Ceylon Journal of Science (*Bio. Sci.*) 42(1): 19-33, 2013
DOI: 10.4038/cjsbs.v42i1.5896

REVIEW PAPER

Methods for Analyzing Diversity of Microbial Communities in Natural Environments

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Received: 30 April 2012 / Accepted: 29 March 2013

ABSTRACT

Difficulties in cultivating most of the microorganisms limit our ability to study microbial ecosystems. Molecular methods are valuable tools for investigating the diversity and structure of bacterial communities. These techniques can be used on culturable as well as non-culturable bacteria. Cultivation independent techniques based on nucleic acids extracted from the environment provide information on community structure and diversity. Analyses of DNA can determine the numbers of different genomes. Ribosomal RNA (rRNA) or rDNA (genes coding for rRNA) fingerprinting, probing and sequencing can be used to detect and identify organisms. The combination of different methods that complement each other is a useful strategy for monitoring changes of microbial communities in natural ecosystems.

Key words: microbial diversity, community, biochemical methods, molecular methods

INTRODUCTION

Our knowledge about bacteria in natural environments is limited, and studying microbial diversity in nature is not an easy task. In natural ecosystems, microorganisms exist in high numbers despite the fact that there are several thousands of microbial species that have not yet been described. One gram of soil or sediment may contain $l0^{10}$ bacteria as counted by fluorescent microscopy after staining with a fluorescent dye. In pure sea water, the number of bacteria is approximately 10^6 per milliliter (Torsvik *et al.,* 1990).

There are bacteria that are adapted to almost all the different environments that exist on the earth, and also bacteria that are able to decompose all the chemical components made by living organisms. Important questions to be addressed when studying bacteria in their natural environment are how do bacterial communities function, and how the qualitative variation in community composition occurs due to environmental changes (Torsvik and Øvreås, 2002). To answer these questions, further studies on basic knowledge regarding the community structure are required. The total bacterial community studied exhibited a tremendous amount of genetic information and therefore a very high genetic diversity (Torsvik *et al.,* 1998).

THE CONCEPT OF MICROBIAL DIVERSITY

Biodiversity has been defined as the range of significantly different types of organisms and their relative abundance in an assemblage or community. The diversity has also been defined according to information theory, as the amount and distribution of information in an assemblage or community (Torsvik *et al.*, 1998). Microbial diversity refers unequivocally to biological diversity at three levels: within species (genetic), species number (species) and community (ecological) diversity (Harpole, 2010). The term species diversity consists of two components; the first component is the total number of species present which can be referred to as species richness. In other words it refers to the quantitative variation among species. The second component is the distribution of individuals among these species, which is referred to as evenness or equability (J). One problem is that evenness often is unknown in bacterial systems because individual cells very seldom are identified to the species level. An attractive

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possibility for the measurement of biodiversity is to use divergence in molecular characters, especially the percentage of either nucleic acid homology or base sequence difference. In the past, diversity has been determined based on taxonomic species, which may limit the scope of information and relationship obtained. The diversity of Operational Taxonomic Unit (OTU) or even communities may give us a better estimation of the functioning of an ecosystem. Diversity studies can be used to retrieve ecological information about community structures. Species diversity is a community parameter that relates to the degree of stability of that community. Essentially, any diversity index must measure the heterogeneity of information stored within the community. Well-organized communities that contain a certain level of diversity are stable (Yannarell and Triplett, 2005). If some kind of stress is introduced to this community, the stability may collapse and the diversity will change. Diversity can therefore be used to monitor successions and effect of perturbations.

FUNDAMENTAL REASONS FOR STUDYING MICROBIAL DIVERSITY

Within natural microbial populations, a large amount of genetic information is "waiting" to be discovered. It has been recorded that culturable bacteria represent a minor fraction of the total bacterial population present (Giovannoni *et al.,* 1990). However, it is important to continue the work both on the culturable as well as the non-culturable bacteria from different environments. Diversity studies are also important for comparison between samples.

Another important reason for studying microbial diversity is the lack of adequate knowledge about the extant and extinct microbes. There is no consensus on how many species exist in the world, the potential usefulness of most of them, or the rate at which they are disappearing or emerging.

The capability of an ecosystem to resist extreme perturbations or stress conditions, can partly be dependent of the diversity within the system. Diversity analyses are therefore important in order to:

- Increase the knowledge of the diversity of genetic resources and understand the distribution of organisms
- Increase the knowledge of the functional role of diversity
- Identify differences in diversity associated with management disturbing
- Understand the regulation of biodiversity
- Understand the consequences of biodiversity. (To what extent does ecosystem functioning and sustainability, depend on maintaining a specific level of diversity)

FACTORS GOVERNING MICROBIAL DIVERSITY

In a bacterial community, many different organisms will perform the same processes and probably be found in the same niches (Zhao *et al.*, 2012). Factors that affect microbial diversity can be classified into two groups, *i.e*., abiotic factors and biotic factors.

Abiotic factors include both physical and chemical factors such as water availability, salinity, oxic/anoxic conditions, temperature, pH, pressure, chemical pollution, heavy metals, pesticides, antibiotics *etc*. (Bååth *et al.*, 1998). In general, all environmental variations affect in different ways and to different degrees, resulting in a shift in the diversity profile.

Biotic factors include plasmids, phages, transposons that are types of accessory DNA that influence the genetic properties and in most cases, the phenotypes of their host and thus have a great influence on microbial diversity (Zhao *et al.*, 2012). In addition, protozoans are also reported as influencing the microbial diversity (Clarholm, 1994).

METHODS FOR DESCRIBING THE DIVERSITY OF MICROBES

Since only a minority of bacterial communities is culturable, only a limited fraction has been fully characterized and named. Prokaryotic organisms are difficult to classify, and the validity of the classification has been often questioned. The morphological characteristics such as cell shape, cell wall, movement, flagella, Gram staining, *etc*. *per se* may not be adequate for establishing a detailed classification of microbes. Advances in molecular and chemical ecology have provided a promising alternative in estimating microbial diversity without having to isolate the organisms (Giovannoni *et al.*, 1990).

Methods to measure microbial diversity in soil can be categorized into two groups, *i.e.*, biochemical techniques (Table 1) and molecular techniques (Table 2).

Conventional and Biochemical Methods

Both conventional and biochemical methods are of high significance in the study of microbial diversity. The diversity can be described using physiological diversity measures too, which avoid the difficulties that may arise in grouping of similar bacteria into species or equivalents. These measures include various indices (tolerance, nutrition *etc*.). Multivariate data analyses have also been used for extracting relevant information in the large data-sets frequently obtained in diversity studies (Sørheim *et al*., 1989). In order to distinguish between different types of microbes, early microbiologists studied metabolic properties such as utilization of different carbon, nitrogen and energy sources in addition to their requirements for growth factors. The phylogenetic distributions of different types of carbon and energy metabolism among different organisms may not necessarily follow the evolutionary pattern of rRNA.

Plate counts

The most traditional method for assessment of microbial diversity is selective and differential plating and subsequent viable counts. Being fast and inexpensive, these methods provide information about active and culturable heterotrophic segment of the microbial population. Factors that limit the use of these methods include the difficulties in dislodging bacteria or spores from soil particles or biofilms, selecting suitable growth media (Tabacchioni *et al.*, 2000), provision of specific growth conditions (temperature, pH, light), inability to culture a large number of bacterial and fungal species using techniques available at present and the potential for inhibition or spreading of colonies other than that of interest (Trevors, 1998).

Table 1. Advantages and disadvantages of conventional and biochemical methods to study microbial diversity (Kirk *et al.*, 2004)

Method	Advantages	Disadvantages
Plate counts	Fast Inexpensive	Unculturable microorganisms not detected Bias towards fast growing individuals Bias towards fungal species that produce large quantities of spores
Community level physiological profiling (CLPP)/ Sole-Carbon-Source Utilization (SCSU) Pattern	Fast Highly reproducible Relatively inexpensive Able to differentiate microbial communities Generates large amount of data Option of using bacterial, fungal plates or site specific carbon sources (Biolog)	Only represents culturable fraction of community Favours fast growing organisms Only represents those organisms capable of utilizing available carbon sources Potential metabolic diversity, not *in situ* diversity Sensitive to inoculum density
Phospholipid fatty acid (PLFA) analysis/Fatty acid methyl ester analysis (FAME)	Culturing of microorganisms is not required Direct extraction from soil Follow specific organisms or communities	If fungal spores are used, more material is needed Can be influenced by external factors Results can be confounded by other microorganisms is possible

These methods select microorganisms with faster growth rate and fungi producing large number of spores (Dix and Webster, 1995). Further, culture methods cannot reflect the total diversity of microbial community.

Sole-carbon-source Utilization (SCSU)

The Sole-Carbon-Source Utilization (SCSU) [also known as Community Level Physiological Profiling (CLPP)] system (for example biochemical identification systems- API and Biolog) was introduced by Garland and Mills (1991). This was initially developed as a tool for identifying pure cultures of bacteria to the species level, based upon a broad survey of their metabolic properties. SCSU examines the functional capabilities of the microbial population, and the resulting data can be analyzed using multivariate techniques to compare metabolic capabilities of communities (Preston-Mafham *et al.*, 2002). However, as microbial communities are composed of both fast and slow growing organisms, the slow growers may not be included in this analysis. Growth on secondary metabolites may also occur during incubation.

A multifaceted approach that includes both functional and taxonomic perspectives represents fertile grounds for future research. A limitation of this methodology is that many of the commercially available kits for measuring physiological diversity have been designed to cover the spectra of human pathogenic bacteria (API and Biolog). Only few research that focus on the optimization of substrate combinations designed for environmental isolates, are reported (Derry *et al.*, 1998). This often leads to problems when identifying the isolates based on the available database. This method has been used successfully to assess potential metabolic diversity of microbial communities in contaminated sites (Konopka *et al.*, 1998), plant rhizospheres (Grayston *et al.*, 1998), arctic soils (Derry *et al.*, 1999), soil treated with herbicides (el Fantroussi *et al.*, 1999) or inocula of microorganisms (Bej *et al.*, 1991).

Advantages of SCSU include its ability to differentiate between microbial communities, relative ease of use, reproducibility and production of large amount of data describing metabolic characteristics of the communities (Zak *et al.*, 1994). However, SCSU selects only culturable portion of the microbial community which limits its application (Garland and Mills, 1991), favours fast growing microorganisms (Yao *et al.*, 2000), is sensitive to inoculum density (Garland, 1996b) and reflects the potential, and not the *in situ*, metabolic diversity (Garland and Mills, 1991). In addition, the carbon sources may not be representative of those present in soil (Yao *et al.*, 2000) and therefore the usefulness of the information can be questioned.

Phospholipid fatty acid (PLFA) analysis

The fatty acid composition of microorganisms has been used extensively to aid microbial characterization. Taxonomically, fatty acids in the range C2 to C24 have provided the greatest information and are present across a diverse range of microorganisms (Banowetz *et al.*, 2006). The fatty acid composition is stable, and is independent of plasmids, mutations or damaged cells. The method is quantitative, cheap, robust and with high reproducibility. However it is important to notice that the bacterial growth conditions are reflected in the fatty acid pattern. This method is also known as the fatty acid methyl ester (FAME) analysis.

One way to examine the entire microbial community structure is to analyze the Phospholipid fatty acid (PLFA) compositions of the organisms since different subsets of a community have different PLFA patterns (Tunlid and White, 1992). It is usually not possible to detect individual strains or species of microorganisms with this method, but changes in the overall compositions of the community can be detected instead. Lipid analysis offers therefore an alternative method for the quantification of community structure that does not rely upon cultivation of microorganisms and is free from potential selections. It does not have the specificity to identify the members of microbial populations to species, rather the method produces descriptions of microbial communities based on functional group affinities (Findlay, 1996). Lipids have been the most often used signature components for determining the community composition of microorganisms in ecological studies (Tunlid and White, 1992). Changes in such lipid profiles may be attributable to alterations in the physiological status of extant populations or to actual shifts in community structure. The estimation of such ‘signatures’ may provide valuable insight to community structure, its nutritional status and activity.

Although FAME analysis is used to study microbial diversity, this fatty acid analysis method might fraught with limitations, when total organisms are used. This may obscure detection of minor species in the population.

Table 2. Advantages and disadvantages of some molecular-based methods to study soil microbial diversity (Kirk *et al.*, 2004)

Method	Advantages	Disadvantages
Mol % Guanine plus Cytosine (G+C)	Not influenced by Polymerase Chain Reaction (PCR) biases Includes all DNA extracted Quantitative Includes rare members of community	Requires large quantities of DNA Dependent on lysing and extraction efficiency Coarse level of resolution
Nucleic acid re-association and hybridization	Total DNA extracted Not influenced by PCR biases Can study DNA or RNA Can be studied *in situ*	Lack of sensitivity Sequences need to be in high copy number for detection Dependent on lysing and extraction efficiency
DNA microarrays and DNA hybridization	Same as nucleic acid hybridization Thousands of genes can be analyzed If using genes or DNA fragments, increased specificity	Only detect the most abundant species Need to culture organisms Only accurate in low diversity systems
Denaturing and Temperature Gradient Gel Electrophoresis (DGGE and TGGE)	Large number of samples can be analyzed simultaneously Reliable, reproducible and rapid	PCR biases Dependent on lysing and extraction efficiency Way of sample handling can influence community, *i.e*. the community can change if stored too long before extraction One band can represent more than one species (co-migration) Only detects dominant species
Single Strand Conformation Polymorphism (SSCP)	Same as DGGE/TGGE No GC clamp No gradient	PCR biases Some ssDNA can form more than one stable conformation
Restriction Fragment Length Polymorphism (RFLP)	Detect structural changes in microbial community	PCR biases Banding patterns often too complex
Terminal Restriction Fragment Length Polymorphism (T-RFLP)	Simpler banding patterns than RFLP Can be automated large number of samples Highly reproducible Ability to compare differences between microbial communities	Dependent on extraction and lysing efficiency PCR biases Type of *Taq* can increase variability Choice of restriction enzymes will influence community fingerprint
Ribosomal Intergenic Spacer Analysis (RISA)/Automated Ribosomal Intergenic Spacer Analysis (ARISA)/ Amplified Ribosomal DNA Restriction Analysis (ARDRA)	Highly reproducible community profiles	Requires large quantities of DNA (for RISA) PCR biases

Cellular fatty acid composition can be influenced by temperature and nutrition, and other organisms can possibly be confound the FAME profiles (Graham *et al.*, 1995). In addition, individual fatty acids cannot be used to represent specific species because individuals can have numerous fatty acids and the same fatty acids can occur in more than one species (Bossio *et al.*, 1998).

Molecular Methods to Study Microbial Diversity

Traditional methods for characterizing microbial communities have been based on analysis of the culturable portion of the bacteria. Due to the non-culturability of the major fraction of bacteria from natural microbial communities, the overall structure of the community has been difficult to interpret (Dokić *et al.*, 2010). Recent studies to characterize microbial diversity have focused on the use of methods that do not require cultivation, yet provide measures based on genetic diversity. The molecular-phylogenetic perspective is a reference framework within which microbial diversity is described; the sequences of genes can be used to identify organisms (Amann *et al.,* 1995). A number of approaches have been developed to study molecular microbial diversity. These include DNA re-association, DNA–DNA and mRNA-DNA hybridization, DNA cloning and sequencing and other PCR-based methods such as Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), Ribosomal Intergenic Spacer Analysis (RISA) and Automated Ribosomal Intergenic Spacer Analysis (ARISA).

Mole percentage guanine + cytosine (mol% G+C)

The first property of DNA used for taxonomical purpose was the base composition expressed as mole percentage guanine + cytosine (mol% G+C). Within bacteria this value ranges from 25% up to 75%, though a value is constant for a certain organism. Closely related organisms have fairly similar GC profiles and taxonomically related groups only differ between 3% and 5% (Tiedje *et al.*, 1999). However, similar base composition is not a confirmation of relationship. On the other hand, if there is a difference in base composition this is a worthy evidence of missing relationship. Mol% G+C can be determined by thermal denaturation of DNA. Advantages of G+C analysis are that it is not influenced by PCR biases, it includes all DNA extracted, it is quantitative and it can uncover rare members in the microbial populations. It does, however, require large quantities of DNA (up to 50 µg) (Tiedje *et al.*, 1999).

Nucleic acid hybridization

Nucleic acid hybridization using specific probes is an important qualitative and quantitative tool in molecular bacterial ecology (Clegg *et al.*, 2000). These hybridization techniques can be done on extracted DNA or RNA, or *in situ*. Oligonucleotide or polynucleotide probes designed from known sequences ranging in specificity from domain to species can be tagged with markers at the 5'-end (Goris *et al.*, 2007).

The sample is lysed to release all nucleic acids. Dot-blot hybridization with specific and universal oligonucleotide primers is used to quantify rRNA sequences of interest relative to total rRNA. The relative abundance may represent changes in the abundance in the population or changes in the activity and hence the amount of rRNA content (Theron and Cloete, 2000). Cellular level hybridization can also be done *in situ*. Valuable spatial distribution information on microbial communities in natural environments can be provided by hybridization methods.

One of the most popular DNA hybridization methods is FISH (Fluorescent *in situ* hybridization). Spatial distribution of bacterial communities in different environments such as biofilms can be determined using FISH (Schramm *et al.*, 1996). Lack of sensitivity of hybridization of nucleic acids extracted directly from environmental samples is the most notable limitation of nucleic acid hybridization methods. If sequences are not present in high copy number, such as those from dominant species, probability of detection is low.

DNA Reassociation

The kinetics of DNA reassociation reflect the variety of sequences present in the environment, thereby reflecting the diversity of the microbial community of the environment. DNA reassociation estimates diversity by measuring the genetic complexity of the microbial community (Torsvik *et al.*, 1996). Total DNA is extracted from environmental samples, purified, denatured and allowed to reanneal. The rate of hybridization or reassociation will depend on the similarity of sequences present. As the complexity or diversity of DNA sequences increases, the rate at which DNA reassociates will decrease (Theron and Cloete, 2000). The parameter controlling the reassociation reaction is concentration of DNA product (C_o) and time of incubation (t), usually described as the half association value, $C_o t_{1/2}$ (the time needed for half of the DNA to reassociate). Under specific conditions, $C_o t_{1/2}$ can be used as a diversity index,

as it takes into account both the amount and distribution of DNA re-association (Torsvik *et al.*, 1998). Alternatively, the similarity between communities of two different samples can be studied by measuring the degree of similarity of DNA through hybridization kinetics (Griffiths *et al.*, 1999).

Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is another tool used to study microbial diversity. This method relies on DNA polymorphisms. In the last couple of years RFLP applications have also been applied to estimate diversity and community structure in different microbial communities (Moyer *et al.*, 1996). In this method, electrophoresed digests are blotted from agarose gels onto nitro-cellulose or nylon membranes and hybridized with appropriate probes prepared from cloned DNA segments of related organisms. RFLP has been found to be very useful particularly in combination with DNA-DNA hybridization and enzyme electrophoresis for the differentiation of closely related strains (Palleroni, 1993), and the approach seems to be useful for determination of intra species variation (Kauppinen *et al.*, 1994). RFLPs may provide a simple and powerful tool for the identification of bacterial strains at and below species level. This method is useful for detecting structural changes in microbial communities but not as a measure of diversity or for detection of specific phylogenetic groups (Liu *et al.*, 1997). Banding patterns in diverse communities become too complex to analyze using RFLP since a single species could have four to six restriction fragments (Tiedje *et al.*, 1999).

However, one should be aware that a similar banding pattern does not necessarily indicate a very close relationship between the organisms compared.

Terminal restriction fragment length polymorphism (T-RFLP).

Terminal restriction fragment length polymorphism (T-RFLP) is a technique that addresses some of the limitations of RFLP (Thies, 2007). This technique is an extension of the RFLP/ ARDRA analysis, and provides an alternate method for rapid analysis of microbial community diversity in various environments. It follows the same principle as RFLP except that one PCR primer is labeled with a fluorescent dye, such as TET (4, 7, 2', 7'-tetrachloro-6-carboxyfluorescein) or 6-FAM (phosphoramidite fluorochrome 5-carboxyfluorescein). PCR is performed on sample DNA using universal l6S rDNA primers, one of which is fluorescently labeled. Fluorescently labeled terminal restriction fragment length polymorphism (FLT-RFLP) patterns can then be created by digestion of labeled amplicons using restriction enzymes. Fragments are then separated by gel electrophoresis using an automated sequence analyzer. Each unique fragment length can be counted as an Operational Taxonomic Unit (OTU), and the frequency of each OTU can be calculated. The banding pattern can be used to measure species richness and evenness as well as similarities between samples (Liu *et al.*, 1997). T-RFLP, like any PCR-based method, may underestimate true diversity because only numerically dominant species are detected due to the large quantity of available template DNA (Liu *et al.*, 1997). Incomplete digestion by restriction enzymes could also lead to an overestimation of diversity (Osborn *et al.*, 2000). Despite these limitations, some researchers are of the opinion that once standardized, T-RFLP can be a useful tool to study microbial diversity in the environment (Tiedje *et al.*, 1999), while others feel that it is inadequate (Dunbar *et al.*, 2000).

T-RFLP is limited not only by DNA extraction and PCR biases, but also by the choice of universal primers. None of the presently available universal primers can amplify all sequences from eukaryote, bacterial and archaeal domains. Additionally, these primers are based on existing 16S rRNA, 18S rRNA or Internal Transcribed Spacer (ITS) databases, which until recently contained mainly sequences from culturable microorganisms, and therefore may not be representative of the true microbial diversity in a sample (Rudi *et al.*, 2007). In addition, different enzymes will produce different community fingerprints (Dunbar *et al.*, 2000).

T-RFLP has also been thought to be an excellent tool to compare the relationship between different samples (Dunbar *et al.*, 2000). T-RFLP has been used to measure spatial and temporal changes in bacterial communities (Lukow *et al.*, 2000), to study complex bacterial communities (Moeseneder *et al.*, 1999), to detect and monitor populations (Tiedje *et al.*, 1999) and to assess the diversity of arbuscular mycorrhizal fungi (AMF) in the rhizosphere of *Viola calaminaria* in a metal-contaminated soil (Tonin *et al.*, 2001). Tiedje *et al.* (1999) reported five times greater success at detecting and tracking specific ribotypes using T-RFLP than DGGE.

Ribosomal intergenic spacer analysis (RISA)/ Automated ribosomal intergenic spacer analysis (ARISA) /Amplified ribosomal DNA restriction analysis (ARDRA)

Similar in principle to RFLP and T-RFLP, RISA, ARISA and ARDRA provide ribosomal-based fingerprinting of the microbial community. In RISA and ARISA, the intergenic spacer (IGS) region between the 16S and 23S ribosomal subunits is amplified by PCR, denatured and separated on a polyacrlyamide gel under denaturing conditions. This region may encode tRNAs and is useful for differentiating between bacterial strains and closely related species because of heterogeneity of the IGS length and sequence (Fisher and Triplett, 1999). Sequence polymorphisms are detected by silver staining in RISA. In ARISA, fluorescently labeled forward primer is detected automatically (Fisher and Triplett, 1999). Both RISA and ARISA method can deduce highly reproducible bacterial community profiles. Limitations of RISA include requirement of large quantities of DNA, relatively longer time requirement, insensitivity of silver staining in some cases and low resolution (Fisher and Triplett, 1999). ARISA has increased sensitivity than RISA and is less time consuming but traditional limitations of PCR also applies for ARISA (Fisher and Triplett, 1999). RISA has been used to compare microbial diversity in soil (Borneman and Triplett, 1997), in the rhizosphere of plants (Borneman and Triplett, 1997), in contaminated soil (Ranjard *et al.*, 2000) and in response to inoculation (Yu and Mohn, 2001).

DNA microarrays

More recently, DNA–DNA hybridization has been used together with DNA microarrays to detect and identify bacterial species (Cho and Tiedje, 2001) or to assess microbial diversity (Greene and Voordouw, 2003). This tool could be valuable in bacterial diversity studies since a single array can contain thousands of DNA sequences (De Santis *et al.*, 2007) with high specificity. Specific target genes coding for enzymes such as nitrogenase, nitrate reductase, naphthalene dioxygenase *etc*. can be used in microarray to elucidate functional diversity information of a community. Sample of environmental 'standards' (DNA fragments with less than 70% hybridization) representing different species likely to be found in any environment can also be used in microarray (Greene and Voordouw, 2003).

Another DNA microarray based technique for analyzing microbial community is Reverse Sample Genome Probing (RSGP). This method uses genome microarrays to analyze microbial community composition of the most dominant culturable species in an environment. RSGP has four steps: (1) isolation of genomic DNA from pure cultures; (2) cross-hybridization testing to obtain DNA fragments with less than 70% cross-hybridization. (DNA fragments with greater than 70% cross-hybridization are considered to be of the same species). (3) Preparation of genome arrays onto a solid support and (4) random labelling of a defined mixture of total community DNA and internal standard (Greene and Voordouw, 2003). This method has been used to analyze microbial communities in oil fields and in contaminated soils (Greene *et al.*, 2000).

Like DNA–DNA hybridization, RSGP and microarrays have the advantages that these are not confounded by PCR biases. Microarrays can contain thousands of target gene sequences but it only detects the most abundant species. In general, the species need to be cultured, but in principle cloned DNA fragments of unculturables could also be used. The diversity has to be minimal or enriched cultures should be used for this method. Otherwise, cross-hybridization can become problematic. Using genes or DNA fragments instead of genomes on the microarray offers the advantages of eliminating the need to keep cultures of live organisms, as genes can be cloned into plasmids or PCR can continuously be used to amplify the DNA fragments (Gentry *et al.*, 2006). In addition, fragments would increase the specificity of hybridization over the use of genomes and functional genes in the community could be assessed (Greene and Voordouw, 2003).

Denaturant gradient gel electrophoresis (DGGE)/Temperature gradient gel electrophoresis (TGGE)

In denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), DNA fragments of same length but with different base-pair sequences can be separated. DNA is extracted from natural samples and amplified using PCR with universal primers targeting part of the 16S or 18S rRNA sequences. The separation is based on the difference in mobility of partially melted DNA molecules in acrylamide gels containing a linear gradient of DNA denaturants (urea and formamide). Sequence variation within the DNA fragments causes a difference in melting behavior, and hence in separation in denaturing gradient gels. The melting of the products occurs in different melting domains, which are stretches of nucleotides with identical melting temperatures (Mühling *et al.*, 2008).

Sequence variations in different fragments will therefore terminate migration at different positions in the gel according to the concentration of the denaturant (Muyzer *et al.*, 1996). Theoretically, DNA sequences having a difference in only one base-pair can be separated by DGGE (Miller *et al.*, 1999). TGGE employs the same principle as DGGE but in this method the gradient is temperature rather than chemical denaturants. Advantages of DGGE/TGGE include reliability, reproducibility, rapidness and low expense. As multiple samples can be analyzed simultaneously, tracking changes in microbial population in response to any stimuli or adversity is possible by DGGE/TGGE (Muyzer, 1999). Limitations of DGGE/ TGGE include PCR biases (Wintzingerode *et al.*, 1997), laborious sample handling (Muyzer, 1999), and variable DNA extraction efficiency (Theron and Cloete, 2000). It is estimated that DGGE can only detect 1–2% of the microbial population representing dominant species present in an environmental sample (MacNaughton *et al.*, 1999). In addition, DNA fragments of different sequences may have similar mobility characteristics in the polyacrylamide gel. Therefore, one band may not necessarily represent one species (Gelsomino *et al.*, 1999) and one bacterial species may also give rise to multiple bands because of multiple 16S rRNA genes with slightly different sequences (Maarit-Niemi *et al.*, 2001).

DGGE profiles have successfully been used to determine the genetic diversity of microbial communities inhabiting different temperature regions in a microbial mat community (Ferris *et al.*, 1996), and to study the distribution of sulphate reducing bacteria in a stratified water column (Teske *et al.*, 1996).

Single strand conformation polymorphism (SSCP)

Single strand conformation polymorphism (SSCP) also relies on electrophoretic separation based on differences in DNA sequences and allows differentiation of DNA molecules having the same length but different nucleotide sequences. This technique was originally developed to detect known or novel polymorphisms or point mutations in DNA (Peters *et al.*, 2000). In this method, single-stranded DNA separation on polyacrylamide gel was based on differences in mobility resulted from their folded secondary structure (Heteroduplex) (Lee *et al.*, 1996). As formation of folded secondary structure or heteroduplex and hence mobility is dependent on the DNA sequences, this method reproduces an insight of the genetic diversity in a microbial community. All the limitations of DGGE are also equally applicable for SSCP. Again, some single-stranded DNA can exist in more than one stable conformation. As a result, same DNA sequence can produce multiple bands on the gel (Tiedje *et al.*, 1999). However, it does not require a GC clamp or the construction of gradient gels and has been used to study bacterial or fungal community diversity (Stach *et al.*, 2001). SSCP has been used to measure succession of bacterial communities (Peters *et al*., 2000), rhizosphere communities (Schmalenberger *et al.*, 2001), bacterial population changes in an anaerobic bioreactor (Zumstein *et al.*, 2000) and AMF species in roots (Kjoller and Rosendahl, 2000).

Other Potential Molecular Methods

Other molecular methods that have the potential to be as equally applicable as the above mentioned methods are Fluorescent *In situ* Hybridization (FISH) (Dokić *et al.*, 2010), DNA sequencing based community analysis such as Pyrosequencing based community analysis (Fakruddin *et al.*, 2012; Lauber*et al.*, 2009), Illumina-based High throughput microbial community analysis (Caporaso *et al.*, 2012; Degnan and Ochman, 2012) *etc*. Though most of these methods are not as applicable as previously mentioned methods, they pose the potential to be methods of choice in future.

With the emergence of next-generation sequencing (NGS) technologies such as pyrosequencing and Illumina-based sequencing, the possibility of discovering new groups of microorganism in complex environmental systems without cultivated strains has been accrued and these real-time sequencing techniques are shedding light into the complexities of microbial populations (Bartram *et al*., 2011). Using NGS, it is possible to resolve highly complex microbiota compositions with greater accuracy, as well as to link microbial community diversity with niche function. Next-generation sequencing strategies involve high throughput sequencing and, can effectively provide deep insights into complex microbial communities in ecological niches (Fakruddin and Mannan, 2012).

Pyrosequencing, developed by Roche 454 Life Science, is one such example and ISA high-throughput sequencing technique which can generate a huge amount of DNA reads (Fakruddin and Chowdhury, 2012). Recently, it has been successfully applied in dissecting complex

microbial environments such as the human gastrointestinal tract, soil, wastewater and marine sediments (Claesson *et al*., 2010). Pyrosequencing has provided a means to elucidate microbial members of the rare biosphere which occur in relatively low abundances. Besides eliminating the use of cloning vectors and library construction, and their associated biases, pyrosequencing can also read through secondary structures and produce vast amount of sequences of up to 100Mb per run (Royo *et al*., 2007). In addition to the sequencing technology itself, various bioinformatics tools have emerged to process and analyze pyrosequenced raw data *in silico* to generate meaningful information. Software such as the Newbler Assembler and RDP Pyrosequencing Pipeline provides a systematic way of analyzing data to rapidly gain insights into the complex microbial composition and structure in environmental samples (Van den Bogert *et al*., 2011).

Metagenomic analysis of microbial communities

Metagenomics is defined as the functional and sequence-based analysis of the collective microbial genomes that are contained in an environmental sample (Zeyaullah *et al*., 2009). In metagenomics, the collective genome (*metagenome* or *microbiome*) of coexisting microbes – called microbial *communities* (Ghazanfar *et al*., 2010) is randomly sampled from the environment and subsequently sequenced (Schloss and Handelsman, 2003). By directly accessing the collective genome of co-occurring microbes, metagenomics has the potential to give a comprehensive view of the genetic diversity, species composition, evolution, and interactions with the environment of natural microbial communities (Simon and Daniel, 2011). Community genomic datasets can also enable subsequent gene expression and proteomic studies to determine how resources are invested and functions are distributed among community members. Ultimately, genomics can reveal how individual species and strains contribute to the net activity of the community (Allen and Banfield, 2005).

Community genomics analyzing methods

Community genomics provides a platform to assess natural microbial phenomena that include biogeochemical activities, population ecology, evolutionary processes such as lateral gene transfer (LGT) events, and microbial interactions (Allen and Banfield, 2005). Applying community genomic data to DNA microarrays allows the analysis of global gene expression patterns and regulatory networks in a rapid, parallel format. Community microarray analyses can uncover apparent linkages between different genes and gene families and the distribution of metabolic functions in the community. Various genome assembly programmes such as ARACHNE, CAP, CELERA, EULER, JAZZ, PHRAP and TIGR assemblers are currently available to analyze community genomics data (Tyson *et al*., 2004).

Recently, sequencing and characterization of metatranscriptomes have been employed to identify RNA-based regulation and expressed biological signatures in complex ecosystems (Zeyaullah *et al*., 2009). Technological challenges include the recovery of high-quality mRNA from environmental samples, short half-lives of mRNA species, and separation of mRNA from other RNA species. Metatranscriptomics had been limited to the microarray/high-density array technology or analysis of mRNA-derived cDNA clone libraries (Simon and Daniel, 2011).

The proteomic analysis of mixed microbial communities is a new emerging research area which aims at assessing the immediate catalytic potential of a microbial community. Mass-spectroscopy-based proteomic methods are rapid and sensitive means to identify proteins in complex mixtures (Schloss and Handelsman, 2003). When applied to environmental samples, 'shotgun' proteomic analyses can produce surveys of prevalent protein species, which allows inferences of biological origin and metabolic function (Ghazanfar *et al*., 2010). Challenges for metaproteomic analyses include uneven species distribution, the broad range of protein expression levels within microorganisms, and the large genetic heterogeneity within microbial communities (Simon and Daniel, 2011). Despite these hurdles, metaproteomics has a huge potential to link the genetic diversity and activities of microbial communities with their impact on ecosystem function.

Statistical methods for assessing functional diversity of microbial communities

Analyzing microbial diversity by metagenomics has limitations in processing the huge amount of data obtained from the community. To improve the efficiency of the analysis programmes, statistical methods have been incorporated. The sequences derived from a mixture of different organisms are assigned to phylogenetic groups according to their taxonomic origins (Tyson *et al*., 2004). Depending on the quality of the metagenomic data set and the read length of the

DNA fragments, the phylogenetic resolution can range from the kingdom to the genus level (Allen and Banfield, 2005). Examples of bioinformatic tools employing similarity-based binning are the Metagenome Analyzer (MEGAN), CARMA, or the sequence ortholog-based approach for binning and improved taxonomic estimation of metagenomic sequences (Sort-ITEMS) (Simon and Daniel, 2011).

Abdo *et al*. (2006) reported a statistical method named 'R' for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. R functions can be implemented for identifying the 'true' peaks, binning the different fragment lengths, and for within cluster sampling.

CONCLUSION

Microbial diversity in natural environments is extensive. Methods for studying diversity vary and diversity can be studied at different levels, *i.e*. at global, community and population levels. The molecular perspective gives us more than just a glimpse of the evolutionary past; it also brings a new future to the discipline of microbial ecology. Since the molecular-phylogenetic identifications are based on sequences, as opposed to metabolic properties, microbes can be identified without being cultivated. Consequently, all the sequence-based techniques of molecular biology can be applied to the study of natural microbial ecosystems. These methods characterize the microbial processes and thereby can be used to reach a better understanding of microbial diversity. In future, these techniques can be used to quantitatively analyze microbial diversity and expand our understanding of their ecological processes.

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