Metagenomic profiling of fungal diversity in avian-influenced freshwaters of vedanthangal bird sanctuary

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Abstract

The microbiome of guano in water bodies includes microbes linked to pathogens, disease transmission, and ecological interactions with soil and water. This study examines the diversity and composition of fungal communities (mycobiomes) in the freshwater ecosystem of Vedanthangal Bird Sanctuary, originating from bird droppings, especially those of migratory species. Metagenomic analysis using Illumina sequencing identified 114,020 and 226,680 reads from two water samples. Among these microbes, fungi were dominant, making up about 64% of the reads, followed by Viridiplantae (32%), Stramenopiles (0.9%), Ichthyosporea (0.7%), and Metazoa (0.6%). In the water of the bird sanctuary, the phylum Ascomycota (43%) appears to play a significant role in organic matter decomposition and nutrient cycling. Basidiomycota (18%) may assist in lignin breakdown and help maintain ecological balance. Rozellomycota (16%), often symbiotic or parasitic, could influence microbial interactions in aquatic environments. However, 1,269 organisational taxonomic units (OTUs) remained unidentified. This aquatic ecosystem hosts a diverse array of fungi. This is the first study from this sanctuary to profile fungal communities through Illumina sequencing. It offers new insights into the variety of fungal populations and their potential ecological roles within the sanctuary. The findings suggest fungal communities in aquatic environments are more complex and diverse than previously recognised, with various phyla contributing to key ecological processes.

Keywords: Fungal metagenomics, Bird Sanctuary, ITS region, Water quality parameters, DNA

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Introduction

Fungi are indispensable components of ecosystems, fulfilling diverse ecological roles such as decomposing organic matter, forming mutualistic relationships, acting as pathogens, and contributing significantly to nutrient cycling in soils (Kendrick, 2000; Jones, 2011). Their ability to synthesize a wide array of secondary metabolites also makes them crucial for pharmaceutical innovations and industrial bioprocesses. Despite estimates suggesting that the global fungal diversity may exceed five million species, a considerable proportion, especially those inhabiting freshwater systems, remains uncharacterized (Lofgren and Stajich, 2021; Paterson *et al*., 2023). Advancements in molecular tools, particularly high-throughput sequencing technologies, have transformed fungal systematics and improved our ability to detect, identify, and classify previously unknown fungal taxa. These methods have enhanced our understanding of fungal ecological roles, including their contributions to biogeochemical cycles and food webs, which underscores their functional importance in both terrestrial and aquatic environments (Gadd, 2007). Fungi in terrestrial systems account for a substantial share of microbial biomass and diversity (Lawley *et al*., 2004; Richards and Bass, 2005), yet equivalent studies in aquatic habitats—especially ornithogenic wetlands are limited. Existing literature shows that DNA sequencing has become the cornerstone of modern mycology, enabling rapid discovery and taxonomic reclassification. Thousands of new fungal species are being described annually, a trend largely driven by the increasing reliance on molecular tools for species delineation (Miralles *et al*., 2020; Cheek *et al*., 2020; Lofgren and Stajich, 2021). The ecological interplay between migratory waterbirds and aquatic environments adds another dimension to microbial diversity. As these birds traverse multiple regions, they may encounter and carry microbial pathogens—including fungi and antibiotic-resistant bacteria—which can subsequently be deposited into water bodies through their droppings (Rashid *et al*., 2015; Islam *et al*., 2021). Prior investigations have established the role of migratory birds in disseminating multidrug-resistant (MDR) microorganisms across aquatic habitats (Najdenski *et al*., 2018; Ramey *et al*., 2018; Lin *et al*., 2020). Despite these concerns, there is a noticeable lack of research on fungal populations within bird sanctuary water bodies. This study aims to address this gap by characterizing fungal communities in the tank ecosystem of Vedanthangal Bird Sanctuary using metagenomic tools. By doing so, it not only contributes to our understanding of fungal diversity in freshwater environments but also assesses their potential as bioindicators for environmental health and water quality monitoring.

Materials and methods

Detailed protocols, including primer sequences, PCR conditions, library preparation steps, and sequencing

parameters, are provided to ensure reproducibility. All reagents and instruments are specified to facilitate replication.

Study area

Vedanthangal Bird Sanctuary, established in 1936, is the oldest wildlife sanctuary in India. Located in Maduranthagam Taluk, Chengalpattu District, Tamil Nadu (12.5657° N, 79.8359° E), it was designated a Ramsar site in 2022 and is recognized internationally as an Important Bird and Biodiversity Area (IBA) (Fig. 1). The sanctuary comprises a primary irrigational tank interconnected with several smaller water bodies, supporting both migratory and resident bird populations. The surrounding landscape is largely flat, with low rocky ridges and denuded hillocks. Soil in the tank is clayey with a fertile guano layer. The marshy wetlands and lush green banks attract large flocks of birds, with surveys recording approximately 50,000 birds during the migratory season annually.

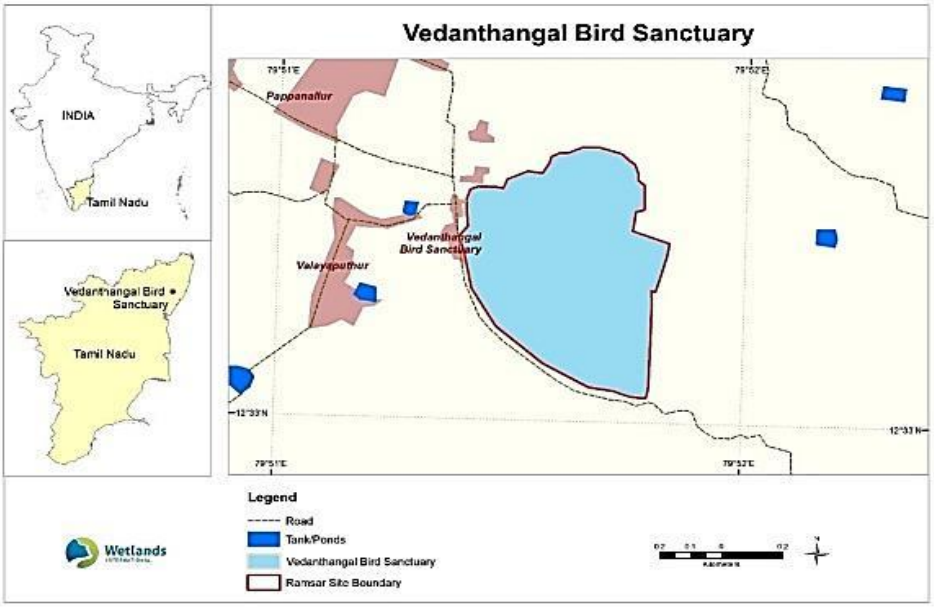

Figure1: Eographical location of Vedanthangal Bird Sanctuary in Tamil Nadu.

Sampling

Sampling was conducted in January 2025 across multiple locations within the sanctuary, capturing varied habitat features. Water samples were collected randomly, and approximately 1 L of semi-solid soil was collected in sterile containers to prevent contamination. DNA extraction was performed, quantified using a Qubit® 4.0 fluorometer, and amplified with ITS1 and ITS4 primers. Gel electrophoresis confirmed the presence of fungal DNA.

DNA isolation and quantification

DNA concentration and quality were assessed using a Qubit® 4.0 fluorometer. ITS-specific primers (ITS1 and ITS4) were used to amplify fungal DNA, and the resulting amplicons were analyzed by

gel electrophoresis. Library preparation followed the Illumina Nextera XT protocol, targeting the ITS2 hypervariable region using ITS2-specific primers. Purification of amplicons was performed using AmPure XP beads. Indexed amplicons were assessed using an Agilent TapeStation 4150 with High Sensitivity D1000 ScreenTape® and quantified with Qubit® Fluorometer 4.0. Sequencing was performed on the Illumina MiSeq platform using a PE300 kit. The Nextera XT Index Kit (Illumina Inc.) was used for library indexing, following the 18S metagenomic sequencing library preparation workflow.

Primer sequences

- ITS2 Forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCATCGATGAAGAACGCAGC
- ITS2 Reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC

Purified PCR products underwent index PCR using the Nextera XT indices kit, followed by quality assessment on an Agilent TapeStation and quantification with the Qubit dsDNA HS Assay Kit. Sequencing libraries were pooled and hybridized to the flowcell, where bridge amplification generated clonally amplified DNA clusters for sequencing.

Sequencing and data analysis

Sequencing of the ITS2 region was performed on the Illumina MiSeq platform using the PE300 kit. Raw sequence data were analyzed using QIIME 2, which supports machine learning-based taxonomic classification and microbiome feature analysis. Denoised sequences were clustered into Operational Taxonomic Units (OTUs), with taxonomic classification assigned from Kingdom to Species. Alpha and beta diversity analyses were conducted, and phylogenetic trees were constructed using FastTree to explore evolutionary relationships.

Taxonomic analysis

Pre-processed sequences were grouped into OTUs using a trained classifier model. Taxa numbers and relative abundances were retrieved at each taxonomic level (Kingdom, Phylum, Class, Order, Family, Genus, and Species). A genus-level heatmap was generated to visualize OTU abundance and distribution.

Phylogenetic tree construction

Phylogenetic trees were generated from aligned sequence data using MAFFT for alignment and masking of highly variable positions to reduce noise. FastTree was then used to construct the final tree, providing insights into the evolutionary relationships among the identified fungal taxa.

Results

A limitation of this study is the relatively small sample size analyzed. While the results offer valuable preliminary insights, future studies with larger sample sets will be necessary to validate

and generalize these findings. Data from Vedanthangal Bird Sanctuary are summarized in Table 1, which shows a total of 114,020 and 226,680 reads. Of these, 57,010 reads in sample A were paired-end, and 113,340 reads in sample B were identical. These 301 bp paired-end reads were stored as FASTQ files under the names 220757_A_R1.fastq.gz, 220757_B_R1.fastq.gz, and S3_R1.fastq.gz.

Table 1: sequencing and filtering statistics for pond water samples.

Sample ID	PE seq	Joined filtered reads	Denoised sequenced (belonging to OTUs)	Filtered sequences* (belonging to OTUs)	Filtered feature count/OTUs
A	152843	114020	38123	35284	1586
B	281585	226680	95017	87732	2068

* Filtered sequences: Total sequences belonging to features remained after removing features consisting of single sequences as well as having mitochondrial hits.

*Filtered feature count: Number of features remained after removing features consisting of single sequences as well as having mitochondrial hits.

For further analysis, the FASTQ files were imported into the QIIME2 pipeline. The DADA2 algorithm was employed to denoise the sequences, effectively distinguishing true biological variation from sequencing errors. Following denoising, a total of 38,123 reads for sample A and 95,017 reads for sample B were retained for downstream analysis. The high number of quality reads highlights the reliability of the sequencing data, which was subsequently used to identify potential genetic variations and gene expression patterns. Advanced bioinformatics tools revealed several single-nucleotide polymorphisms (SNPs) and differentially expressed genes between the two samples, offering insights into their underlying biological differences.

Operational Taxonomic Units (OTUs) were then generated using a classifier trained on UNITE version 8. OTUs were categorized at multiple taxonomic levels, including phylum, class, order, family, genus, and species. Before this step, the feature table was aggregated to the genus level. A heatmap was constructed to visualize OTU abundance, normalizing the data by adding a pseudo count of 1 and applying a base-10 logarithm. Clustering was performed along both feature and sample axes. In the heatmap (Fig. 2), each row represents an OTU, and each column represents a sample; yellow indicates higher abundance, whereas blue indicates lower abundance.

α-diversity

Alpha diversity, or within-sample diversity, was calculated to assess species richness (Table 2). Various metrics were used to summarize the diversity of organisms in each sample with a single value, providing an overview of community composition.

Rarefaction analysis

Rarefaction curves (Fig. 3) were generated to estimate species richness as a function of sampling effort.

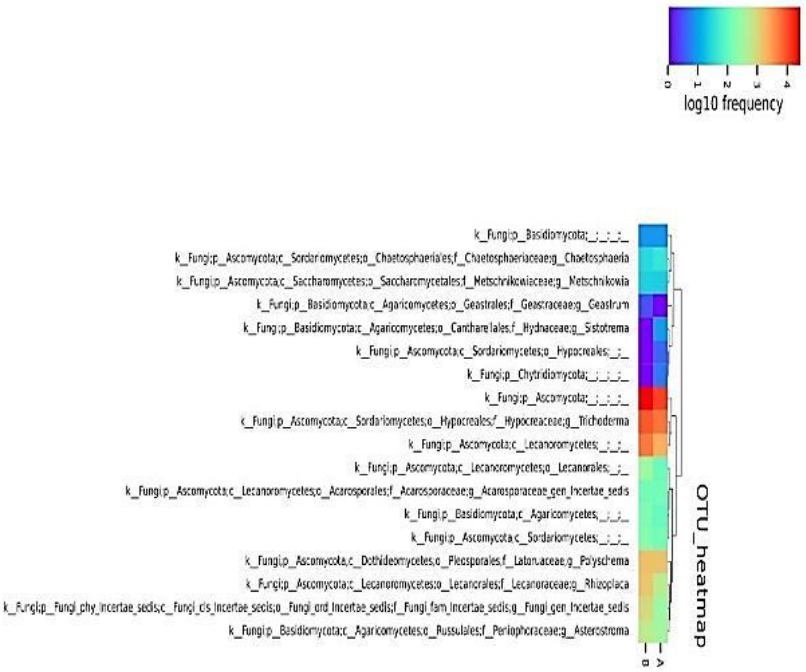

Figure 2: Heatmap of operational taxonomic units (OTUs) illustrating the log[10] transformed frequency of fungal taxa identified in the metagenomic analysis.

Table 2: comprehensive comparison of the Alpha-diversity metrics for the analyzed samples, providing insights into the richness, evenness, and overall diversity of the microbial communities present in each sample.

Samples	Chao1	Shannon entropy	Observed features
A	1586.88	7.9345	1586
B	2068.14	7.1544	2068

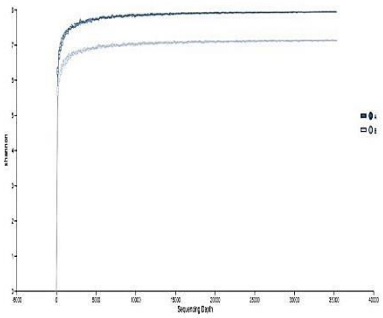

Figure 3: Rrarefaction curve showing the Shannon diversity index as a function of sequencing depth for two distinct samples.

The curves illustrate the relationship between the number of sequences and the observed OTUs. A steep slope on the left side of the curves indicates that additional species are likely yet to be detected. Each curve represents a different sample, with the X-axis denoting sequence counts and the Y-axis denoting observed OTUs.

A total of 1,315,767 full-length ITS1 reads were generated. Taxonomic assignment revealed reads belonging to Basidiomycota (Agaricomycetes), Ascomycota (Hypocreales), and Chytridiomycota. A sunburst chart (Fig. 4) visualizes the taxonomic composition of the fungal communities identified.

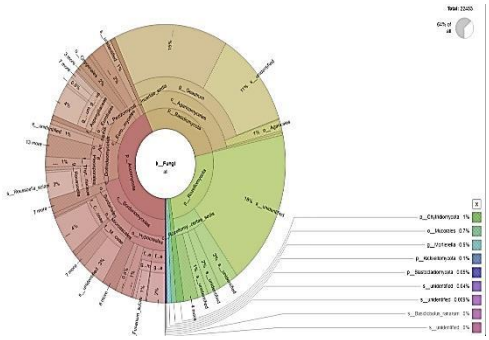

Figure 4: Sunburst chart depicting the taxonomic classification of fungal communities identified in the metagenomic analysis.

The dominant phylum was Ascomycota, followed by Basidiomycota and Rozellomycota. Uncharacterized sequences represented a substantial fraction, particularly within Rozellomycota (18%) and Basidiomycota (11%). Within Ascomycota, the most abundant orders were Hypocreales and Sordariomycetes incertae sedis, with prominent genera including *Roussoella* and *Fusarium*. Minor phyla such as Chytridiomycota, Kickxellomycota, and Blastocladiomycota contributed only a small proportion of the community. Early-diverging lineages were also present, including *Mortierella* (0.6%) and Mucorales (0.7%).

A Venn diagram (Fig. 5) at the genus level showed that 14 genera were common to all samples, while 17 genera were unique to sample A and 15 to sample B. Beta diversity analysis (Table 3) was performed to assess differences between samples, where a value of 0 indicates identical species composition and a value of 1 indicates no shared species. Results suggest partial overlap in species composition between samples, indicating an even representation of fungal taxa across the two sites (Fig. 4).

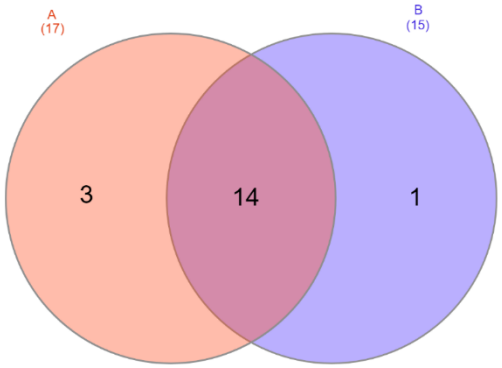

Figure 5: Venn diagram illustrating the degree of similarity and dissimilarity between samples A and B at the Genus level.

Table 3: Detailed analysis of the beta diversity indices within the samples, offering valuable insights into the variations in microbial community composition between the different samples and highlighting the extent of dissimilarity or similarity in their overall microbial structures.

Bray-Curtis distance matrix		
Samples	A	B
A	0	0.35968
B	0.35968	0

Jaccard distance matrix		
Samples	A	B
A	0	0.68798
B	0.68798	0

Unweighted unifrac distance matrix		
Samples	A	B
A	0	0.43110
B	0.43110	0

Weighted unifrac distance matrix		
Samples	A	B
A	0	0.31847
B	0.31847	0

Minor differences were observed in the abundance of Agaricomycetes and fungal Incertae sedis (Table 4, Fig. 6).

Table 4: Comprehensive list of fungal classes that have been identified from both Sample A and Sample B, offering a detailed overview of the taxonomic classification of fungal communities present in each sample and providing insights into their distribution, abundance, and potential ecological significance.

Class	A	B
Lecanoromycetes	2881	6899
Ascomycota__	11096	26228
Fungi_cls_Incertae_sedis	288	937
Agaricomycetes	448	725
Sordariomycetes	5606	8195
Dothideomycetes	1545	1583
Basidiomycota	7	7
Saccharomycetes	22	23
Chytridiomycota	4	0

Overall, the rank abundance curves suggest a stable and diverse fungal community within the studied environment. The presence of various ecological niches likely supports a wide range of fungal species. Similar abundances of Agaricomycetes and fungal Incertae sedis suggest complementary ecological roles, while the general homogeneity of the communities indicates little variation in species composition between the two ponds.

omogeneous, with little species variation.

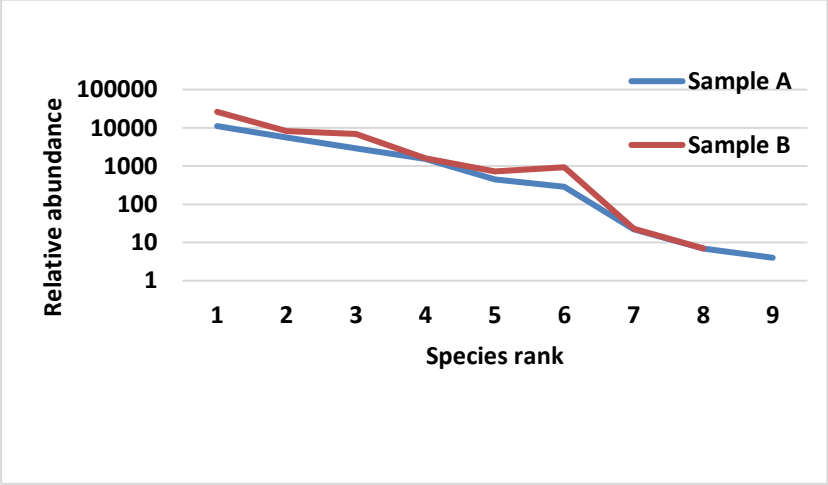

Figure 6: Rank abundance curve illustrating the comparative relative abundance of species across various ranks for two samples. The logarithmically scaled y-axis indicates species abundance, whereas the x-axis signifies species rank, demonstrating community evenness and diversity.

Discussion

This study is the first of its kind to utilize metagenomics analysis to study the diversity and composition of freshwater fungi in the wetlands inside the Vedanthangal Bird Sanctuary. provided valuable insights into the fungal communities present in this unique ecosystem, shedding light on their potential roles and interactions within the environment. The findings are expected to have implications for the management strategies meant to conserve the

biodiversity of freshwater fungi in similar habitats.

Total dissolved solids (TDS) primarily refer to the diverse array of minerals found in water. and the permissible value it is 500 mg/l as established by IS10500, BIS, and FAO. During this investigation, the tank water samples showed an elevated TDS, indicating significant mineral content and relatively higher levels of contaminants as reported in a similar study in a bird sanctuary (Kumar and Dua, 2009). Turbidity might indirectly affect the nutrient levels and sedimentation (Österling *et al*.,, 2010). This may be attributed to the shallower depth at these places, which promotes the abundant development of submerged plants, especially *Hydrilla* and *Vallisneria*. Dissolved oxygen levels may rise in areas with a higher rate of subsurface photosynthetic activity, which has also been observed and shown in similar studies (Haritash *et al*., 2015; Shan *et al*., 2021). The CO_2 level of surface water in bodies of water like lakes, tanks, and rivers may decrease as pH increases (Sahu and Mani, 2020). During the nighttime, plants emit CO_2, which leads to a decrease in pH or an increase in acidity. During daylight hours, when plants consume CO_2, the water's alkalinity may increase, resulting in a rise in pH.

Sequencing the small subunit ribosomal RNA (SSU rRNA) and its internal transcribed spacer (ITS) region provides valuable information and aid in phylogenetic analysis and species identification due to their highly conserved and variable regions. Sequences of ribosomal regions, the ribosomal cassette of repetitive genes (18S ITS1–5.8S ITS2-28S) are of great utility and are widely employed for species identification (Luo *et al*., 2019; Phukhamsakda *et al*., 2022) thus have been used to study and establish evolutionary relationships and genetic diversity among different organisms (Bruns *et al.,* 1991; Raja *et al*., 2011, Raja *et al*., 2017). Analysis of sequences identified ascomycota and basal clades of fungi as the most abundant fungal groups in the samples collected from the study site. This result align with previous studies on lakes, which have consistently shown that the Ascomycota phylum is the most abundant (Lepére *et al*., 2019, de Souza *et al*., 2021). Further, it suggests that these fungal groups play a crucial role in the decomposition of organic matter and nutrient cycling in aquatic ecosystems. In addition, the present study also revealed that the most fundamental fungal groups, including the *Rozellomycota, Mortierellomycota, Chytridiomycota, Mucoromycota, Zoopagomycota, Kickxellomycota, Monoblepharomycota, Blastocladiomycota, and Basidiobolomycota. Cryptomycota,* often called *Rozellomycota or Rozellida*, is the 62 most basic members of the Kingdom Fungi phylum (Lara *et al*., 2010; Jones *et al*., 2011; Jones *et al*., 2011; Livermore and Mattes, 2013; Mangot *et al*., 2013; Ishida *et al*., 2015; Ishii *et al*., 2015; Lazarus and James, 2015; Richards *et al*., 2015; Benny *et al*., 2016; Rojas-Jimenez *et al*., 2017; Bass *et al*., 2018; Hirakata *et*

al., 2019; Lepère *et al*., 2019; Rosa *et al*., 2022; Corsaro, 2022). lists aquatic taxa (such as *Blastocladiomycota, Chytridiomycota, and Neocallimastigomycetes)* and terrestrial taxa (such as *Entomophthoromycota and Glomeromycota)* as the basic lineages of fungi or basal clades of fungi.

The fungi are traditionally classified into two advanced lineages, namely the Ascomycota and the Basidiomycota, as well as two primitive lineages. There are many distinct lineages of fungus found in freshwater environments, and this diversity in genetics makes the Ascomycota group that these fungi belong to more accurately described as an ecological assembly than a taxonomic category. In freshwater environments, these groups are known to break down organic decomposers (Gessner and Van Ryckegem, 2003). Lotic aquatic environments (streams, rivers, and brooks) and lentic aquatic habitats (lakes, swamps, and bogs) are home to freshwater ascomycetes, which live on submerged or partly submerged substrates (Wong *et al*., 1998; Shearer 2001). This aligns with prior discoveries that the majority of fungi are classified into two fungal phyla in numerous aquatic ecosystems, and the Basidiomycota are less prevalent. This suggests that there may be specific environmental factors or ecological niches that favour the growth and proliferation of certain fungal phyla over others in aquatic environments. Further research could investigate these factors to better understand the distribution and abundance of fungi in different ecosystems.

This study establishes that the three main classes of freshwater fungi—the Ascomycota, the Chytridiomycota, and the Basidiomycota—are mostly recognised for their roles as parasites, symbionts, or significant plant pathogens. Nonetheless, their existence in water is connected to their crucial function as organisms that break down the biomass of dead plants and animals (Kuehn *et al*., 2011; Wurzbacher *et al*., 2014; Gulis *et al*., 2019). Further, we found out that the second biggest group of Ascomycota is the Sordariomycetes. Some species of Sordariomycetes have asci that are not operculate, unitunicate, or nonfissitunicate, and the ascomata around the edges of the cell are not lichenized. These fungi are common in soil, dung, and plant material, playing important roles in decomposition processes. Some species within the Sordariomycetes group are also known to cause plant diseases (Maharachchikumbura *et al*., 2016,; Reblova *et al*., 2016; Zhang *et al*., 2017a;Luo *et al*., 2018a, b).

Both Ascomycota and Basidiomycota are essential to the integrity of ecosystems and play significant roles in their maintenance of ecosystem (Hanson *et al*., 2008, Challacombe *et al*., 2019). There are several aspects of microbial ecosystems that need to be considered when estimating alpha diversity for microbiomes (Arbel *et al*., 2016; Zhang and Grabchak, 2016; Willis and Martin, 2018). Weiss *et al*. (2017) found that using these identical subsamples allowed

them to compute diversity indices that could compare ecosystems "fairly," regardless of variations in sample sizes. The fungal communities and their link with the environmental factors are the subject of a few studies that have been conducted on ponds (Guo *et al*., 2015; Brodie *et al*., 2018).

Conclusion

This study reveals a diverse fungal community within Vedanthangal Bird Sanctuary's tank ecosystem, dominated by phyla such as Ascomycota, Basidiomycota, and Rozellomycota. It offers essential baseline data to better understand fungi's role in nutrient cycles and the ecological balance of freshwater habitats. Due to the limited existing knowledge of fungal populations in aquatic environments, particularly in ornithogenic wetlands, this research emphasizes the necessity for further investigations into seasonal changes and various environmental conditions. Future research should also examine interactions between fungi and other organisms—including bacteria, algae, invertebrates, and birds—to clarify fungi's contribution to ecosystem sustainability. Understanding aquatic fungi's functions could improve ecological models and guide conservation strategies that protect biodiversity and ecosystem health in wetlands like Vedanthangal. Recognizing fungi as key to wetland resilience highlights their significance in environmental management. Additionally, ongoing research might discover new fungal species or biochemical processes with potential ecological, environmental, or pharmaceutical benefits, supporting both ecosystems and human well-being. Although these findings are preliminary due to limited samples, they form a foundational step toward more comprehensive studies. Expanding sampling and environmental variables will be necessary to validate and extend these results. Despite these limitations, this marks the first metagenomic investigation of fungal diversity in avian guano-affected freshwater ecosystems at Vedanthangal, an important stopover for migratory birds. The data indicate fungi as the dominant eukaryotic group (about 64% of total reads), with Ascomycota, Basidiomycota, and Rozellomycota being the primary phyla. The detection of numerous classes, orders, families, genera, and species, along with over a thousand uncertain OTUs, suggests a largely unexplored fungal reservoir in guano-impacted wetlands. These insights offer a baseline understanding of community structure and hint at fungi's roles in nutrient cycling, organic matter processing, and ecosystem stability. They also propose that bird-derived guano deposition influences microbial diversity. Future efforts should include expanding reference databases, exploring functional roles, and conducting long-term monitoring to deepen our understanding of these fungal communities and their importance for ecosystem health. Overall, this research supports future biodiversity efforts and conservation initiatives aimed at

preserving these biodiversity-rich sanctuaries like Vedanthangal.

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Author contributions

SV and MG supervised, provided resources, and wrote the original draft of the paper, conceptualization, data curation, formal analysis, manuscript outline, and validation. MM, MV and MGE performed the writing, review, and editing of the paper. SV did the process of investigation. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The data supporting this study will be made available upon request

Code Availability

Not applicable.

Declarations

Conflicts of Interest: There is no conflict of interest to declare.

Ethics declaration: Not applicable.

Consent to Participate: Not applicable.

Consent for Publication: Not applicable.

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