

Comparison of bacterial diversity from two mangrove ecosystems from India through metagenomic sequencing

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Running title:

Comparative mangrove bacterial diversity using metagenomics

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Abstract

In the present study, we explored the microbial diversity of two mangrove regions, Mangalavanam (MAN) and Puthuvype (PVY), in Cochin, India. While PVY is a coastal island, MAN lies in the heart of the mainland of Cochin, making these two ecosystems different. Illumina MiSeq metagenomic analysis using V3-V4 region of 16S rRNA gene was employed to study the microbial diversity. Based on the sequence analysis, the most abundant prokaryotic domain was bacteria (99.94% and 99.87% in sample PVY and MAN, respectively). Proteobacteria was the most dominant phylum in both MAN(48.19%) and PVY (65.66%). Alphaproteobacteria (18.27 %) was the most abundant class found in MAN, whereas Gammaproteobacteria (43.94%) was dominant in PVY. Principal component analysis was used to determine factors that modulate the bacterial community structure. In both environments, bacterial communities showed significant variation due to variation in sediment composition and location. At the phylum level, both sampling sites showed similarities but minor communities and species showed distinct variations in the distribution. The observed differences in the bacterial community structure in these ecosystems could be attributed to the specific environmental characteristics of each sampled mangrove ecosystem.

Keywords: Mangroves, Metagenomics, non-culturable, microbial diversity, Proteobacteria

1. Introduction

Mangroves are estuarine ecosystems that constitute 60-70% of the coastline in the tropics and subtropics. They play significant protective and functional role in ecological balancing. Hence, mangroves form a crucial component of the food chain in the saline coastal biome. The dynamic environment of the mangrove ecosystem due to tidal influx, salinity, pH, temperature, availability of light, degree of soil anoxia, soil texture, rainfall pattern and organic and inorganic nutrient availability provides an excellent environment for a wide range of organisms with heterogeneous functions (Holguin et al., 2001). Mangroves are natural carbon sinks storing enormous amount of CO₂ trapped from the atmosphere in their biomass (Ray and Jana, 2017). In addition, mangroves are important reservoirs of microbial diversity (Imchen et al., 2018). The microbial communities in mangroves suggests that they play a significant role in biogeochemical cycles and ecosystem functions.

Less than 1% of the total microbial communities from environmental samples are culturable and conventional methods are inadequate for assessing the taxonomic and functional diversity of these ecosystems (Handelsman, 2004; Steen et al., 2019). The metagenomic analysis of environmental samples allow us to shed light on these unexplored communities (Stewart, 2012; Parvathi et al., 2019). Moreover, the species richness in mangrove sediments is far greater than that of the water column (Kirchman et al., 2010; Ramírez-Flandes et al., 2019). Apart from the mere diversity, microbial communities in mangrove sediments also play vital role in the biogeochemical cycles of coastal ecosystems (Thorsten and José, 2001). Alternate respiratory pathways like denitrification and sulfate reduction are favoured in the organic carbon rich mangrove sediments, with anaerobic conditions (Krishnan and LokaBharathi, 2009). The biomes from such anoxic environments are involved in methanogenesis, and many redox processes such as nitrogen fixation, denitrification, sulfur oxidation, and hydrogen oxidation (Ramírez-Flandes et al., 2019).

The present work compares the microbial diversity in two mangrove ecosystems in an urban area along the southwest coast of India, using the metagenomic approach. The growth of urban areas might severely impact the mangroves and result in deterioration of biodiversity in these ecosystems. The microbial diversities of these mangrove regions along with their predicted functional profiling in the urban area of Cochin is presented in this paper.

2. Materials and methods

2.1. Site description and sampling

We selected two major mangroves along the southwest coast of India, namely Puthuvypeen (PVY) and Mangalavanam (MAN) (Fig S1). Puthuvypeen mangrove region is situated on the western side of Cochin with Kodungallur Strait on the North, Cochin backwaters and Cochin Port on the South, River Periyar and Cochin city on the East and Arabian Sea on the west. The ecology of this island is unique, endowed with large canals and a network of small canals over 50 km long (Chatturvedi, 2005). The area covered by this study has geographical coordinates of 09°58' N latitude and 76°23' E longitude. Similarly, the Mangalavanam ecosystem located at 9°59' N latitude and 76°16' E longitude, is only a small patch of greenery with a tidal wetland with an extent of 2.74 ha. It has been declared as a protective area on 31st August, 2004 (Madhusudhanan and Jayesh, 2011). Sediment samples were collected from two mangrove ecosystems using core sampling. A core with a length of 30 cm and 7 cm diameter was used for the collection of samples. Five core samples separated by a distance of 2 m from each other were collected from each mangrove region in December, 2017, and the individual samples were processed and pooled for metagenome analysis.

2.2 Environmental parameters

In situ measurements of pH, temperature and salinity were performed using a multi-parameter analyser. Total nitrogen (TN), total sulphur (TS) and total carbon (TC) in sediment were determined using standard protocol (Flash 2000 CHN elemental analyser, Thermo Fisher Scientific Incorporation, US) (Grashoff et al., 1999).

2.3 DNA isolation and amplicon library construction

The DNA was extracted from the sediment using Ultraclean Soil DNA isolation Kit, (MoBio lab, Geneworks, Australia). Five subsamples from each location were used in the study. DNA extracted from the subsamples were pooled and were considered as a single sample representing a particular location. The sample DNA was quantified using a Nanodrop (Nanodrop technologies, USA), and DNA was stored at -80°C and an average concentration of 25 ng/μl of DNA was used for PCR reaction. The specific primers (for V3-V4 region) used for PCR amplification were 16S Forward Primer-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and 16S Reverse Primer-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTCHVGGGTATCTAATCC) and library was constructed according to Illumina MiSeq metagenomic-library-prep-guidelines.

https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

2.4 Illumina sequencing

PCR products were purified using PureLink PCR Purification kit (Invitrogen, CA, USA). The purified, pooled samples were subjected to several preparation steps with dilution, denaturation, neutralization and again pooled with other libraries prepared for NGS according to Illumina MiSeq next generation sequencing guidelines (Illumina, CA, USA) at Genotypic Technology Pvt. Ltd (Bangalore, India)(Ramanan et al., 2016; Parvathi et al., 2019).

<https://www.illumina.com/content/dam/illumina-marketing/documents/products/datasheets/hiseq-3000-4000-specification-sheet-770-2014-057.pdf>.

2.5 Data analysis

The automated Perl code was used to remove raw reads with adapter sequences. Sequences with high-quality reads (more than 70% bases) and with aPhred score greater than 20 were used for further analysis using Illumina BaseSpace 16S Metagenomics App. Greengenes (v 13.5) database was used for data analysis. The alpha diversity was calculated using QIIME python script (alpha_diversity.py). Hierarchical clustering dendrogram was generated using Illumina 16s App and heat map using R scripts. A Venn diagram was constructed to identify common and unique species in the two mangrove sediment samples(Wang et al., 2007).

2.6 Statistical analysis

For comparing the relative complexity of the bacterial communities, different statistical tools such as Goods coverage (QIIME, alpha_diversity.py), richness and diversity statistics (DOTUR, abundance-based coverage estimator ACE (S_{ACE}), bias-corrected Chao1 (S_{chao1}) and the Shannon-Weaver and Simpson diversity index were used (Chao, 1987; Gotelli and Colwell, 2011). The comparative metagenomic study was performed using a web-based analytical pipeline, METAGEN assist (Arndt et al., 2012) <http://www.metagenassist.ca/METAGENassist/faces/Home.jsp>. The heatmap was generated using R script where the relative abundance of species of two samples were arranged in matrix format and linkages were generated using hierarchical clustering based on Bray-Curtis distance based dendrogram for each sample. Principal component analysis was performed to understand the relationship between environmental parameters and various bacterial phyla using PAST (version 3.0) after normalisation (x-mean/standard deviation). Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt), was used to predict the functional composition of a metagenome using marker gene data and a database of reference genomes.

PICRUSt uses an extended ancestral-state reconstruction algorithm/ evolutionary modelling to predict metagenomes from 16S data and a reference genome database (Greengenes)(https://picrust.github.io/picrust/tutorials/algorithm_description.html#metagenome-prediction).

2.7 Nucleotide sequence accession number

The Illumina sequencing data were deposited into the NCBI Sequence Read Archive (SRA, <https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP106765>) under the bioproject number, PRJNA383283, and SRA study number, SRP106765.

3 Results and Discussion

3.1 Environmental parameters and sediment characteristics

Environmental parameters varied greatly between the selected mangrove ecosystems, MAN and PVY. The major physico-chemical parameters analysed are enlisted in the Table 1. The sediment of MAN was nitrogen and sulphur rich with a value of 2.072 and 0.263% respectively. Interestingly, sulphur was present in very low amount (0.04%) in PVY sample. The PVY sample was carbon rich (14.331%) which could be due to the associated aquaculture activities around these mangroves. Similarly, the sediment pH and salinity were 7.2 and 22.6psuat MAN and 7.4 and 25.8psuat PVY, respectively.

3.2 Taxonomic richness and diversity of the prokaryotic community

A total of 6,08,852 sequence reads were detected from two mangrove sediments with a processed sequence read length of 1,11,172 for MAN and 1,11,890 for PVY. Based on the results, about 6687 and 3064 OTUs were detected for MAN and PVY, respectively. The number of species detected was significantly high for MAN(5494) compared to PVY(2462) (Table 2). The abundance and evenness of the species and bacterial community diversity were higher for MAN compared to PVY based on Shannon, Simpson diversity indices and rarefaction analysis (S Fig 2). The Venn diagram showed 247 and 155 genera unique to MAN and PVY, respectively. Only 56 genera were common in MAN and PVY samples. The Mangalavanam mangrove system is located in the heart of the Cochin city. It is a popularly known as the “green lung of Cochin”. This system is a tidal wetland area with shallow tidal lake with thick mangrove vegetation. This region has lot of anthropogenic influences and houses a large variety of resident and migratory birds. The area happens to be a roosting place for exotic varieties of resident and migratory birds. This mangrove is also a rich feeding zone, due to the presence of various fish and other aquatic organisms. This may be the reason for high diversity in this region compared to PVY.

3.3 Taxonomic composition of the prokaryotic community

Based on sequence analysis, the most abundant prokaryotic domain was bacteria in PVY (99.94%) and MAN (99.87%). The prokaryotic phylogenetic diversity at the phyla level is shown in Fig.1a. At Phylum level, OTUs could be classified into 41 phyla in PVY and 55 in MAN sediment samples. Proteobacteria was the major phyla in both (MAN-48.19% and PVY-65.66%) sediment samples. Several studies on mangroves located on the coast of Saõ Paulo State-Brazil, Yunxiao mangrove ecosystem of China, Mangrove areas of Kerala (Kumbla, Valpadananam, Kavayi and Bangramanjeshwar) (Imchen et al., 2018), Sundarban, Netidhopani (Ghosh et al., 2010), and Red Sea mangroves of Saudi Arabia (Alzubaidy et al., 2016; Imchen et al., 2018) reported on the dominance of Proteobacteria. The microbial diversity at the phylum level were comparable with mangrove microbiome diversity reported from other parts of India (Nair et al., 2013; Fernandes et al., 2014; Basak et al., 2016; Basak et al., 2016; Khandeparker et al., 2017).

Among Proteobacteria, class Gammaproteobacteria (43.94%) was dominant in PVY while it was Alphaproteobacteria (18.27%) in MAN (Fig 1b). Dominance of Gammaproteobacteria have been reported from Chinese mangrove ecosystem (Liang et al., 2007) and in mangroves of Red Sea coast of Saudi Arabia (Ullah et al., 2017). These Gammaproteobacteria were related to free-living and symbiotic sulfur-oxidizing bacteria (Liang et al., 2007). The oxidative pathway of nitrogen in the mangrove sediments are more likely to be mediated by members belonging to the class Gammaproteobacteria (Li et al., 2011). In PVY, Alphaproteobacteria was the most abundant (14.87%), followed by Deltaproteobacteria (3.29%). Whereas, in MAN, Deltaproteobacteria (13.28%) and Gammaproteobacteria (9.93%) were lower in their abundance. This was in concurrence with previous reports from Mangalavanam mangrove and Mandovi-Zuari estuary (Nair et al., 2013; Khandeparker et al., 2017), but in contrast with the Brazilian mangrove sediments, where Beta-, Gamma-, and Deltaproteobacteria were most abundant. Deltaproteobacteria are sulfate reducers and the typical anoxic conditions in the mangroves with very low redox potential provide an ideal habitat for sulfate reducers and methanogens (Dar et al., 2008; Taketani et al., 2010). However, in another tropical mangrove swamp metagenome study, Deltaproteobacteria (43.88%) was the major class, followed by Alphaproteobacteria and Gammaproteobacteria (Ismail et al., 2017). MAN and PVY were different in terms of its proximity to the Arabian Sea. MAN lies in the heart of the urban Cochin city, whereas PVY is closer to the sea with more tidal influence and hence higher salinity was observed in this sampling location (26 psu against 22 psu at MAN). The changes in the carbon, nitrogen and sulphur contents along with changes in pH and salinity could have attributed to variations in the bacterial community structure in two mangrove ecosystems. In fact, bacterial

community structure is driven by salinity gradients and availability of organic and inorganic compounds in estuarine environments (Smith and Kemp, 2003).

3.4 Species level variations

There was distinct variation in the species distribution at MAN and PVY. The *Desulfococcus* spp., *Flavobacterium* spp., *Geobacter* spp., *Hyphomicrobium* spp., *Mycobacterium* spp., *Plantomyces* spp., *Pseudomonas* spp., *Thermomonas* spp. etc. were few of the unique species in MAN. *Arcobacter* spp., *Clostridium* spp., *Clostridiisalibacter* spp., *Microbulbifers*spp., *Pseudoalteromonas* spp., *Photobacterium* spp., *Vibrio* spp. etc. were unique to PVY (Fig 2). This difference in species level makes these two ecosystems different in its biological functions. In MAN, there were dominance of denitrifiers like *Rhodoplanes* spp. (1.428%) and *Plantomyces* spp. (1.184%) probably involved in nitrogen cycle in this region. The dominance of *Desulfococcus*spp. (1.354%) may be attributed to the involvement in sulphur cycle. In the case of PVY, *Vibrio* spp. (6.12%) were predominant, followed by *Microbulbifers*spp. (4.79%), *Photobacterium* spp. (3.39%), *Erythrobacter nanhaisediminis* (1.33%), *Muricauda* spp. (1.20%), *Clostridiisalibacter* spp. (0.979) etc. Based on the principal component analysis, microbial diversity of these samples were also dependent on the salinity, C, N and S content (Fig.3).

3.5 Functional roles of mangrove microbial community

PICRUSt was used to make predictions on functions of microbial communities. Databases such as KEGG and COG were used for annotations. KEGG ortholog (KO) and COG annotations were from v3.5 of IMG that had identifiers in the Greengenes reference tree. Based on the KO analysis, both sites had similar functional patterns (fig. 4). Majority of the genes present were associated with metabolic functions, followed by cellular functions, genetic functions, and environmental functions. In both MAN and PVY mangroves, the human diseases associated genes were not detected and organismal system were low in number. This results point out the existence of critical features for competitive microbial life within mangroves, as observed in Brazilian mangroves (Andreote et al., 2012). Functions such as amino acid transport and metabolism, carbohydrate transport and metabolism, translation, ribosomal structure and biogenesis, post translational modification, energy production and conservation etc. were determined. High occurrences of sequences related to carbohydrate and amino acid metabolism, metabolism of cofactors and vitamins, and energy metabolism were detected in the Brazilian mangrove samples (Andreote et al., 2012). In the species level diversity, we observed abundant denitrifiers in MAN. There were several reports stating that mangrove sediments were predominantly anoxic, but in this environment, aerobic to

anaerobic shift occur constantly, providing appropriate environment for nitrification process, and this will possibly favour nitrate source for anaerobic ammonium oxidation and denitrification processes (Dias et al., 2011; Andreote et al., 2012). Based on the COG analysis, there was a great variation in the abundance of genes associated with the transport and metabolic activities such as signal transduction mechanism and amino acid transport and metabolism (fig. 4).

4. Conclusion

Bacterial diversity in mangrove sediments may be dependent on the environmental parameters as well as the anthropogenic effects like pollution. The present study has revealed important insights into the bacterial community structures in two closely located mangrove ecosystems in the southwest coast of India. PVY is a coastal ecosystem influenced directly by tidal influx. But, MAN is a bird sanctuary known as the “green lung of Cochin” that lies in the heart of the urban city of Cochin with more anthropogenic influences. The mangrove ecosystems in the MAN and PVY are exceedingly diverse in their microbial content due to variations in nutrient components in the sediments. In both these environments, Proteobacteria were the dominant phyla but the dominant classes in these environments were extremely different. The minor communities also were distinctly different in these environments. The functional analysis also revealed a similar pattern except for gene abundance associated with transport and metabolism of amino acids, signal transduction processes. Our observation suggests that different mangrove ecosystems support different bacterial community structure and has improved our understanding on the biodiversity in Mangalavanam and Puthuvypemangrove ecosystems.

Author Contributions

JV, NVK and AP have collected and analysed samples. NVK have performed analysis of chemical parameters. JV, NVK and PA has collected the samples, performed statistical analysis of the data and preparation of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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List of figure legends

Fig1. Phylum level (a) and Class level (b) classification of bacteria present in mangrove sediments from Mangalavanam (MAN) and Puthuvype (PVY)

Fig 2. Heatmap and clustering (Bray-Curtis dissimilarity) analysis for 36 dominant bacterial species present in mangrove sediments from Mangalavanam (MAN) and Puthuvype (PVY)

Fig3.Principal component analysis showing relationship with environmental parameters and bacterial diversity. Major phyla contributing to >90% of total phyla in Mangalavanam (MAN) and Puthuvype (PVY) were used for the analysis.

Fig4.Functional analysis of Mangalavanam (MAN) and Puthuvype (PVY) using PICRUSSt based on COG analysis.

Supplementary Figures

Fig S1.Station map showing the two mangrove ecosystems – Puthuvypeen (PVY) and Managalavanam (MAN)

Fig S2. Rarefaction curve for the sediment samples of Puthuvypeen (PVY) and Managalavanam (MAN)

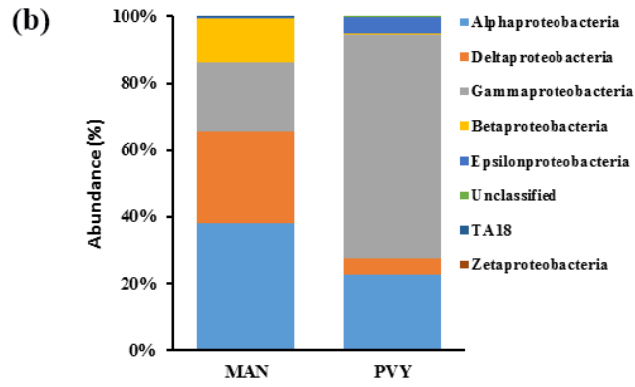
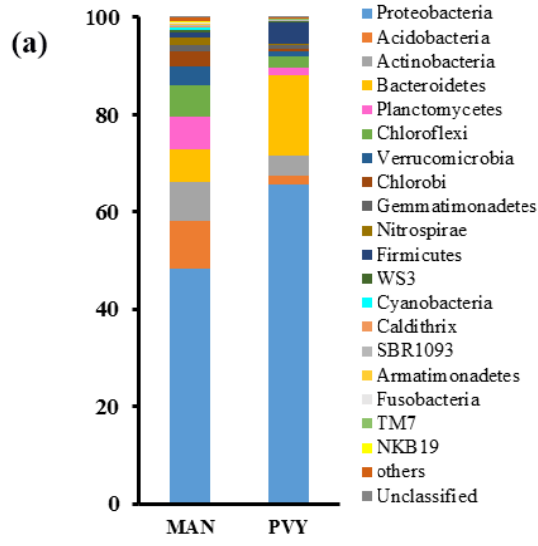


Figure 1

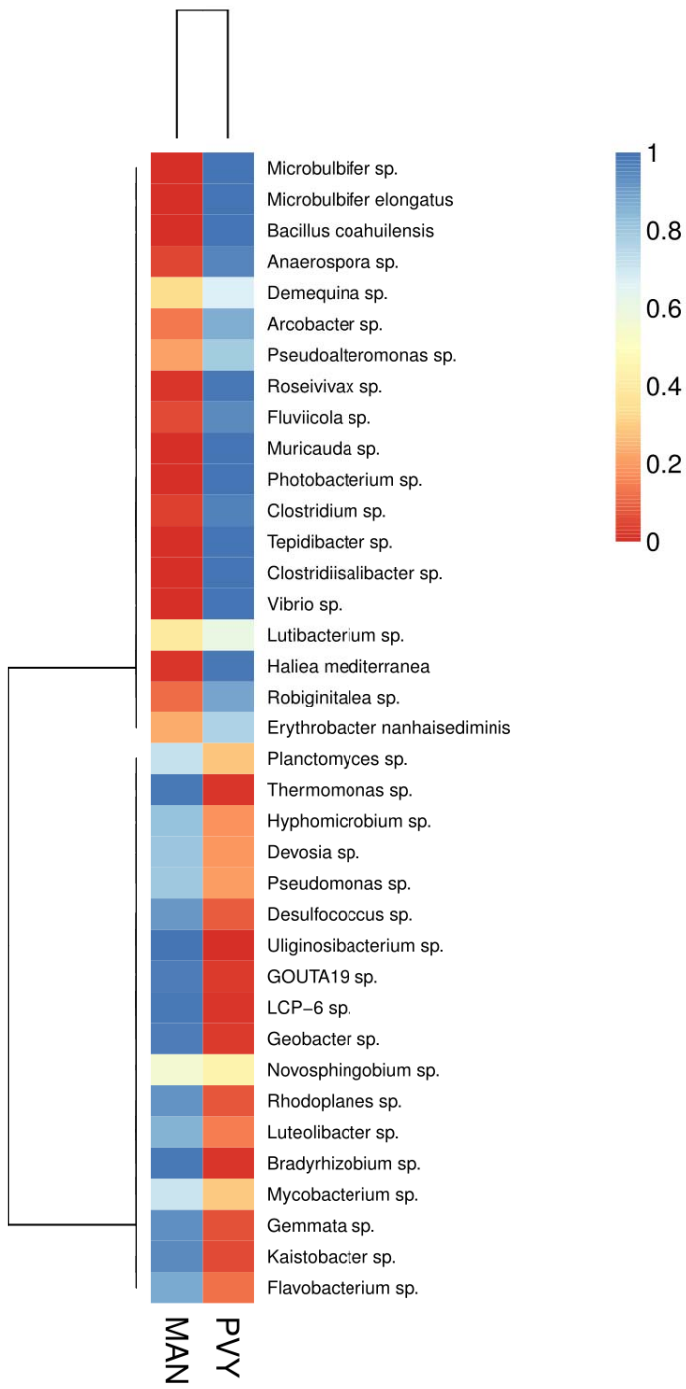


Figure 2

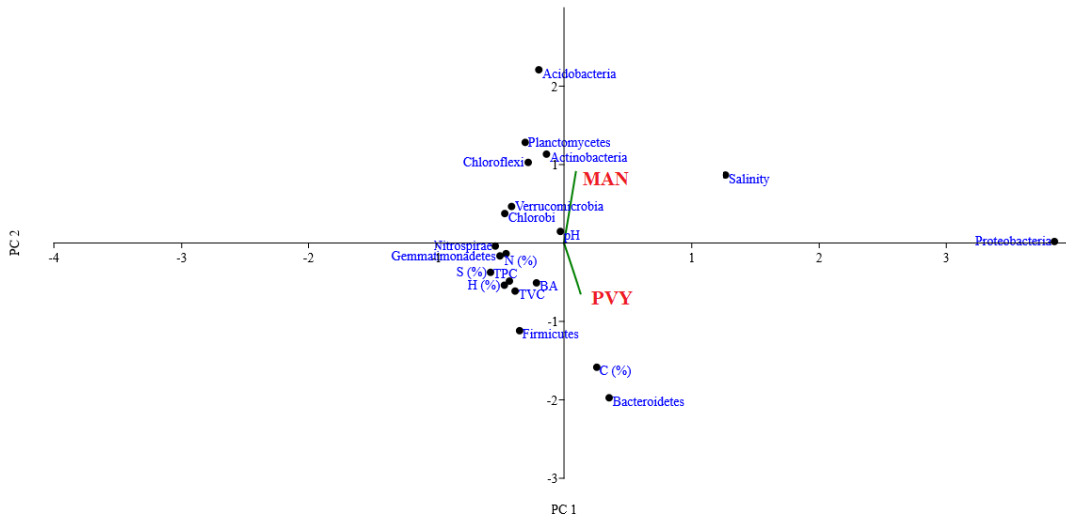


Figure 3

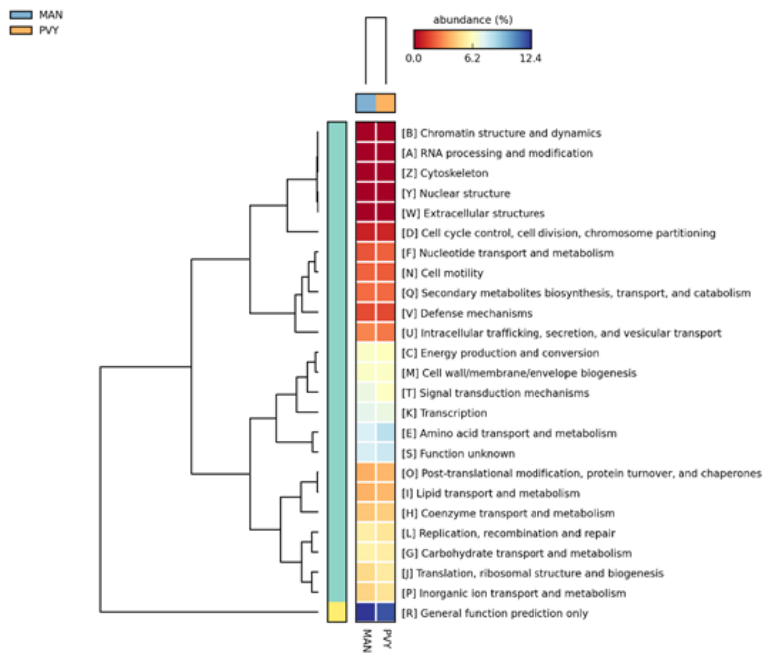


Figure 4

Table 1: Physico-Chemical parameters of mangrove sediments from Mangalavanam (MAN) and Puthuvype (PVY)

Parameters	MAN	PVY
TN (%)	2.072	1.472
TC (%)	6.529	14.331
TH (%)	1.066	1.887
TS ((%)	0.263	0.04
Temp (°C)	28.77	30.5
pH	7.2	7.4
Salinity	22.6	25.8

Table 2: Statistical analysis of sequence from V3-V4 regions of two mangrove sediments.

Parameters	Samples	
	MAN	PVY
Total paired end reads	335684	273168
Processed reads	284803	237566
Total Identified rRNA sequences	111172	111890
Total OTUs Picked	6687	3064
Shannon	10.56	7.70
chao1	7905.88	3797.47
observed_species	5494	2462
goods_coverage	0.97	0.98

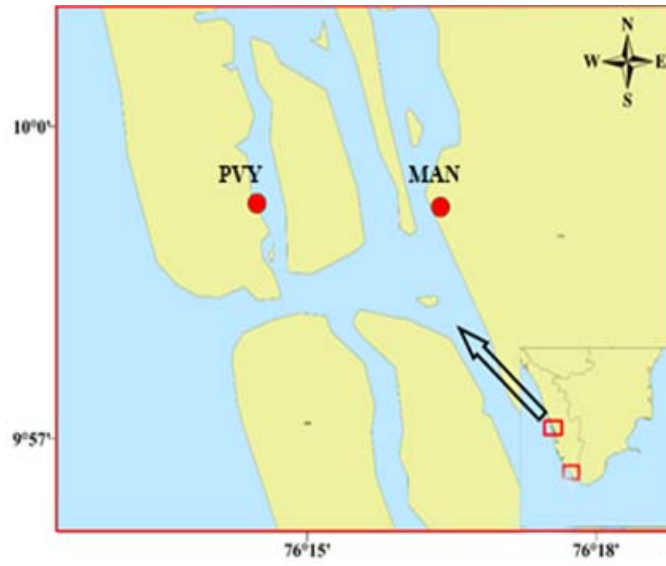


Fig S1

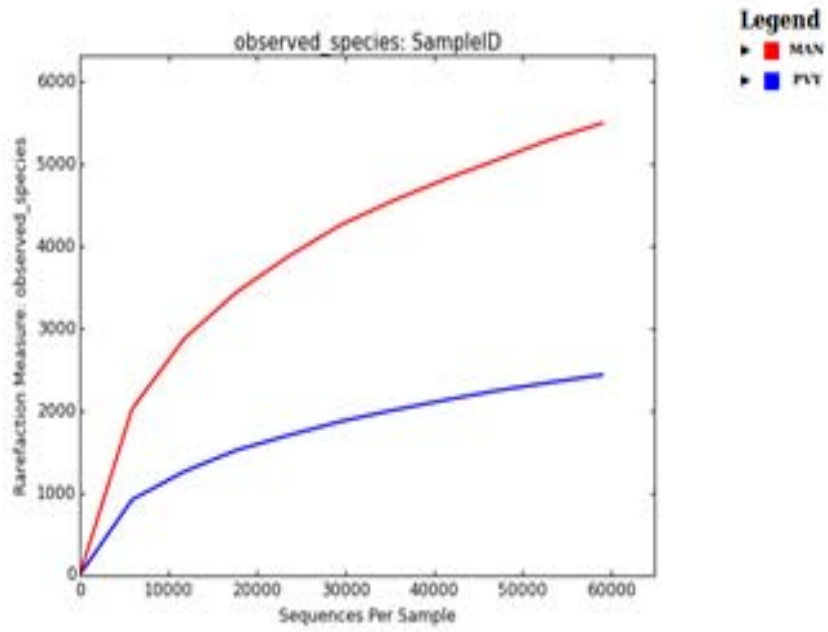


Fig S2