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Microbial Ecology in the Era of Next Generation Sequencing

Surajit De Mandal[1], Amrita Kumari Panda[2], Satpal Singh Bisht[2] and Nachimuthu Senthil Kumar[1]*

[1]*Department of Biotechnology, Mizoram University, Aizawl-796004, Mizoram, India*
[2]*Department of Zoology, Kumaun University, Nainital-263002, Uttarakhand, India*

Abstract

Microorganisms are the major part of earth's biological diversity. Due to most of the microbes being non-culturable, it is necessary to use culture independent techniques to study the uncultured microbes. Metagenomics is the molecular tool which helps to understand the genetic makeup of the wide variety of uncultivable microorganisms. Currently, Next Generation Sequencing (NGS) is one of the most advanced technologies used in metagenomics studies and different computational tools have been developed for the analysis of large metagenomic dataset. This review demonstrates the different tools used in NGS analysis and its applications in microbial ecology.

Keywords: Biological diversity; Metagenomics; Next generation sequencing

Introduction

Natural environments harbor a large number of microorganisms representing the major reservoir of undescribed biodiversity [1] and occupy diverse habitats from deep sea sediments to high up in the atmosphere [2]. But, most of the microbes are unculturable by standard techniques [3,4]. Thus, their ecology and functional roles are unknown and our understanding of the composition of the natural microbial world is therefore rudimentary. The reason for uncultivable nature may be the fact that they depend on other organisms for critical processes, fail to grow *in vitro* or have even become extinct in fossil records [5]. Some are metabolically active, even though they were unculturable in laboratory [6]. Culturing these microbes in artificial environment needs in-depth knowledge of the nutrient and growth requirements. Although some work has attempted to culture these uncultivable bacteria [7,8], the technique is time consuming and laborious and therefore needs some alternative methods to study them.

DNA sequence-based methods can overcome these problems since we can isolate the genetic material directly from live or dead cells from various environmental samples, allowing a new emerging field called metagenomics. Metagenomics involves the culture independent approach to study the unculturable microorganisms directly from an environmental samples such as soil [9,10], seawater [11], ground water [12], antarctic desert soil [13] etc. In the mid-1980s, Norman Pace [14] suggested a culture-independent approach which was later named as "metagenomics" by Handelsman and first appeared in publication in 1998. This field is also called as environmental genomics, ecogenomics or community genomics. In this technique, the isolation of environmental DNA directly resulted in the isolation of a number of novel genes and was designed with several practical gains such as the discovery of new genes and gene products that would lead to agricultural innovations, medicinal chemistry and industrial processes. It can also be used to reveal diversity patterns of microorganisms, horizontal gene transfer analysis, identification of novel metabolic pathways and examining genes/operons for desirable enzyme candidates (e.g., cellulases, chitinases, lipases, antibiotics) and other natural products [3,15,16].

Ribosomal RNA for the identification of uncultured bacteria and its community

Ribosomal RNA is largely used for the study of uncultured bacteria as it has several advantages. The 16S gene occurs in all living organisms with the notable exception of viruses and represents more than 80% of total bacterial RNA found in every living cell and generally stable and long enough (approx. 1500 nt), contains independently evolving domains, i.e. variable regions. They are shorter than 23s (approx. 2300 nt) and easier to sequence. The 16S rRNA gene structure consists of interspersed conserved and variable regions which is suitable for PCR amplification and sequencing. As a molecular chronometer, these molecules have preserved their evolutionary history. Most of the studies have been carried out with 16S rRNA and has been used as a phylogenetic marker as it is present in all prokaryotes and contains different hypervariable regions separated by highly conserved gene segments. They have highly conserved portion which carries the information on early evolutionary events and more recent changes are documented within less conserved position or stretches. The degree of divergence of present day rRNA sequence gives an estimate of their phylogenetic distance. They donot have any ortholog - paralog issues or horizontal gene transfer. It is easy to PCR amplify and they have a very slow rate of mutation. The new era of metagenomics was ushered in by studies using 16S rRNA as a phylogenetic marker of microbial taxa. Focusing on a small part of the microbial genome brings down the sequencing costs dramatically [17].

It is important to choose the appropriate variable region of the 16S rRNA for microbial ecological studies mainly for biogeography, metacommunity theory or microbiome analysis. Previous studies selected the hypervariable region which can exhibit sufficient phylogenetic signals to classify at the genus level [18]. Liu et al. [19] suggested for the 250 bp region between the V2 and V3 hypervariable regions for microbial ecological studies. The use of tags of the V3 and V6 regions in 454 pyrosequencing also provides sufficient taxonomic assignments [20]. Analysis by Wang et al. [21] proved that V2 and V4 regions can results best taxonomic assignment at the genus level, where as for the study of microbial community complex ecosystem it is recommended to use V7-V8 region [22,23].

***Corresponding author:** Nachimuthu Senthil Kumar, Department of Biotechnology, Mizoram University, Aizawl-796004, Mizoram, India, Tel: +91-9436352574; Email: nskmzu@gmail.com

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Analysis of metagenome using DGGE and TGGE

Sanger sequencing (first generation sequencing) was widely used for molecular biology studies with no or minimum errors. Based on the Sanger sequencing approach, different PCR based methods have been developed to study the microbial communities. Denaturing Gradient Gel Electrophoresis (DGGE) or Terminal Restriction Fragment Length Polymorphism (T-RFLP) has been developed to analyze the uncultured microbial communities in diverse environments [24,25]. These methods allow differentiating gene molecules (in polyacrylamide gels) based on the decreased electrophoresis mobility of a partially melted double stranded DNA molecule at the level of a single base containing a linear gradient of DNA denaturants (a mixture of urea and formamide) or a linear temperature gradient. However, these techniques present several drawbacks as the dominant populations are better revealed and bands from more than one species may be hidden behind a single band resulting in underestimation of bacterial diversity. The same isolate can have different identifiable bands because multiple copies of 16S rRNA gene are present in a single genome of species. The Sanger sequencing based methods present several drawbacks since it is expensive and cannot detect rare microorganisms [26,27].

Next generation sequencing technology (NGS)

NGS allows massively parallel sequencing with thousands to millions of sequences in one experiment at considerably low cost compared to Sanger method. There are no requirements of bacterial cloning of DNA fragments and electrophoresis, since NGS libraries preparation process are carried out in a cell-free system and the sequence output is directly detected. However, it produces shorter reads with a higher error rate than Sanger sequencing [28-30]. Moreover, secondary structure and thermal stability of the genome affects the efficiency of PCR amplification of the genomic fragments. There are different NGS platforms (e.g. Illumina, Pacific Biosciences, Ion Torrent, SOLiD) with different principle and divergent features such as run times, yields and read lengths [31] (Table 1).

Advances in NGS have revolutionized the field of microbial ecology. Any NGS study follow some common steps such as sample and metadata collection, DNA extraction, library construction, sequencing and read preprocessing followed by quantitative analysis and functional binning [32,33]. Collection of environment sample is the first step in any metagenomic study. But, the problem faced in microbial ecology studies are that no information is available about the amount of required sampling that will comprise entire population in an environment. Usually rarefaction curve, a plot of number of species versus number of individuals sampled, which typically defines the population richness and evenness is used to estimate the fraction of species sequenced.

Another important aspect for any successful metagenomic study is the extraction of high quality DNA from the environmental samples [34]. Environmental samples contain DNA in a variety of packages like free DNA, virus particle, and prokaryotic and eukaryotic cells. These can be suspended in water, bound to a solid matrix like soil, or encased in a biofilm or tissue. So extraction methods must be chosen carefully based on the medium and the DNA population of interest (Susannah Green Tringe) and DNA should come from all the microbial representative present in the sample without any shearing or contamination [35]. Although many commercial kits are available for metagenome isolation, it is required to develop our own method to optimize extraction and reducing bias caused by unequal lysis of different members of the soil microbial community [36,37]. Beat beating, sonication, detergents or enzymatic lysis have been used for the isolation purpose. Since some samples contain high humic acid, additional purification is required. Polyphenol compound often co-purified along with the DNA can enzymatically modify the isolated DNA [38]. One strategy may be the use of Skimmed milk which can aid in DNA extraction process from soil [39]. Multiple displacement amplification can be used to increase template DNA for samples with very less amount of DNA [40,41].

Two kind of NGS strategy is applied in microbial community analysis: 1) deep amplicon sequencing and 2) complete metagenome or metatranscriptome analysis. Shotgun sequencing is applied to know the function of the community. The analysis procedure involves assembly (merging overlapping short reads into contigs), binning (grouping reads or contigs into individual genomes and assigning the groups to specific species, subspecies, or genus), functional annotation (for the prediction of CDS, CRISPR, noncoding RNAs) and functional assignment to the predicted protein coding sequences. On the other hand, amplicon sequencing is used for community profiling using marker genes (e.g., 16S rRNA gene) in different ecosystems. Procedure involves denoising (filtered the 'noise' sequences from the raw data), Chimera detection (recombinants which are formed when prematurely terminated fragments reanneal to other template DNA during PCR), OTU clustering (alignment is done by either aligning query sequences against pre-aligned reference sequences (42) or using pairwise and multiple sequence alignments), Taxonomic classification (taxonomic

Technology (company)	*Amplification*	Chemistry	Sequencing method	Yield (Gb/run)	*Highest Average Read Length*	Error rate	Output file	Disadvantage	Advantage	Website
Illumina	Bridge amplification	Reversible dye terminator (seq-by-synthesis)	incorporation of fluorescent nucleotides	1- 60	300 bp (overlapping paired-end sequencing available	≥0.1	Fastq (Phred +64 & 33, Illumina +1.8)	Short reads and long runtime	High throughput and low cost	www.illumina.com
SOLiD (Life Technologies)	Emulsion PCR	Sequencing by ligation (SBL)	fluorescent short linkers	3	75 bp (paired-end sequencing available)	>0.06	Fastq (Phred +33)	Long runtime	Low error rate	www.appliedbiosystems.com
454 (Roche)	Emulsion PCR	Pyrosequencing (seq-by-synthesis)	incorporation of normal nucleotides	0.7	700 bp	1	SFF, fasta, fastq	High error rate in homopolymer	Long read	www.454.com
SMAT (Pacific Bio)	N/A (single molecule)	Single Molecule Real Time (SMRT™)	incorporation of fluorescent nucleotides	0.3-0.5	5,000 bp average; maximum read length ~22,000 bases	16	Fastq (Phred +33)	No PCR longest reads	High error	www.pacificbio.com
Ion Torrent (Life Technology)	N/A	Proton detection (seq-by-synthesis)	measuring pH change	1	400 bp (bidirectional sequencing available)	1	Fastq (Phred +33)	new	Short read	www.iontorrent. com

Table 1: Summary of the major next-generation sequencing platform.

assignment of OTUs) and Statistical analysis, (alpha and beta-diversity analysis, multidimensional scaling and analysis of similarities (ANOSIM), hypothesis testing etc). A flow diagram for the analysis of metagenomic data is shown in Figure 1 and Table 2 represents metagenomics tools available for Microbial Ecology Studies.

Third-generation sequencing

Third-generation sequencing (SMRT sequencing) is based on the sequencing by synthesis approach and allows the detection of single molecules, but also enables real-time sequencing. The biggest advantage is that it allows 20,000 reads or more, with average read lengths of 5 kilobases. Moreover, it can also directly detect epigenetic modifications such as 4-methylctosine (mC), 5-mC and 6-methyladenine [42].

Application of NGS in microbial ecology

Analysis of microbial community composition and its function is a

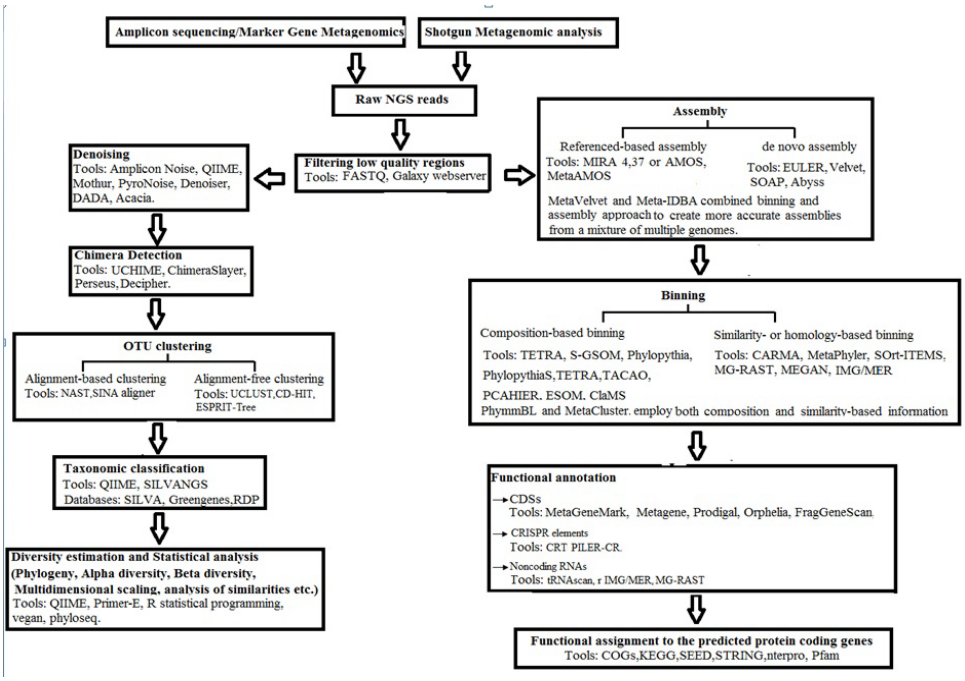

Figure 1: Flow diagram for the analysis of Metagenomics data.

Metagenomics tools	website
General Software package	
STAMP	http://kiwi.cs.dal.ca/Software/STAMP
CD-HIT-OUT	http://weizhong-lab.ucsd.edu/cd-hit-otu/
GAAS	http://sourceforge.net/projects/gaas/
Megan	http://www-ab.informatik.unituebingen.de/software/megan/ welcome.html
MetaPhlAn	http://huttenhower.sph.harvard.edu/metaphlan
MetaSim	http://www-ab.informatik.uni-tuebingen.de/software/metasim
Mocat	http://vm-lux.embl.de/~kultima/MOCAT/index.htm
Livermore Metagenomics Analysis Toolkit	https://computation-rnd.llnl.gov/lmat
Straine	http://www.hsls.pitt.edu/obrc/index.php?page=URL1221230034
Metagenome assembly	
Velvet	http://www.ebi.ac.uk/~zerbino/velvet/
Celera	http://www.cbcb.umd.edu/research/assembly.shtml#software
Metasim	http://ab.inf.uni-tuebingen.de/software/metasim/welcome.html #Download
Euler	http://nbcr.sdsc.edu/euler/ JAZZ
Mapping to reference genome	
Bowtie	http://bowtie-bio.sourceforge.net/index.shtml
BWA	http://bio-bwa.sourceforge.net/
MAPLE:	http://www.genome.jp/maple-bin/mapleSubmit.cgi?aType=sDirect
Quality analysis	
FastQC	http://www.bioinformatics.babraham.ac.uk/projects/download.html#fastqc
Prinseq	http://edwards.sdsu.edu/cgi-bin/prinseq/prinseq.cgi
Gene calling	
Genemark.	hmm http://exon.gatech.edu/GeneMark/metagenome/Prediction/

Microbial diversity Analysis	
MLST	http://www. mlst.net) http://www.mlst.net/
MOTHUR	http://www.mothur.org/
EstimateS	http://viceroy.eeb.uconn.edu/EstimateS/
QIIME	http://qiime.org/install/virtual_box.html
PHACCS	http://phaccs.sourceforge.net/
Binning	
TETRA	http://www.megx.net/tetra/index.html
Phylopathia	http://cbcsrv.watson.ibm.com/phylopythia.html
MEGAN	http://ab.inf.uni-tuebingen.de/software/megan/
CARMA	http://www.cebitec.uni-bielefeld.de/brf/carma/carma.html
Phymm	http://www.cbcb.umd.edu/software/phymm/
Functional Annotation	
MEX (Motif Extraction)	http://adios.tau.ac.il/SPMatch/
RAMMCAP	http://weizhong-lab.ucsd.edu/rammcap/cgi-bin/rammcap.cgi
Analysis of quantitative metagenomics data.	
FANTOM:	http://www.sysbio.se/Fantom/
Comparitive Metagenomics	
MEGAN	http://metagenomics.anl.gov/
MG-RAST	http://metagenomics.anl.gov
Camera	http://camera.calit2.net/
Shotgun FunctionalizeR	http://shotgun.math.chalmers.se/
MetaStats	http://metastats.cbcb.umd.edu/detection.html
UniFrac	http://bmf.colorado.edu/unifrac/
Galaxy	https://main.g2.bx.psu.edu/u/aun1/w/metagenomic-analysis
MetaMine	http://www.megx.net/metamine/
MetaLook	http://www.megx.net/metalook/index.php
IMG/M	http://img.jgi.doe.gov/cgi-bin/m/main.cgi
Online tools for NGS analysis	
Parallel Meta	http://www.computationalbioenergy.org/parallel-meta.html
SOrt-ITEMS	http://metagenomics.atc.tcs.com/binning/SOrt-ITEMS/
PANGEA	http://www.ohloh.net/p/pangea-plus
Genohub:	https://genohub.com/bioinformatics/17/metagenomic-analysis
Additional Metagenome analysis links	
EBI Metagenomics:	https://www.ebi.ac.uk/metagenomics
MetaPhlAn:	http://huttenhower.sph.harvard.edu/metaphlan
Amphora	Net http://pitgroup.org/amphoranet

Table 2: Metagenomics tools available for microbial ecology studies

major objective in modern microbial ecology studies. NGS has already been applied in such studies in diverse ecosystem such as caves, forests, hot springs, deserts etc [43-47]. A pyrosequencing based approach revealed that the soil stratification and resource availability can impact the taxonomic and functional diversity of bacterial communities [48]. NGS based studies have proved that the agriculture management system can significantly affect the microbial diversity [49,50]. Chhabra et al. [51] identified genes and operons responsible for mineral phosphate solubilization in the barley rhizosphere using pyrosequencing. A NGS based study of soil bacterial communities showed that the conversion of primary Amazonian forest to pasture increases bacterial alpha-diversity, but decreases beta diversity resulting in the homogenization of communities across space. This homogenization is driven by the loss of forest soil bacteria with restricted ranges (endemics) and results in a net loss of diversity [52]. Analysis using 227 million Illumina shotgun sequences from forest soil and 246 million from deforested soils revealed that bacterial taxonomic and functional adaptations at the bacterial community level is due to an increase in nutrient availability from slash and burn clearing of Amazon forest [53]. Analysis of 62081 pyrosequencing reads from 10 soil samples revealed that bacterial diversity and soil physicochemical properties did not show consistent changes along the elevation gradients in southwestern highlands of Saudi Arabia [54]. An NGS study carried out in temperate deciduous forest of Northeast Ohio, USA found that the vernal pool microbial communities may rely on their metabolic plasticity for growth and survival during limited resources [55]. Analysis of NGS reads shown that microbial functional activity increased throughout decomposition in spring, summer and winter, while it decreased in autumn in a Midwest temperate forest in Morris Bean Reserve of Greene County, Ohio, USA [56]. Pyrosequencing of V3-V4 regions described the bacterioplankton communities in a coastal Antarctic lake which is under long-term environmental change [57]. Fierer et al. [47] used Illumina shotgun sequencing in different ecosystem and found that desert microbial communities had high relative abundance of genes associated with osmoregulation and dormancy, whereas genes linked with nutrient cycling and catabolism of plant-derived organic compounds was less. This study also revealed that abiotic factors are more important in harboring the desert microbial communities. Another shotgun study detected identification of genes related to phytic acid utilization [58]. Analysis of total of 1,294,216 raw 16S rRNAV6 sequences revealed that Actinobacteria, Acidobacteria, Nitrospirae, and Verrucomicrobia were abundant in nutrient-rich inner mangrove sediments, while Proteobacteria and Deferribacterias were high in outer mangrove sediments [59]. Based on pyrosequencing of the V2-

V3 16S rRNA gene regions, Nacke et al. [60] found that soil bacterial community composition and diversity of the six analyzed management types showed significant differences between the land use patterns in grassland and forest ecosystems and more over bacterial community structure was largely driven by tree species and soil pH.

NGS technologies are improving day by day with more accuracy and longer sequence reads which are applied in many microbial ecology studies. Due to the continuous decrease in NGS cost, a huge number of environmental samples has been sequenced to solve research problems in microbial ecology. This paper will be helpful in understanding the basics of next generation sequencing as well as for designing new metagenomics projects. Future research with the help of NGS technologies will further unzip the unseen microbial diversity and its function useful in solving ecological problems.

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