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Metagenomic Characterization of Fungal Diversity in Lotic and Lentic Freshwater Ecosystems of Palghar District, India

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ABSTRACT

Freshwater fungal communities remain poorly characterized in many tropical regions, particularly through culture-independent approaches. The present study employed shotgun metagenomic sequencing to characterize fungal diversity in selected lotic and lentic freshwater habitats of Palghar District, Maharashtra, India. Four environmental samples representing two lotic and two lentic habitats were sequenced on an Illumina NovaSeq X Plus platform, generating approximately 25 Gb of paired-end data per sample. Fungal taxa were identified using Kaiju, and community diversity was evaluated using species richness, Shannon diversity, Simpson diversity, Pielou evenness and Bray–Curtis dissimilarity. Estimated fungal relative abundance ranged from 0.98% to 2.82%, with the highest abundance observed in Lentic 1. Basidiomycota and Chytridiomycota were prominent among the resolved fungal groups. Chytridiomycota occurred in all four samples and showed comparatively greater representation in the lotic habitats, while Lentic 1 was characterized by a strong contribution of Agaricales, particularly Coprinopsis cinerea. Lentic 1 showed the highest resolved species richness but the lowest diversity and evenness, whereas the lotic samples exhibited higher Shannon and Simpson diversity and greater evenness. Bray–Curtis analysis showed the greatest similarity between Lotic 1 and Lotic 2, while Lentic 1 was compositionally distinct. The findings demonstrate that Palghar freshwater habitats support heterogeneous fungal assemblages comprising both aquatic-associated and terrestrially derived fungal signatures and highlight the value of shotgun metagenomics for assessing understudied freshwater mycobiomes.

Keywords: Aquatic fungi, Chytridiomycota, Lotic and lentic habitats, Metagenomics, Shotgun Sequencing

INTRODUCTION

Freshwater fungi are ecologically important components of aquatic ecosystems, where they participate in decomposition of plant-derived organic matter, nutrient cycling and transfer of energy through detrital food webs. Their community composition can also respond to environmental variables and anthropogenic stress, making them potentially useful indicators of freshwater ecosystem condition. Despite these functions, aquatic fungal diversity remains considerably less characterized than terrestrial fungal diversity, partly because many taxa are difficult to culture or identify using conventional morphological approaches (Barros et al., 2024). Major aquatic fungal groups include aquatic hyphomycetes, which are predominantly associated with submerged leaf litter and play an important role in decomposition within streams (Ingold, 1942; Koivusaari et al., 2019). Chytridiomycota represent another important group and occur mainly as saprotrophs or parasites in the pelagic zones of standing freshwater bodies (Wurzbacher et al., 2010). Yeasts are also widely distributed in freshwater pelagic environments. In addition, oomycetes, although taxonomically distinct from true fungi, are commonly considered alongside aquatic fungi because of their similar ecological roles; they include saprobic species as well as important parasites and pathogens of aquatic plants and animals.

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Palghar District represents a suitable region for investigating freshwater fungal diversity because of its heterogeneous hydrological landscape and the ecological and socioeconomic importance of its freshwater resources. The Vaitarna river system and its tributaries, including the Surya River, drain substantial parts of Palghar, while associated reservoirs contribute to drinking-water supply for the Mumbai Metropolitan Region. The region extends from the Western Ghats through the Konkan terrain toward the Arabian Sea, providing diverse lotic and lentic habitats that may support distinct fungal assemblages (D'Silva et al., 2025). At the same time, portions of Palghar are subject to considerable anthropogenic pressure. Previous investigations around the Tarapur industrial area have documented contamination of groundwater, drainage channels and adjoining aquatic environments, highlighting the need for baseline ecological information from the region (Naik et al., 2007).

Despite this ecological importance, freshwater fungi remain substantially less characterized than terrestrial fungal communities, particularly in tropical and geographically underrepresented regions. Culture-dependent and morphology-based methods may underestimate diversity because many fungi occur at low abundance, fail to grow under routine laboratory conditions or do not produce diagnostic structures during examination. Culture-independent sequencing therefore offers an important complementary approach.

Shotgun metagenomics provides the additional advantage of recovering fungal sequence signatures without reliance on cultivation or amplification of a single marker region and can reveal both aquatic-associated taxa and terrestrially derived components entering freshwater through runoff, leaf litter and catchment inputs (Sanyal et al., 2025).

Conventional isolation and microscopy provide valuable information on cultivable and sporulating freshwater fungi but may underestimate the actual community because many fungal taxa occur at low abundance or remain uncultured. Culture-independent high-throughput sequencing can overcome some of these limitations. In particular, shotgun metagenomics permits characterization of fungal sequence diversity without amplification of a single taxonomic marker and can simultaneously reveal aquatic-associated fungi as well as terrestrial fungal signatures entering freshwater through runoff, litter and catchment inputs.

Recent shotgun-metagenomic studies have demonstrated its potential for characterizing freshwater fungal communities while also highlighting the limited representation of aquatic fungal genomes in existing reference databases. Considering the ecological importance of freshwater fungi, the environmental heterogeneity of Palghar District and the limited information available on its freshwater mycobiome, the present study employed shotgun metagenomic sequencing to characterize fungal communities in lotic and lentic freshwater habitats of Palghar District, Maharashtra, India. The study aimed to determine the fungal taxa represented in these habitats, examine their distribution between lotic and lentic environments, and evaluate patterns of fungal community diversity.

Against this background, characterization of fungal communities in the lotic and lentic freshwater habitats of Palghar District is warranted to generate baseline information on an understudied component of regional biodiversity and to improve understanding of fungal distribution across contrasting freshwater environments. The present study therefore employed shotgun metagenomic sequencing to characterize fungal taxa associated with selected lotic and lentic freshwater habitats of Palghar District.

MATERIAL AND METHOD

Study Area and Sampling Design

The study was conducted as part of a broader investigation of freshwater fungal diversity in Palghar District, Maharashtra, India. The study area included both lotic freshwater habitats, such as streams, rivers and creeks, and lentic habitats, including ponds, lakes and reservoirs. The broader investigation involved the examination of freshwater habitats for fungi associated with water, submerged leaf litter, woody debris and foam. For shotgun metagenomic analysis, four environmental samples representing the two major freshwater habitat categories were selected. Two samples represented lotic habitats and two represented lentic habitats.

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The samples were designated as Lotic 1 (RRSID-28999), Lotic 2 (RRSID-29000), Lentic 1 (RRSID-29001) and Lentic 2 (RRSID-29002) (Table 1).

Table 1 Sample details

Sample	Site	Habitat	GPS	Season	Sample material
Lotic 1	Vaitarna and Tansa river	River	Vaitarna river- 19.7227° N and 73.1268° E Tansa river-19.52054° N and 72.88036° E	Late Rainy	Foam, submerged leaf litter and herbaceous debris
Lotic 2	Surya, Pinjal and Deherja river	River	Surya river- 19°38′51″ N, 72°51′26″ E Pinjal river- 19.64213° N and 73.08282° E Deherja river- 19.731° N and 73.026° E	Late Rainy	Foam, submerged leaf litter and herbaceous debris
Lentic 1	Khand, Wandri and Kelwa dam	Lake	Khand dam- 19.7731° N Wandri dam- 19.614858° N and 72.953854° E Kelwa dam- 19.633° N and 72.825° E	Late Rainy	Foam, submerged leaf litter and herbaceous debris
Lentic 2	Aswali, Kurje and Dhamni dam	Lake	Aswali dam- 20.0926° N and 72.7954° E Kurje dam- 20.0757235° N and 72.9492619° E Dhamni dam- 19.925122°N and 73.060799°E	Late Rainy	Foam, submerged leaf litter and herbaceous debris

Metagenomic DNA Extraction, Library Preparation, Sequencing and Sequence Processing

Total environmental DNA was extracted from the four freshwater samples using the QIAamp DNA Mini Kit. DNA concentration was measured using a Qubit fluorometer, while DNA integrity was assessed by TapeStation analysis. All four DNA preparations satisfied the quality-control requirements and were processed further for library preparation (Sanyal et al., 2025). Shotgun metagenomic libraries were prepared using the KAPA HyperPlus library preparation kit.

Extracted DNA was subjected to enzymatic fragmentation, followed by end repair, A-tailing and sequencing-adapter ligation. Libraries were amplified using minimal PCR cycles, purified and subjected to quality assessment. TapeStation analysis indicated representative library fragment sizes of approximately 450 bp. The normalized libraries were subsequently pooled and sequenced on an Illumina NovaSeq X Plus platform using paired-end sequencing with a read length of 2 × 150 bp, generating approximately 25 Gb of sequence data per sample.

The quality of raw sequencing reads was evaluated using FastQC v0.11.9, and overall sequencing quality was summarized using MultiQC. Adapter contamination identified in the raw reads was removed using Trimmomatic, and the resulting high-quality paired-end reads were retained for downstream analysis. Potential human-derived reads were identified by alignment against the GRCh38 human reference genome using Bowtie2 and removed prior to taxonomic analysis.

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The sequencing report indicated that only approximately 0.01–0.04% of reads aligned to the human reference genome. Quality-filtered reads were assembled de novo using MEGAHIT v1.2.9. Assembly quality was evaluated using QUAST/MetaQUAST, based on assembly parameters such as the number of contigs, N50, L50 and GC content. The resulting high-quality reads and assembled contigs were subsequently used for taxonomic characterization of the freshwater microbial communities.

Fungal Taxonomic Profiling, Diversity Analysis and Habitat-Wise Distribution

Taxonomic classification of the overall freshwater metagenomic community was initially performed using Kraken2 v2.1.3, which was used to determine the proportion of reads that could be assigned to known microbial taxa. Because the present investigation focused particularly on the fungal component of the freshwater metagenome, Kaiju was employed for fungal taxonomic profiling. Kaiju-based assignments were used to identify fungal taxa detected in each of the four samples and to estimate their relative representation within the metagenomic datasets (Sanyal et al., 2025). The Kaiju analysis revealed an overall low abundance of fungal sequences compared with the total microbial metagenome but detected multiple fungal taxa across the lotic and lentic samples. The identified taxa included freshwater-associated fungi as well as predominantly terrestrial fungal taxa whose sequences may have entered aquatic habitats through surrounding soil, riparian vegetation, decaying plant material, runoff or other allochthonous inputs. For ecological interpretation, the detected fungi may therefore be grouped as aquatic or aquatic-associated fungi, predominantly terrestrial fungi detected in freshwater, and taxa with broader or uncertain ecological associations. Because metagenomic sequencing detects DNA rather than cultured organisms, the identified taxa should be described as “detected,” “taxonomically assigned,” or “represented by sequence reads,” rather than as fungal isolates.

Community diversity was assessed using the Shannon and Simpson diversity indices for alpha diversity, while Bray–Curtis dissimilarity was used to evaluate differences in community composition among samples. Principal Coordinates Analysis (PCoA) was employed to visualize patterns of similarity and dissimilarity among the metagenomic communities. Differences between the lotic and lentic groups were statistically evaluated using PERMANOVA implemented through Adonis2 with 999 permutations, and dispersion among groups was examined using the betadisper function. For a paper specifically addressing freshwater fungal diversity, the relative abundance of each fungal taxon should be compared among Lotic 1, Lotic 2, Lentic 1 and Lentic 2 using the numerical Kaiju output. Taxa may further be categorized as shared between the two habitat types or predominantly associated with either lotic or lentic samples. Where fungal-only abundance data are available, alpha-diversity and beta-diversity indices should preferably be recalculated using the fungal subset alone so that conclusions refer specifically to the freshwater mycobiome rather than the entire microbial community.

RESULTS

Shotgun metagenomic sequencing generated approximately 25 Gb of paired-end sequence data per sample. All four datasets showed satisfactory sequencing quality and were retained for downstream taxonomic analysis. Fungal sequences were subsequently identified using Kaiju, which revealed a comparatively low but diverse fungal component across the lotic and lentic samples.

Fungal Community Composition

Kaiju-based profiling revealed a taxonomically diverse fungal community across the four freshwater samples. Among the resolved taxa, the major phyla represented were Basidiomycota, Chytridiomycota, Zoopagomycota, Mucoromycota, Calcarisporiellomycota, and Glomeromycota. Basidiomycota showed a pronounced representation in Lentic 1 (0.963%), largely due to members of Agaricales, whereas its contribution was considerably lower in Lentic 2 (0.047%), Lotic 1 (0.010%) and Lotic 2 (0.026%). In contrast, Chytridiomycota was consistently detected in all four samples, with a comparatively higher representation in the lotic habitats. Its estimated abundance was highest in Lotic 2 (0.251%), followed by Lotic 1 (0.204%), Lentic 1 (0.152%) and Lentic 2 (0.126%). Zoopagomycota and Mucoromycota occurred at lower abundances, whereas Calcarisporiellomycota was principally represented in Lotic 1.

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Glomeromycota was represented by Rhizophagus-associated sequences, although its abundance could not be reliably resolved from the available graph.

At the order level, Agaricales was the most prominent resolved order in Lentic 1 (0.963%) but occurred only at low abundance in the remaining samples. The chytrid component comprised several orders, including Rhizophydiales, Synchytriales, Spizellomycetales, and Polychytriales. Spizellomycetales showed greater representation in the lotic samples (0.084–0.089%) than in the lentic samples, while Rhizophydiales ranged from approximately 0.042% to 0.073% and Synchytriales from 0.042% to 0.058% across the samples. Polychytriales was also more evident in the lotic samples. Other represented orders included Kickxellales, Umbelopsidales, Mucorales, Calcarisporiellales, and Glomerales. At the family level, Psathyrellaceae was the most prominent resolved family in Lentic 1 (0.749%). Other Agaricales-associated families included Agaricaceae, Hydnangiaceae, and Hymenogastraceae. Among the aquatic-associated chytrid lineages, Batrachochytriaceae occurred at approximately 0.042–0.073%, while Synchytriaceae contributed approximately 0.042–0.058% across the samples. Spizellomycetaceae showed greater representation in the lotic samples (0.063% each), whereas Powellomycetaceae occurred at comparatively lower abundance. Additional families included Kickxellaceae, Umbelopsidaceae, Phycomycetaceae, Calcarisporiellaceae, and Glomeraceae.

Distribution of Fungal Taxa in Lotic and Lentic Habitats

Kaiju profiling revealed clear variation in fungal representation among the four freshwater samples. The estimated total fungal relative abundance ranged from 0.98% to 2.82%, with the highest representation in Lentic 1 (2.82%), followed by Lotic 1 (1.70%) and Lotic 2 (1.69%), while Lentic 2 showed the lowest fungal abundance (0.98%). The pooled Others category constituted the largest proportion of the fungal profile in all samples. Among the individually resolved taxa, Coprinopsis cinerea showed a pronounced contribution in Lentic 1 (~0.75%), whereas Synchytrium microbalum and Batrachochytrium dendrobatidis occurred at low but comparable abundances across multiple samples. Polychytrium aggregatum and Spizellomyces punctatus showed comparatively greater representation in the lotic samples. Thus, although the two lotic samples had similar overall fungal abundance, their taxonomic composition differed from that of the lentic samples, particularly from Lentic 1.

The detected fungal assemblage comprised taxa with contrasting ecological affiliations. Aquatic-associated chytrid fungi included Synchytrium microbalum, Polychytrium aggregatum, and Batrachochytrium dendrobatidis, whereas Coprinopsis cinerea, Agaricus bisporus, Laccaria bicolor, Rhizophagus irregularis, and Umbelopsis ramanniana represented predominantly terrestrial-associated fungi. Overall, the metagenomic profiles demonstrated distinct sample-wise differences in fungal abundance and community composition across the lotic and lentic freshwater habitats.

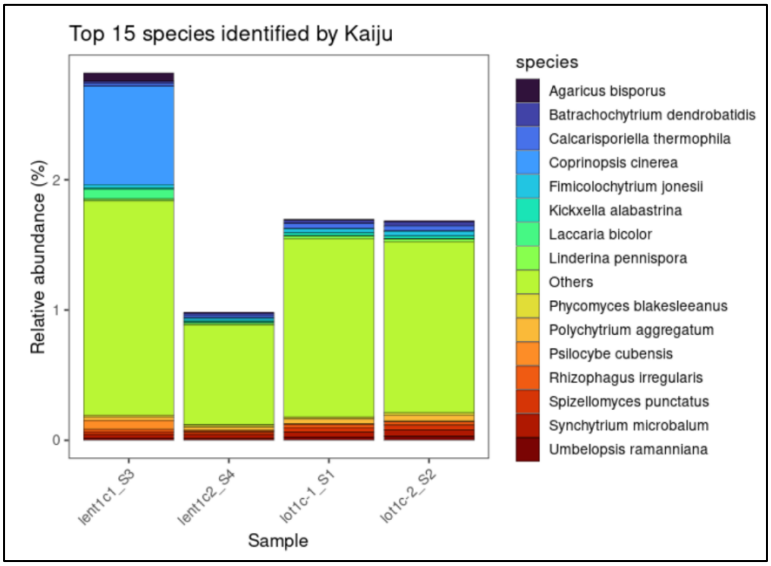

Figure 1 Relative abundance of fungi across all the samples

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Table 2 Relative abundance of all the reported fungal taxon across all the samples

Fungal taxon	Lentic 1 (S3)	Lentic 2 (S4)	Lotic 1 (S1)	Lotic 2 (S2)
Agaricus bisporus	0.073	0.016	0	0.016
Batrachochytrium dendrobatidis	0.042	0.068	0.047	0.073
Calcarisporiella thermophila	0	0	0.037	0
Coprinopsis cinerea	0.749	0	0	0
Fimicolochytrium jonesii	0.021	0	0.021	0.026
Kickxella alabastrina	0.016	0	0.026	0.026
Laccaria bicolor	0.073	0.005	0.01	0.005
Linderina pennispora	0.016	0.016	0.021	0.021
Others	1.649	0.764	1.377	1.314
Phycomyces blakesleeanus	0.005	0.01	0	0.01
Polychytrium aggregatum	0.021	0	0.031	0.042
Psilocybe cubensis	0.068	0.026	0	0.005
Rhizophagus irregularis	0	0	0	0.000*
Spizellomyces punctatus	0.021	0	0.063	0.063
Synchytrium microbalum	0.047	0.058	0.042	0.047
Umbelopsis ramanniana	0.021	0.021	0.026	0.037
Total fungal relative abundance	2.822	0.984	1.702	1.686

Alpha Diversity

Alpha-diversity analysis showed clear variation among the four freshwater samples. Lentic 1 had the highest resolved species richness (S = 13) but the lowest Shannon diversity (H′ = 1.474), Simpson diversity (1−D = 0.577), and Pielou evenness (J′ = 0.575), indicating greater dominance by a limited number of taxa. In contrast, the lotic samples showed higher overall diversity and more even taxon distribution.

Lotic 2 recorded the highest Shannon diversity (H′ = 2.243), while Lotic 1 showed the highest Simpson diversity (1−D = 0.880) and Pielou evenness (J′ = 0.957). Lentic 2 exhibited the lowest richness (S = 8) but comparatively high evenness (J′ = 0.865).

Table 3 Alpha Diversity

Sample	Resolved species richness (S)	Shannon index (H′)	Simpson diversity (1−D)	Pielou evenness (J′)
Lentic 1	13	1.474	0.577	0.575
Lentic 2	8	1.799	0.799	0.865
Lotic 1	10	2.203	0.88	0.957
Lotic 2	12	2.243	0.878	0.903

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Beta diversity and comparison between lotic and lentic habitats

Bray–Curtis dissimilarity indicated substantial variation in fungal community composition among the four samples. The lowest dissimilarity was observed between Lotic 1 and Lotic 2 (0.178), indicating the greatest similarity between the two lotic fungal communities. In contrast, Lentic 1 was highly dissimilar from the other samples, with values of 0.745 against Lentic 2, 0.794 against Lotic 1, and 0.751 against Lotic 2. Lentic 2 showed intermediate similarity to the lotic samples, particularly Lotic 2 (0.427). Overall, the beta-diversity pattern suggested greater similarity between the two lotic fungal communities and marked compositional distinctness of Lentic 1.

Table 4 Beta Diversity

	Lentic 1	**Lentic 2**	**Lotic 1**	**Lotic 2**
Lentic 1	0	0.745	0.794	0.751
Lentic 2	0.745	0	0.558	0.427
Lotic 1	0.794	0.558	0	0.178
Lotic 2	0.751	0.427	0.178	0

Gene Prediction and Functional Annotation

A large number of open reading frames (ORFs) were predicted from the assembled metagenomes. The number of predicted ORFs ranged from approximately 1.96 million to 2.25 million per sample. Lotic 2 showed the highest number of predicted ORFs, whereas Lentic 1 showed the lowest. Functional annotation using eggNOG-mapper successfully assigned functions to a substantial proportion of the predicted protein sequences.

Sample	**Predicted ORFs**	**eggNOG-annotated sequences**
Lentic 1	19,64,728	15,29,438
Lentic 2	20,72,064	8,51,383
Lotic 1	20,13,649	15,41,738
Lotic 2	22,51,425	15,67,662

The number of annotated sequences was highest in Lotic 2 and lowest in Lentic 2. These results indicated substantial functional potential within the freshwater metagenomes. However, because gene prediction and eggNOG annotation were performed on the entire microbial metagenome, these functional assignments cannot be attributed specifically to fungal taxa without further fungal-specific sequence extraction or taxonomic binning.

DISCUSSION

Shotgun metagenomic profiling revealed a relatively small but taxonomically heterogeneous fungal species across the lotic and lentic freshwater samples. The estimated overall fungal contribution ranged from approximately 0.98% to 2.82%, with the highest representation in Lentic 1 and comparable values in the two lotic samples. A low proportional representation of fungal sequences is consistent with several molecular surveys of aquatic microbial communities. Comeau et al. (2016) analysing more than 300 marine and freshwater samples, reported an average fungal contribution of approximately 1.35% of high-quality eukaryotic sequences, although abundance varied substantially among habitats. More recently, Sanyal et al. (2025) using shotgun metagenomes from 26 lakes and one tropical reservoir, found that fungi constituted only a small proportion of the total classified metagenome, despite exhibiting considerable taxonomic diversity. Thus, the relatively low fungal abundance observed in the present study is consistent with the

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general pattern that fungi can represent a quantitatively minor but ecologically important fraction of freshwater microbial communities.

A notable component of the present fungal assemblage was Chytridiomycota, which was detected in all four samples and showed greater resolved representation in the two lotic samples. Chytrid-associated taxa included Batrachochytrium dendrobatidis, Synchytrium microbalum, Polychytrium aggregatum, Spizellomyces punctatus and Fimicolochytrium jonesii but the last two fungi are usually terrestrial fungi. The importance of Chytridiomycota in freshwater ecosystems is well established. Jobard et al. (2012) reported that approximately 50% of fungal sequences recovered from three European lakes belonged to Chytridiomycota. Similarly Khomich et al. (2017), examining 77 Scandinavian lakes, found that although Dikarya contributed the greatest OTU richness, Chytridiomycota accounted for approximately 63% of fungal reads. Comeau et al. (2016) likewise observed widespread dominance of chytrid-like sequences across diverse aquatic environments, while Zhang et al. (2016) identified 196 chytrid OTUs among 641 fungal OTUs from streams, ponds, meltwater and estuarine habitats in Svalbard. These studies support the consistent occurrence of zoosporic fungi in freshwater systems. Monchy et al. (2011) found Chytridiomycota to dominate fungal sequences in two French lakes, although their spatial distribution differed strongly between the lakes.

The prominence of chytrid-associated taxa is ecologically relevant because these fungi include both saprotrophic and parasitic forms. Many aquatic chytrids infect phytoplankton, whereas others utilize particulate organic material. Their zoospores can also form an important trophic link between otherwise poorly edible phytoplankton and zooplankton, thereby contributing to carbon and nutrient transfer through the so-called mycoloop. Frenken et al. (2017) emphasized that chytrid parasitism can alter phytoplankton succession and bloom dynamics, while Grossart et al. (2019) identified aquatic fungi as important participants in organic-matter transformation and aquatic food-web interactions. Thus, even comparatively low chytrid sequence abundance may represent organisms with disproportionately important ecosystem functions.

Nevertheless, the proportional contribution of resolved chytrid taxa in the present dataset was lower than that reported in several targeted amplicon studies. This difference is not necessarily ecological. Rojas-Jimenez et al. (2017), for example, found that Cryptomycota and Chytridiomycota overwhelmingly dominated fungal communities in Antarctic ice-covered lakes; Chytridiomycota represented approximately 26% of fungal reads and 40% of detected fungal OTUs. In the recent shotgun-metagenomic study of Sanyal et al. (2025), Chytridiomycota averaged only about 4.9% of fungal reads, although individual samples reached approximately 16%, whereas Ascomycota and Basidiomycota were substantially more abundant. Climate, trophic status, substrate availability, water-column characteristics and sequencing methodology can all influence the apparent community profile.

The prominent contribution of Agaricales in Lentic 1, particularly sequences assigned to Coprinopsis cinerea, is another important feature of the present study. Agaricales are primarily associated with terrestrial substrates, but their occurrence in freshwater sequence datasets is not unusual. Sanyal et al. (2025) identified Agaricales as the most abundant fungal order across their lake metagenomes, followed by Saccharomycetales, Polyporales, Eurotiales and Hypocreales. Lepère et al. (2019) similarly found a substantial representation of Dikarya across 25 lakes and four rivers, demonstrating that filamentous Ascomycota and Basidiomycota are more common in freshwater molecular datasets than traditional aquatic-fungal surveys had suggested. Khomich et al. (2017) also recovered both true aquatic and apparently terrestrial fungal components from lake water. Matsuoka et al. (2019) recovered nearly 2,000 fungal OTUs from river water in a Japanese forest landscape, including both aquatic fungi and terrestrial groups such as decomposers and mycorrhizal fungi.

The detection of other predominantly terrestrial taxa, including Agaricus, Laccaria, Rhizophagus and Umbelopsis, can reasonably be interpreted within the context of terrestrial–aquatic connectivity. Terrestrial fungal DNA, spores, hyphal fragments and plant-associated material can be transported into freshwater through runoff, rainfall, litter fall and inflowing streams. Sugiyama et al. (2024) directly demonstrated that river-water eDNA can recover surrounding terrestrial fungal communities; aquatic eDNA contained

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approximately 1.5 times as many OTUs as sporocarp surveys and detected numerous inconspicuous terrestrial fungi. At a broader taxonomic level, Deiner et al. (2016) described river networks as conveyer belts of biodiversity information, showing that flowing water transports environmental DNA originating from both aquatic and terrestrial environments. Likewise, Lepère et al. (2019) proposed that some fungi recovered from freshwater represent allochthonous material introduced from catchments and sediments. This interpretation is also consistent with the large-scale agricultural-water survey of Pham et al. (2024), which recovered more than 6,500 fungal OTUs from 503 freshwater samples and demonstrated strong connections between aquatic mycobiota, surrounding land use and environmental conditions.

This interpretation is particularly relevant to the Palghar landscape, where freshwater bodies receive substantial terrestrial organic material from surrounding vegetation and catchments. Previous studies from the Western Ghats of India have demonstrated strong links between fungal communities, riparian vegetation and local water characteristics. Rajashekhar and Kaveriappa (2003), investigating six rivers and a sulphur spring, found that aquatic hyphomycete richness was significantly related to riparian vegetation and that differences among fungal communities were associated with factors such as pH, dissolved oxygen, hardness, temperature and iron concentration. Raviraja et al. (1998), examining foam and leaf samples from ten Western Ghats streams, likewise found that stream category was an important determinant of fungal community composition. These regional studies support the view that local habitat conditions can generate substantial variation in fungal assemblages even within the same broad geographic region.

Consequently, detection of terrestrial-associated fungi should not be considered an artefact that must necessarily be excluded from the freshwater mycobiome. Rather, such taxa can provide information on connectivity between freshwater bodies and their surrounding catchments. At the same time, sequence detection alone does not demonstrate that these organisms were actively growing or completing their life cycles in water. This distinction is important for taxa such as Laccaria and Rhizophagus, which have well-established terrestrial ecological associations. Accordingly, the present study is better interpreted as describing the fungal DNA assemblage of freshwater environments, comprising both aquatic-associated and terrestrially derived components, rather than assuming that every detected species represents a resident aquatic fungus.

Interestingly, classical aquatic hyphomycetes were not conspicuous among the dominant taxa recovered by shotgun metagenomics. This contrasts with substrate-focused investigations in streams, where aquatic hyphomycetes are frequently dominant decomposers of submerged leaves. da Silva et al. (2019), for example, identified 20 aquatic hyphomycete taxa in a tropical stream and 13 in a nearby lentic system, with fungal biomass and diversity generally greater in the lotic habitat. However, the absence or scarcity of classical hyphomycetes in a water-column molecular dataset is not unprecedented. Porter et al. (2026), using ITS sequencing across rivers, creeks, lakes and dams in semi-arid Australia, recovered 344 fungal OTUs but found aquatic hyphomycetes to be virtually absent, while zoosporic and unclassified fungal lineages were prominent. This suggests that the fungal groups detected by molecular analysis depend strongly on the sampled compartment; leaf-litter or woody-substrate surveys may preferentially recover aquatic hyphomycetes, whereas water-column DNA can emphasize zoosporic fungi, yeasts, transient terrestrial fungi and fungal environmental DNA. Regional studies also demonstrate that freshwater fungal communities can respond sharply to unusual water chemistry. Rajashekhar and Kaveriappa (1996) found no aquatic hyphomycetes within a sulphur spring in the Western Ghats, whereas 16 species occurred downstream; elevated temperature and sulphide concentrations inhibited fungal growth and sporulation. Such studies emphasize that apparently similar freshwater habitats may support very different fungal assemblages when water chemistry differs

The alpha-diversity pattern observed in the present study further demonstrates that richness and community evenness need not follow the same trend. Lentic 1 showed the highest resolved richness (S = 13) but the lowest Shannon diversity, Simpson diversity and Pielou evenness, consistent with a community strongly influenced by the comparatively high representation of C. cinerea. Conversely, both lotic samples exhibited greater Shannon and Simpson diversity and substantially higher evenness. This pattern broadly agrees with da Silva et al. (2019), who found higher fungal diversity indices and biomass in their lotic environment than

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in the adjacent lentic habitat. Hydrological turbulence, continued replenishment of organic substrates and heterogeneous microhabitats in streams can promote diverse fungal assemblages.

However, the present results should not be interpreted to indicate that lotic habitats are inherently more diverse than lentic habitats. Several studies demonstrate substantial fungal diversity in lakes. Khomich et al. (2017) identified 232 fungal OTUs across 77 oligotrophic lakes, while Jobard et al. (2012) revealed diverse chytrid, ascomycete and basidiomycete communities in three European lakes. Zhang et al. (2016) similarly observed high fungal diversity across different standing and flowing aquatic environments. More importantly, Sanyal et al. (2025) found significant variation in Shannon, inverse Simpson and Pielou indices among lake layers and locations, illustrating that within-habitat environmental heterogeneity may be as important as broad classification into lotic and lentic ecosystems.

The beta-diversity results support this interpretation. Lotic 1 and Lotic 2 showed the lowest Bray–Curtis dissimilarity (0.178), suggesting relatively similar resolved fungal communities, whereas Lentic 1 was markedly different from all other samples. Comparable habitat-specific structuring has been observed elsewhere. da Silva et al. (2019) reported that fungal communities from tropical lotic and lentic habitats differed by at least 40%, despite sharing several taxa. Zhang et al. (2016) found distinct fungal assemblages among streams, ponds, meltwater and estuarine environments, while Lepère et al. (2019) reported that only a relatively small proportion of fungal OTUs was shared among freshwater ecosystems. Sanyal et al. (2025) likewise demonstrated significant compositional differences among lakes, depths and seasons. Collectively, these observations suggest that freshwater fungal communities are strongly structured by local environmental conditions and are not determined solely by whether the habitat is lotic or lentic.

The distinctiveness of Lentic 1 may therefore reflect differences in organic-matter inputs, water chemistry, surrounding vegetation, nutrient availability or anthropogenic influence. Although these variables could not be directly tested in the present dataset, several studies provide support for such environmental control. Ortiz-Vera et al. (2018), investigating fungal communities along a polluted tropical river, found that fungal diversity was associated with pH and dissolved iron, whereas community composition responded to dissolved oxygen, nitrate, BOD, TOC, metals and seasonality. Bai et al. (2018) showed that river fungal communities differed significantly along mountainous, urban wastewater-affected and agricultural sections and demonstrated strong relationships between fungal composition and anthropogenic nutrient inputs. In lake metagenomes, Sanyal et al. (2025) identified total organic carbon and total nitrogen as significant predictors of fungal community composition, with oxygen also contributing to variation.

Additional evidence comes from long-term freshwater surveys. Pham et al. (2024) analysed 503 water samples collected over several years from an agriculturally dominated watershed and identified 6,571 fungal OTUs spanning 15 phyla, demonstrating that land use, water physicochemistry, hydrology and weather collectively influence freshwater fungal assemblages. Likewise, fungal-community studies of lake sediments in the Yellow River headwater region identified dissolved organic carbon, total nitrogen, temperature and C: N characteristics as important predictors of fungal community structure. These studies indicate that the pronounced differences observed between Lentic 1 and the other samples could plausibly result from site-specific environmental filtering rather than the lentic condition alone.

Published studies also show that anthropogenic disturbance does not always produce a simple decrease in fungal richness. Ortiz-Vera et al. (2018) observed major compositional shifts across a severe pollution gradient, whereas Pietryczuk and colleagues reported greater fungal abundance or taxonomic diversity in some organic-rich and polluted rivers. In northeastern Poland, fungal abundance was greatest in a strongly anthropogenically affected river, and the highest taxonomic diversity occurred in waters rich in organic matter. Thus, nutrient and organic-matter enrichment may favour some fungal taxa while excluding others, leading to community restructuring rather than a uniform loss of diversity.

A further point of contrast concerns the relative representation of major fungal phyla. Sanyal et al. (2025) found Ascomycota to be the dominant phylum in their lake metagenomes, followed by Basidiomycota and Chytridiomycota. Ortiz-Vera et al. (2018) also recovered Ascomycota, Basidiomycota and Chytridiomycota as major components of a tropical river. Conversely, Jobard et al. (2012), Khomich et al. (2017) and Comeau

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et al. (2016) reported strong quantitative representation or dominance of Chytridiomycota. Such contrasting outcomes emphasize the influence of ecosystem type, season, sampling compartment, molecular marker and analytical pipeline. The comparatively conspicuous Agaricales component in Lentic 1 and the higher resolved chytrid representation in the lotic samples therefore fall within the broader range of fungal-community structures reported from freshwaters rather than indicating an anomalous assemblage.

The substantial Others category in the Kaiju profile also deserves consideration. Freshwater sequencing studies repeatedly recover large fractions of unidentified fungal diversity. Ortiz-Vera et al. (2018) reported that approximately 68% of fungal OTUs could not be identified to known species-level lineages, while investigations of the Laurentian Great Lakes found that approximately 49–72% of OTUs lacked matches to described fungal groups below the kingdom level. Most strikingly, Porter et al. (2026) reported that about 73% of sequences from Australian freshwater systems could not be confidently assigned even to a fungal phylum. These observations indicate that unresolved reads are a recurrent feature of aquatic fungal surveys and may contain substantial dark fungal diversity.

This problem is especially relevant to shotgun metagenomics. Sanyal et al. (2025) demonstrated that species-level classification of freshwater fungal metagenomic reads remains constrained by inadequate representation of aquatic fungal genomes in reference databases. Better-characterized terrestrial species may therefore receive more confident assignments than poorly characterized aquatic relatives. Consequently, species-level identifications in the present study should be interpreted as reads taxonomically assigned to particular reference species, particularly for unexpected terrestrial taxa, rather than unequivocal evidence that every reference species was biologically established at the sampling site.

Despite these limitations, the occurrence of multiple chytrid lineages alongside Basidiomycota and other fungal groups emphasizes the ecological complexity of the Palghar freshwater mycobiome. Chytrids can function as saprotrophs and parasites and can link otherwise poorly edible phytoplankton biomass to zooplankton through zoospores, while filamentous fungi contribute to decomposition of terrestrial and aquatic organic matter. Jobard et al. (2012) demonstrated that zoosporic, yeast and mycelial fungal forms occupy distinct ecological niches in lakes, and Comeau et al. (2016) found that many abundant aquatic chytrid sequences were related to phytoplankton parasites. Thus, even though fungal sequences constituted only a small proportion of the present metagenomes, their ecological significance may be disproportionate to their sequence abundance.

Overall, the present study demonstrates that the freshwater fungal assemblage of Palghar contains both aquatic-associated zoosporic fungi and terrestrially derived fungal signatures, with substantial variation among individual samples. The relatively similar composition and high evenness of the two lotic samples contrast with the distinctive, less-even assemblage of Lentic 1, suggesting that hydrology interacts with local environmental conditions in structuring fungal communities. These findings agree with a growing body of molecular evidence demonstrating that freshwater mycobiomes are diverse, spatially heterogeneous and strongly connected to their surrounding catchments, while differences from studies dominated by Ascomycota or Chytridiomycota illustrate the substantial context dependence of aquatic fungal community structure.

CONCLUSION

Shotgun metagenomics revealed a diverse but relatively low-abundance fungal component across the freshwater habitats of Palghar District. Chytridiomycota was consistently detected and showed greater representation in the lotic samples, whereas Lentic 1 was characterized by strong representation of Agaricales. The lotic samples exhibited higher fungal diversity and evenness, while Lentic 1 showed greater richness but stronger taxon dominance. Bray–Curtis analysis further indicated high similarity between the two lotic communities and marked distinctness of Lentic 1. The occurrence of both aquatic-associated and terrestrial fungal taxa highlights strong connectivity between freshwater habitats and surrounding catchments. Overall, the study provides baseline metagenomic information on the freshwater mycobiome of Palghar and demonstrates the usefulness of shotgun sequencing for uncovering fungal diversity that may be overlooked by conventional methods.

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