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METAGENOMICS ANALYSIS OF EUKARYOTIC COMMUNITY IN SOIL USING 18S AND 28S rRNA GENE HIGH THROUGHPUT SEQUENCING

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Abstract- The present study applied metagenomics to characterize the diversity and relative occurrence of eukaryotic organisms in the soils of Chandan plant (*Santalum album;* MS1) and Bamboo plant (*Bambussa bambus;* MS2). DNA-based methods do not depend on the culturability of microbes, and therefore they offer an effective method for the study of metagenomics profiling. For this purpose, we used PCR primers that allow the specific amplification of 18S-ribosomal-DNA (rDNA) and 28S-ribosomal-DNA sequences. The metagenome samples were subjected to sequencing by Ion torrent PGM which resulted in 431,425 (MS1) and 303,564 (MS2) reads respectively. The taxonomic profile obtained by comparison with SILVA SSU database showed predominance of the phyla: Ascomycota (32.22% in MS2), Streptophyata (31.49% in MS2), Annelida (27.99% in MS 1), Nematoda (16.6% in MS1). At the genus level Enchytraeus (26% in MS1), and Aspergillus (22.4% in MS2) were predominant. The taxonomic assignment based nuclear 18S and 28S ribosomal sequences showed discrepancy with the SSU based assignments possibly due to the absence of most eukaryotic genomes in the public databases. The present study provides a preliminary snapshot of the diversity and relative abundance of the metazoan within the soil samples and expands our knowledge of these multicellular eukaryotes present in the soil ecosystem and these consortiums may be helpful in soil fertility and enhance plant productivity.

Keywords- Soil, 18S r RNA and 28S r RNA gene, High throughput sequencing

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Introduction

Soil is a complex environment and a reservoir of microbial diversity with several thousands of different bacterial species [1]. Soil also hosts numerous eukaryotic micro organisms that can represent a significant fraction of the microbial biomass in some ecosystems. Soil bacterial and eukaryotic communities are involved in ecosystem functions such as decomposition and geochemical cycling [2,3] and strongly influence soil physical characteristics [4,5] as well as plant health and nutrition [6]. Soils are complex and provide a vast diversity of habitats that result from structural networks, physicochemical conditions and biological interactions. Eukaryotic and bacterial community structure may be influenced by a range of environmental parameters, including: pH [7], temperature [8], moisture content [9], and nutrient status [10], substrate availability and complexity [11], exposure to the roots of different plant species [12].

This environmental heterogeneity is thought to contribute to the maintenance of soil microbial communities that typically represent the largest fraction of below-ground biomass [13] and are estimated to constitute somewhere in the order of tens of thousands of microbial 'species' per gram of soil [14,15]. Nonetheless, the relative influence of these parameters on microbial activities is poorly understood.

Metagenomic analyses based on second-generation sequencing have many advantages, as they can generate huge numbers of sequences from single samples [16]. However, to date most metagenomics studies have focused on bacterial and eukaryotic community [17-19] and only a limited number of studies have been carried out for metazoans [20-25]. Microbial community has been well characterized using 16S ribosomal DNA sequence [26], but signature gene for metazoan metagenomics studies is less clear [21]. Over the past many years, several studies have reported to characterized eukaryotic and metazoan community [27-29], but the extent of compatibility between the primers and the target regions of these primers has not been estimated thoroughly. Hills and Dixon [27] reported universal primers for nuclear ribosomal DNA regions to study the metazoans using suitable sequences.

Studying the impact of global change on soil ecosystems is one of the major challenges for metagenomics analysis. The study of microbial community and multicellular organisms is still in progress using well-established approaches, however analyses dynamics of microbial eukaryotic communities in soil is very limited [30,31] although remarkable advances have been made recently using culture-independent, molecular approaches in aquatic environment [32-36]. Next-generation DNA sequencing technology has dramatically reduced the cost of data generation [37]. This has

been greatly benefited genetic biosciences, especially metagenomics. Metagenomics [38] is the study of microbial communities. It typically needs additional depth of sequencing; thus a low sequencing cost is beneficial. In the meantime, however, the computational costs of analysis have grown unsustainably as large amounts of data have been generated. As a result, the bottleneck in metagenomics has moved from sample collection and data generation to data analysis [39]. None of these studies have included eukaryotic micro-organisms possibly because they were in a minority in the studied ecosystems or because they were physically excluded (by filtration or centrifugation on density gradients) from the biomass before DNA extraction.

The enormous sequence coverage required to analyze less dominant species within a habitat has severely challenged the current computational resources. In response, keeping in mind the importance of metazoan in the soil ecosystem and the practical power of the metagenomics approach was used to study the diversity and relative abundance of eukaryotes present in the soil metagenomics.

Materials and Methods

Sample Collection

The soil used in this study was collected from agricultural land of Modasa (Chandan and Bamboo) at (23.47°N 73.3°E), Gujarat, India. The soil was collected by digging 1 cm deep and collected in aseptic plastic bags [40]. Two different types of soil samples (Chandan and Bamboo) were collected in sterile container and transported to the laboratory in cold condition and stored at -20^{0}C for further analysis.

DNA Extraction

DNA extraction was carried out from 0.5gm of each soil samples using CTAB (cetyltrimethyl-ammonium bromide) and SDS(sodium dodecyl sulfate) described by Robert [41], with some modification followed by washing of soil with 1X PBS and filtration of soil using filter paper, to remove some large molecule followed by phenol: Chloroform classical method. In addition DNA was also isolated with XcelGen Soil g DNA isolation kit (XG2413-01). Finally isolated DNA from both methods was pooled in equilmolar conc. and used for downstream application.

PCR based Analysis using Ion Tags

PCR amplification of the short variable region V9 of 18S and D9-D10 region of 28S rRNA gene was performed with primers specific for domain eukaryotes [Table-1]. The 5'-ends of the forward primers were fused with the barcoded adaptor plus key sequence, whereas the reverse primers were fused with the truncated P1-adapter sequence (trP1), respectively. For amplicon library preparation 100 ng of genomic DNA, 0.33 pM of each primer, 0.58 mM of dNTPs, 1X PCR buffer 0.15 mM MgCl2 and 1 U of Taq Polymerase enzyme (genie) were used in 30 µl amplification reaction. The PCR conditions were as follows: 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, and a final extension step of 72°C for 5 min.

Amplicon products were purified and exact fragment size and concentration was determined using Agilent High Sensitivity DNA Chip kit on Agilent Bioanalyser 2100 (Agilent Technologies, USA). The libraries were adjusted to a final concentration of 26pM in equimolar concentration and attached to the surface of Ion Sphere particles (ISPs) using Ion One Touch 200 Template v2 sequencing kit (Life Technologies, USA) according to the manufacturer's instructions. Clonally amplified ISP was then enriched by using Ion One Touch ES System resulting in ISPs >95% templated-ISPs. Templated-ISPs were sequenced on Ion 318 semiconductor chip using the Ion Torrent Personal Genome Machine (Life Technologies, USA) for 130 cycles. After sequencing, all raw reads were filtered within the PGM software to remove low quality and polyclonal sequences. All good quality filtered data were exported as *.sff files and subsequently used for bioinformatics analysis.

Table 1- List of primers targeting regions of 18S rRNA and 28S rRNA gene used in this study for Amplicon sequencing

Primer	Oligo Sequence (5' to 3')*	Product size (bp)	Group
1380F	CCCTGCCHTTTGTACACAC	176	18S Eukaryotes
1510R	CCTTCYGCAGGTTCACCTAC		
9F	AAGACCCTGTTGAGYTTGACTCT	228	28S Eukaryotes
10R	CCGCCCCAGYCAAACTCCC		

Metagenomics Analysis

All resulting sequencing data sets were uploaded to the Metagenomics - Rapid Annotation using Subsystem Technology (MG-RAST) server (http://metagenomics.anl.gov/) checked for low-quality reads prior to dereplication, annotation and phylogenetic identification. Taxonomic analysis in MG-RAST consisted of comparing the metagenomics sequences with the Non-Redundant Multi -Source Protein Annotation database (M5NR) and finally data was analyzed with SILVA Small Subunit rRNA database (SSU) on the MG-RAST server [43]. Finally data were analyzed with SILVA r RNA SSU data set.

Result and Discussion

To investigate the diversity and relative abundance of eukaryotic species present in the soil.Metagenomic DNA from soil was sequenced using the IonTorrent PGM machine. Metagenome sequencing resulted 431,425 reads with an average read length of 114 ± 14 bp length in Chandan (MS1) and 303,564 reads with an average read length of 112 ± 11 bp length in Bamboo(MS2). The taxonomic profiling at phylum level of both samples are depicted in [Fig-1].

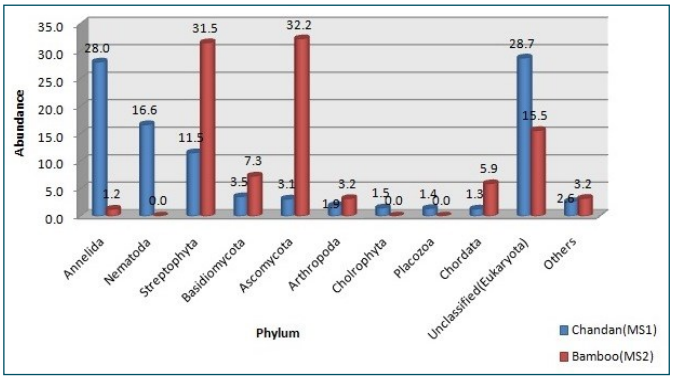

Fig. 1- Phylogenetic classification at Phylum level

Community structure of eukaryota in soil metagenomics were studied on the basis of SILVA SSU rRNA database for 18S rRNA and 28 S rRNA genes at maximum e-value of 1e-20, a minimum identity of 60 %, and a minimum alignment length of 50 bp. According to SILVA SSU database and sequences features were

identified in the given metagenome dataset having putative rRNA regions.

At phyla level, Ascomycota (32.2%) and Streptophyta (31.5%) were dominant in soil 2 (MS2) as compared to soil 1(MS1). However, Annelida was over abundant in soil 1 (MS1) only. In addition, many sequences were unclassified [Fig-1]. Fungi are ubiquitous in the environment and play an important role in nutrient and carbon cycling processes in soil [44]. Fungi play an important role in the soil ecosystem as major decomposers of biomass and stimulate plant growth. However, some fungi possess antagonistic properties towards plant pathogens [45]. Predomininace of phylum Ascomycotas have been also reported in environmental soil [46].The abundance of Nematoda in soil 1(MS1) (16.6%) where absent in soil 2(MS2). Occurrence of Nematoda, could be having role in agriculture sector [47].

Tagged 18 S rRNA and 28 S rRNA Gene sequencing of soil metagenomics revealed the sequences related to 20 genera in soil 1 (MS1) and 17 genera in soil 2 (MS2), there were many other genera below 1% and they may represent different eukaryotic species or phenotypes. It was observed that, genera, Enchytraeus (26%) in soil 1(MS1) and Aspergillus were most dominant in the soil 2(MS2) that's 22.4%, while sequences assign to Mazuz (14.1%) and zea (2.8%) *were* dominant in soil 2(MS2) as compared to soil 1 (MS1). However, presence of other eukaryotes in both the samples shown in [Fig-2] and [Fig-3] at genus level. Eukaryotic rRNA genes and their associated transcribed spacers have been used as marker genes [48-51].

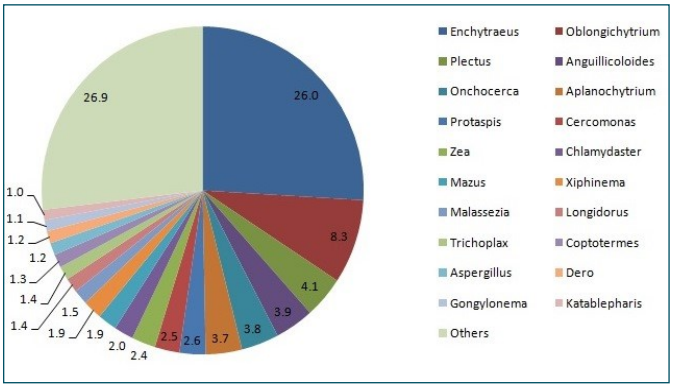

Fig. 2- Abundance genera in Chandan soil sample (MS1)

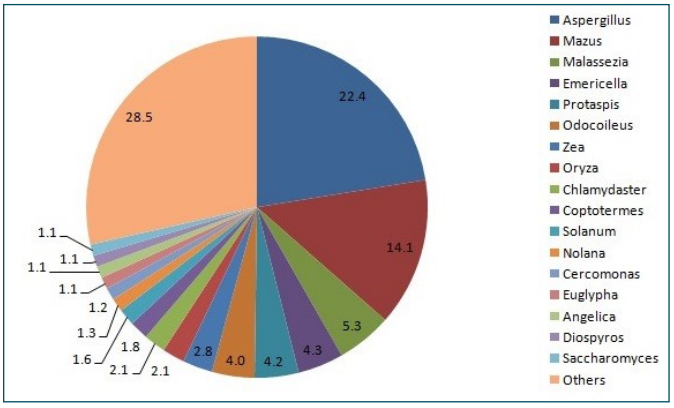

Fig. 3- Abundance of genera in Bamboo soil sample (MS2)

We identified sequences related to organisms (protists and metazoan) commonly found in soil samples. Contradictory to fungi, metazoan, protists form an unnatural taxonomic eukaryotes group and belonging to different kingdoms [52,53]. The survey of 18S sequences gives a local view of the eukaryotic diversity in the studied soil. The ideal represented taxonomic group of sequences related to the basidiomycete group known to account for a majority of ectomycorrhizal and saprobic fungal species in soils [54].

Bailly et al., [55] evaluated an experimental approach based on the construction and screening of a cDNA library using polyadenylated mRNA extracted from a forest soil and they identified ascomycete and a basidiomycete fungal species. They act as plant pathogens, mycorrhizal symbionts are major decomposers of plant materials [44,56] and also represent a dominant component of the soil biomass [56].

Several studied have been reported the occurrence of fungal species in the various soils in different ecosystems, such as forests [57], grasslands [58,59], stream sediments [60] and agricultural fields [61] and fungal community is affected by plant growth [61] and fertilizers and pesticides [62].

Conclusion

The soil metagenomics study revealed the eukaryotic organisms present in soil sample. The fungal community were prevalent in both biomaterials. The present study provides a baseline for understanding the complexity of the microbial ecology of the soil sample with special reference to fungal and metazoan. Eukaryotic microorganisms cloud plays important roles in regulating soil fertility, plant health and other nutrients. Further more study to be required in large data set to draw a solid conclusion in area of applied agriculture sector.

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Conflicts of Interest: None declared.

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