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Molecular Identification and Phylogenetic Analysis of Dominant Fungal Pathogens in Ginger (*Zingiber officinale Rosc.*) Rhizosphere of Chhatrapati Sambhajinagar District, Maharashtra, India

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Abstract
Ginger (*Zingiber officinale Rosc.),* an economically important spice crop belonging to the family *Zingiberaceae,* is extensively cultivated in the Marathwada region of Maharashtra, particularly in the Chhatrapati Sambhajinagar district. In recent years, ginger cultivation in this region has been severely affected by fungal diseases, resulting in substantial yield losses and reduced crop productivity. The present study aimed to identify dominant fungal pathogens associated with ginger rhizospheric soils using Internal Transcribed Spacer (ITS) region sequencing.A total of 20 fungal isolates obtained from rhizospheric and soil samples were subjected to molecular characterization. ITS sequence analysis and BLAST comparison revealed four major pathogenic species belonging to the genus *Fusarium*, namely *Fusarium nematophilum*, *F. proliferatum*, *F. solani*, and *F. chlamydosporum*. In addition, *Talaromyces purpureogenus* was identified as an associated soil fungus. Phylogenetic relationships inferred using the Neighbor-Joining method grouped the isolates into distinct, well-supported clades, confirming species-level identification. Accurate molecular identification of rhizospheric fungal pathogens is essential for understanding disease epidemiology and designing targeted disease management strategies. This study provides baseline molecular data on ginger-associated fungal diversity in the Chhatrapati Sambhajinagar agro ecological zone.

Keywords: Rhizosphere soil fungi, Chhatrapati Sambhajinagar, Molecular identification, Phylogeny

Introduction
The rhizosphere the narrow region of soil directly influenced by plant roots represents one of the most dynamic interfaces in terrestrial ecosystems. It hosts a dense and metabolically active microbial community that profoundly influences plant growth, nutrient uptake, stress tolerance, and disease interactions. Microorganisms in this zone, particularly fungi, play essential ecological roles, including decomposition of organic matter, nutrient cycling, and the formation of symbiotic or antagonistic relationships with plants (Robert *et al*., 2017; Schoch *et al*., 2012) [5, 8]. Fungi are often considered keystone members of the rhizosphere microbiome due to their ability to modulate plant immunity, mobilize nutrients, create mutualistic networks, and in many cases, act as devastating pathogens capable of limiting crop productivity (Schoch *et al*., 2012; Rosangkima *et al*., 2018) [8, 6].
Ginger (Zingiber officinale Rosc.), a perennial rhizomatous herb belonging to the family Zingiberaceae, holds substantial economic significance in tropical and subtropical agriculture. India ranks among the world's largest producers of ginger, and the crop plays a critical role in domestic markets, export sectors, and traditional medicinal systems (Rahman *et al*., 2009) [4]. The Marathwada region of Maharashtra, particularly the Chhatrapati Sambhajinagar district, has emerged as a notable ginger-growing zone due to its favourable agro-climatic conditions and expanding market demand. However, ginger is highly susceptible to a range of soil borne diseases that threaten both yield and quality. Rhizome-dwelling pathogens, especially fungi, frequently proliferate under warm and humid environments typical of the region, making ginger cultivation challenging for small and medium-scale farmers.

Previous research has shown that the rhizosphere mycobiome exerts a significant influence on ginger growth and disease susceptibility. Beneficial fungi such as Trichoderma and Talaromyces contribute to nutrient acquisition and biological control, whereas pathogenic taxa such as Pythium, *Fusarium*, Aspergillus, Penicillium, Rhizopus, and Mucor cause severe pre- and post-harvest losses (Rosangkima *et al*., 2018; Robert *et al*., 2017) [6, 5]. Among these, *Fusarium* species are considered particularly destructive. Members of this genus are notorious for their wide host range, ability to survive as chlamydospores in soil, and capacity to cause chronic wilt, rhizome rot, and vascular discoloration (Robert *et al*., 2017) [5]. Reports from various ginger-producing regions of India highlight *Fusarium* solani, F. oxysporum, and related taxa as major threats associated with soft rot complexes and yield reduction (Rahman *et al*., 2009; Rosangkima *et al*., 2018) [4, 6]. Despite their recognized importance, accurate species level identification remains limited in many agro-ecological regions, including Maharashtra's ginger belts. One of the major challenges in understanding ginger pathology lies in the morphological similarity among fungal taxa. Many *Fusarium* and Trichoderma species exhibit substantial phenotypic overlap, making classical identification based solely on morphology unreliable. This has driven the adoption of molecular markers, particularly the Internal Transcribed Spacer (ITS) region of ribosomal DNA, as a universal fungal barcode (Schoch *et al*., 2012) [8]. ITS sequencing enables high resolution discrimination of fungal species, even in complex soil environments where traditional culturing techniques may overlook cryptic or slow-growing taxa. Advances in phylogenetic reconstruction methods such as Neighbor-Joining (NJ) approaches with bootstrap validation have further strengthened our ability to characterize fungal diversity and evolutionary relationships (Felsenstein, 1985; Saitou & Nei, 1987; Nei & Kumar, 2000; Tamura *et al*., 2021) [1, 7, 3, 9]. Despite these available tools, there remains a significant knowledge gap concerning the fungal diversity associated with ginger cultivation in Chhatrapati Sambhajinagar district. Farmer reports and field observations indicate a rising incidence of rhizome rot, poor germination, and slow plant establishment, all of which may be linked to soil borne fungal pathogens. Yet, comprehensive molecular-level studies investigating both rhizospheric and non-rhizospheric fungi are scarce for this region. Without species-level pathogen identification, it is difficult to formulate targeted management strategies or predict disease outbreaks under changing climatic and soil conditions. Given these challenges, the present study was undertaken to identify and characterize fungal taxa associated with ginger rhizosphere soils in Chhatrapati Sambhajinagar district using ITS sequencing and phylogenetic analysis. By integrating molecular data with evolutionary relationships, this research aims to provide a foundation for future disease management strategies, guide cultivar selection, and support sustainable ginger cultivation in the region.

Materials and Methods

Study Area and Field Survey

The study was conducted in the major ginger-growing regions of the Chhatrapati Sambhajinagar (Aurangabad) district, located in the Marathwada region of Maharashtra, India. The district is characterized by semi-arid climatic conditions, moderate rainfall, and diverse soil types, including clay loam and black cotton soil, which support ginger cultivation. A preliminary survey was carried out to identify fields with active ginger cultivation, disease symptoms, and suitable access for sampling. Based on these criteria, 12 distributed across key agricultural zones were selected for systematic soil sampling. The survey recorded information on geographical coordinates, cropping history, soil conditions, irrigation type, and disease occurrence. Visual inspection was performed to record any disease symptoms such as rhizome rot, wilt, leaf yellowing, or stunted growth, which served as indicators of potential fungal infection.

Sample Collection

Rhizospheric soil sampling

Rhizospheric soil samples were collected from five sites by gently uprooting ginger plants and manually dislodging loosely adhering soil. Soil tightly adhering to the roots within a depth of 0-15 cm was collected using sterilized spatulas. Approximately 500 gm of rhizospheric soil was collected from each site. Total twenty samples were collected and molecular identification has been done.

Transport and storage

All samples were placed in sterile, labelled polyethylene bags and transported in an insulated container to maintain microbiological integrity. Samples were stored at 4 °C until further processing. All tools used during sampling were sterilized with 70% ethanol to prevent cross-contamination.

Isolation of fungi

Soil samples were processed using a serial dilution technique. One gram of soil was suspended in 9 mL sterile distilled water and serially diluted up to 10^{-3}. Aliquots of 0.1 mL were plated on Potato Dextrose Agar (PDA) supplemented with streptomycin sulfate (50 mg/L) to inhibit bacterial contamination. Plates were incubated at 27 ± 2 °C for 5-7 days. Distinct fungal colonies were sub-cultured onto fresh PDA plates to obtain pure cultures. Morphological features such as colony color, texture, margin type, spore morphology, and sporulation patterns were recorded as preliminary identification parameters.

Genomic DNA extraction

Genomic DNA was extracted from freshly grown fungal mycelia using the Progenome DNA Extraction Kit, following the manufacturer's protocol. Approximately 50-100 mg of fungal biomass was harvested, lysed, and subjected to chemical and enzymatic digestion to release genomic DNA. The DNA was purified through silica column-based adsorption and elution steps.

DNA quantification and quality assessment

The concentration and purity of extracted DNA were assessed using a Quantus™ Fluorometer (Promega, USA). DNA quality was verified by electrophoresis on a 1% agarose gel stained with ethidium bromide and visualized under a UV transilluminator (Himedia Gel Doc System). Samples showing bright, intact genomic DNA bands without smearing were selected for PCR amplification.

ITS-Region amplification

Marker selection

The ITS region of nuclear ribosomal DNA was selected as the universal fungal barcode due to its high interspecific variability and robust amplification properties.

PCR reaction mixture
The ITS region was amplified using primers ITSu3 and ITSu4. The 25 µL reaction volume consisted of:

Table 1: Details of ITS polymerase chain reaction composition and Primer used for ITS gene amplification and its sequencing of ITSu3 and ITSu4.

Component	Volume
GoTaq® Green Master Mix	12.5 µL
ITSu3 Primer (5′-CAWCGATGAAGAACGYAGC-3′)	1.5 µL
ITSu4 Primer (5′-RGTTTCTTTTCCTCCCGTTA-3′)	1.5 µL
Template DNA	3.0 µL
Nuclease-Free Water	6.5 µL

A subset of samples was also tested using Hi-PCR® REDy Mix to ensure amplification consistency.

Thermal cycling conditions
PCR amplification was performed in a thermal cycler using the following program:

- **Initial denaturation:** 95 °C for 3 min, 35 cycles.
- **Denaturation:** 95 °C for 45 sec
- Annealing: 48 °C for 50 sec.
- **Extension:** 72 °C for 70 sec.
- **Final extension:** 72 °C for 10 min.
- **Hold:** 4 °C

PCR products were resolved on a 1.3% agarose gel, and a single discrete band (approximately 550-650 bp) indicated successful ITS amplification.

PCR product purification and sequencing
Enzymatic clean-up
Amplified PCR products were purified using a combination of Exonuclease-I and Shrimp Alkaline Phosphatase (1 unit/µL each) to remove unincorporated primers and dNTPs. Clean-up reactions were incubated at 37 °C for 30 min, 80 °C for 15 min.

Cycle sequencing
Purified amplicons were sequenced in both forward and reverse directions using Big Dye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730xl Genetic Analyzer. Sequencing was performed with the same primer pair (ITSu3 and ITSu4). Thermal cycling conditions such as 96 °C for 2 min, 35 cycles of: 96 °C for 30 sec, 55 °C for 15 sec & 60 °C for 4 min.

Template preparation for capillary electrophoresis
Sequencing reaction products were ethanol-precipitated, dried, and re-suspended in Hi-Di™ formamide. Samples were denatured at 95 °C for 2 min prior to electrophoresis.

Sequence assembly and identification
Forward and reverse sequence chromatograms were manually inspected for quality using Sequence Aligner Software. Clean, high-quality reads were assembled into consensus sequences.

BLAST Analysis
Consensus ITS sequences were subjected to the Blast algorithm against the NCBI GenBank database. Species identification was based on Maximum identity (%), Max score and total score
Query coverage (%), Only matches with ≥99% identity and high coverage were considered reliable.

Multiple Sequence Alignment and Phylogenetic Analysis
Alignment
All ITS sequences were aligned using CLUSTALO integrated in MEGA 11. Sequences were manually trimmed to remove low-quality regions.

Phylogenetic Tree Construction
A Neighbour-Joining (NJ) phylogenetic tree was constructed using 1,000 bootstrap replicates for statistical support, p-distance model to calculate evolutionary divergence & inclusion of all positions containing gaps and missing data.

Dataset Composition
The final alignment consisted of: 20 nucleotide sequences, 432 aligned positions & Clades with bootstrap values ≥70% were considered strongly supported.

Results and Discussion
Genomic DNA yield and quality
High quality genomic DNA was successfully extracted from freshly grown fungal mycelia using the Progenome DNA Extraction Kit. The DNA yield ranged from moderate to high concentrations, sufficient for downstream PCR amplification. Quantification using the Quintus™ Fluorometer confirmed adequate DNA concentrations with acceptable purity levels. Agarose gel electrophoresis (1% agarose) revealed intact, high-molecular weight genomic DNA with minimal or no shearing. The absence of smearing and the presence of sharp DNA bands indicated effective cell lysis and purification. Only samples exhibiting intact genomic DNA were selected for further molecular analysis.

PCR amplification of the ITS region
The internal transcribed spacer (ITS) region of nuclear ribosomal DNA was successfully amplified using the universal fungal primers ITSu3 and ITSu4. PCR amplification yielded single, clear, and reproducible bands for the majority of samples when visualized on a 1.3% agarose gel. The amplified fragments were consistently observed within the expected size range of approximately 550-650 bp, confirming successful and specific amplification of the ITS region. Comparable amplification efficiency was observed when reactions were performed using both Goat® Green Master Mix and Hi-PCR® REDy Mix, demonstrating the robustness and reproducibility of the PCR protocol.

PCR product purification
PCR products subjected to enzymatic cleanup using Exonuclease-I and Shrimp Alkaline Phosphatase showed improved sequencing quality, as evidenced by the absence of primer-dimer peaks and background noise in subsequent chromatograms. The purification method efficiently removed residual primers and unincorporated dNTPs without loss of amplicon integrity.

DNA sequencing and read quality
Bidirectional sequencing of purified ITS amplicons was successfully performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730xl Genetic Analyzer. Sequencing reactions generated high-quality chromatograms with well-resolved peaks and minimal background interference. Forward and reverse sequence reads were trimmed and assembled to obtain consensus ITS sequences of appropriate length. The sequences exhibited clear base

calling across most of the read length, indicating efficient cycle sequencing and capillary electrophoresis.

ITS sequence validation and taxonomic identification

The obtained ITS consensus sequences were suitable for downstream bioinformatics analyses, including sequence alignment and database comparison. Preliminary sequence inspection confirmed the presence of conserved ITS motifs flanking variable regions, characteristic of fungal ribosomal DNA. The high quality and length of the ITS sequences indicated their suitability for accurate taxonomic identification and phylogenetic analysis using reference databases such as GenBank or UNITE. (table:1)

ITS sequence similarity and BLAST based species identification

Amplification of the Internal Transcribed Spacer (ITS) region using primers ITSu3 and ITSu4 produced clear, single amplicons of the expected size (~550-650 bp), confirming the suitability of ITS as a universal fungal barcode (Schoch *et al*., 2012) [8] (table:1). Subsequent BLASTn analysis against the NCBI GenBank database enabled precise species-level identification of the fungal isolates.

The BLAST results revealed high sequence identity scores (99.67-100%), confirming the accuracy of species identification. The dominant fungal species identified from the rhizospheric and soil samples included *Fusarium nematophilum*, *Fusarium proliferatum*, *Fusarium solani*, *Fusarium chlamydosporum*, *Talaromyces purpureogenus* and some additional isolates such as *Trichoderma afroharzianum*, *Coprinellus radians*, and *Colletotrichum sp.* A representative summary of BLAST results is provided below:

Table 2: Sample IDs showing Similarity Searches in Sequence Alignment

Sample ID	Max Score	Identified Species	% Identity	GenBank Accession
KH03	492	*Fusarium nematophilum*	100%	KJ512156
KUM03	547	*Fusarium proliferatum*	100%	KR906694
SB02	562	*Fusarium solani*	100%	MW375794
SB03	547	*Fusarium chlamydosporum*	100%	ON207928
KH02	497	*Talaromyces funiculosus*	100%	MZ014551
KH01	547	*Coprinellus radians*	99.67%	MK843954
SB04	521	*Colletotrichum sp.*	100%	ON153393
KKH04	562	*Trichoderma afroharzianum*	100%	OM515010
KHO4	577	*Schizophyllum*	100%	KU836577
KUMO1	542	*Corynascus sepedoniuum*	100%	MG250420
KUMO2	614	*Dichomitus*	100%	MZ544377
KUMO4	529	*Cladosporium pseudotenuissiumum*	100%	PP385418
KKH01	499	*Uncultered endophytic fungi*	100%	EF505066
KKH02	562	*Coprinellus radians*	100%	KM246171
KKH05	582	*Flavodon flavus*	100%	MZ636989
SB01	562	*Fusarium solani*	100%	MW375794
KM02	510	*Teleromyces Purpureogenus*	100%	KY230505
KM03	499	*Myceliophthora verrucosa*	100%	KR105943
KM04	209	*Teleromyces purpureogenus*	100%	MF380979

Dominance of *Fusarium* spp.

The identification of four major *Fusarium* species *F. nematophilum*, *F. proliferatum*, *F. solani*, and *F. chlamydosporum* demonstrates the prevalence of this genus in ginger soils of Chhatrapati Sambhajinagar district. The genus *Fusarium* is widely known for its destructive pathogenicity and ability to cause wilt, root rot, and rhizome decay in ginger and other crops (Robert *et al*., 2017; Rosangkima *et al*., 2018) [5, 6]. The present results align with earlier findings from Northeast India and Mizoram, where *F. solani* and related species were identified as major causal agents of ginger soft rot (Rosangkima *et al*., 2018) [6]. The dominance of multiple *Fusarium* species in a single region highlights the complex pathogenic landscape, suggesting the possibility of mixed infections and synergistic disease impacts on ginger plants.

Phylogenetic Analysis

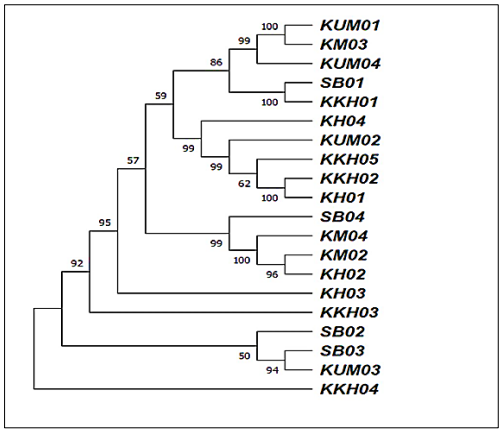

Fig 1: Phylogenetic analysis by Neighbour Joining Tree model method

A Neighbour-Joining (NJ) phylogenetic tree constructed from 20 ITS sequences (432 aligned nucleotide positions) provided insights into the evolutionary relationships among the fungal isolates. The tree displayed strong bootstrap values (70-99%) supporting key clades, in agreement with classical molecular evolutionary methods (Felsenstein, 1985; Saitou & Nei, 1987; Nei & Kumar, 2000; Tamura *et al*., 2021) [1, 7, 3, 9].

Major Phylogenetic Clades

The phylogenetic tree clearly delineated the isolates into distinct clusters:

A. *Fusarium* Clade (High Bootstrap Support)

A prominent clade comprising *F. nematophilum*, *F. proliferatum*, *F. solani*, and *F. chlamydosporum* exhibited >90% bootstrap support, confirming their close evolutionary relationships and validating BLAST-based identification.

F. solani occurred at a basal position in the clade, reflecting its genetically diverse and widely distributed nature.

F. nematophilum and *F. chlamydosporum* formed a strongly supported subclade, indicating their molecular similarity.

These findings support earlier conclusions that ITS sequencing provides robust phylogenetic resolution for *Fusarium* species (Schoch *et al*., 2012) [8].

B. *Talaromyces purpureogenus* clustering

Samples KH02 and KH03, identified as *Talaromyces purpureogenus*, and clustered together with 96% bootstrap support, reflecting species-level consistency.

Talaromyces spp. are increasingly recognized as both soil saprophytes and biocontrol agents, and their presence may influence soil microbial equilibria.

C. *Coprinellus radians* Clustering

Isolates KH01 and SB04 grouped with 99% bootstrap support, indicating strong molecular coherence. Although *Coprinellus* spp. are typically saprophytic, their presence indicates active organic decomposition processes in ginger soils.

D. Presence of Biocontrol Species

The identification of *Trichoderma afroharzianum*, a well-known beneficial fungus used in biocontrol, suggests the natural presence of antagonistic microorganisms that could potentially suppress soilborne pathogens (Robert *et al*., 2017) [5].

The phylogenetic relationships observed in the study strongly corroborate the ITS-based BLAST identifications and reflect the ecological diversity of fungal species associated with ginger cultivation in the study region.

Ecological and pathological significance

Implications of *Fusarium* dominance

The widespread presence of multiple *Fusarium* species indicates high disease pressure in ginger fields of Chhatrapati Sambhajinagar. Similar findings have been reported in other Indian states where *Fusarium*-induced soft rot and wilt significantly reduce rhizome yield (Rahman *et al*., 2009; Rosangkima *et al*., 2018) [4, 6]. Given that *F. solani* and related species can survive in soil for extended periods, their detection underscores the need for region-specific disease management strategies.

Role of saprophytic and beneficial fungi

Species such as *Talaromyces* and *Trichoderma* may contribute positively to soil health by competing with pathogens, producing antimicrobial compounds, and improving nutrient cycling (Robert *et al*., 2017) [5]. Their identification in ginger rhizosphere implies potential natural suppression of pathogens.

Mixed fungal community dynamics

The coexistence of pathogenic, saprophytic, and beneficial fungi highlights the complexity of ginger soil ecosystems. Understanding these interactions is essential for designing integrated soil health management practices.

Comparison with previous studies

The findings of this study agree with earlier molecular characterizations of ginger pathogens reported from other regions of India (Rosangkima *et al*., 2018) [6], supporting the dominance of *Fusarium* spp. in ginger rhizosphere soils. Additionally, the detection of *Talaromyces* and *Trichoderma* species supports existing literature demonstrating a diverse fungal community influencing ginger health (Robert *et al*., 2017; Schoch *et al*., 2012) [5, 8].

Conclusion

The present study successfully employed ITS-based molecular characterization to elucidate the diversity and ecological composition of fungal communities associated with ginger rhizospheric and non-rhizospheric soils of the Chhatrapati Sambhajinagar district. High-quality genomic DNA was consistently obtained from fungal isolates, enabling robust PCR amplification, sequencing, and downstream bioinformatics analyses. The ITS region proved to be a reliable universal fungal barcode, yielding clear amplicons, high-quality bidirectional sequences, and precise species-level identification.

BLAST-based sequence analysis revealed a predominance of *Fusarium* species, including F. nematophilum, F. proliferatum, F. solani, and F. chlamydosporum, confirming the widespread occurrence of this pathogenic genus in ginger-growing soils of the study area. The high sequence identity values (99.67-100%) and strong bootstrap support in phylogenetic analyses validated the accuracy of molecular identification and highlighted the close evolutionary relationships among the *Fusarium* isolates. The dominance of multiple *Fusarium* species within a single agro-ecosystem underscores the likelihood of complex disease etiology, mixed infections, and sustained soil-borne inoculum pressure affecting ginger productivity.

In addition to pathogenic fungi, the detection of saprophytic and beneficial taxa such as Talaromyces purpureogenus, Coprinellus radians, and Trichoderma afroharzianum reflects the ecological heterogeneity of ginger soils. The presence of Trichoderma, a well-known biocontrol agent, suggests the existence of naturally occurring antagonistic microorganisms that may influence pathogen dynamics and soil health. Such beneficial fungi could be strategically exploited for developing eco-friendly disease management practices.

Overall, the integration of ITS sequencing, BLAST analysis, and phylogenetic inference provided comprehensive insights into the fungal community structure associated with ginger cultivation in the region. The findings emphasize the need for integrated disease management strategies that consider

both pathogenic dominance and beneficial microbial interactions. Future studies incorporating multi-locus sequencing, population genetics, and functional assays will further enhance understanding of pathogen diversity and microbial interactions, ultimately contributing to sustainable ginger production and soil health management.

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