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Insights into freshwater ciliate diversity through high throughput DNA metabarcoding

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Abstract

The freshwater bodies of India are highly biodiverse but still understudied, especially concerning ciliates. Ciliates constitute a significant portion of eukaryotic diversity and play crucial roles in microbial loops, nutrient recycling, and ecosystem maintenance. The present study aimed to elucidate ciliate diversity in three freshwater sites in the Delhi region of India: Okhla Bird Sanctuary (OBS), Sanjay Lake (SL), and Raj Ghat pond (RJ). This study represents the first investigation into the taxonomic diversity and richness of freshwater ciliates in India using a high-throughput DNA metabarcoding approach. For the analysis, total environmental DNA was extracted from the three freshwater samples, followed by sequencing of the 18S V4 barcode region and subsequent phylogenetic analyses. Operational taxonomic units (OTU) analyses revealed maximum species diversity in OBS (106), followed by SL (104) and RJ (99) sites. Ciliates from the classes Oligohymenophorea, Prostomatea, and Spirotrichea were dominant in the three sites. The study discusses the ability of the metabarcoding approach to uncover unknown and rare species. The study highlights the need for refined reference databases and cautious interpretation of the high-throughput sequencing-generated data while emphasizing the complementary nature of molecular and morphological approaches in studying ciliate diversity.

Keywords: ciliates; diversity; DNA metabarcoding; freshwater; India

Introduction

Ciliates represent a remarkably diverse clade of eukaryotic microorganisms, showcasing the utmost morphological complexity and differentiation among single-celled organisms (Chen et al. 2017). With an extensive species diversity, there are over 4000 described free-living ciliates (Gao et al. 2017, Canals et al. 2020). These microorganisms exhibit distinctive structural and functional features, including intricate subcellular and organellar structures, functionally diverse macronuclear and micronuclear genomes, sexual reproduction through conjugation, whole-genome duplications, unique patterns of cortical structure with semi-autonomous mechanisms of inheritance and morphogenesis. Additionally, there is evidence of epigenetic mechanisms, reflecting a broad array of ecological niches, lifestyles, coupled with rapid evolutionary radiation at the population level due to their short generation time (Clamp and Lynn 2017). Ciliates play a vital role in freshwater ecosystems, being the richest and relatively most abundant group (Carvalho da Silva and Fernandes 2023). They serve as major contributors to the microbial loop, acting as recyclers, and remineralizers of organic material. They also prey upon bacteria and smaller protists, contributing significantly to maintaining ecosystem balance (Abraham et al. 2019).

The taxonomic identifications of ciliates traditionally relies on microscopic techniques involving observation of live or fixed cells, with a focus on ciliature or silver-line system (Foissner 2016). However, relying solely on microscopy for identification presents certain limitations, including the time-intensive process of culturing ciliates, complex staining procedures, and the existence of morphospecies and cryptic species that can only be distinguished at the genetic level, posing challenges to accurate identifications (Rajter et al. 2022). Consequently, the identification of ciliates and other microorganisms has proposed DNA-based methods as an alternative taxonomic approach to unveil their vast diversity (Medinger et al. 2010, Cahoon et al. 2018, Pitsch et al. 2019, Antil et al. 2023).

DNA barcoding is a method of identifying species on the based on short DNA sequences linked to morphologically identified species (Hebert et al. 2003). DNA metabarcoding, or metabarcoding, is a method of identifying multiple species from bulk samples by relying on reference barcode sequences (Cordier et al. 2021). Environmental DNA can be directly extracted from soil or water and sequenced using high-throughput sequencing techniques such as 454-pyrosequencing, Ion torrent, and Illumina (Goodwin et al. 2017, Burki et al. 2021). The choice of a barcoding marker that provides the desired taxonomic resolution is crucial for ciliate metabarcoding studies. Hypervariable regions of the 18S ribosomal DNA (V4 or V9), COI gene, ITS1-5.8S-ITS2 region, and D1-D2 region of the 28S rDNA have been suggested as barcoding genes for ciliates (Zhao et al. 2016, Abraham et al. 2019, Antil et al. 2023). The method generates millions of short sequence reads (metabarcodes) of less than 500 bps in a single run (Slatko et al. 2018). These barcodes have created opportunities for discoveries from diverse environments, including the detection of rare, overlooked, and unculturable taxa (Andreoli et al. 2009, de Vargas et al. 2015, Pernice et al. 2016, Boenigk et al. 2018, Oliverio et al. 2020, Mugnai

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et al. 2023). The technique of metabarcoding has revolutionized the study of genomics and molecular biology, proving especially useful in the study of microbial diversity (Gimmler et al. 2016, Pawlowski et al. 2016, George et al. 2019, Sieracki et al. 2019, Zhao and Langlois 2022).

It is well known that the Indian Subcontinent is rich in biodiversity due to its diverse ecosystems. Despite this richness, data concerning ciliate diversity from India is rather scarce; only a few reports based on morphological identifications are available (Bhatia and Sewell 1936, Mahajan and Nair 1971, Kalavati and Raman 2008, Somasundaram et al. 2015, Rakshit and Sarkar 2016, Purushothaman et al. 2017, Bindu et al. 2018, Elangovan and Gauns 2018, Bharti and Kumar 2019, Chanda et al. 2019, Kaur et al. 2021). These studies have significantly contributed to the knowledge of the biodiversity of ciliates in India. However, to date, no eDNA metabarcoding studies have been conducted in India to study ciliate diversity, despite their worldwide application in studying microbial diversity and biomonitoring. In the present study, we compared three different types of water bodies: a river, lake, and pond. No previous study has compared ciliate communities in these habitats in Delhi using DNA metabarcoding approach. Hence, the present study aimed to: (i) unravel ciliate diversity from the three freshwater bodies and (ii) to examine the potential of using DNA metabarcoding for ciliate diversity studies in the future.

Materials and methods

Study area

The study was conducted at three sites in the catchment of River Yamuna in Delhi.

(1) Okhla Bird Sanctuary (OBS) (28°34′12″N, 77°18′8.28″E), is a bird sanctuary located at the Okhla barrage over River Yamuna. The site is situated at the point where the river enters Uttar Pradesh. It spreads over 4 km^2, with 20% area having a depth of 2–3 m and the rest of the area with a depth of more than 3 m.
(2) Sanjay Lake (SL) (28°36′51.12″N, 77°18′14.04″E) is an artificial lake developed by the Delhi Development Authority (DDA). It is situated at Trilokpuri in East Delhi and is surrounded by the residential areas of Kalyanpuri on the eastern side and Mayur Vihar on the western side. The lake's surface area is approximately 0.17 km^2, with a depth ranging from 1 m to 2.5 m, and it exhibits extensive growth of water hyacinth.
(3) Raj Ghat Pond (RJ) (28°38′26.16″N, 77°14′57.84″E) is a man-made pond located inside Raj Ghat, a memorial to Mahatma Gandhi and a major tourist spot in India. The pond is small and shallow, covering a surface area of about 0.01 km^2 with a depth of approximately 2 m.

DNA extraction, PCR amplification, and high-throughput sequencing

Surface water samples were collected from the littoral zone using a beaker. Sampling took place from the 11th to the 15th in the month of July 2019 at each site during the early morning hours around 8 am, as ciliates tend to be found near the shore due to reduced sunlight. The collected samples were transported to the laboratory in 500–1000 ml bottles and filtered through a Nytex net/mesh with a pore size of 120 µm to remove large crustaceans, debris, and other unwanted materials. These filtered samples were immediately transferred to 500–1000 ml beakers and the mouths of the beakers were covered with aluminum foil. The samples were kept at room temperature for enrichment, and boiled cabbage pieces were added. After the culture was enriched, DNA was extracted from each sample separately. The samples were filtered again using a 120 µm filter, removing the cabbage pieces from the medium. The cells were then centrifuged to obtain a cell pellet with a minimal volume of the medium. Total environmental DNA was extracted according to the manufacturer's protocol using a DNeasy blood and tissue kit (Qiagen, India) in 150 µl Elution buffer. The purity and concentration of the extracted DNA sample were assessed using Nanodrop. The samples were sequenced using 18S V4 amplicon sequencing using specific primers (18S V4: 528–706R as the barcode) (Hadziavdic et al. 2014). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The mixed PCR products were purified, and sequencing libraries were generated using Ion Plus Fragment Library Kit 48 reactions (Thermo Scientific) following the manufacturer's protocol. The library was sequenced on an Ion S5™ XL platform, and 400 bp/600 bp single-end reads were generated.

Sequence data processing

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH: fast length adjustment of short reads to improve genome assemblies (Magoč and Salzberg 2011). High-quality clean tags were obtained from raw tags through specific filtering conditions according to the QIIME (Caporaso et al. 2010) quality control process. Chimera sequences were identified and removed using UCHIME Algorithms (Edgar et al. 2011). Sequence analysis was performed by Uparse software, Uparse v7.0.1001 (Edgar 2013). Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs). The representative sequence for each OTU was screened for further annotation. The Silva database was employed for annotating taxonomic information. Phylogenetic information based on the OTUs was estimated/predicted using the MUSCLE software, Version 3.8.31 (Edgar 2004).

Statistical analysis

Alpha diversity was estimated to determine the complexity of each sample, analyzed through the following indices: Observed-species, Shannon index, and Chao1 index. All these indices were calculated using QIIME (Version1.7.0) and displayed with R software (Version 2.15.3). Community richness was identified by the Chao1 index, utilizing the Chao1 estimator (http://www.mothur.org/wiki/Chao), and community diversity was estimated using the Shannon index (http://www.mothur.org/wiki/Shannon). Beta diversity analysis was employed to evaluate differences between the samples in species complexity. Beta diversity, both weighted and unweighted unifrac, was calculated using QIIME software (Version 1.7.0). The weighted unifrac distance provides quantitative analysis, measuring the relative abundance of ciliate communities in the samples, while the unweighted unifrac distance offers qualitative analysis, determining the presence or absence of taxa in the samples. Cluster analysis was performed using the principal component analysis (PCA) to estimate differences in the samples. This analysis utilized the FactoMineR package and the ggplot2 package in R software (Version 2.15.3). The unweighted pair group method with arithmetic means (UPGMA) clustering, a hierarchical clustering method, was performed to estimate differences in the samples.

Table 1. Sequence details obtained from three sites with diversity indices, Okhla Bird Sanctuary (OBS), Sanjay Lake (SL), and Raj Ghat Pond (RJ).

Sample name	Raw reads	Qualified reads	Average length (nt)	OTUs	Shannon Index	Chao1 Index
OBS	1,57 307	1,49 219	298	106	2.808	103.500
SL	1,52 357	1,44 920	295	104	2.592	100.176
RJ	1,43 475	1,35 060	292	99	0.675	98.500

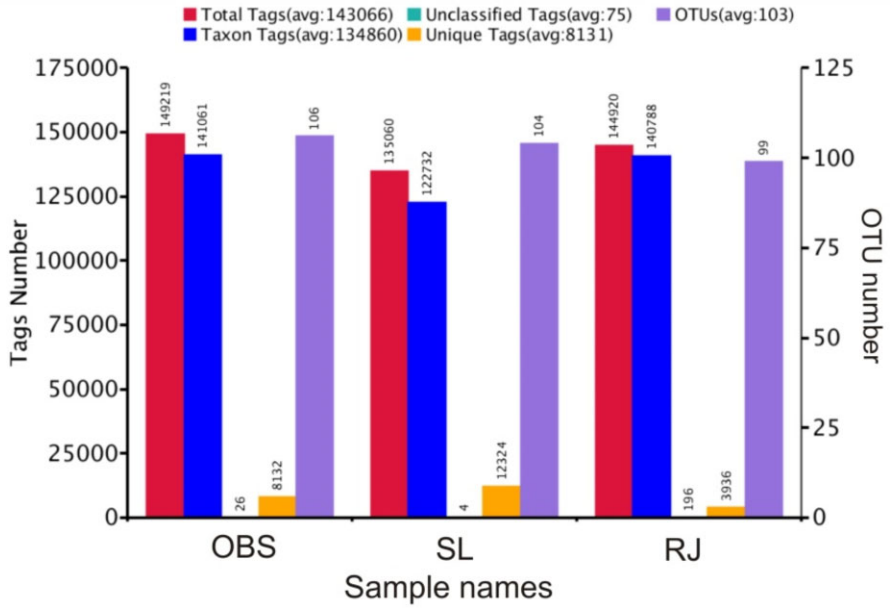

Figure 1. Summary of the tags and OTUs obtained from three sampling sites, Okhla Bird Sanctuary (OBS), Sanjay Lake (SL), and Raj Ghat Pond (RJ).

Results

Environmental DNA (eDNA) from three sites

Environmental DNA (eDNA) was extracted from the three selected freshwater sites during July 2019. Number of qualified reads obtained for each sample were as follows: OBS (149219), SL (144920), and RJ (135060). The average nucleotide length for each site was 292–298 nt. Operational taxonomic units (OTUs) were obtained by clustering all the effective tags with 97% identity. The maximum number of OTUs was obtained from the OBS sample (106), followed by SL (104) and RJ (99) (Table 1, Fig. 1).

Alpha diversity

Alpha diversity refers to the diversity of species observed within a particular ecosystem; it includes the number of species and their richness (Whittaker 1972). In the present study, alpha diversity was assessed for all the sites by species number calculated based on the OTUs. The OTUs were maximum in OBS (106), followed by SL (104), and lowest in RJ (99). Species diversity was assessed by calculating various diversity indices. Shannon diversity index was found to be maximum in OBS (2.80), followed by SL (2.59), and lowest in RJ (0.67). Chao1 estimator values, reflecting species abundance, were highest in OBS (103.50), followed by SL (100.17) and lowest in RJ (98.50). The Shannon index and Chao1 values collectively indicate that OBS has the highest ciliate diversity and abundance, followed by SL site, while RJ exhibits the least diversity (Table 1).

A rarefaction curve was obtained to equalize the sample size of all three sites by selecting a specified number of samples equal to or less than the number of reads in the smallest sample and randomly discarding reads from larger samples until all the samples were of equal size (Weiss et al. 2017). The rarefaction curve shows steep slopes in all the sites initially but later reached a plateau, indicating high species diversity initially but later on saturated as the number of species started to repeat. In OBS and SL the slope started to flatten a little later than in RJ, indicating more species diversity in OBS and SL samples (Fig. 2A). The relative abundance curve also showed a steep decrease in slope for all the three samples, with OBS exhibiting the maximum relative abundance, followed by SL and RJ (Fig. 2B). The Venn diagram, based on OTUs revealed a greater number of overlapping OTUs between OBS and SL (17 OTUs) compared to OBS and RJ (4 OTUs) (Fig. 2C). The maximum number of the overlapping regions was observed between SL and RJ (18 OTUs), indicating that SL and RJ may have identical ciliate communities.

Beta diversity

Beta diversity is the comparison of species diversity between different ecosystems and is usually measured as the difference in species number and composition between these ecosystems. Beta diversity indices were calculated to determine the differences between microbial communities based on their composition. The weighted and unweighted pair-group method with arithmetic means (UPGMA) based on weighted and unweighted Unifrac matrices were analyzed, and the dissimilarity coefficients were determined. The dissimilarity coefficients, represented by Beta diversity heatmap based on weighted (quantitative) and unweighted unifrac (qualitative) distance between OBS and RJ were 0.466 and 0.609; between OBS and SL were 0.429 and 0.447; between SL and RJ were 0.180 and 0.502, respectively (Fig. 3). This indicates that

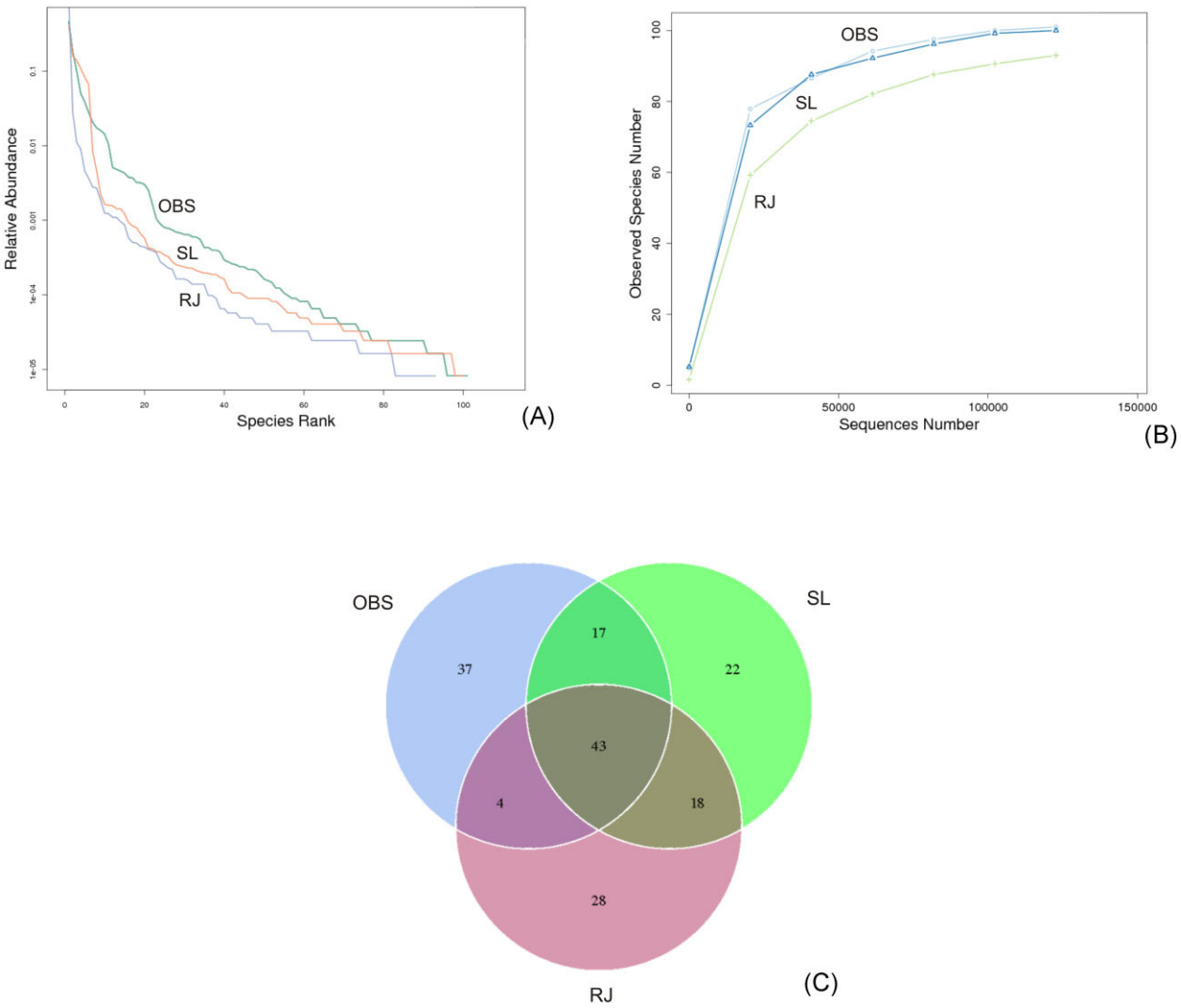

Figure 2. Rarefaction curves representing (A) relative abundance and (B) observed species number based on ciliate OTUs in each sample, and (C) Venn's diagram showing number of OTUs in different samples, Okhla Bird Sanctuary (OBS), Sanjay Lake (SL), and Raj Ghat Pond (RJ). Overlapping regions represent species similarity in different sites.

the SL and RJ site were more similar in terms of species diversity and composition.

Beta diversity was further represented using a hierarchical clustering method called the unweighted pair group method with arithmetic averages (UPGMA) and by using principal coordinate analysis (PCoA). UPGMA based on weighted unifrac distance indicated that SL and RJ clustered together, suggesting identical community structure and species abundance. UPGMA based on unweighted unifrac distance, on the other hand, showed that OBS and SL clustered together indicating a similar group of taxa. Similarly, the PCoA plot based on weighted unifrac distance showed that SL and RJ were placed together on the right side of the plot, at the positive coordinate of the PC1 axis, while the OBS site was positioned separately. The PCoA plot based on unweighted unifrac distance revealed that OBS and SL were placed together on the left side of the plot, at the negative coordinates of PC1, whereas the RJ site was positioned separately (Fig. 4).

Microbial community analysis

Nine different eukaryotic phyla, namely, Ciliophora, Gastrotricha, Rotifera, Ochrophyta, Diatomea, Protalveolata, Cryptomycota, Basidiomycota, and Chlorophyta were observed in the three freshwater samples. In OBS, 4% of species belonged to the Phylum Gastrotricha (Kingdom Metazoa), while this phylum was poorly distributed in the other two samples, with 0.01% and 0.02% in SL and RJ, respectively. The phylum Rotifera (Kingdom Metazoa) constituted 2.09% in the OBS sample, with a significantly lower abundance in the other two samples (0.20% in SL and 0.01% in RJ). The % abundance of other phyla belonging to Kingdom Eukaryota (Ochrophyta, Diatomea, Protalveolata), Kingdom Fungi (Cryptomycota, Basidiomycota), and Kingdom Chloroplastida (Chlorophyta) was relatively very low in all the three samples, ranging from 0.01 to 0.2% (Figs 5 and 6).

Ciliate community composition

In the OBS sample, ciliates belonging to the class Oligohymenophorea were identified to have high % abundance (66.29%) followed by the class Prostomatea (3.25%), Spirotrichea (0.66%), Litostomatea (0.30%), Phyllopharyngea (0.08%), Nassophorea (0.08%) and Colpodea (0.01%). The sample also had unidentified eukaryotes contributing to 20.78% of the total species. OBS is observed to have a high % abundance of unidentified eukaryotes

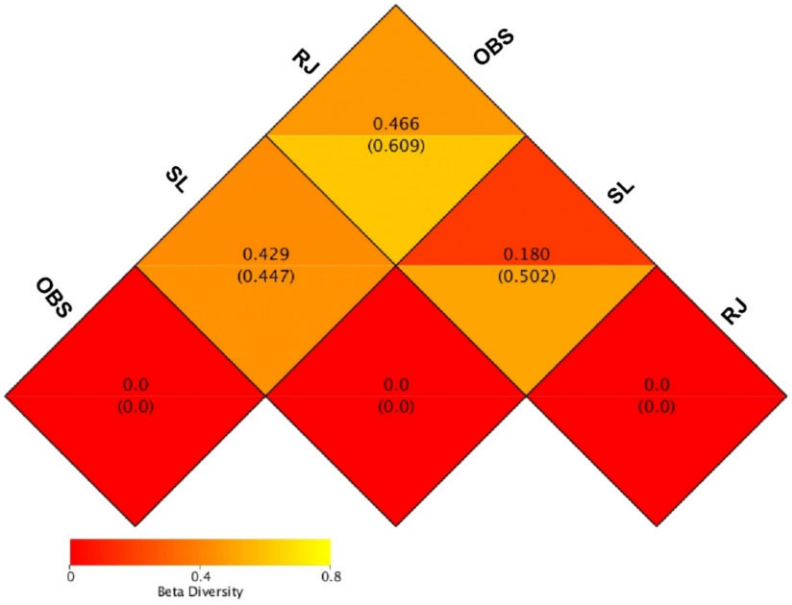

Figure 3. Beta diversity heatmap based on weighted and unweighted unifrac distances. Each grid represents the pairwise dissimilarity coefficient between samples, in which weighted unifrac distance is displayed above and unweighted unifrac distance below. OBS = Okhla Bird Sanctuary, SL = Sanjay Lake, RJ = Raj Ghat Pond.

as compared to the other two samples which indicates that OBS may have a high percentage of microbial diversity as compared to SL and RJ. In SL sample, a high % abundance of ciliates belonging to the class Oligohymenophorea (97.73%) followed by the class Litostomatea (0.94%), Spirotrichea (0.41%), Prostomatea (0.11%), Karyorelictea (0.04%), Phyllopharyngea (0.03%), and Nassophorea and Heterotrichea with 0.01%. Around 0.09% contributed to unidentified eukaryotes. In RJ sample, a high % abundance of ciliates belonging to the class Oligohymenophorea (97.01%), followed by the class Prostomatea (1.26%), Spirotrichea (0.97%), Phyllopharyngea (0.22%), Litostomatea (0.18%), Colpodea (0.04%) and Nassophorea (0.02%). This sample also had unidentified eukaryotes having a % abundance of 0.02% (Figs 5 and 6).

Overall, ciliates belonging to the class Oligohymenophorea (87.01%) were predominant, followed by the class Prostomatea (1.54%), Spirotrichea (0.68%), Litostomatea (0.48%), Phyllophryngea (0.11%), Nassophorea (0.04%), Colpodea (0.02%), Karyorelictea (0.01%), and Heterotrichea (<0.01%), observed in all the three samples (Fig. 7). Based on the relative species abundance and the clustered heatmap showing taxonomic abundance, it was observed that species from the genus *Paramecium* of class Oligohymenophorea, *Coleps* of class Prostomatea, and *Tetmemena* of class Spirotrichea were present predominantly in all three samples. Thus, these genera, representing different classes of the phylum Ciliophora, can be considered key components of the ciliate communities identified from the freshwater samples of Delhi, India (Fig. 7).

Phylogenetic analyses

A total of 309 OTU representative sequences from all the three sites were assigned to Ciliophora to infer a maximum likelihood tree. The tree topology revealed nine clusters representing nine classes of the phylum Ciliophora. The class Oligohymenophorea represented the maximum number of genera, followed by the class Spirotrichea, Litostomatea, Prostomatea, Colpodea, Phyllopharyngea, Nassophorea, Karyorelictea, and Heterotrichea. Thus, the tree indicated clustering of three superclades which include CONThreeP (Colpodea, Oligohymenophorea, Nassophorea, Prostomatea, and Phyllopharyngea), superclade SAL (Spirotrichea, Litostomatea), and superclade Postciliodesmatophora (Karyorelictea and Heterotrichea).

Representative species belonging to the classes Oligohymenophorea, Spirotrichea, and Phyllopharyngea were identified. The class Oligohymenophorea was represented by a few important genera such as *Paramecium*, *Dexiotricha*, and *Cyclidium*, Phyllopharyngea by *Chilodonella* and *Trithigmostoma*, Spirotrichea by *Hypotrichidium*, *Oxytricha*, *Paraurostyla*, *Pseudokeronopsis*, and *Tetmemena*. Some genera were identified up to species level: *Cyclidium glaucoma*, *Dexiotricha granulosa*, *Paramecium multimacronucleatum*, *P. polycaryum*, *P. tetraurelia*, *Chilodonella uncinata*, *Trithigmostoma steini*, *Hypotrichidium paraconicum*, *Oxytricha granulifera*, *Paraurostyla weissei*, *Pseudokeronopsis erythrina*, and *Tetmemena pustulata* (Fig. 8).

Discussion

Taxonomic diversity from the three freshwater sites

The diversity of ciliates observed in the three freshwater sites of the Delhi, India, was explored for the first time using DNA metabarcoding approach. Notably, a high number of ciliate species were identified from the classes Oligohymenophorea, Spirotrichea, and Prostomatea. Previous reports indicate that these classes are highly diverse and dominate freshwater ecosystems (Amaral-Zettler et al. 2009), marine ecosystems (Gimmler et al. 2016), deep-sea hypersaline anoxic basins (Filker et al. 2013), and bromeliad tanks (Simão et al. 2017). It was observed that during July and September months (monsoon and postmonsoon seasons in Delhi), temperature, pH, and electrical conductivity are in a favourable range in all three sites. These factors could have encouraged the growth of and abundance of these classes. Other than physical factors, the abundance of oligohymenophorean and spirotrich ciliates is due to their nature as planktonic grazers. The monsoon and post-monsoon seasons favour the growth of bacteria and smaller phytoplanktons (<20 µm), on which these grazer

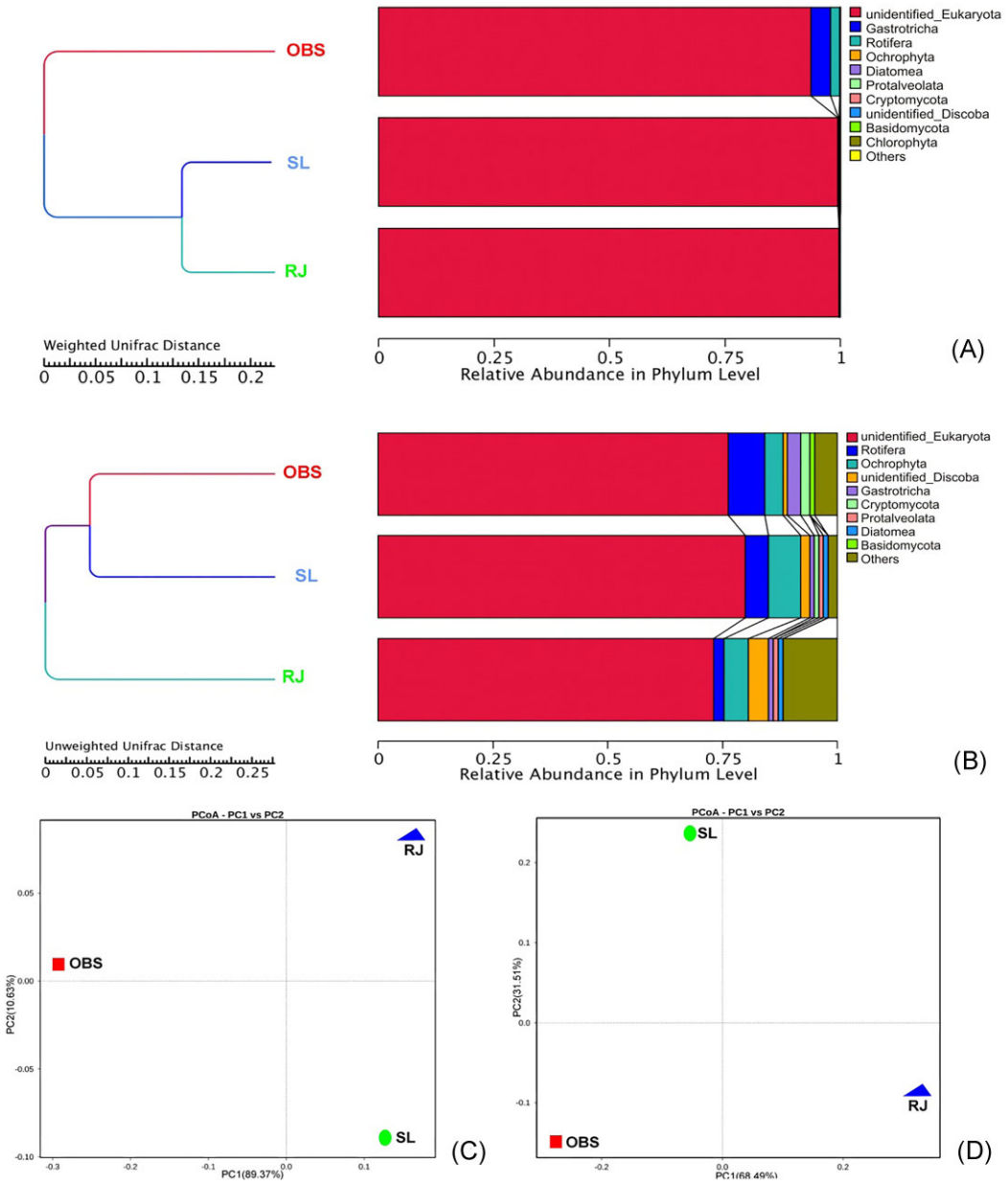

Figure 4. The Unweighted Pair-group Method with Arithmetic Mean (UPGMA) cluster tree based on (A) weighted unifrac distance and (B) unweighted unifrac distance. Principal coordinates analysis (PCoA) based on (C) weighted unifrac distance and (D) unweighted unifrac distance. The percentage on each axis indicates the contribution value to discrepancy among samples. OBS = Okhla Bird Sanctuary, SL = Sanjay Lake, RJ = Raj Ghat Pond.

ciliates feed, providing conditions for their growth and abundance during this season (Haraguchi et al. 2018). Another adaptive strategy employed by species of these classes is the formation of cysts in unfavourable conditions, which hatch when the conditions are favourable. This may also contribute to their abundance in the monsoon and post-monsoon seasons when the conditions are suitable (Verni and Rosati 2011). The high abundance of oligohymenophorean species such as *Paramecium* and spirotrichean species such as *Oxytricha*, *Paraurostyla*, *Pseudokeronopsis*, and *Tetmemena* could be correlated with the 18S rDNA copy number and cell size. Large species, more than 100 µm in size, have a large macronucleus or consists of multiple macronuclear nodules, hence, possessing multiple copies of the 18S rDNA (Zhu et al. 2005). The correlation between cell size and 18S rDNA copy number has been reported in many protistan studies (Medinger et al. 2010, Stoeck et al. 2014). Interestingly, three species from the genus *Paramecium*, namely, *Paramecium multimacronucleatum*, *P. polycaryum*, *P. tetraurelia*, were observed to be highly abundant in all three sites (Fig. 8), and this could be attributed to multiple reasons. *Paramecium* species are common worldwide in ponds, lakes, and streams, and are reported to tolerate pollutants, heavy metals, and organic matter, making them good bioindicator species (Miyoshi et al. 2003). Most of the *Paramecium* morphospecies consist of cryptic species, which are morphologically similar and can be delimited by molecular methods (Fokin 2010). In the present study, ciliates belonging to the genus *Paramecium* are identified up to the species level due to the availability of a well-characterized and updated *Paramecium* database (Arnaiz et al. 2019). Another reason for the abundance of *Paramecium* species, especially *P. polycaryum* and *P. tetraaurelia*, in the samples (more than 35%) could be

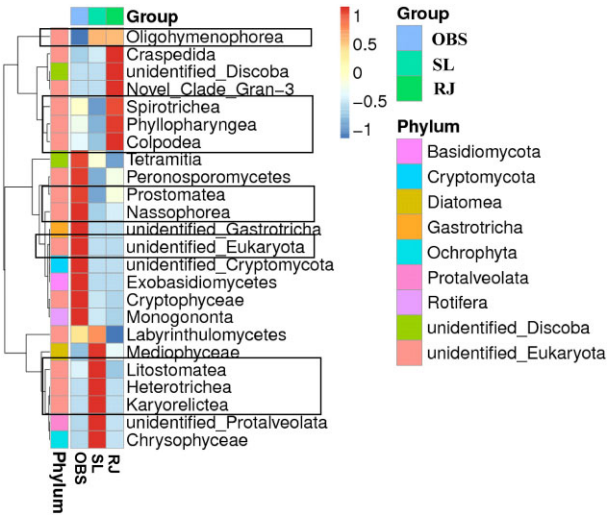

Figure 5. Clustered heatmap showing the relative abundance of different classes belonging to different phyla present in the three studied samples. The class marked with boxes represents the classes of Phylum Ciliophora. OBS = Okhla Bird Sanctuary, SL = Sanjay Lake, RJ = Raj Ghat Pond.

due to the well-known mechanism of autogamy in these species (Diller 1954, Nowak et al. 2011). This mechanism allows them to swiftly respond to environmental factors, helping them to maintain their life and vigor. *Coleps* and *Colpoda* from classes Prostomatea and Colpodea respectively, were also observed in the present study in all the sites. *Levicoleps*, *Cryptocaryon*, and *Prorodon* were observed from the class Prostomatea, which were not observed in microscopic study from the same sites, possibly due to their small size, making them difficult to capture (Dolan and Marro 2020).

The other most represented class was Litostomatea, with 0.9% species in SL sample and 0.16% species in RJ sample. *Dileptus* sp., *Amphileptus* sp., *Didinium* sp., and *Aceneria* sp. were identified from the class Litostomatea. Litostomateans are easily detected even if their abundance is low due to their large size and a higher number of copies of 18S rDNA (Vd'ačný et al. 2011, Vd'ačný and Foissner 2012). Litostomateans (mainly haptorians) commonly lead a predatory lifestyle and are present in polysaprobic conditions because these organisms prefer microaerophilic environments (Kaur et al. 2021). Strikingly, nassophoreans were also present in the sample but were less than 0.1% in all three samples with the representative genus being *Leptopharynx*. Anaerobic armophoreans and plagiopylids were absent from the samples, which may be attributed to dissolved oxygen levels exceeding 2 mg/L in the sampling sites. About 1% of the OTUs were unassigned to any group of ciliates, most likely due to an extensive gap in available reference sequences, as numerous ciliate species are not represented in molecular databases (Boscaro et al. 2017).

Comparing high-throughput sequencing (HTS) and traditional methods for studying ciliate diversity

Ciliate community structure and distribution have been studied for a long time using both traditional microscopic methods and modern molecular methods. The accuracy and precision of both the techniques vary, hence, have been long debated (McManus and Katz 2009, Santoferrara et al. 2016). Microscopic methods are useful for characterizing the species by studying morphological features, which vary among different groups of ciliates. The identification process is mostly cumbersome and requires expertise (Will and Rubinoff 2004). DNA-based methods are widely used and are comparatively easier for identifying species in a sample based on the operational taxonomic units (OTUs) obtained from the samples using one or more available barcodes (Hebert et al. 2003, Bucklin et al. 2007, Lin et al. 2009). DNA-based methods, such as high throughput sequencing techniques, are known to detect the rarest of species in an assemblage, revealing diversity not evident through other methods (Stoeck et al. 2009, Santoferrara et al. 2014, 2020).

In the present study using the high throughput sequencing method (HTS), the maximum number of species were observed in the OBS site (106), followed by SL (104), and RJ (99). In microscopic observations from the same sites, ciliates from eight classes, Spirotrichea, Oligohymenophorea, Prostomatea, Litostomatea, Phyllopharyngea, Karyorelictea, Heterotrichea, and Colpodea were observed. Predominantly, ciliates were observed from the classes Spirotrichea, Oligohymenophorea, and Prostomatea. Spirotrichea was observed to be the most diverse class. Through HTS analyses, in addition to the eight aforementioned classes, ciliates from the class Nassophorea were also observed, forming a total of nine classes. OTUs from the class Oligohymenophorea were predominantly present in the three samples, followed by class Prostomatea, and class Spirotrichea. The rest of the classes were observed in less abundance. The genera observed exclusively in the present study using HTS analysis and that escaped microscopic observations were *Hypotrichidium, Amphileptus, Protocyclidium, Levicoleps, Trithigmostoma, Tokophrya, Aceneria, Cryptocaryon, Prorodon, Bromellothrix,* and *Leptopharynx*.

It is noted that DNA-based methods can identify only those species with available 18S rDNA sequences in the databases. Hence, this factor may have contributed to the variations in ciliates identified by both methods. Previous studies also reveal differences in the number of ciliate taxa suggested by morphological and high-throughput sequencing techniques (Medinger et al. 2010, Stoeck et al. 2014). Most of the time, the number of ciliate taxa suggested by molecular methods were more than double of that indicated by microscopy alone (Doherty et al. 2007, Dopheide et al. 2009, Tucker et al. 2017). Metabarcoding often leads to the overestimation of diversity because it retrieves more species or OTUs than morphologically identified species. This phenomenon holds true when estimating the diversity of diatoms (Zimmermann et al. 2015, Mora et al. 2019), euglenids (Lax and Simpson 2013), seagrass communities (Cowart et al. 2015), algal communities (Manoylov 2014), cyanobacteria (Mackeigan et al. 2022), and the total eukaryotic SAR clade (Grattepanche et al. 2018). However, metabarcoding is a valuable approach to identifying gaps in described groups of species and can lead to refinement of diversity assessments (Mora et al. 2019). The main reason for the incongruence of data is the incompleteness and lack of accuracy of reference databases. Missing taxa in reference databases would not be identified in environmental sequences, whereas sequences with incorrect identifications in databases will generate inaccuracies in taxonomic identifications (Santoferrara et al. 2016). Richness overestimation in metabarcoding data is due to the intraspecific and intragenomic variability of the barcoding marker. This becomes problematic when a single traditionally characterized species or bioindicator taxon has multiple genotypes at the barcoding region (Pawlowski et al. 2018). In cases where a single species exhibits multiple genotypes at the barcoding region, it may cluster into different OTUs, artificially creating taxonomic richness in the data. High intraspecific variation is common in ciliates due to the polyploid somatic macronuclei (Weisse and Lettner 2002, Wang et al.

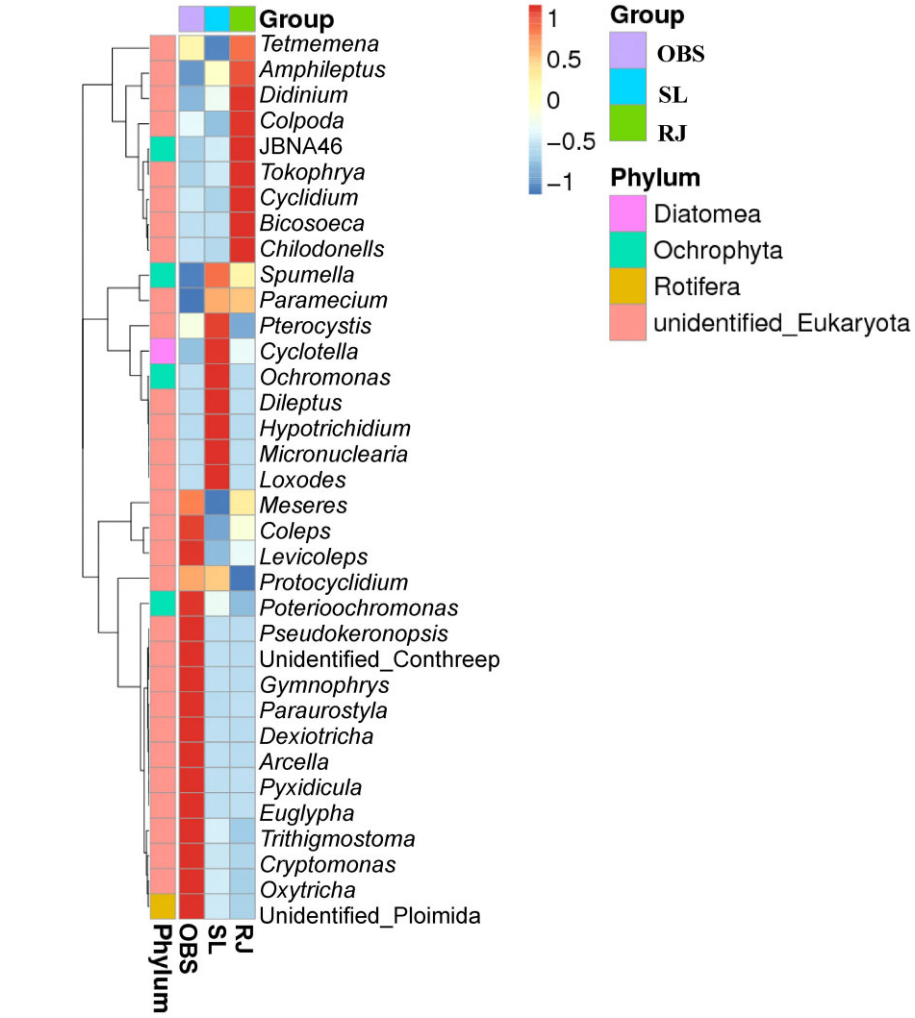

Figure 6. Clustered heatmap showing relative species abundance where majority of the genera are from Phylum Ciliophora (unidentified_Eukaryota). OBS = Okhla Bird Sanctuary, SL = Sanjay Lake, RJ = Raj Ghat Pond.

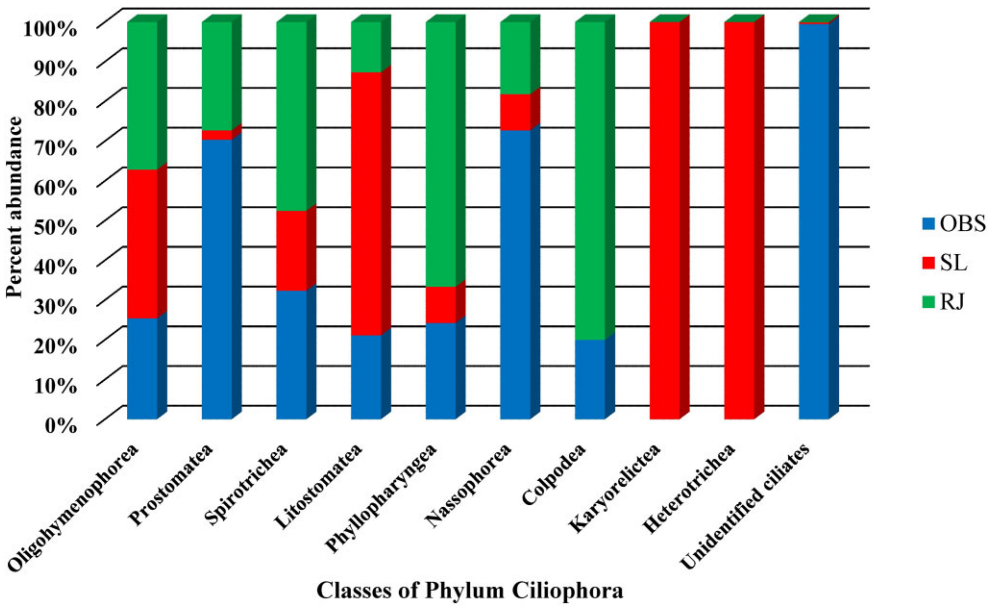

Figure 7. Graph showing the % abundance of different classes of Phylum Ciliophora determined from the three samples, Okhla Bird Sanctuary (OBS), Sanjay Lake (SL), and Raj Ghat pond (RJ).

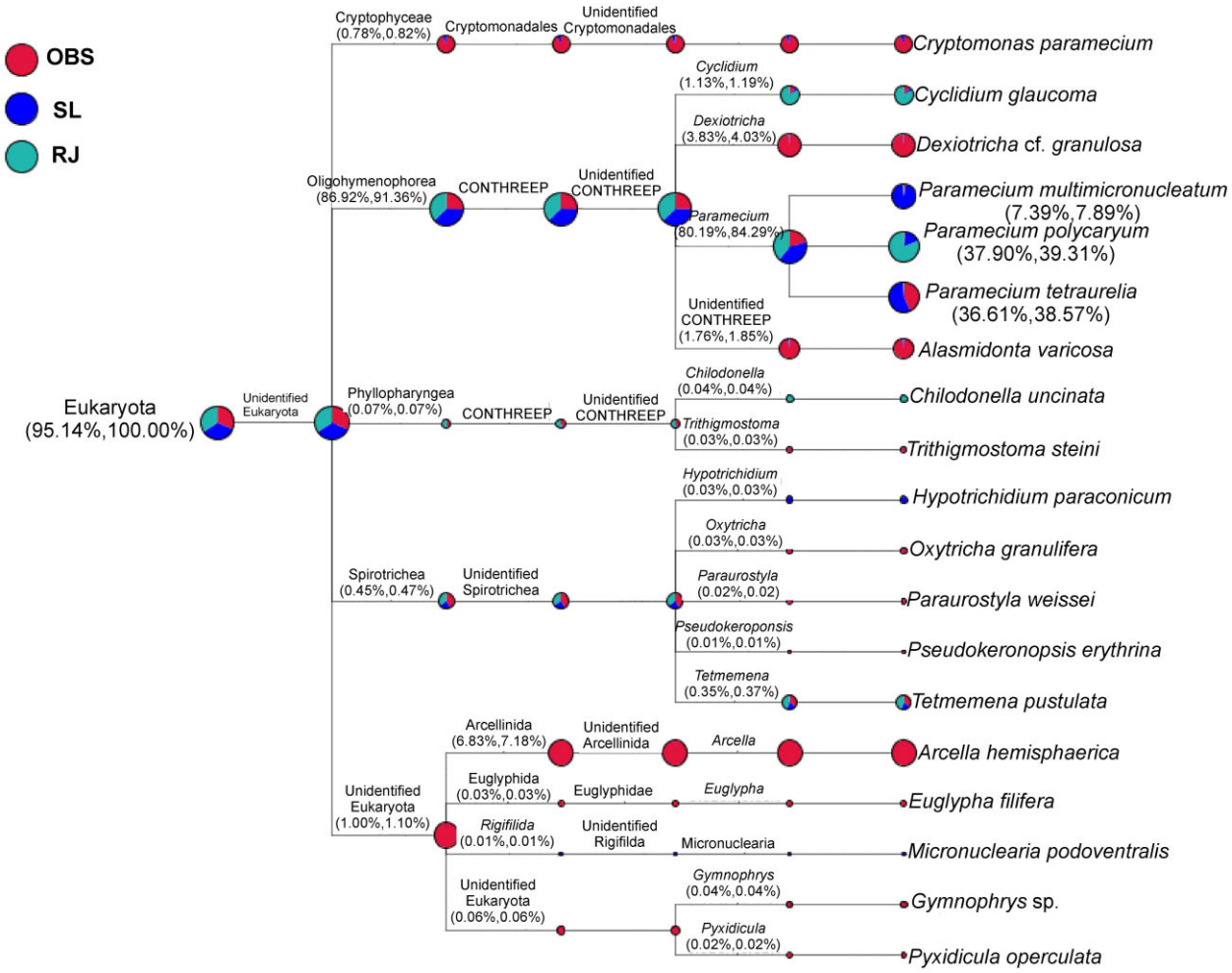

Figure 8. Phylogenetic tree showing specific ciliate species in the three samples. Different colour represents different samples (OBS in red, SL in blue, and RJ in green) and the size of the circles stand for the relative abundance of the species. The first number below the taxonomic name represents the percentage in the whole taxon and the second number represents the percentage in the selected taxon. OBS = Okhla Bird Sanctuary, SL = Sanjay Lake, RJ = Raj Ghat pond.

2017). This phenomenon is also prevalent among other bioindicator groups, such as diatoms (Pérez-Burillo et al. 2020) and aquatic insects (Elbrecht et al. 2014). It is worth noting that OTUs do not necessarily correspond to species and, as a result, require phylogenetic analyses to assign OTUs to specific taxa (Santoferrara et al. 2014).

The over- or underestimation of data from morphological observations is attributed to various factors involved in the complex staining and identification processes. Taxa that are low to very low in abundance are either lost in multiple steps involved in staining, or remain unnoticed. Sometimes, cells are inevitably contracted, swollen, or broken after the fixation or bleaching process, leading to discrepancies in morphology-based identifications (Dunthorn et al. 2014, Abraham et al. 2019, Rajter et al. 2022). Moreover, some ciliates groups, such as free-living litostomateans, members of the genera *Pseudoprorodon* and *Cryptolophosis* cannot be sufficiently stained at all (Abraham et al. 2019). The "cryptic species" or "cryptic diversity" problem is prevalent in both methods. Cryptic species are those that are morphologically similar but genetically diverse. Cryptic diversity refers to the presence of unseen species, rare, or inactive forms in an environmental sample. These species may be morphologically diverse but go unnoticed (Fenchel et al. 1997). In microscopic observations, there could also be an issue with polymorphic species which are different types or forms of individuals of the same species (Dolan 2015). Another problem is the presence of shared derived characters in morphologically similar species (Santoferrara et al. 2016). Dormant or inactive stages of the ciliates in the form of cysts can contribute to the additional diversity, hence, some species may be excluded from the microscopic observations or counts (Pawlowski et al. 2014). Most of the ciliates present in extreme environments such as hypersaline lagoons or sites of extreme salinity are in the form of cysts hidden in the 'seed bank', awaiting favourable growth conditions (Fenchel et al. 1997, Esteban and Finlay 2003, Fenchel 2005, Galotti et al. 2014). Ciliate community composition changes rapidly in short periods due to environmental conditions, the availability of suitable food resources, competition, or predation (Finlay and Esteban 1998). Some species groups are prevalent at a certain time of the year, and others at different times, altering the community dynamics of sampling sites (Salmaso et al. 2020). The "collector's curve" is another source of variation; it states that as one increases the sampling effort, the number of organisms caught increases, resulting in varying abundance of organisms (Heip et al. 1998). Various enrichment techniques can also increase the number of species and can produce biases in the estimates of abundance (Fenchel et al. 1997).

Future scope of high throughput DNA metabarcoding to assess ciliate diversity

Taxonomy is a science that is constantly improving its ability to identify species and assign position of species in the classification system. Ciliate diversity assessments use taxonomy as a tool to provide accurate diversity estimates from undiscovered habitats. With the advancement of technology diversity assessments have improved. According to the present study, the high throughput DNA metabarcoding technique proves to be an easy, powerful, and innovative tool with immense potential to revolutionize the study of ciliate diversity in complex ecosystems. With this method, researchers can efficiently identify and quantify ciliate taxa present in environmental samples without time-consuming culturing and microscopic examinations. It can identify unknown and rare ciliates and can offer vital clues about ecosystem health and stability, as ciliates are bioindicators of environmental conditions and pollutant levels. However, numerous hurdles persist in the process of establishing standardized barcoding markers for ciliates to yield better resolution beyond the genus level. The suitability of the 18S V4 barcode region as a marker for discriminating closely related species also remains a subject of ongoing scientific debate and scrutiny, and therefore, multiple barcoding loci can be preferred over the use of a single barcode (Zhan et al. 2019). Multiple primer pairs can be employed targeting specific taxonomic groups and amplification bias can be prevented (Cristescu 2014). The reliability and accuracy of "blind metabarcoding" need to be tested wherever possible combining morphological community descriptions from the same area sampled for molecular analysis (Cowart et al. 2015). To minimize variability in DNA quantity, DNA extraction process, amplification rates in different species, barcodes used, sequencers, runs, and 18S rDNA copy numbers, these parameters must be evaluated and standardized prior to the study. The number of replicates can be increased and sampling at different seasons of the year can be done to curtail the influence of physicochemical parameters on the diversity, abundance, and richness estimates of the ciliates.

Conclusion

The present study represents a pioneering utilization of DNA metabarcoding to elucidate ciliate diversity within freshwater samples from Delhi, India. The study reveals ciliate diversity, abundance, and composition from the three freshwater sites: OBS, SL, and RJ. The OBS site exhibited the maximum ciliate diversity and abundance compared to SL and RJ sites. Additionally, similarities in ciliate composition were observed between SL and RJ sites. Ciliates from the classes Oligohymenophorea, Prostomatea, and Spirotrichea were dominant in all three sites. The study discusses specific challenges associated with studying ciliate diversity using solely DNA-based methods or by traditional microscopic methods. Future studies employing DNA metabarcoding approach could benefit from integrating environmental data such as water quality parameters, temperature, and nutrient levels to enhance our understanding of the factors influencing ciliate diversity. Beyond taxonomic identification, future studies could focus on understanding the functional roles of ciliates within ecosystems. In conclusion, the future of high-throughput DNA metabarcoding for assessing ciliate diversity is promising, and continued efforts will contribute to utilizing ciliates as potential bioindicators for diversity assessments, monitoring environmental changes, and understanding climatic variations across different regions.

Author contributions

S Makhija and R Toteja designed and supervised the research study. JS Abraham, S Somasundaram, S Maurya, and U Sood collected samples, performed experiments and analyzed the data. JS Abraham wrote the original draft. S Makhija, R Toteja, and R Lal revised and improved the manuscript.

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Supplementary data

Supplementary data is available at *FEMSMC Journal* online.

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