

Molecular characterization of *Fusarium oxysporum* by PCR amplification of 18S rRNA gene region

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The genus *Fusarium* is one of the most diverse and pathologically important fungi with affinities established to Ascomycotina. Usual identification of *Fusarium* species based on their micro and macroscopic features and morphological characters alone may lead to incorrect species designation. In order to identify the correct species, we amplified the 18S rRNA gene region by PCR, sequenced and analyzed for sequence similarity among the NCBI data. *Fusarium oxysporum*, a soil borne plant pathogen was isolated from the *Allium cepa*. The isolate was cultured in fresh potato dextrose agar (PDA) plates for 7 days. Morphological identification was done by observing under microscope after lacto-phenol cotton blue staining. Genomic DNA isolation was carried on the grown *Fusarium oxysporum*. Further, PCR amplification of ITS regions was performed using Universal ITS primers. The amplified product of 18S rRNA gene was sequenced and submitted to NCBI database. The soil borne fungal pathogen of onion was identified as *Fusarium oxysporum* based on its morphological and molecular characteristics.

Keywords: 18S rRNA, *Allium cepa*, BLAST, DNA extraction, ITS primers, Morphological identification

The genus *Fusarium* inhabits the soil and organic substrata and is widely distributed throughout the world¹. *Fusarium* basal rot (FBR) is a root and bulb fungal disease of onions² caused by *Fusarium oxysporum* Schlechtend: *F. o.f. sp. cepae*³. *Fusarium* species were traditionally classified in the Deuteromycotina/Fungi Imperfecti although affinities to Ascomycotina have been established. Within the Hyphomycetes, the genus *Fusarium* is among the most diverse and pathologically important³. There is no universally accepted species delineation concept in *Fusarium*, and as a consequence, taxonomists disagree on the number of species in the genus⁴. Identification

of *Fusarium* species is commonly done based on their micro and macroscopic features. These include physical macroscopic description of colonies on appropriate media (based on colony growth, texture, colour and pigment) and microscopic description of hyphae, phialides, conidiogenous cells, conidia and microconidia⁵. However, these features are mostly reported to be unstable⁶. These methods are time consuming and have proved to be partial and unsatisfactory.

Presently, identification of eukaryotic organisms is basically done based on the nucleotide sequence information from conserved regions using polymerase chain reaction (PCR) amplification. Use of DNA markers in fungal diagnostics and molecular taxonomy is now well established⁷. DNA-based genetic markers provide a genetic diagnostic tool that permits direct identification of pathotypes in any developmental stage in environment independent manner⁸. DNA sequences amplification through the PCR has found widespread application in the diagnosis and detection of fungi. This method relies on the conserved nature of rDNA such that isolates from the same species maintain the same sequence, whereas the more phylogenetically diverse the species is, the greater is the difference in the sequences of rDNA⁹⁻¹³.

Valuable sequences in distinguishing species and origins of *Fusarium* include internal transcribed spacer (ITS) region from the conserved ribosomal RNA genes, intergenic spacer (IGS), translation elongation factor (EF-1a), β -tubulin region and the mitochondrial small subunit (mtSSU)¹⁴⁻¹⁶. The ITS primers make use of conserved regions of the 18S, 5.8S and 28S rRNA genes to amplify the noncoding regions between them¹⁷. Sequence analyses of ITS regions are employed to study intrageneric relationships within *Pythium*¹⁸ and *Phytophthora*¹⁹⁻²².

Accurate identification of species is crucial, as an aid in disease management and genetic diversity studies. Correct species name of a plant pathogenic fungus is important for designing effective disease control management, quarantine purposes and as a basis for making decisions to protect agricultural crops as well as other natural resources from fungal pathogens. Therefore, the present study was

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conducted to identify *Fusarium* species using morphological characteristics and DNA sequencing of 18A rRNA gene. ITS region was chosen for amplification and identification of *Fusarium*.

Materials and Methods

Allium cepa infected samples were cut into small pieces and surface sterilized with 1% sodium hypochloride solution for 1 min. Thereafter, the samples were washed with sterilized distilled water before placing them in Petriplates containing potato dextrose agar (PDA) (pH. 8) medium. The sealed plates were incubated at the room temperature (38°C) for 4 days until the fungus growth appeared. The isolated fungal pathogen was subcultured by single spore technique for culture purification in fresh PDA plates²³. The pure isolate was preserved at 4°C.

Morphological characterization

Identification was carried out on the basis of morphological characteristics described by Burgess *et al.*¹. Species was identified according to the morphology of the macroconidia, microconidia and their arrangement in chains or false heads, the size and type of conidiophore, and the presence or absence of chlamydospores²⁴.

DNA extraction by modified CTAB extraction method

In preparation for DNA extraction, the isolate was grown as shake culture (200 rpm) in potato dextrose broth for 7-10 days at 38°C. According to Moller *et al.*²⁵ 50 mg of fungal mycelia was scrapped from 7-10 days old potato dextrose broth and manually ground in 1.5 mL of microfuge tubes with micropestle adding 500 µL of pre warmed (60°C) TES lysis buffer (100 mM Tris pH 8.0; 10 mM EDTA; pH 8.0; 2% SDS). About 50 µL of proteinase K was added to the ground material, incubated in 60°C for 60 min. Further, 140 µL of 5M NaCl and 64 µL of 10% w/v of CTAB were added to the suspension incubated at 65 C for 10 min. DNAs were extracted by adding equal volume of chloroform: isoamyl alcohol (24:1) centrifuged at 14000 rpm for 10 min. DNA was precipitated by adding 0.6 volume of cold isopropanol and 0.1 volume of 3M sodium acetate pH 5.2 and maintained at -20°C, centrifuged and washed twice with 70% ethanol suspended in 100 µL of TE (10 mMTris pH 8.0; 1mM EDTA pH 8.0).After extraction, the DNA sample was run on 2% agarose gels in 0.5 M Tris borate EDTA (TBE) buffer (pH 8) at the room

temperature at a constant voltage of 100 V to determine the quality.

Molecular characterization using sequencing of the ITS region

Universal Primers ITS1 (5'-TCCGTAGGTGAACCT GCGG-3') (Forward) and ITS2 (3'-GCTGCGTTCTT CATCGATGC-5') (Reverse)¹⁷ were used to amplify the ITS region of *Fusarium oxysporum*. PCR reaction mixture consisted of 20 µL viz., 9.7 µL of PCR master mix, 2 µL of forward primer and 2 µL reverse primer, template DNA 2 µL and 4.3 µL of sterile water. The amplification was carried out in Thermocycler. The PCR reaction conditions were as follows: Initial denaturation at 94°C for 3 min, 36 cycles of amplification denaturation at 94°C for 1 min, annealing step at 55°C for 1 min, and extension at 72°C for 30 s followed by a final extension step at 72°C for 5 min. The PCR product was resolved on 2% agarose gels in TBE buffer and visualized by staining with ethidium bromide (0.5 µg/mL) and photographed under ultraviolet light using an ultraviolet transilluminator. After gel electrophoresis, PCR products were purified with PCR Klenzol™ (GeNei, Bangalore, India) and sequenced with an ABI 3700 DNA sequencer at Acme Progen Biotech Pvt. Ltd (Salem, Tamilnadu, India).

Nucleotide data analysis

DNA sequences were aligned using ClusterW multiple alignment in molecular evolutionary genetic analysis 4 or MEGA 4²⁶ and adjusted manually. The sequence obtained was then compared with the GenBank (NCBI) database using BLAST tool²⁷.

Results and Discussion

Isolation and morphological identification of *Fusarium oxysporum*

Fusarium oxysporum was isolated from diseased samples of *Allium cepa* in fresh PDA plates (Fig. 1). Isolated *F. oxysporum* produced white and brown colored colonies with aerial mycelium. Canoe-shaped Macroconidia with a long apical cell and a foot shaped basal cell formed with 3 to 5 septa. Uni or bicellular, ovoid to ellipsoid microconidia were abundant²⁸ as seen in Fig. 2. Based on the morphological characters it was identified as *Fusarium oxysporum* with the description of Burgess LW¹.

Genomic DNA isolation and PCR amplification

DNA was successfully extracted from *F.oxysporum* by CTAB method. In agarose gel electrophoresis

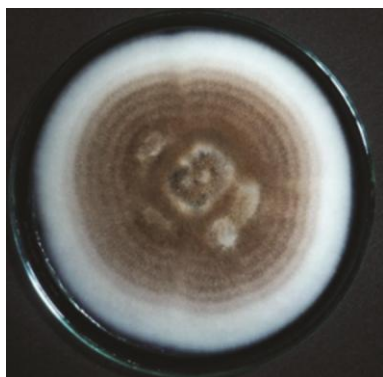


Fig. 1 — *Fusarium oxysporum* cultured in PDA plate

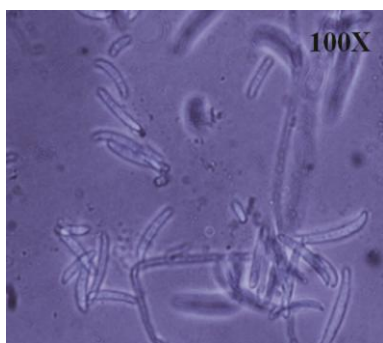


Fig. 2 — Macroconidia structures of *Fusarium oxysporum* by lactophenol-cotton blue staining method

profile, DNA extracted from *F. oxysporum* (Fig. 3) was compared with Lambda DNA/HindIII marker. The size of the extracted DNA from *F. oxysporum* was approximately 23000 bp in size. The PCR products generated using the ITS 1 and ITS 4 primers were analyzed on agarose electrophoresis gel (Fig. 4). Amplification of the ITS region of strain *F. oxysporum* had a size of 1646 bp. It was submitted to the NCBI and the accession number KC 119197 was received. PCR amplification of ITS region was performed using the universal primers. The *Fusarium* genus was amplified as a fragment of 540 bp corresponding to the region of the 18S rRNA intervening sequence for *Fusarium* spp. (Plate 4).

Sequence analysis

The isolate of *F. oxysporum* was sequenced and submitted in NCBI database. Based on the closest match of BLAST analysis, it showed 100% homology with *F. oxysporum* with the accession numbers of AY667487.1, AY669123.1, AY667488.1, AY669119.1, AY669126.1, and AY669121.1. Hence, the pathogen was confirmed as *Fusarium oxysporum*. Sequencing the ITS gene region is effective for identifying some species of *Fusarium*.

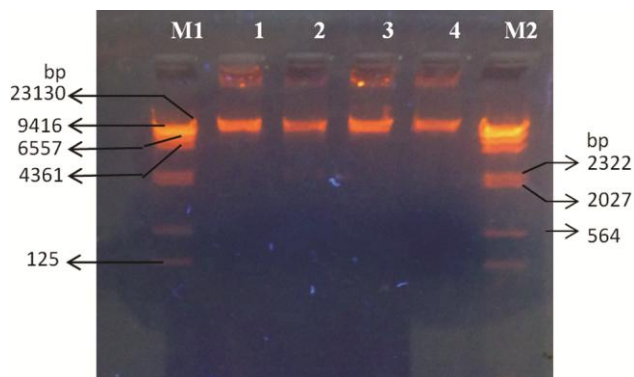


Fig. 3 — Agarose gel electrophoresis profile of DNA extracted from *F. oxysporum*. [Lanes: M1 and M2 = λ DNA/HindIII Marker; 1, 2, 3 and 4 = *F. oxysporum* DNA (approximately 23 Kb)]

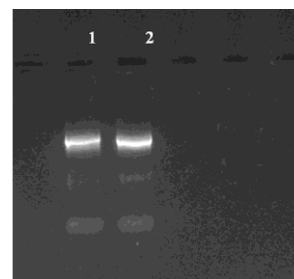


Fig. 4 — Amplified PCR products. [Lane 1 and 2 PCR products of *F. oxysporum*. Amplification of conserved ribosomal regions of *Fusarium* sp. using the primers ITS-1 and ITS-4]

The current fungal taxonomic systems have used macroconidia and microconidia in the asexual stages to identify fungal species. However, the plasticity and intergradation of the phenotypic traits has presented difficulties in identifying the filamentous fungi²⁹. Detection of *F. oxysporum* is of concern because it causes *Fusarium* basal rot (FBR)². This highlights the importance of exact identification of *F. oxysporum*. Similar studies by Ashwathi *et al.*³⁰ which involved isolation of 10 fungal species from infected coriander and performed PCR amplification of ITS gene region using universal ITS primers. The soil borne fungal pathogen of coriander was identified as *F. oxysporum* based on its cultural, morphological and molecular characteristics. Molecular profiling using ITS region sequencing is an indispensable method for identification studies as studied by Singha *et al.*⁶. They isolated *F. oxysporum* from infected tomato collected from Assam.

Analyses of rDNA sequences constitute an important complement of the morphological criteria needed to allow fungi to be more easily identified³¹. Many workers globally have used DNA based markers extensively to evaluate genetic diversity in

this important fungus. The advent of DNA-based molecular methods has provided useful tools with which to study the phylogeny of *Fusarium* and to differentiate species, formaespeciales, races, and strains³². Mohammed *et al.*³³ isolated 27 strains of *Fusarium* from the stems, crown and roots of infected tomato plants and did morphological identification. Further to confirm the identity of the fungus, the isolates were identified using analysis based on morphological criteria and sequencing of the translation elongation factor 1-alpha (TEF) gene using ef1 and ef2 primers. Twenty three strains belonged to *F. oxysporum*, 3 to *Fusarium solani* and 1 strain to *Fusarium redolens*.

In this study, the sequencing of the 1646 bp ITS fragment revealed 100% genetic similarity to other *Fusarium oxysporum* species. This suggests that species designation based on morphological data alone could be unreliable. Similar work done by Young-Mi *et al.*³⁴, where sequences of the internal transcribed spacers of the ribosomal DNA among 13 species from *Fusarium* sections *Elegans*, *Liseola* and *Dlaminia* were compared, also revealed discordant results. Similar results have been obtained by Dhoro⁴, El-Kazzaz *et al.*³⁵, Balali & Iranpoor²⁸; Stewart *et al.*³⁶ and Hsuan *et al.*²⁷.

High intraspecies diversity and 100% genetic similarity was noted between *Fusarium oxysporum* and *Fusarium fujikuroi* from the NCBI database suggesting that species designation can be unreliable if based on morphological data alone. Based on the overall results, the use of molecular methods constitutes an important complement of the morphological criteria needed to allow fungi to be more easily identified⁴. According to the report by Kistler³⁷, genetic relationships based on rDNA sequences suggest *F. oxysporum* to be a species complex consisting of at least five phylogenetically distinct species.

The small subunit (SSU) 18S rRNA gene is one of the most frequently used genes in phylogenetic studies and an important marker for random target PCR in environmental biodiversity screening³⁸. In general, rRNA gene sequences are easy to access due to highly conserved flanking regions allowing for the use of universal primers. Their repetitive arrangement within the genome provides excessive amounts of template DNA for PCR, even in smallest organisms³⁹. Hence, in this study, 18S rRNA gene region was amplified and used for molecular identification and confirmation of *F. oxysporum*.

Conclusion

The molecular technique used in this study revealed the genetic diversity within *Fusarium*. However, further analysis is required using bioinformatics tools to predict primers specific to *Fusarium oxysporum*. It may help in early identification of this fungus and thereby in disease control management to protect agricultural crops. The primers showed good specificity for the genus *Fusarium*, and approximately 1646 bp product was amplified. ITS primers 1 and 4 were used to amplify the genus specific PCR assay for rapid identification of *Fusarium*. The soil-borne fungal pathogen of *Allium sativa* was identified as *Fusarium oxysporum* based on its morphological and molecular characteristics. This study revealed that the use of molecular methods constitutes an important complement to the morphological criteria needed to allow fungi to be more easily identified. The sequencing of rDNA sequences using the *Fusarium* specific primers is more reliable as a diagnostic technique as well as revealing genetic relatedness of *Fusarium oxysporum*.

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