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MYCOBIOME OF ANJUR FOREST SOIL: A METABARCODING APPROACH

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Abstract: *Soil fungi are crucial for nutrient cycling and plant health. The knowledge pertaining to the diversity of fungi reveals the soil health. However, conventional classification methods pose challenges in comprehensively identifying the complete fungal community. The metabarcoding approach offers a powerful and comprehensive alternative for studying fungal diversity in soil ecosystems. This study investigated the diversity of fungal species in soil samples collected from undisturbed zones within the Anjur Reserve Forest, located in the Chengalpattu District of Tamil Nadu, which falls under the East Deccan Dry Evergreen Ecoregion. Metabarcoding analysis of the soil samples clustered the sequences into 151 operational taxonomic units (OTUs), yielding a total of 195,908 reads. The dominant fungal phyla identified were Ascomycota, Basidiomycota, Glomeromycota, and Mortierellomycota. At the species level, a total of 78 species were identified, with Asterostroma sp. (18654), Muyocopron ficinum (8369), Phaeobotryon mamani (1703), and Verrucaria sp. (1138) being the most abundant. Notably, a significant portion of the reads (84,338) could only be classified at the kingdom level (Fungi). Additionally, the study detected DNA from opportunistic, pathogenic, ectomycorrhizal, and endophytic fungi, suggesting potential introduction by wild animals, birds, or wind. This study highlights the diverse fungal communities present in the Anjur Reserve Forest and their potential influence on soil dynamics within this forest ecosystem. The dominance of specific fungal taxa likely contributes to enhanced nutrient cycling and improved soil structure, playing a crucial role in mitigating soil erosion and maintaining ecosystem stability. Further research focusing on the functional roles of these individual fungal species across different environmental contexts will provide a deeper understanding of their potential in ecosystem management and restoration. Expanding the study to include a greater number of samples across the East Deccan Dry Evergreen Forest ecoregion would further elucidate the significant role of fungi in this unique ecosystem.*

Keywords: *Fungal Diversity, Metabarcoding, East Deccan Dry Evergreen Ecoregion, ITS, rRNA, QIIME2, NCBI, Asterostroma sp.*

INTRODUCTION

The Anjur Reserve Forest, situated within the geographical influence of the Eastern Ghats, inherits the region's unique ecological significance, characterized by diverse flora and fauna despite increasing environmental pressures. This study explores the diversity of fungal species in soil samples collected from Anjur Reserve Forest, Chengalpattu District of the State of Tamil Nadu, falls under the East Deccan Dry Evergreen Ecoregion. This makes Anjur a critical, yet understudied, microcosm of Eastern Ghats biodiversity (Ritz and Young, 2004; Devi et al., 2020; Ramachandran et al., 2020). The Eastern Ghats are characterized by varied terrain and forest types, and the Anjur Reserve Forest is a part of this complex ecosystem, represents a unique and valuable ecosystem, Despite the ecological importance of Anjur Reserve Forest, there is a notable absence of comprehensive studies on its soil microbial communities.

Forest soil fungal diversity is crucial for ecosystem health, driving decomposition and nutrient cycling by breaking down organic matter and releasing vital elements (Luo et al., 2021; Djiemel et al., 2021). Furthermore, Mycorrhizal fungi form symbiotic relationships with plants, enhancing water and nutrient uptake, while fungal hyphae improve soil structure and reduce erosion (Li et al., 2022). Diverse fungal communities ensure ecosystem resilience, with each species contributing unique functions, maintaining overall stability and health (Wagg et al., 2019). Traditional fungal culturing methods present significant limitations, hindering our ability to fully grasp the complexity of soil fungal diversity. Issues like media

selection, varying environmental requirements, and the difficulty in identifying fungi based solely on morphology create substantial obstacles (Nam et al., 2023).

Metabarcoding provides a culture-independent approach, allowing for the comprehensive analysis of even rare and unculturable fungal species, thus revealing the true extent of soil fungal diversity and their functional roles within the ecosystem (Bahram et al., 2021; Wu et al., 2023). Molecular taxonomy, particularly High-throughput next-generation sequencing (NGS) of the fungal internal transcribed spacer (ITS) region, specifically ITS3 and ITS4, has become a reliable alternative to traditional methods for assessing fungal diversity in various environments (Martin et al., 2005). This method exploits variable sequences among species, significantly enhancing our ability to assess fungal diversity (Garlapati et al., 2019).

The presence of humic acids and phenolic compounds in the soil can interfere with both the quality and quantity of template DNA, which plays a crucial role in the amplification of barcoding genes (Guerra et al., 2020). Therefore, robust DNA extraction methods, such as the cetyl trimethylammonium bromide (CTAB) and polyvinylpyrrolidone (PVP) method, are essential for removing these inhibitory compounds, resulting in better quality and higher yields of intact DNA (Kachiprath et al., 2018). This study aimed to characterize the fungal diversity within the soils of Anjur Reserve Forest using high-throughput sequencing of the fungal internal transcribed spacer ITS region, to establish a critical baseline for future ecological studies and conservation efforts (Sommermann et al., 2018), this research provides a baseline understanding of the fungal biodiversity within this important forest ecosystem, contributing to the knowledge base on the ecological significance of fungi in this unique environment.

Materials and Methods

Soil Sampling

Soil samples were collected from different zones of Anjur reserve forest area in the Chengalpattu district of Tamil Nadu, India (coordinates: 12.718349° N 80.041825° E, - Figure 1). Nine soil samples were collected by following standard guidelines (approximately 5 cm depth and 250 g each). Equal amounts of soil, free of stones and dust particles, were mixed into a single sterile airtight plastic bag and transported to the laboratory and processed immediately.

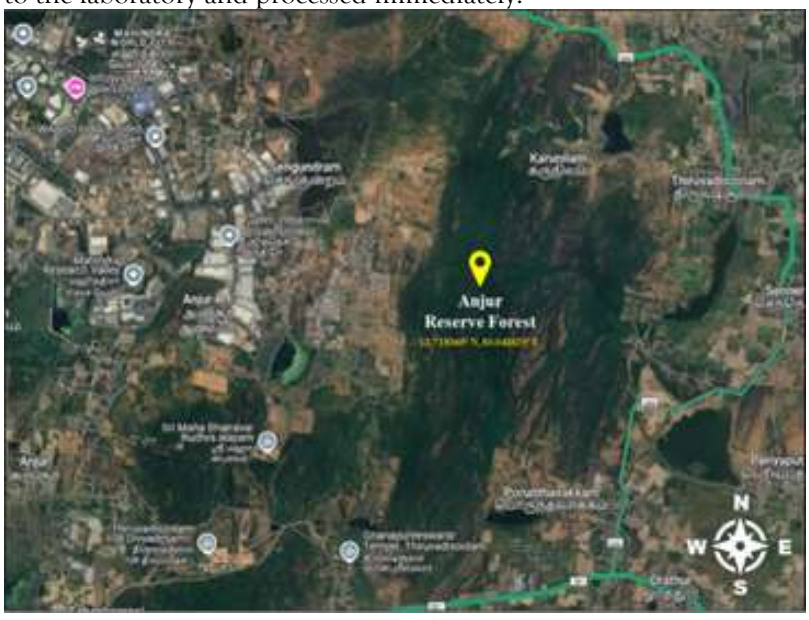

Figure 1: Anjur Reserve Forest Google Map view

Total DNA extraction and ITS region amplification

From the mixture a gram of soil was taken in triplicates in 2 ml autoclaved tubes and resuspended in 500 µl of Tris-EDTA (TE) buffer (pH 8.0) (Sigma-Aldrich,Missouri, USA). The samples were vortexed at 2400 rpm for 2 minutes and followed CTAB method for environmental DNA (eDNA) extraction. The quality of the purified eDNA was assessed using 1.5% agarose gel electrophoresis, while the quantity was measured with Invitrogen Qubit DNA BR Assay (Carlsbad, CA). The purified eDNA samples were

normalized to equal concentrations and pooled into a single vial. The ITS2 region was amplified via Polymerase Chain Reaction (PCR) using validated primers: forward ITS3 (5' GCATCGATGAAGAACGCAGC 3') and reverse ITS4 (5' TCCTCCGCTTATTGATATGC 3'). The purified amplicon product was quantified using the Qubit DNA BR Assay (Carlsbad, CA), and its quality was assessed using the Agilent Technologies 4200 TapeStation (Singapore).

NGS Library preparation and Sequencing

The purified amplicons from each sample were pooled into a single tube to achieve equimolar concentrations and then used for NGS library preparation. The library was prepared with unique dual indexing adapters using the KAPA HyperPrep kit (Hoffmann-La Roche Ltd) according to the manufacturer's protocol. The purified libraries were diluted and then submitted for NGS on the Illumina NextSeq (San Diego, CA).

Data analysis and fungal identification

The demultiplexed sequences were imported to the QIIME2 Amplicon tool (https://qiime2.org/) for bioinformatics analyses. Chimeric filtering and sequence truncation were conducted with the qiime2-dada2 plugin. Taxonomic assignments were determined for amplicon sequence variants (ASVs/ OTUs) using qiime2-feature-classifier classify-sklearn against the UNITE fungal ITS database version 10.15156 and trained with Naive Bayes classifier and a confidence threshold of 99%.

Factors such as extraction methods, PCR variability, and primer biases can influence read counts, potentially impacting abundance estimates. However, these biases generally do not disrupt the correlation between read counts and cell abundance, implying that higher read counts indicate higher abundance. Consequently, we used the number of reads as an indicator of relative abundance for comparative purposes.

Fungal diversity

The statistical analysis of species diversity was calculated using Shannon alpha diversity and Pielou's evenness index calculated using QIIME (Feranchuk et al., 2018; McPhersen et al., 2018). Fungal taxa present in the soil were calculated based on the sequencing reads of each OTU. The sequence reads of each OTU are considered more than 1000 as dominant, between 100 to 1000 as moderate, below 100 reads minor and below 10 reads are rare taxon.

Functional Annotation

For the identified genera/species further analysis was conducted to assign putative functional roles and biological characteristics. This involved the following steps:

Functional Role Assignment: Putative primary trophic roles (saprotroph, pathogenic, mycorrhizal, endophytic, and lichenized fungi) were assigned to each identified taxon using the FUNGuild database.

Cellularity Determination: The cellularity of each taxon (unicellular or multicellular) was determined based on information available in the MycoBank and Index Fungorum databases.

Micro- and Macrofungi Classification: Taxa were broadly classified as micro- or macrofungi based on their typical size and visibility as described in mycological literature. Microfungi were defined as those primarily existing as microscopic mycelia or forming microscopic fruiting bodies, while macrofungi were defined as those forming macroscopic fruiting bodies.

Mode of Reproduction Assessment: Information regarding the mode of reproduction (including both asexual and sexual mechanisms, e.g., conidia, ascospores, basidiospores) for each taxon was compiled from mycological literature and databases. For taxa where the mode of reproduction was not well-established or information was limited, this was noted."

Results

Fungal Taxonomy

Analysis of soil fungi from the Anjur reserve forest area revealed 1,729 features with 195,908 frequencies of reads (Figure-2) and 151 amplicon sequence variants (ASVs) (Supplementary table-1). At the phylum level, the community was dominated by Ascomycota, Basidiomycota and Glomeromycota (Figure-3). Notably, the less abundant phyla Chytridiomycota, Mortierellomycota, and Mucoromycota were also present at moderate levels. The most dominant identified ASV at the genus/species level was Asterostroma sp., with 18,654 reads. Additionally, Muyocopron ficinum, Phaeobotryon mamane, and

Verrucaria each yielded over 1,000 reads. In total, ten ASVs had more than 1,000 reads and twenty-seven ASVs had more than 100 reads when compared with the UNITE database. A substantial number of ASVs were only identifiable at the kingdom level (Fungi, 84,338 reads) or phylum level (12,118 reads), suggesting the potential presence of novel fungal taxa and new records for the East Deccan Dry Evergreen Forest ecoregion.

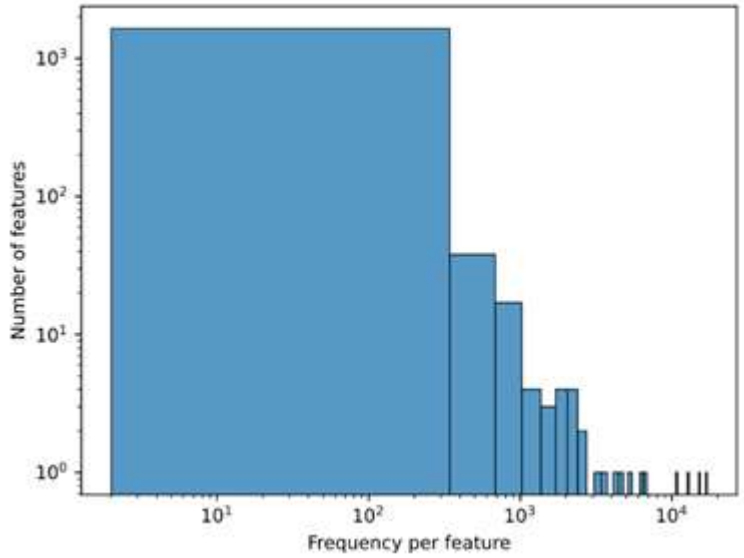

Figure-2: Total number of features with the sequence frequency

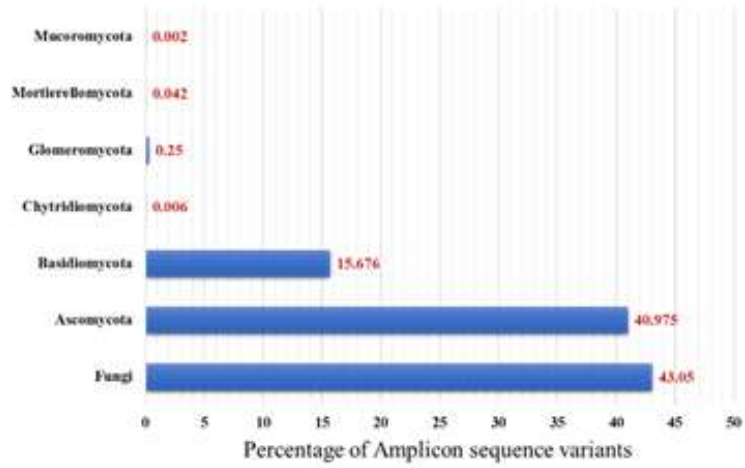

Figure-3: Percentage of fungal amplicon sequence variants (ASVs) at phylum level identified from soil of Anjur reserve forest

Fungal diversity

The Alpha rarefaction curve suggests a good representation of the present diversity. The Shannon diversity index value of 6.86 (Figure-4a) and Pielou's evenness index value of 0.7958 indicate a relatively high level of species diversity and even distribution within the Anjur forest soil. These observations align with the sufficient sequencing depth achieved across fifteen rarefaction steps (Figure-4b). From the identified 151 Amplicon Sequence Variants (ASVs), 78 taxa were classified at the species or genus level, and the corresponding sequences have been submitted to NCBI, receiving the accessions listed in Table-1.

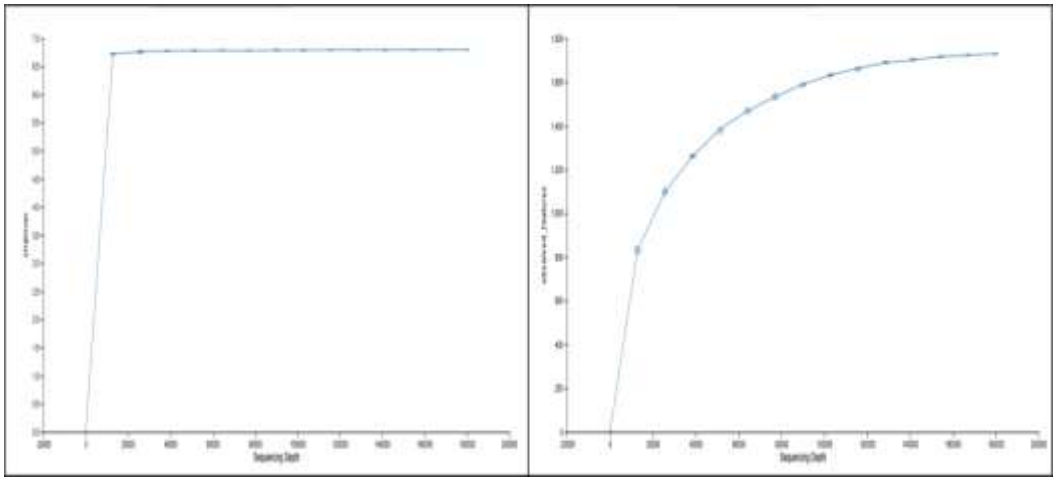

Figure-4: (a) Shannon diversity index (b) observed features with the sequence depth plotted using QIIME2

Table-1: Identified Genera/Species with NCBI Accession ID

S.No	Genera/Species	NCBI Accession ID
1	*Aaosphaeria arxii*	PQ536616
2	*Allocanariomyces tritici*	PQ536617
3	*Arxiella celtidis*	PQ536618
4	*Aspergillus glabripes*	PQ536619
5	*Aspergillus sp.*	PQ536620
6	*Botryosphaeria corticis*	PQ536621
7	*Candida parapsilosis*	PQ536622
8	*Cephaliophora tropica*	PQ536623
9	*Coniella sp.*	PQ536624
10	*Coniochaeta prunicola*	PQ536625
11	*Crinipellis brunnescens*	PQ536626
12	*Crinipellis sp.*	PQ536627
13	*Cryptocoryneum japonicum*	PQ536628
14	*Curvularia tsudae*	PQ536629
15	*Dentiscutata savannicola*	PQ536630
16	*Dentiscutata sp.*	PQ536631
17	*Diaporthe sp.*	PQ536632
18	*Entoloma sp.*	PQ536633
19	*Exserohilum sp.*	PQ536634
20	*Fusarium sp.*	PQ536635
21	*Fusarium tonkinense*	PQ536636
22	*Glomus sp.*	PQ536637
23	*Gymnopilus ochraceus*	PQ536638
24	*Gymnopilus sp.*	PQ536639
25	*Helminthosporium genistae*	PQ536640
26	*Helminthosporium sp.*	PQ536641
27	*Helminthosporium velutinum*	PQ536642
28	*Humicola sp.*	PQ536643
29	*Linnemannia hyalina*	PQ536644
30	*Lycoperdon rupicola*	PQ536645
31	*Malassezia sp.*	PQ536646
32	*Microascus chinensis*	PQ536647
33	*Muyocopron ficinum*	PQ536648
34	*Neocucurbitaria ribicola*	PQ536649

35	*Ossicaulis lignatilis*	PQ536650
36	*Penicillium sp.*	PQ536651
37	*Periconia macrospinosa*	PQ536652
38	*Phaeobotryon mamane*	PQ536653
39	*Phaeopezia calongei*	PQ536654
40	*Phaeosphaeriopsis glaucopunctata*	PQ536655
41	*Phomatospora dinemasporium*	PQ536656
42	*Pichia mandshurica*	PQ536657
43	*Poaceascoma lochii*	PQ536658
44	*Preussia sp.*	PQ536659
45	*Pseudothielavia arxii*	PQ536660
46	*Rhizophagus sp.*	PQ536661
47	*Robillarda sp.*	PQ536662
48	*Roussoella mediterranea*	PQ536663
49	*Saitozyma sp.*	PQ536664
50	*Seiridium marginatum*	PQ536665
51	*Stropharia rugosoannulata*	PQ536666
52	*Sublophiostoma thailandicum*	PQ536667
53	*Talaromyces sp.*	PQ536668
54	*Truncospora macrospora*	PQ536669
55	*Verrucaria sp.*	PQ536670
56	*Westerdykella ornata*	PQ536671
57	*Antella niemelaei*	PQ561782
58	*Anthostomella lamiacearum*	PQ561783
59	*Archaeorhizomyces sp.*	PQ561784
60	*Aspergillus clavatophorus*	PQ561785
61	*Ceratobasidium sp.*	PQ561786
62	*Chlorociboria argentinensis*	PQ561787
63	*Cortinarius rubrophyllus*	PQ561788
64	*Cunninghamella blakesleeana*	PQ561789
65	*Kiskunsagia ubrizsyi*	PQ561790
66	*Melanconiella sp.*	PQ561791
67	*Vararia breviphysa*	PQ586975
68	*Notholepista fistulosa*	PQ586976
69	*Clitocybula lignicola*	PQ586977
70	*Asterostroma sp.*	PQ589725
71	*Serusiauxiella sp.*	PQ589726
72	*Tylospora asterophora*	PQ589727
73	*Mortierella sp.*	PQ589728
74	*Macrophomina phaseolina*	PQ589729
75	*Gymnopilus maritimus*	PQ589730
76	*Buergenerula spartinae*	PQ589731
77	*Cortinarius sp.*	PQ589732
78	*Curvularia sp.*	PQ589733

The identified species exhibit diverse trophic roles, categorized as saprotrophic (47), pathogenic (17), mycorrhizal (6), endophytic (7), and lichenized (1). Most of these fungi are multicellular. However, notable exceptions include the unicellular yeasts *Saitozyma*, *Candida parapsilosis*, and *Pichia mandshurica*, found within the saprotrophic and plant pathogen groups.

Morphologically, the identified fungi range from microscopic forms to macroscopic structures such as mushrooms and visible fruit bodies. Interestingly, some fungi with names suggesting large structures

(macrospora) can still be inconspicuous. Saprotrophs primarily utilize ascospores and basidiospores for reproduction, with asexual methods also being common. Plant pathogens frequently reproduce asexually via conidia, although some also produce sexual ascospores. In contrast, mycorrhizal, endophytic, and lichenized fungi predominantly rely on sexual spores like basidiospores, zygospores, or ascospores, with asexual reproduction also observed. The assigned trophic roles, cellularity, micro/macro classification, and mode of reproduction were compiled into Table-2 with their corresponding phylum. This allowed for a comprehensive overview of the functional and biological diversity of the soil fungal community in the Anjur Reserve Forest.

Table-2: Assigned trophic roles, cellularity, micro/Macro classification and mode of reproduction for the identified genera/species from the Anjur Reserve Forest

S.No	Taxa	Phylum	Trophic roles	Uni/Multicellular	Micro/Macro fungi	Mode of Reproduction
	Saprotrophs (Decomposers)-nutrient cycling					
1	*Asterostroma sp.*	Basidiomycota	saprophytic	Multicellular	Micro-small and inconspicuous	Ascospores
2	*Muyocopron ficinum*	Ascomycota	saprophytic	Multicellular	Micro-Forms small, black ascomata on leaves	Ascospores
3	*Ossicaulis lignatilis*	Basidiomycota	saprophytic	Multicellular	Small mushrooms, Inconspicuous	Basidiospores
4	*Cryptocoryneum japonicum*	Ascomycota	saprophytic	Multicellular	Forms small, dark structures	Ascospores
5	*Humicola sp.*	Ascomycota	saprophytic	Multicellular	Micro	Asexual, producing conidia
6	*Antella niemelaei*	Basidiomycota	saprophytic	Multicellular	considered microscopic to small macroscopic	Ascospores
7	*Penicillium sp.*	Ascomycota	saprophytic	Multicellular	Micro	Asexual, producing conidia; some species have known sexual stages in genera like Eupenicillium, Talaromyces
8	*Poaceascoma lochii*	Ascomycota	saprophytic	Multicellular	Forms small ascomata on grasses	Ascospores
9	*Roussoella mediterranea*	Ascomycota	saprophytic	Multicellular	Forms small ascomata on plant material	Ascospores
10	*Dentiscutata savannicola*	Glomeromycota	saprophytic	Multicellular	Micro	Ascospores
11	*Truncospora macrospora*	Basidiomycota	saprophytic	Multicellular	While it has "macrospora," the fruit body is a poroid crust, often inconspicuous	Basidiospores
12	*Serusiauxiella sp.*	Ascomycota	saprophytic	Multicellular	Forms small, often lichenicolous structures	Ascospores
13	*Aspergillus sp.*	Ascomycota	saprophytic	Multicellular	Micro	Asexual, producing conidia; some species have

						known sexual stages in genera like Emericella, Eurotium, etc
14	*Aspergillus clavatophorus*	Ascomycota	saprophytic	Multicellular	Micro	Asexual, producing conidia
15	*Gymnopilus sp.*	Basidiomycota	saprophytic	Multicellular	Mushrooms, macroscopic	Basidiospores
16	*Stropharia rugosoannulata*	Basidiomycota	saprophytic /edible mushroom	Multicellular	Mushrooms, macroscopic	Basidiospores
17	*Preussia sp.*	Ascomycota	saprophytic /association with animal dung	Multicellular	Forms small ascomata	Ascospores
18	*Gymnopilus ochraceus*	Basidiomycota	saprophytic	Multicellular	Mushrooms, macroscopic	Basidiospores
19	*Dentiscutata sp.*	Glomeromycota	saprophytic	Multicellular	Micro	Ascospores
20	*Entoloma sp.*	Basidiomycota	saprophytic	Multicellular	Mushrooms, macroscopic	Ascospores
21	*Periconia macrospinosa*	Ascomycota	saprophytic	Multicellular	Micro	Primarily asexual via conidia; sexual stage unknown or rare
22	*Lycoperdon rupicola*	Basidiomycota	saprophytic	Multicellular	Macro	Basidiospores
23	*Westerdykella ornata*	Ascomycota	saprophytic	Multicellular	Micro	Ascospores
24	*Mortierella sp.*	Mortierellomycota	saprophytic	Multicellular	Micro	Zygospores
25	*Melanconiella sp.*	Ascomycota	saprophytic	Multicellular	Forms small stromata	Ascospores
26	*Microascus chinensis*	Ascomycota	saprophytic	Multicellular	Micro	Ascospores
27	*Pseudothielavia arxii*	Ascomycota	saprophytic	Multicellular	Micro	Ascospores
28	*Gymnopilus maritimus*	Basidiomycota	saprophytic	Multicellular	Mushrooms, macroscopic	Basidiospores
29	*Notholepista fistulosa*	Basidiomycota	saprophytic	Multicellular	Small mushrooms, Inconspicuous	Basidiospores
30	*Archaeorhizomyces sp.*	Ascomycota	Undefined Saprotroph (mycorrhizal or mycorrhiza-like interactions)	Multicellular	Micro	Sexual reproduction not yet observed, presumed to be ascomycete-like
31	*Aaosphaeria arxii*	Ascomycota	saprophytic	Multicellular	Micro	Ascospores
32	*Linnemannia hyalina*	Mortierellomycota	saprophytic	Multicellular	Micro	Zygospores
33	*Talaromyces sp.*	Ascomycota	saprophytic	Multicellular	Micro	Ascospores
34	*Clitocybula lignicola*	Basidiomycota	saprophytic	Multicellular	Small mushrooms, borderline but often	Basidiospores

					considered small macroscopic	
35	*Buergenerula spartinae*	Ascomycota	saprophytic	Multicellular	Micro	Ascospores
36	*Aspergillus glabripes*	Ascomycota	saprophytic	Multicellular	Micro	Asexual, producing conidia
37	*Coniochaeta prunicola*	Ascomycota	saprophytic	Multicellular	Micro	Ascospores
38	*Phaeopezia calongei*	Ascomycota	saprophytic	Multicellular	Small, cup-shaped ascomycete	Ascospores
39	*Saitozyma sp.*	Basidiomycota	Undefined Saprotroph	Unicellular	Micro	Asexual budding; some species can form basidiospores
40	*Anthostomella lamiacearum*	Ascomycota	saprophytic	Multicellular	Micro	Ascospores
41	*Chlorociboria argentinensis*	Ascomycota	saprophytic	Multicellular	fruiting body is visible, the main organism is the microscopic mycelium	Ascospores
42	*Vararia breviphysa*	Basidiomycota	saprophytic	Multicellular	Forms small, resupinate fruit bodies	Basidiospores
43	*Cephaliophora tropica*	Ascomycota	saprophytic	Multicellular	Micro	Sexual reproduction not well-documented, primarily known for asexual conidia
44	*Cunninghamella blakesleeana*	Mucoromycota	saprophytic	Multicellular	Micro	Ascospores
45	*Crinipellis brunnescens*	Basidiomycota	saprophytic	Multicellular	Small mushrooms, borderline	Ascospores
46	*Robillarda sp.*	Ascomycota	saprophytic	Multicellular	Forms small pycnidia	Primarily asexual via conidia; sexual stage unknown or rare
47	*Crinipellis sp.*	Basidiomycota	saprophytic	Multicellular	Small mushrooms, borderline	Ascospores
Pathogenic fungi						
48	*Macrophomina phaseolina*	Ascomycota	plant pathogen-causing charcoal rot	Multicellular	Forms microsclerotia	Primarily asexual via sclerotia and pycnidia producing conidia; sexual stage known as Macrophomops is phaseolina producing ascospores
49	*Helminthosporium sp.*	Ascomycota	plant pathogens-	Multicellular	Micro	Primarily asexual via

			causing leaf spots and blights			conidia; sexual stages in Cochliobolus or Pyrenophora
50	*Fusarium sp.*	Ascomycota	causing wilts, rots, and other diseases	Multicellular	Micro	Primarily asexual via conidia; many have known sexual stages in Gibberella or related genera
51	*Phaeosphaeriopsis glaucopunctata*	Ascomycota	cause leaf spot and necrosis	Multicellular	Forms small pycnidia	Ascospores
52	*Botryosphaeria corticis*	Ascomycota	Causes cankers on woody plants	Multicellular	mycelium is microscopic	Ascospores
53	*Curvularia sp.*	Ascomycota	plant pathogens	Multicellular	Micro	Asexual, producing conidia; sexual stage known in some species as Cochliobolus
54	*Neocucurbitaria ribicola*	Ascomycota	cause cankers on Ribes species	Multicellular	Forms small, black pseudothecia on branches	Ascospores
55	*Seiridium marginatum*	Ascomycota	canker-causing pathogen of conifers	Multicellular	Forms small acervuli	Primarily asexual via conidia; sexual stage unknown or rare
56	*Diaporthe sp.*	Ascomycota	causing cankers and dieback	Multicellular	Mycelium is microscopic, forms small pycnidia	Ascospores
57	*Exserohilum sp.*	Ascomycota	leaf spot and bligh	Multicellular	Micro	Primarily asexual via conidia; sexual stage known in some species as Setosphaeria
58	*Fusarium tonkinense*	Ascomycota	causing wilts, rots, and other diseases	Multicellular	Micro	Primarily asexual via conidia; sexual stage known as Gibberella
59	*Helminthosporium velutinum*	Ascomycota	plant pathogen	Multicellular	Micro	Primarily asexual via conidia; sexual stages in Cochliobolus or Pyrenophora
60	*Curvularia tsudae*	Ascomycota	plant pathogens	Multicellular	Micro	Asexual, producing conidia; sexual stage known in some species as Cochliobolus

61	*Helminthosporium genistae*	Ascomycota	Pathogen specific to Genista plants	Multicellular	Micro	Primarily asexual via conidia; sexual stages in Cochliobolus or Pyrenophora
62	*Malassezia sp.*	Basidiomycota	animal commensals and pathogens, their presence in soil might be due to shedding	Multicellular	Yeast	Asexual budding
63	*Candida parapsilosis*	Ascomycota	opportunistic pathogen in animals	Unicellular	Micro	Asexual budding
64	*Pichia mandshurica*	Ascomycota	opportunistic pathogen in animals	Unicellular	Micro	Asexual budding; some species can form ascospores
Mycorrhizal Fungi-nutrient uptake						
65	*Tylospora asterophora*	Basidiomycota	Ectomycorrhizal	Multicellular	Forms small, resupinate fruit bodies	Basidiospores
66	*Rhizophagus sp.*	Glomeromycota	Arbuscular mycorrhizal fungus	Multicellular	Mycorrhizal fungi, microscopic structures in roots	Primarily sexual reproduction via zygospores
67	*Glomus sp.*	Glomeromycota	Arbuscular mycorrhizal fungus	Multicellular	Micro	Asexual reproduction via spores is dominant; sexual reproduction is rare or unknown
68	*Ceratobasidium sp.*	Basidiomycota	orchid mycorrhizal	Multicellular	microscopic hyphal sheaths	Basidiospores
69	*Cortinarius rubrophyllus*	Basidiomycota	Ectomycorrhizal	Multicellular	Mushrooms, macroscopic	Basidiospores
70	*Cortinarius sp.*	Basidiomycota	Ectomycorrhizal	Multicellular	Mushrooms, macroscopic	Basidiospores
Endophytes-provide benefits to the host plant						
71	*Phaeobotryon mamane*	Ascomycota	endophytic	Multicellular	Forms small, black ascomata on leaves	Ascospores
72	*Coniella sp.*	Ascomycota	endophytic	Multicellular	Micro	Asexual, producing conidia
73	*Phomatospora dinemasporium*	Ascomycota	endophytic	Multicellular	Forms small perithecia	Ascospores
74	*Sublophiostoma thailandicum*	Ascomycota	endophytic	Multicellular	Forms small ascomata	Ascospores
75	*Arxiella celtidis*	Ascomycota	endophytic	Multicellular	Micro	Ascospores

76	*Allocanariomyces tritici*	Ascomycota	endophytic	Multicellular	Micro	Asexual, producing conidia
77	*Kiskunsagia ubrizsyi*	Ascomycota	endophytic (Symbiotroph)	Multicellular	Micro	Ascospores
Lichenized Fungi (lichens with algae or cyanobacteria)						
78	*Verrucaria sp.*	Ascomycota	lichen-forming fungi	Multicellular	Lichens with crustose thalli, often appearing as colored stains on surfaces	Ascospores within perithecia

DISCUSSION

Fungal taxonomy and diversity

This research, employing amplicon sequencing of environmental DNA (eDNA), explored the fungal diversity within the soil of the Anjur Reserve Forest, India, with a focus on characterizing the taxonomic composition of the fungal community. Traditional culturing methods often fail to capture the substantial proportion (70-90%) of soil fungi. While many soil fungal studies have historically concentrated on cultivable diversity, metabarcoding offers a more comprehensive view, revealing the diversity of both culturable and non-culturable taxa. The high proportion of classified amplicon sequence variants (ASVs) assigned solely to the kingdom Fungi suggests the presence of numerous currently unrecognized fungal taxa within this ecosystem.

Analysis of read abundance indicated that Ascomycota was the dominant phylum, followed by Basidiomycota, Glomeromycota, Mortierellomycota, Chytridiomycota, and Mucoromycota. Within these assemblages, the genera *Asterostroma*, *Muyocopron*, *Phaeobotryon*, and *Verrucaria* exhibited relative abundance. Notably, *Asterostroma* was highly abundant, yet species-level identification within this genus was limited (Muller et al., 2000; Suhara et al., 2010), suggesting at the potential presence of novel *Asterostroma* species, possibly new to science.

The dominance of *Asterostroma* suggests its potentially crucial role in the soil ecosystem of the Anjur Reserve Forest. *Muyocopron ficinum* is a relatively understudied species with limited information available in the scientific literature (Tennakoon et al., 2021; Ferro et al., 2023), underscoring a significant knowledge gap. The presence of *Phaeobotryon mamane*, an endophytic and plant pathogenic fungus, in the soil could originate from fallen leaves or plant parts, or it may persist in the soil as spores, mycelial fragments, or even as residual DNA after the organism is no longer viable.

The identification of Acarosporaceae, a lichen family commonly found in rocky and soil-based environments, particularly in mountainous regions, highlights the potential contribution of these organisms to ecosystem processes such as soil formation and nutrient cycling (Park et al., 2023).

A higher Shannon index signifies a greater variety of species and a more even distribution of their abundances. In ecological terms, this often suggests a more stable and complex ecosystem (Gorelick, 2006). The presence of a high percentage of unidentified sequences highlights the potential for novel fungal discoveries within this ecosystem. This underscores the need for further taxonomic and phylogenetic studies to accurately identify these unknown taxa and expand our understanding of fungal diversity in the Anjur Forest. The role of individual fungal species focusing on the functional role of these fungal communities across different environmental contexts gains deeper understanding of their potency in ecosystem management and restoration. Studying the diversity of fungi from the ecoregion, i.e. East Deccan Dry Evergreen Forest using a greater number of samples provide the significance of the fungi in detail in this unique ecoregion.

Functional Annotation

The dominant saprotrophic community, primarily composed of multicellular Ascomycota and Basidiomycota, plays a vital role in decomposition and nutrient cycling. This group encompasses a range of sizes, from microscopic molds like *Penicillium* and *Aspergillus* that reproduce both asexually via conidia and sexually via ascospores, to small macroscopic fungi like *Ossicaulis lignatilis* and inconspicuous crust-

like forms such as *Truncospora macrospora*, which reproduce via basidiospores. Notably, the potentially novel *Asterostroma* species, a multicellular basidiomycete with a likely basidiospore-based reproduction, exhibited high abundance within this saprotrophic assemblage.

Beyond decomposition, the soil harbors a suite of pathogenic fungi, predominantly multicellular ascomycetes. These microfungi employ diverse reproductive strategies, often with an emphasis on asexual dispersal through conidia, as seen in *Fusarium* and *Curvularia*, while also possessing sexual stages resulting in ascospore formation. The presence of both unicellular yeasts like *Malassezia* and *Candida*, reproducing via budding, and filamentous pathogens underscores the varied life forms contributing to potential plant and animal interactions within the ecosystem.

The presence of mycorrhizal fungi, all multicellular, highlights their crucial role in plant nutrient acquisition. The ectomycorrhizal basidiomycetes, such as *Tylospora asterophora* and *Cortinarius*species (macroscopic mushrooms), reproduce via basidiospores and establish symbiotic relationships with plant roots. Similarly, arbuscular mycorrhizal glomeromycetes like *Rhizophagus* and *Glomus* (microscopic root colonizers) predominantly reproduce asexually through spores, facilitating nutrient exchange.

Endophytic fungi, primarily multicellular ascomycetes, represent another significant functional group. These microfungi, exemplified by *Phaeobotryon mamane* and *Coniella*, exhibit varied reproductive modes, including both ascospore and conidia production, and are known to provide benefits to their host plants.

Finally, the detection of the lichenized fungus *Verrucaria*, a multicellular ascomycete forming macroscopic crustose thalli, indicates the presence of symbiotic relationships with algae or cyanobacteria, contributing to unique microhabitats within the soil environment through ascospore dispersal. This functional and structural diversity underscores the intricate ecological network within the Anjur Reserve Forest soil.

CONCLUSION

eDNA analysis unveiled a rich fungal diversity in Anjur Reserve Forest, dominated by Ascomycota and Basidiomycota, with the rarer phyla Chytridiomycota and Mucoromycota also present. Genera like Asterostroma were abundant, hinting at potential novel species. The high number of unclassified sequences suggests significant undiscovered fungal diversity. Saprotrophs dominated, indicating crucial roles in decomposition and nutrient cycling. The presence of pathogenic fungi, potentially influenced by animal shedding, further illustrates the ecosystem's complexity. Mycorrhizal and endophytic fungi also contribute to this intricate web. The presence of lichenized fungi adds to this ecological diversity. A high Shannon index points to a stable ecosystem. Understanding these fungal roles is vital for ecosystem management. Long-term studies are required for further in-depth analysis of these fungal communities.

Author Contributions

The authors confirm contribution to the paper is as follows: study conceptualization and design: Udaya Prakash NK, Bhuvaneswari S; data collection: Sivaraj I, Madhankumar D; analysis and interpretation of results: Sivaraj I, Madhankumar D, Sripriya NS; draft manuscript preparation: Sivaraj I, Madhankumar D, Sripriya NS. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of Interests

The authors declare that they have no conflict of interest

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