | *F. incarnatum*         | JX974650         | ND               |

**MANIPUR**

| UOMMF-45    | 24°49’N & 93°54’E   | *F. verticillioides*         | *F. verticillioides*     | JX974638         | 6.23             |
| UOMMF-46    | 29°08’N & 75°44’E   | *F. verticillioides*         | *F. proliferatum*       | JX974640         | 89.23            |

Similar to the tef-1α phylogeny, isolates UOMMF-7 (*F. solani*) and UOMMF-59 (*F. incarnatum*) maintained diversity from the other examined isolates with the lowest ISSR similarity coefficients (0.26 and 0.294, respectively). The similarity coefficients for the remaining isolates ranged from 0.302 to 0.96. Cluster Ic had four isolates from the second haplotype in the phylogenetic TEF tree. Based on fragment patterns, we observed a breakdown of associations by species. For example, Clade I from the analysis of tef-1α (Fig. 1) was entirely *F. verticillioides*, but in (Fig. 2) we observed three *F. verticillioides* isolates (UOMMF-13, UOMMF-19, UOMMF-54) with low similarity coefficients compared to
other isolates of the same species. Alternatively, we observed isolate UOMMF-2 (F. andiyazi) having a high similarity coefficient (> 0.882) with F. verticillioides isolates. Most of the F. verticillioides isolates exhibited high similarity coefficients ranging from 0.78 to 0.96.

3.2.3. FUM Gene Deletion Analysis

Investigation of fumonisin gene presence/absence revealed the presence of all examined genes for 40 of the F. verticillioides isolates. The remaining 16 F. verticillioides isolates showed deletion variation based on the absence of examined genes (Table 4). Isolate UOMMF-16 showed the greatest number of missing genes (9 out of 12), but for most isolates there were only one or two absent genes. The gene found to be most absent across all examined isolates was FUM6, which was absent in 75% of the isolates exhibiting gene deletions, followed by FUM1 which was absent in 31% of the deletion isolates. None of the other Fusarium species were examined for the presence or absence of fumonisins.

3.3. Analysis of Total Fumonisin Production By CD-ELISA

The results obtained from the CD-ELISA are presented in (Table 2). Among the 56 F. verticillioides isolates tested, we observed fumonisin production by 46 strains while 10 were fumonisin-negative. Among the other Fusaria tested, the F. proliferatum isolate also tested positive for fumonisin production. The remaining species did not produce any detectable fumonisins when tested by CD-ELISA. Among the F. verticillioides isolates, the highest concentration of fumonisins (762.2 μg/g) was produced by isolate UOMMF-39 while the lowest measurable concentration of fumonisins (0.2 μg/g) was produced by UOMMF-6 and UOMMF-33. The concentration of fumonisins produced by the F. proliferatum isolate was 89.23 μg/g.

4. DISCUSSION

Fusarium spp. infect and cause disease in 81 of the 101 economically-important plants [50]. Species within this genus also produce an intriguing array of secondary metabolites that are associated with diseases of plants, and when ingested often cause cancer or other growth defects in humans and animals [39, 51, 52]. To differentiate F. verticillioides, VERT-1 and VERT-2 primers were used which yielded an 800 bp amplicon only for F. verticillioides isolates. Our finding that the VERT primers did not amplify for the F. andiyazi, F. incarnatum, F. proliferatum, F. sacchari or F. solani isolates was expected since similar results have been reported by other researchers [53]. The tef-1α gene has emerged as a key diagnostic tool for identification of Fusarium species [12]. With this locus we were able to support/confirm the morphological identities of 50 isolates, and refute/refine the morphological identities of 12 isolates (Table 1). The tef-1α locus has been proven its usefulness for accurate identification of Fusarium species infecting sorghum from India [23]. The finding that a majority of our sampled species were F. verticillioides isolates. The observed subdivision was mostly species, or species complex, related. Fusarium andiyazi, F. incarnatum, F. proliferatum, F. sacchari and F. verticillioides are part of the Fusarium fujikuroi species complex (FFSC), which could explain their close phylogenetic proximity [57]. The remaining isolates belong to different

Fig. (1). Phylogenetic tree inferred using tef-1α sequences from 62 Fusarium isolates sampled across different geographical locations in India. A sequence for an F. redolens strain (AY714109.1), downloaded from GenBank, was used as the out-group species.

We also observed more clustering of adjacent branches in the ISSR dendrogram based on geography which was different from our cladal observations in (Fig. 1). For example most of the isolates of cluster Ia were from Karnataka, while 50 % of the isolates of cluster Ib were from Andra Pradesh and 50 % of the isolates cluster Ic were from Rajasthan.

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Table 3. Primers used for ISSR analysis and their respective characteristics.

| Primer Name | Repeat Pattern | Fragment Length (bp) | Tm (°C)a | Ta (°C)b | Monomorphic Bands | Polymorphic Bands |
|-------------|----------------|----------------------|----------|----------|-------------------|-------------------|
| ISSR02      | (CT)AC         | 200-2200             | 40       | 40       | 0                 | 18                |
| ISSR03      | (CT)GC         | 200-2200             | 40       | 43       | 0                 | 14                |
| ISSR04      | (CA)AC         | 200-2200             | 45       | 43       | 0                 | 12                |
| ISSR05      | (CA)GT         | 300-1950             | 45       | 43       | 0                 | 10                |
| ISSR06      | (CA)AG         | 200-2000             | 45       | 43       | 1                 | 13                |
| ISSR07      | (CA)GC         | 200-2200             | 45       | 43       | 0                 | 17                |
| ISSR09      | (GT)GG         | 200-1800             | 37       | 40       | 0                 | 12                |
| ISSR10      | (GA)CC         | 200-1900             | 48       | 40       | 0                 | 11                |
| ISSR12      | (CAC)GC        | 200-2000             | 32       | 40       | 0                 | 16                |
| ISSR13      | (GAG)GC        | 200-1700             | 32       | 46       | 2                 | 11                |
| ISSR14      | (CTC)GC        | 200-2200             | 45       | 43       | 0                 | 17                |
| ISSR16      | (GA)T          | 200-2200             | 18       | 43       | 0                 | 13                |
| ISSR19      | (GACA)         | 200-2200             | 40       | 43       | 0                 | 17                |
| ISSRA1      | (GA)T          | 250-1000             | 42.9     | 46       | 2                 | 3                 |
| ISSRA2      | (AC)T          | 300-2200             | 49.3     | 48       | 0                 | 10                |
| ISSRA3      | (AG)C          | 200-2200             | 41.1     | 41       | 0                 | 17                |
| ISSRA6      | (CCAT)         | 200-2200             | 69.6     | 68       | 0                 | 16                |
| ISSRA7      | (AG)G          | 200-2200             | 46.6     | 44       | 0                 | 14                |
| ISSRA8      | (ATG)          | 200-2200             | 51.3     | 50       | 0                 | 15                |
| ISSRA9      | (GA)T          | 400-1000             | 43.3     | 46       | 0                 | 3                 |

*a Melting temperature for primer pair.

*b Annealing temperature for primer pair.

species complexes for which each respective species is the namesake. For example, there is the *Fusarium incarnatum-equiseti* species complex or FIESC (isolate UOMMF-59), the *Fusarium solani* species complex or FSSC (isolate UOMMF-7), and the out-group species is the namesake for the *Fusarium redolens* species complex or FRSC [58]. Species within the FFSC are readily associated with diseases of maize, hence the large sampling of *F. verticillioides*; however, *F. incarnatum* and *F. solani* are not often associated with maize infection and this may correlate with their genetic diversity. A multi-locus sequence analysis might offer better resolution of the genetic diversity among the many *F. verticillioides* isolates sampled. Our finding of little to no grouping of isolates by geographic origin; particularly for *F. verticillioides*, could indicate gene flow circumventing geographic boundaries through interstate transport of infected grains across India.

Greater intra-species diversity was observed by examining ISSR patterns. Similar ISSR studies involving 98 isolates of *Fusarium oxysporum* f.sp. *cubense* isolated from banana, from different geographical locations in India, resulted in seven genotype clusters with wide intra-species diversity among the isolates [59, 60]. ISSR marker were used to study the genomic analyses of pathogenic and non-pathogenic *F. solani* isolated from *Dalbergia sissoo* [61]. The low similarity coefficients for the *F. solani* and *F. incarnatum* isolates support the diversity observed in the sequence analysis of *tef-1α*. We observed that pattern similarity among the isolates partially corresponded to geographic origin, but our observation of similarity between isolates from various parts of the country, representing different races, still supported evidence of gene flow across geographic boundaries.

Our findings confirmed a previous report that the fumonisin biosynthetic gene cluster is conserved in fungal species such as *F. verticillioides* and *F. proliferatum* [37]. To date, few other species of *Fusarium* have been reported to contain a fumonisin biosynthetic gene cluster [23, 62]. Previous studies revealed the existence of non-toxigenic *F. verticillioides* isolates that resulted from gene deletions of, or mutations in, fumonisin biosynthesis genes [23, 33, 49]. We were able to observe FUM gene deletions in 16 of our sampled *F. verticillioides* isolates. However, not all of these deletion strains exhibited a fumonisin-negative phenotype. Six isolates of *F. verticillioides* tested positive for fumonisin in the CD-ELISA (UOMMF-1, UOMMF-14, UOMMF-27, UOMMF-30, UOMMF-33 and UOMMF-39), but exhibited
Fig. (2). An inferred UPGMA dendrogram based on DICE similarity estimates from the ISSR marker analysis of 62 Fusarium isolates. Species other than F. verticillioides are shown with thickened branches and noted to the right of the UOMMF number. Geographic origins for the sampled isolates are color-coded.

Table 4. Fumonisin production and patterns of FUM gene deletion among the F. verticillioides isolates.

| Isolate   | CD-ELISA | FUM1 | FUM2 | FUM3 | FUM4 | FUM5 | FUM6 | FUM7 | FUM8 | FUM9 | FUM10 | FUM11 | FUM12 | FUM13 | FUM14 | FUM15 |
|-----------|----------|------|------|------|------|------|------|------|------|------|-------|-------|-------|-------|-------|-------|-------|
| UOMMF-1   | +        |      |      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| UOMMF-3   | -        |      |      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| UOMMF-4   | -        |      |      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| UOMMF-5   | +        |      |      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| UOMMF-6   | +        |      |      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| UOMMF-8   | +        |      |      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| UOMMF-9   | +        |      |      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| UOMMF-10  | +        |      |      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| UOMMF-11  | -        |      |      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| UOMMF-12  | +        |      |      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| UOMMF-13  | -        |      |      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| UOMMF-14  | +        |      |      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| UOMMF-15  | +        |      |      |      |      |      |      |      |      |      |       |       |       |       |       |       |
| Isolate   | CD-ELISA | \( FUM_3 \) | \( FUM_5 \) | \( FUM_6 \) | \( FUM_8 \) | \( FUM_{10} \) | \( FUM_{13} \) | \( FUM_{14} \) | \( FUM_{19} \) | \( FUM_{21} \) |
|-----------|----------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| UOMMF-16  | -        | -            | -            | -            | -            | +            | -            | -            | -            | -            |
| UOMMF-17  | +        | +            | +            | +            | +            | +            | +            | +            | -            | -            |
| UOMMF-19  | +        | +            | +            | +            | +            | +            | +            | +            | +            | -            |
| UOMMF-21  | +        | +            | +            | +            | +            | +            | +            | -            | +            | +            |
| UOMMF-22  | +        | +            | +            | -            | -            | -            | +            | +            | +            | -            |
| UOMMF-23  | +        | +            | +            | +            | +            | +            | +            | +            | +            | +            |
| UOMMF-24  | +        | +            | +            | +            | +            | +            | +            | +            | +            | -            |
| UOMMF-25  | +        | +            | +            | +            | +            | +            | +            | -            | +            | +            |
| UOMMF-26  | +        | +            | +            | +            | +            | +            | +            | +            | +            | +            |
| UOMMF-27  | +        | +            | +            | +            | +            | +            | -            | -            | -            | +            |
| UOMMF-28  | +        | +            | +            | +            | +            | +            | +            | +            | +            | +            |
| UOMMF-29  | +        | +            | +            | +            | +            | +            | +            | -            | +            | +            |
| UOMMF-30  | +        | +            | +            | +            | -            | +            | +            | +            | -            | +            |
| UOMMF-31  | +        | +            | +            | +            | +            | +            | -            | -            | +            | +            |
| UOMMF-32  | +        | +            | +            | +            | +            | +            | +            | +            | +            | -            |
| UOMMF-33  | +        | +            | +            | +            | +            | +            | +            | +            | -            | -            |
| UOMMF-34  | -        | -            | +            | -            | -            | +            | +            | -            | +            | +            |
| UOMMF-35  | +        | +            | +            | +            | +            | +            | +            | -            | +            | +            |
| UOMMF-36  | -        | +            | -            | -            | +            | +            | +            | +            | +            | +            |
| UOMMF-37  | +        | +            | +            | +            | +            | +            | +            | +            | +            | +            |
| UOMMF-38  | +        | +            | +            | +            | +            | +            | +            | +            | -            | +            |
| UOMMF-39  | +        | +            | +            | +            | -            | +            | +            | +            | +            | +            |
| UOMMF-40  | +        | +            | +            | +            | +            | +            | +            | +            | -            | +            |
| UOMMF-41  | +        | +            | +            | +            | +            | +            | +            | +            | -            | +            |
| UOMMF-42  | +        | +            | +            | +            | +            | +            | -            | +            | +            | +            |
| UOMMF-43  | +        | +            | +            | +            | +            | +            | +            | -            | +            | +            |
| UOMMF-44  | +        | +            | +            | +            | +            | +            | +            | +            | -            | +            |
| UOMMF-45  | +        | +            | +            | +            | +            | +            | +            | +            | -            | +            |
| UOMMF-47  | +        | +            | +            | +            | +            | +            | +            | +            | -            | +            |
| UOMMF-48  | +        | +            | +            | +            | +            | +            | +            | +            | -            | +            |
| UOMMF-49  | -        | -            | +            | -            | +            | +            | -            | +            | +            | +            |
| UOMMF-50  | +        | +            | +            | +            | +            | +            | +            | +            | -            | +            |
| UOMMF-51  | -        | -            | +            | -            | -            | +            | +            | +            | -            | +            |
| UOMMF-52  | -        | +            | +            | -            | -            | -            | +            | +            | +            | +            |
| UOMMF-53  | +        | +            | +            | +            | +            | +            | +            | +            | +            | +            |
| UOMMF-54  | -        | -            | -            | -            | -            | -            | -            | -            | -            | -            |

(Table 4) contd....
Table 4 contd. . .

| Isolate | CD-ELISA a | FUM1 b | FUM2 b | FUM3 b | FUM4 b | FUM5 b | FUM6 b | FUM7 b | FUM8 b | FUM9 b | FUM10 b | FUM11 b | FUM12 b | FUM13 b | FUM14 b |
|---------|------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| UOMMF-54 | +          | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       |
| UOMMF-55 | +          | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       |
| UOMMF-56 | +          | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       |
| UOMMF-57 | +          | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       |
| UOMMF-58 | +          | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       |
| UOMMF-60 | +          | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       |
| UOMMF-61 | +          | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       |
| UOMMF-62 | +          | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       | +       |

aCD-ELISA: presence (+) or absence (-) of fumonisins.
bPCR Diagnostic: presence (+) or absence (-) of FUM gene.

gene deletions that included FUM6, FUM10, FUM13, FUM19, FUM14 genes. These genes are involved in the modification of produced fumonisins, or the production or increase of some isoforms and analogs of FB1, FB2 and FB3 [31]. The exact reason for this is unknown and warrants further study. Among the various fumonisins produced by strains of F. verticilloides in culture, FB1 typically accounts for 70% to 80% of those produced, while FB2 accounts 15% to 25%, FB3 accounts for 3% to 8% and FB4 occurs rarely [62]. If the CD-ELISA is detecting the presence of non-specific fumonisins, then perhaps the reason we did not amplify certain genes related to primer mismatches due to variation in a particular strain [63]. Alternatively, if each of the fumonisins relies on different sets of genes for its synthesis, then non-specific fumonisin detection would still be possible despite absence of genes. Another reason their genotype did not correlate with the CD-ELISA may be due to the inability of the assay to detect different isoforms which are produced by gene deletions or mutations that result in detection of false positives [64]. Similar findings were reported by several researchers in F.verticilloides isolated from different crops which failed to produce detectable quantities of FB1 [65, 66]. Among the 62 isolates we studied, only the F. verticilloides and F. proliferatum isolates produced detectable levels of fumonisins. This data correlated well with previous studies which reported the major fumonisin producers are F. verticilloides, F. proliferatum, F. nygamai and Aspergillus niger [67-69].

5. CONCLUSIONS/PERSPECTIVES

The present study provided relevant information on the current status of Fusarium infection across different maize-growing regions of India. We showed variation in fumonisin production among Fusarium spp. that serve as potential threats for agricultural production. The genotype study of our sampled F. verticilloides isolates revealed the occurrence of non-toxigenic strains, and confirmed that their fumonisin-negative phenotype was likely due to deletion of genes which are required for fumonisin biosynthesis. Likewise, from the results of ISSR it was concluded that the distribution of lineages of Fusarium spp. across India is random, and these strains cannot be wholly subdivided based on their geographic origins or fumonisin producing capability. Future studies involving a more holistic approach should be conducted to better understand the distribution and diversity of Fusarium spp. and fumonisin contamination in agricultural crops. This will aid in developing suitable strategies for the management of seed health.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to Department of Biotechnology (DBT), Govt. of India (Project- BT/PR9864/AGR/05/ 391/2007 Dated: 6 June 2008) for financial assistance. The authors are also thankful to institute of excellence (MHRD), University of Mysore for providing the equipment to carry out this work.

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