Section A-Research paper

PCR-Based Identification of *Fusarium napiforme*: Unveiling the Genetic Signature

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Abstract

Fusarium napiforme is a phytopathogenic fungus that causes substantial economic losses in various crops worldwide. Accurate and timely identification of this pathogen is crucial for effective disease management strategies. The present study performs a molecular identification method using polymerase chain reaction (PCR) to detect and identify *Fusarium napiforme*. In this study, specific primers targeting the internal transcribed spacer (ITS) region of the fungal rRNA gene were designed and used in PCR amplification. *Fusarium napiforme* was isolated from ridge gourd (*Luffa acutangula*) seeds by agar plate method. DNA was extracted from the isolates and subjected to PCR using the designed primers. The resulting PCR products were then analyzed by gel electrophoresis to determine the presence or absence of *Fusarium napiforme*. The PCR assay demonstrated high specificity and sensitivity in detecting *Fusarium napiforme*. The designed primers exclusively amplified the target DNA fragment from *Fusarium napiforme* isolates. The PCR method provides a rapid and reliable tool for the accurate identification of *Fusarium napiforme*. This technique can significantly aid in early disease diagnosis and facilitate prompt implementation of appropriate management strategies to control *Fusarium napiforme* infections. Additionally, it can assist in quarantine measures to prevent the spread of the pathogen to unaffected areas.

Keywords: Disease management, *Fusarium napiforme*, Internal transcribed spacer, Molecular identification, Polymerase chain reaction, Phytopathogenic fungus.

Introduction

Fungal plant pathogens are detrimental microorganisms that infect and damage various plant species, causing severe agricultural losses worldwide. These parasitic fungi invade plants through wounds, stomata, or direct penetration, proliferating within their hosts and disrupting vital physiological processes. They spread rapidly through air, soil, or contaminated materials, attacking crops such as wheat, rice, and corn, as well as fruit trees and ornamental plants. Common fungal pathogens include powdery mildews, rusts, and smuts. The devastation caused by these pathogens includes reduced yields, stunted growth, and quality deterioration. Effective management strategies, including crop rotation, resistant cultivars, and fungicides, are essential to safeguarding global food security.[1,2,3,4]

Fusarium napiforme is a fungal pathogen that can have a significant impact on various plants. This fungus primarily affects the roots and stems of plants, causing a range of symptoms and damage. One of the major impacts of *Fusarium napiforme* is the development of root rot. The fungus invades the roots, leading to their decay and subsequent reduction in nutrient uptake. This can result in stunted growth, wilting, and yellowing of leaves. Plants affected by *Fusarium napiforme* may also exhibit damping-off, a condition where seedlings become weak and eventually die. Furthermore, *Fusarium napiforme* produces toxins that can further harm plants. These toxins can interfere with the plant's physiological processes, disrupt cell membranes, and induce oxidative stress. As a result, the overall health and vigor of the plant decline, making it more susceptible to other diseases and environmental stresses. Controlling *Fusarium napiforme* is challenging as it can persist in soil and plant debris. Crop rotation, sanitation practices, and the use of resistant plant varieties are commonly employed to manage the disease. Fungicides may also be used, but their effectiveness can vary. *Fusarium napiforme* poses a threat to plants by causing root rot, inhibiting nutrient uptake, and producing toxins. Its impact can result in reduced growth, wilting, and increased

susceptibility to other diseases. Effective management strategies are essential to mitigate the damage caused by this fungal pathogen.[5,6,7,8]

Polymerase Chain Reaction (PCR) is a revolutionary molecular biology technique used to amplify specific segments of DNA. Developed in the mid-1980s, PCR has become an essential tool in various fields, including genetics, forensics, and medical research. The process involves multiple cycles of DNA denaturation, primer annealing, and DNA synthesis, all carried out by a heat-stable DNA polymerase enzyme. During denaturation, the DNA sample is heated to separate the double-stranded DNA into single strands. Next, short DNA primers, complementary to the target region, anneal to the separated strands. The DNA polymerase then extends the primers, synthesizing new DNA strands. Each cycle effectively doubles the number of DNA copies, leading to exponential amplification of the target region. PCR's precision, sensitivity, and speed have made it indispensable for a wide range of applications, such as identifying genetic disorders, detecting pathogens, and analyzing ancient DNA samples.[9,10,11]

Polymerase Chain Reaction (PCR) has revolutionized the field of molecular biology and has become an essential tool for various scientific disciplines. Its importance stems from its ability to amplify specific DNA sequences exponentially, allowing researchers to detect and analyze small amounts of genetic material with high precision and sensitivity. PCR has numerous applications in research, diagnostics, forensics, and biotechnology. In research, PCR enables the study of gene expression, genetic mutations, and microbial diversity, among other investigations. In diagnostics, PCR plays a critical role in the identification and detection of pathogens, including viruses, bacteria, and parasites, aiding in the timely diagnosis and treatment of infectious diseases. In forensics, PCR allows for the analysis of minute amounts of DNA evidence, assisting in criminal investigations and providing crucial information in court cases. Moreover, PCR is pivotal in biotechnology, where it facilitates DNA cloning, gene expression analysis, and the production of recombinant proteins. Overall, the Polymerase Chain Reaction has had a transformative impact on various scientific fields, providing researchers with a powerful and versatile tool for studying DNA, diagnosing diseases, solving crimes, and advancing biotechnological applications.[10,12,13,14]

In the present study *Fusarium napiforme* was isolated from ridge gourd seeds by agar plate method and subcultured for further study. The pathogen was identified as *Fusarium* by direct microscopy using lactophenol cotton blue method. For the confirmation of species molecular techniques were used. The present study performs a molecular identification method using polymerase chain reaction (PCR) to detect and identify *Fusarium napiforme*. In this study, specific primers targeting the internal transcribed spacer (ITS) region of the fungal rRNA gene were designed and used in PCR amplification. The resulting PCR products were then analyzed by gel electrophoresis to determine the presence or absence of *Fusarium napiforme*.

Materials and Methods

1) Reagents used
   - 0.2% aqueous sodium hypochlorite
   - Insta Gene™ Matrix Genomic DNA isolation kit (Catalog # 732-6030)
   - 1% agarose gels
   - Ethidium bromide

- Distilled water
- Potato dextrose agar media
- Amoxicillin

2) Sample collection

Seeds sample of ridge gourd were collected from Indian Institute of Horticultural Research, Bengaluru, Karnataka, India.

3) Sample Preparation

Seeds of ridge gourd were surface sterilised with 0.2% aqueous sodium hypochlorite solution followed by rinsing in distilled water and drying in the hood of laminar air flow and 10 surface sterilised seeds were arranged on the potato dextrose agar media on agar plate.[15] Agar plates were prepared by preparing potato dextrose agar media followed by autoclaving at 15 pounds per square inch at 250°F (121°C) for 30 minutes. Amoxicillin was added to avoid bacterial contamination. After inoculation the plates were sealed and incubated at 25 ± 2°C for 48 hours. After 48 hours fungus was isolated, subcultured and identified based on morphology as *Fusarium* with the help of direct microscopy as described by Barnet and Hunter,[16] Nelson et al.,[17] Pitt and Hocking,[18] Leslie and Summerall[19]To validate the species, molecular techniques such as polymerase chain reaction and agarose gel electrophoresis were used.

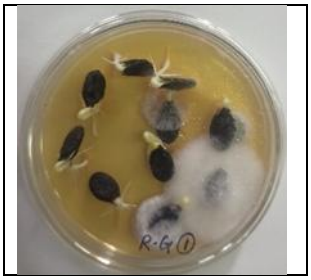

Fig 1: Rigde gourd seeds infected with *Fusarium*

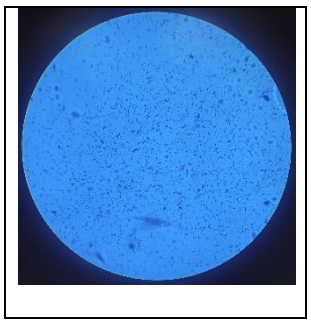

Fig 2: *Fusarium napiforme* isolated from ridge gourd seeds

4) Genomic DNA extraction**:** Genomic DNA was isolated by using the Insta GeneTM Matrix Genomic DNA isolation kit (Catalog # 732-6030)

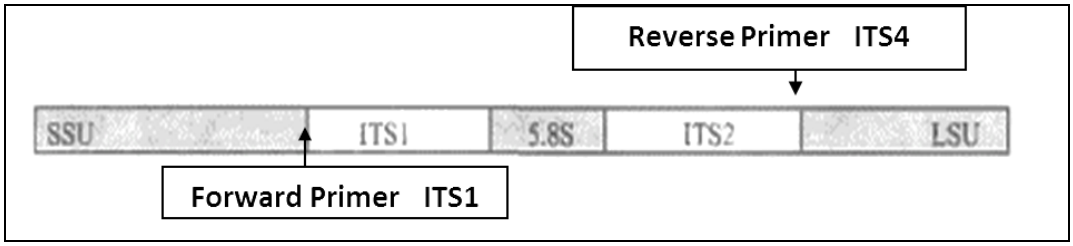

Fig 3: Ribosomal Gene organization and Target region amplified

Table 1: Primer Details - Ribosomal RNA ITS Region Universal primers

ITS Primer for Fungi	Sequence Details	Amplicon size (bp)
Forward Primer ITS1	GGAAGTAAAAGTCGTAACAA GG	**620 bp**
Reverse Primer ITS4	TCCTCCGCTTATTGATATGC	

5) Polymerase Chain Reaction: Target gene fragment was amplified using Thermo Scientific Veriti Thermal Cycler

(a) PCR Protocol: DNA fragments are amplified using 1 µl of template DNA in 10 µl of total PCR reaction mixture using ITS1F/ITS4Rprimers (50 pmol) and 30 amplification cycles with following program:

Table 2: PCR Protocol

Initial denaturation	95℃ for 5 mins
Denaturation	95℃ for 1mins
Annealing	55℃ for 30 sec
Extension	72℃ for 1mins
Final Extension	72℃ for 7 mins

(b) Purification of PCR products: Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).

(c) Sequencing: The PCR product was sequenced using the 1492R ITS1F/ITS4R primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq®DNA polymerase (FS enzyme) (Applied Biosystems). Single-pass sequencing was performed on each template using18S rRNA gene universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were re suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Bio systems).

Results

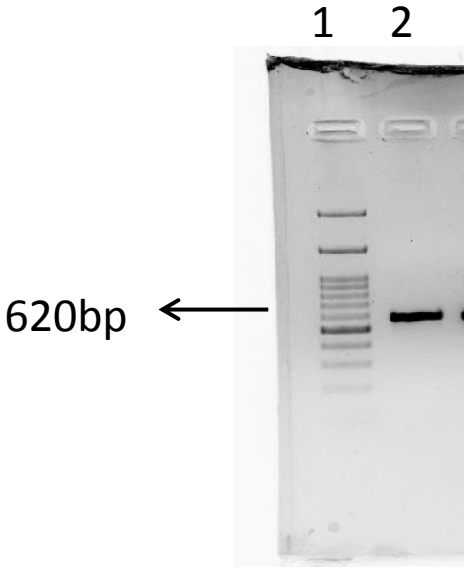

Fig 4: PCR Amplicon of ITS 1-4

Lane 1-Molecular size ladder, lane 2 – Sample *Fusarium*

Forward Sequence

>0723_472_1_PCR_FU_FORWAED_A01.ab1

CCGAAGGCTGACAGCGGAGGGACATTACCGAGTTTACAACTCCCAAACCCCTGTG
AACATACCAATTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGC
CCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCATAA
ATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA
GCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
ACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCA
ACCCTCAAGCCCAGCTTGGTGTTGGGACTCGCGAGTCAAATCGCGTTCCCCAAAT
TGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGT
AATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCAG
GTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCCGGAGGAAA

Reverse sequence

>0723_472_002_PCR_FU_REVERSE_A07.ab1

GCCCTCGGGGGAATTCCTACCGGGGATCCGAGGTCAGCATTCAGAAGTTGGGGTT
TAACGGCGTGGCCGCGACGATTACCAGTAACGAGGGTTTTACTACTACGCTATGGA
AGCTCGACGTGACCGCCAATCAATTTGGGGAACGCGATTTGACTCGCGAGTCCCA
ACACCAAGCTGGGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAG
AATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAA
TTCACATTACTTATCGCATTTTGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATC
CGTTGTTGAAAGTTTTGATTTATTTATGGTTTTACTCAGAAGTTACATATAGAAACA
GAGTTTAGGGGTCCTCTGGCGGGCCGTCCCGTTTTACCGGGAGCGGGCTGATCCG
CCGAGGCAACAATTGGTATGTTCACAGGGGTTTGGGATTTGTAAGCTCGCTAATGA
TCCCTCCGCTGTTTCACCAACGGATACCTTGTTACGACTTTTACTTACA

>contigous sequence

TGTAAGTAAAAGTCGTAACAAGGTATCCGWWGGCTGAAACAGCGGAGGGATCAT
TASCGAGYTTACAAMTCCCAAACCCCTGTGAACATACCAATTGTTGCCTCGGCGG
ATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGT
TTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGAT
CTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATT
GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCT
GGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAGCTTGGTGTTGG
GACTCGCGAGTCAAATCGCGTTCCCCAAATTGATTGGCGGTCACGTCGAGCTTCC
ATAGCGTAGTAGTAAAACCCTCGTTACTGGTAATCGTCGCGGCCACGCCGTTAAAC
CCCAACTTCTGAATGYTGACCTCGGATCCCMGGTAGGAATWCCCSCGWGRRCTTA
AGCATATCAATAAGCCGGAGGAAA

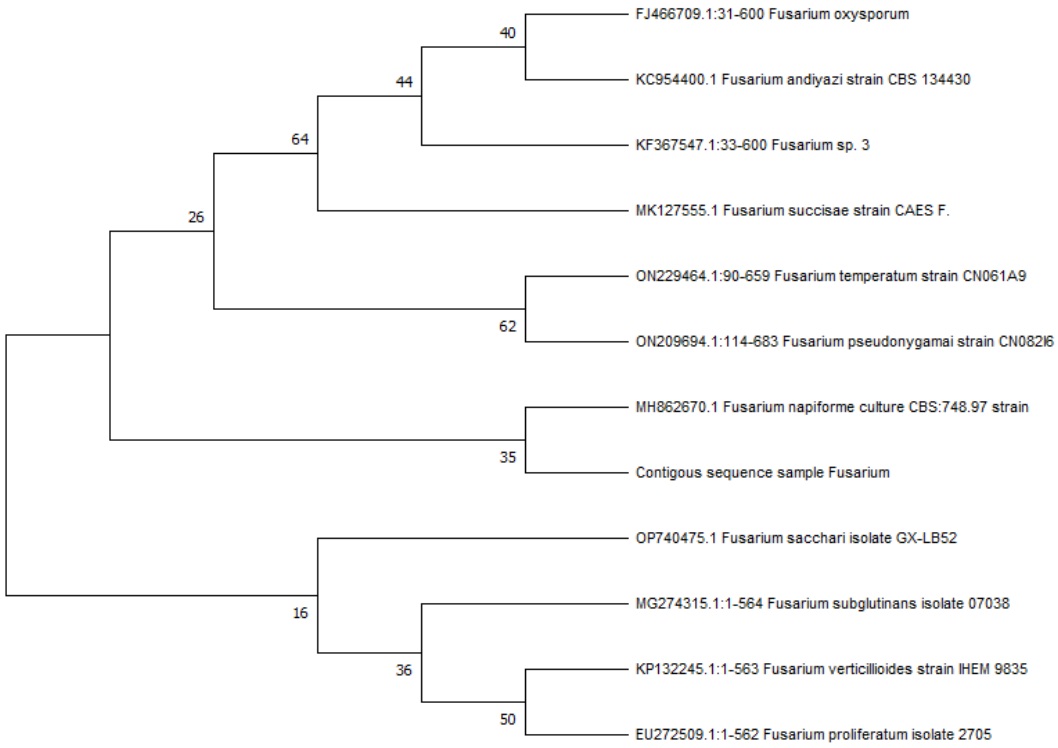

Fig 5: Phylogenetic tree of fungal isolate of sample *Fusarium*

Discussion

In the present study, we employed a PCR-based approach to identify *Fusarium napiforme* and revealed its distinctive genetic signature. The accurate identification of this fungal species is crucial due to its potential impact on various agricultural crops[5] and its role as an opportunistic human pathogen[20]. Through the utilization of specific primers targeting the ITS [21,22], we successfully amplified and sequenced the DNA of *Fusarium napiforme* isolates from ridge gourd seeds. The obtained results demonstrated the specificity and sensitivity of the PCR assay in distinguishing *Fusarium napiforme* from closely related species. The development of a reliable diagnostic tool is of paramount importance, as misidentification of this fungus could lead to erroneous management practices and compromised human health outcomes. Our findings contribute to the growing body of knowledge on *Fusarium napiforme*

and enable researchers and clinicians to promptly and accurately detect and differentiate this species from others in various environmental and clinical settings. Moreover, the genetic analysis allowed us to gain insights into the genetic diversity and population structure of *Fusarium napiforme*. The identification of distinct genetic signatures among isolates from different geographical locations and hosts suggests possible regional variations in the distribution and virulence of the fungus. This information is crucial for understanding the epidemiology and pathogenic potential of *Fusarium napiforme* and guiding appropriate disease management strategies.

DNA sequencing of fungi is crucial for identification because it allows for accurate and precise species determination. Fungi can exhibit similar morphological characteristics, making traditional identification methods challenging. DNA sequencing provides a reliable and objective way to distinguish between closely related species, helping researchers, biologists, and environmentalists better understand fungal diversity, distribution, and ecological roles. Additionally, it aids in detecting potential pathogenic or beneficial fungi, leading to improved management strategies in agriculture, medicine, and environmental conservation.[25,26]

Molecular identification of plant fungal pathogens is essential due to its numerous advantages in the field of plant pathology.[23] Traditional methods of identification, based on visual symptoms and morphological characteristics, can be imprecise and time-consuming. Molecular techniques, on the other hand, offer a more accurate and rapid approach to identify and characterize fungal pathogens. By targeting specific genetic markers unique to each pathogen, such as DNA sequences or gene expression patterns, molecular methods provide a reliable means of distinguishing between closely related species. This precision is critical in detecting emerging or exotic pathogens that may pose significant threats to agricultural and natural ecosystems. Additionally, molecular identification allows for early and accurate diagnosis of diseases, enabling prompt and targeted management strategies to be implemented, reducing the risk of widespread outbreaks and minimizing economic losses. Furthermore, understanding the genetic diversity and population structure of fungal pathogens aids in assessing their potential for resistance to fungicides and developing appropriate disease control strategies. Overall, molecular identification is a crucial tool in plant pathology, facilitating effective disease management and safeguarding global food security and environmental health.[2,24,24]

Conclusion

In conclusion, the present study has successfully employed PCR-based techniques to identify and unveil the genetic signature of *Fusarium napiforme*, shedding light on crucial aspects of this elusive fungal species. Through a meticulous combination of molecular methodologies, we have provided substantial evidence supporting the accurate identification and differentiation of *Fusarium napiforme* from other closely related Fusarium species. The development and validation of specific primers targeting key genetic markers have enabled us to achieve a high level of sensitivity and specificity in detecting this pathogen, thus facilitating its rapid and reliable identification in various environmental samples and infected plant tissues. The genetic signature of *Fusarium napiforme* revealed in this study represents a significant step towards a comprehensive understanding of its biology, ecology, and pathogenicity. By pinpointing unique genetic sequences specific to this fungal species, our

findings will aid in its early detection and monitoring in agricultural and natural ecosystems, potentially preventing and controlling its detrimental impacts on crops and plants. Moreover, the robust PCR-based approach presented in this research serves as a valuable model for the identification of other elusive and closely related fungal species, which have proven challenging to differentiate through traditional morphological and cultural methods. The integration of genetic information in the identification process not only enhances accuracy but also expedites the diagnostic process, making it more accessible and applicable to diverse research and diagnostic laboratories.

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