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Review

Molecular approaches towards assessment of cyanobacterial biodiversity

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Cyanobacteria, a diverse group of photosynthetic prokaryotes, often play central roles in carbon and nitrogen assimilation which makes their environment productive. Despite their significant agronomic values, biodiversity assessment and conservation of these important microbes have not been given much attention by researchers. This review tries to bring together the important aspects of molecular approaches being used for cyanobacterial biodiversity assessment. Molecular approaches have been divided into 2 classes: PCR independent and PCR based approaches. While the former includes guanine plus cytosine (G+C) content, nucleic acid re-association and hybridization and DNA microarrays, the latter basically uses DNA cloning and sequencing, DGGE (denaturing gradient gel electrophoresis), TGGE (temperature gradient gel electrophoresis), SSCP (single strand conformation polymorphism), RFLP (restriction fragment length polymorphism), ARDRA (amplified ribosomal DNA restriction analysis), T-RFLP (terminal restriction fragment length polymorphism), RISA (ribosomal intergenic spacer analysis), ARISA (automated ribosomal intergenic spacer analysis) and RAPD (random amplified polymorphic DNA). Molecular assessment of cyanobacterial biodiversity frequently uses markers like 16S rDNA, phycocyanin locus, *nif* gene, *rpo* gene, ITS region etc. This article examines and compares various conventional and modern methods and their short comings, if any. It attempts to provide a general overview of biodiversity assessment, molecular techniques and markers used for biodiversity assessment and also recommends combinatorial approach with different molecular markers. It is likely to improve the degree of resolution and provide as possible the broadest picture and indepth information about biodiversity documentation.

Key words: Cyanobacteria, biodiversity, molecular approaches, molecular markers, DGGE, TGGE, SSCP, RFLP, ARDRA, T-RFLP, RISA, ARISA, RAPD, 16S rDNA, phycocyanin locus, *nif* gene, *rpo* gene.

INTRODUCTION

Biological diversity or biodiversity, as it is recently called, is a “cluster of concepts” (Contoli, 1991) which encompasses many interrelated aspects, from genetics and molecular biology to community structure and habitat heterogeneity (Battaglia, 1996, Boero, 1996). However, the most fundamental meaning of biodiversity probably lies in the concept of species richness (Baltanas, 1992), that is, the number of species occurring at a site, in a region or ecosystem. Ecologists are used to measuring diversity through a number of indices all of which relate, more or less directly, the number of species to their abundance and/or numerical dominance (Magurran, 1988).

India is one of the mega-biodiversity countries of the world, having almost all possible kind of climatic variations, with a great variety of plants and microbes. Of the total 40,000 species of algae reported so far, about 6500 species are found in India. Of this 1,500 species belong to 150 genera of cyanobacteria (Litavitis, 2002).

Cyanobacteria, the first prokaryotic organisms believed to have evolved approximately 3.8 billion years ago on

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this planet earth, are one of the most widespread groups of gram-negative bacteria. They display considerable morphological diversity and unusual capabities for cellular differentiation, while their basic metabolism is fairly uniform. Cyanobacteria are capable of having photoautotrophic growth, performing oxygenic photosynthesis similar to those of eukaryotic algae and plants (Gorl et al., 1998). Cyanobacteria are believed to be responsible for the conversion of the primitive anaerobic reducing environment to modern aerobic oxidizing environment (Lukow et al., 2000). During the course of evolution they adapted to almost every ecological niche, including the most extreme ones (Schopf, 2000). They can be found in freshwater lakes, hot and cold springs examples of which are: the arctic ice (Torsvik et al., 1996), deserts (Garcia-Pichel et al., 2001), oceans, that is marine cyanobacteria, (Thajuddin and Subramanian, 2005) and even the arctic hot springs (Rios et al., 2007) and Antarctic granite rocks (Roeselers et al., 2007). The adaptability of cyanobacteria to different environmental conditions is ascribed to the plasticity of their genome. They are used as biofertilizers, single cell protein (SCP), food, biomass for grazers and in the reclamation of saline and alkaline soils (Singh, 1961). They are also known to produce important biomolecules such as carotene, scytonemin and glycine betaine which act as UV protectant and antidesiccant, respectively. Cyanobacteria play a major role in the primary productivity as well as nitrogen economy of various ecosystems. About 20 - 30% of the total carbon fixed on the earth is attributed to cyanobacteria only.

In view of the above, it becomes very important to have an accurate knowledge of their morphology, systematics and biodiversity in order to explore their hidden wealth and potential for human welfare. Biodiversity of cyanobacteria has been reported to decline due to various anthropogenic activities like habitat loss and fragmentation, disturbance and pollution, construction of dams, chemical pollution and global climatic changes. Further, in view of the limitations of morphology in biodiversity assessment and lack of information on genetic diversity of cyanobacteria, molecular assessment of biodiversity has emerged as a reliable tool for biodiversity documenttation (Lee et al., 1996). Rapid decline of biodiversity has generated an unprecedented urge for speedy inventory and monitoring of regional and global biodiversity to help in the formulation of priorities for conservation of economically useful species (Singh, 2002).

Historically, the taxonomy of the cyanobacteria has been formulated under the International Code of Botanical Nomenclature based on phenotypic characters (Bourrelly, 1985; Marsh et al., 1997; Banerjee et al., 2000). Identification of cyanobacteria using the above proposals of classification is primarily based on features such as morphology of the filament, vegetative cells, heterocyst and akinete (Komárek and Anagonistidis, 1989). The form of the colony, shape of the terminal cells, presence or absence of sheath and gas vesicles, as well as life cycle are additional features used for identification of some genera (Rajaniemi et al., 2005). However, the reliability of these phenotypic characters and their use in the taxonomy has been questioned by Anagnostidis and Komárek (1989). This is because these characters are prone to environmental changes. For example, the presence of nitrogen sources like NH_4^+, NO_3^- inhibits the formation of heterocyst and presence of phosphate inhibits the formation of akinetes in cyanobacteria. Due to the above reasons, the assessment of biodiversity using morphological and physiological variables provides misleading information. Moreover, cyanobacteria in nature may be present in the form of vegetative cells, akinetes, spores, hormogones and other resting bodies, which may not regenerate in culture so quickly. The identification of these resting bodies is not possible under microscope (Komárek and Anagonistidis, 1989). Another important drawback of this approach is the unavailability of a universal culture medium that can support the growth of all cyanobacteria. Despite the above, morphological approaches were used by Thajuddin and Subramanian (2005) to estimate marine cyanobacterial biodiversity. Narayan et al. (2006) have analysed biodiversity of selected cyanobacteria.

Contrary to the above, much effort has been directed towards assessing the biodiversity at molecular level (Lee et al., 1996; Teske et al., 1996; Peters et al., 2000, Garcia-Pichel et al., 2001, Rasmussen and Svenning, 2001; Gugger et al., 2002; Teaumroong et al., 2002; Narayan et al. 2006; Roeselers et al., 2007). A number of approaches have been developed to study microbial diversity at molecular level such as DNA-DNA hybridization, DNA cloning and sequencing, PCR based DNA fingerprinting methods like DGGE/TGGE, RFLP, RAPD, SSCP, RISA/ARISA, T-RFLP as well as PCR independent methods like G+C content, DNA microarrays and DNA reassociation analysis. These methods are extremely useful for documenting the biodiversity at molecular level. However, it is pertinent for all to know that most of the PCR based methods use 16S rDNA sequences for diversity analysis (Woese, 1987), *nif* gene (Lee et al., 1996), phycocyanin locus (Neilan et al., 2002). RNA polymerase gene (Neilan et al., 1995) has also been used for assessing cyanobacterial biodiversity. The molecular characterization of cyanobacteria in general (Rocap et al., 1999), identification and phylogenetic analysis of toxigenic cyanobacteria (Neilan et al., 1995) and phenotypic and genotypic analysis of symbiotic and free living cyanobacteria from a single site have been done thoroughly (West and Adams, 1997). However, only a small number of cyanobacteria have been analyzed using molecular techniques (Neilan et al., 2002; Lyra et al., 1997). While assessing the patterns of species diversity in inland marshes of Northern Belize (Central America), Rejmankova et al. (2004) did realise that cyanobacteria are a neglected component of biodiversity. Diversity of *Synechococcus* and *Prochlorococcus* popu-

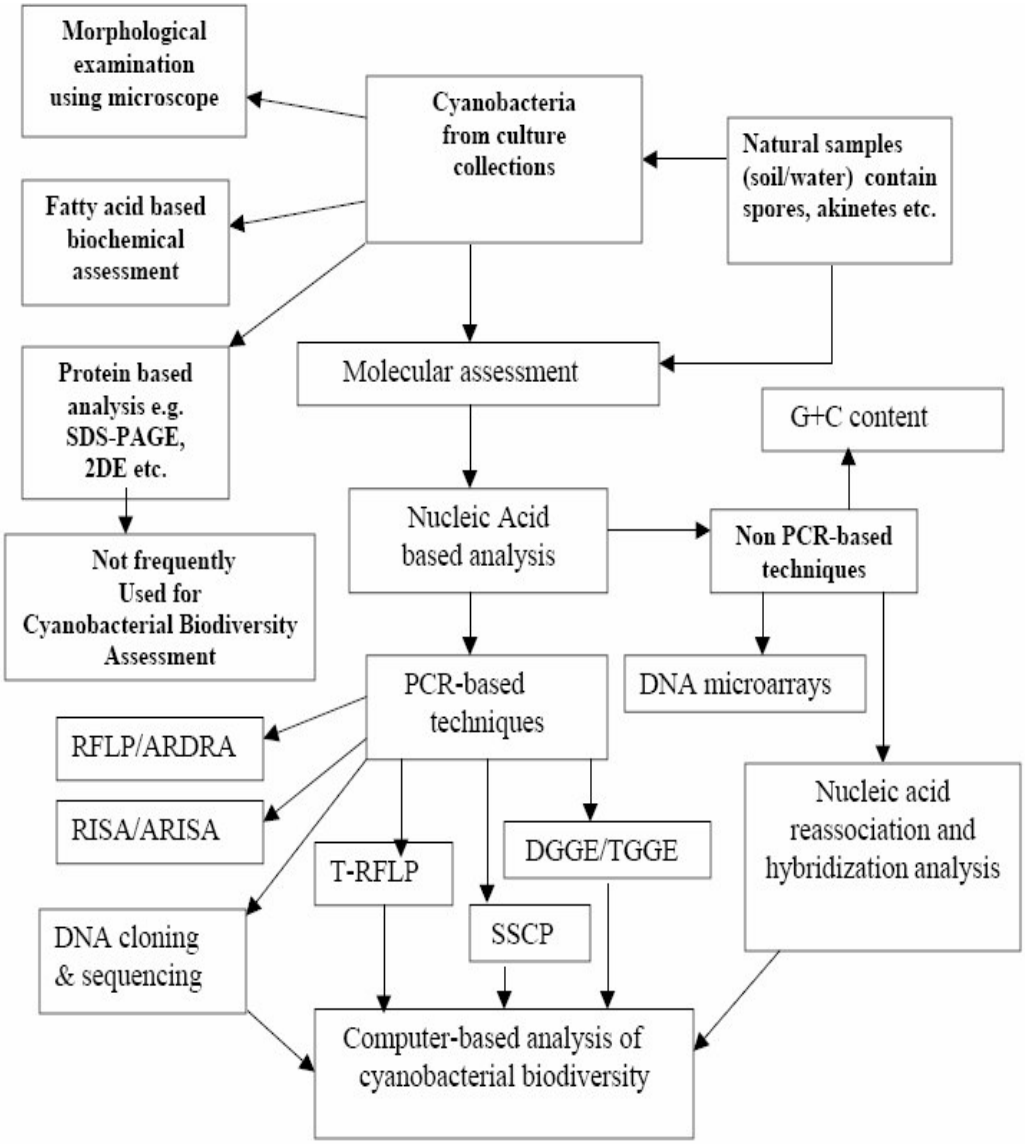

Figure 1. An overview of techniques used for cyanobacterial biodiversity assessment.

lations has been determined from DNA sequences of the N-regulatory gene ntcA by Penno et al. (2006). Recently, Tsygankov (2007) has reviewed nitrogen-fixing cyanobacteria for nitrogenases and hydrogenases enzymes and their biodiversity has been assessed by Subramaniyan et al. (2007) in effluents from dye, paper mill, pharmaceutical and sugar industries. Chinnasamy et al. (2007) have also assessed cyanobacterial biodiversity from different freshwater ponds of Thanjavur, Tamilnadu. They have tried to establish relationship between physico-chemical properties and biological parameters of water such as plankton, pH, dissolved oxygen, net productivity, alkalinity, nitrate, nitrite, total phosphorous and inorganic phosphorus and total cyanobacterial species.

This review provides a brief account of the biodiversity assessment of cyanobacteria inhabiting diverse environments and compares the various methods used to study cyanobacterial biodiversity from natural system.

GENERAL APPROACHES USED FOR BIODIVERSITY ASSESSMENT

Biodiversity assessment has been done at morphological, physiological, biochemical and molecular levels (Figure 1). The cyanobacteria have been broadly classified into unicellular and multicellular (filamentous) types. The filaments may be unbranched, truly branched or have false branching. They have also been classified on the basis of presence or absence of heterocyst and position of akinetes (Marsh et al., 1997; Geitler, 1932). The morphological assessment of biodiversity done by using light

microscope has certain limitations, as it fails to identify the spores or akinetes and hormogonia or small fragments. Further, the biodiversity assessment at biochemical level has been done by using fatty acid composition. The fatty acid composition of 66 cyanobacterial strains has been analysed and used for characterization (Kenyon, 1972, Kenyon et al., 1972). Highly polar but unknown glycolipids have also been reported from 3 heterocystous strains (Sallal et al., 1990). Marine picoplanktonic *Synechococcus* strains depicted similar fatty acid composition as the fresh water *Synechocystis* sp. strain PCC 6308 (Merritt et al., 1991). The lipid analysis of *Prochloron* sp indicated a closer relationship with the cyanobacteria than with eukaryotic algae (Perry et al., 1978). However, data are relatively scarce and several studies are still preliminary. Problems of inconsistency and variations due to factors such as growth conditions have not always been systematically investigated (Holton, 1981).

Irrespective of the above problem, the protein profiling of cell extract or membrane by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) has been found to be a useful tool in the identification of species and subspecies of microorganisms. It is very widely used in the identification of bacteria such as species of *Lactococcus, Lactobacillus*, *Leuconostoc* (Perez et al., 2000), *Staphylococcus* (Berber et al., 2003) and cyanobacteria (Woese, 1987). However, it is still not so widely applied in classification of cyanobacteria because of the requirement of huge amount of culture and difficulty in protein profiles comparison. Nevertheless, in future it may be used for the assessment of cyanobacterial biodiversity. Nowadays, the phylogeny determined by protein domain content is being used to assess genetic relationships (Yang et al., 2004). A simple classification scheme that uses only the presence or absence of protein domain architecture has been used to determine the phylogeny of 174 complete genomes of various microorganisms including cyanobacteria.

The above methods employed for the assessment of cyanobacterial biodiversity at morphological and biochemical levels have some drawbacks and limitations in studying cyanobacterial diversity like spatial heterogeneity, inability to culture soil microorganisms and taxonomic ambiguity of microbes etc. The problems associated in studying cyanobacterial biodiversity arose not only from methodological limitations, but also from a lack of taxonomic knowledge. Many a times cyanobacteria show such variations that even trained taxonomists fail to identify them correctly. It is difficult to study the diversity of a group of microorganisms because the understanding to identify the species present is lacking. To overcome these drawbacks, cyanobacterial biodiversity assessment is being done at molecular level. Since the genetic constituent of the species does not show quantifiable change over time, the nucleic acid based analyses are used for this kind of study (according to C-value paradox) with respect to the environment. As a result it is considered the most accepted tool.

TECHNIQUES USED FOR BIODIVERSITY ASSESSMENT

The molecular techniques being used to study cyanobacterial biodiversity can be broadly classified into PCR independent and PCR-based approaches. The PCR-independent approach includes guanine plus cytosine (G+C) content estimation, nucleic acid reassociation and hybridization and DNA microarrays whereas PCR-based approach includes denaturing gradient gel electrophoresis(DGGE), temperature gradient gel electrophoresis(TGGE), single strand conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP), amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism(T-RFLP), ribosomal intergenic spacer analysis (RISA), automated ribosomal intergenic spacer analysis (ARISA), and randomly amplified polymorphic DNA (RAPD) etc. Figure 1 presents a consolidated view of the different approaches used to assess the microbial diversity.

PCR independent approach

Molecular approaches targeting DNA that do not include PCR may be categorized into the following types.

G+C content

Differences in the guanine plus cytosine (G+C) content of DNA have been used to study the bacterial diversity of soil communities. It is based on the fact that microorganisms differ in their G+C content and taxonomically related groups only differ between 3 and 5% (Tiedje et al., 1999). This method provides a coarse level of resolution as different taxonomic groups may share almost the same G+C range. Advantages of G+C analysis are that it is not influenced by PCR biases, employs all DNA extracted, quantitative in nature and can uncover rare members in the microbial populations. It does, however, require large quantities of DNA (up to 50 µg) (Tiedje et al., 1999). Some workers have examined the G+C content of planktonic species of *Anabaena* and attested to its taxonomic significance (Baudouin-Cornu et al., 2004). In general, all the cyanobacterial strains assigned to *Anabaena* species showed similar DNA base composition. Baudouin-Cornu et al. (2004) used the G+C content in conjunction with protein and found appreciable correlation of cyanobacterial proteins with its genome based composition which can be further used for phylogenetic studies.

Nucleic acid reassociation and hybridization analysis

DNA reassociation is a measure of genetic complexity of the microbial community and has been used to estimate

diversity (Torsvik et al., 1990, 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 re-associates decreases (Theron and Cloete, 2000). Under specific conditions, the time required for half of the DNA to reassociate (the half association value Cot1/2) can be used as a diversity index, as it takes into account both the amount and distribution of DNA reassociation (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). This technique is a measure of genetic variation of the natural populations and is used to compare two communities (Torsvik and Ovreas, 2002). While comparing two communities, the DNA of one community may be radioactively labeled and used as a template. Cross hybridization between the two DNA samples is carried out. The rate of reassociation or hybridization is directly proportional to similarity. The major shortcoming of this technique is that it requires large quantity of highly purified DNA, which prevents the routine use of this technique in biodiversity analysis.

Nucleic acid hybridization using specific probes is an important qualitative and quantitative tool in molecular bacterial ecology (Threon and Cloete, 2000; Griffiths et al., 1999;, Schramm et al., 1996, Guo et al., 1997; 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 (Threon and Cloete, 2000). Fluorescent markers commonly used include derivatives of fluorescein or rhodamine. Hybridization can also be conducted at the cellular level *in-situ*. This provides valuable spatial distribution information on microorganisms in environmental samples. The widely used technique for proper estimation of nucleic acid hybridization that is, FISH (Fluorescence *in situ* Hybridization) uses rRNA targeted fluorescent probes to investigate the composition of soil microbial communities. Furthermore, this technique has been modified and renamed as TSA-FISH (Tyramide Signal Amplification of FISH) which increases the fluorescent signals from hybridized cells 20 -40 times more than the background. Probes are designned complementary to either species specific or group specific. Notwithstanding numerous uses of this technique to explore diversity, this may sometimes produce false positive and negative results due to methodological or environmental factors. Lehtimaki et al. (2000) have characterized some of the *Nodularia* strains of cyanobacteria from brackish waters, by genotypic and phenotypic methods. DNA-DNA hybridization has been used by Suda et al. (2002) to clarify and revise the taxonomic position of several strains of bloom-forming species of oscillatorioid cyanobacteria.

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 (Muyzer and Smalla, 1999) with high specificity. The microarray can either contain specific target genes such as nitrate reductase, nitrogenase or naphthalene dioxygenase that provide functional diversity information or can contain a sample of environmental standards (DNA fragments with less than 70% hybridization) representing different species found in the environmental samples (Greene and Voordouw, 2003). Microarray is a great technique used for the investigation of environmental response of cyanobacteria. Its real significance is appreciated while investigating organisms like *Prochlorococcus* which is a dominant autotroph in titanic areas of the open ocean (Muyzer and Smalla, 1999).

PCR- based approaches

DNA cloning and sequencing

Initially, molecular methods for biodiversity studies relied on cloning of target genes isolated from environmental samples. In the initial step of this approach PCR products are first cloned and then sequenced. By comparing the sequences with those available in sequences databases (GenBank, EMBL, DDBJ), the information about the identity and relatedness of the new sequences to known species is obtained. Giovannoni et al. (1998) first used the cloning sequencing approach by targeting 16S rDNA for the determination of bacterioplankton diversity in Sargasso Sea. Semenova et al. (2001) have analyzed nucleotide sequences of fragments of 16S rDNA of the Baikal natural populations and laboratory cultures of cyanobacteria by DNA cloning and sequencing. The cloning sequencing strategy has been used in various ecosystems for studying prokaryotic diversity (Zwart et al., 2002). Svenning et al. (2005) studied the diversity of symbiotic cyanobacterial strains within the genus *Nostoc* based on 16S rDNA sequence analysis with the help of DNA cloning and sequencing. This technique is good enough for assessing biodiversity, but in more diverse ecosystems this approach becomes laborious, time consuming and expensive.

DGGE/TGGE

Denaturing gradient gel electrophoresis and thermal

gradient gel electrophoresis are two routinely used almost similar techniques used in assessing the biodiversity of microbial communities from environmental samples as well as monitoring of their dynamics (Muyzer et al., 1996). These methods were originally developed to detect gene mutation by medical research community. Muyzer et al. (1993) were the first to expand the use of PCR-DGGE to study microbial diversity. In most of the electrophoretic fingerprinting methods, nucleic acid fragments are separated by their size but DGGE/TGGE separates DNA strands by their sequence composition (Hovig et al., 1991). The PCR- DGGE protocol consists of 6 major steps, sample collection, nucleic acid extraction, PCR amplification of target gene, separation of these PCR amplicons by DGGE, staining and visualization of profiles and data analysis. DNA extracted from the soil samples is amplified using PCR with primers targeting part of 16S rDNA sequences. The PCR product is separated on a gel that is composed of a linear gradient of denaturant. In DGGE, the chemical denaturant urea and formamide are used while in TGGE, temperature is used (Lessa and Applebaum, 1993). Differential migration occurs as more denaturant is needed to separate sequences with high GC content. A high GC sequence (35-40 bp GC clamp) is attached to the 5 end of the forward primer to prevent complete strand separation (Sheffield et al., 1989). On denaturation, DNA melts in domains which are sequence specific and it migrates differentially through the polyacrylamide gel (Muyzer et al., 1996). The fingerprints obtained on the denaturing gradient gel represent the community structure, an approximation of number of population (represented by each band) and their relative abundance (represented by band intensity). More specific information of population composition can be obtained by secondary analysis of the DGGE/TGGE bands via sequencing or hybridization. DGGE/TGGE bands can be excised from gels, reamplified and sequenced or transferred to membranes and hybridized with specific probes to get more diversity information (Kirk et al., 2004). In addition to community structure information, the DGGE method is amenable to semiquantitative assessment of activity. One of the most general measures of cellular activity is the ribosome content. The ratio of rRNA to rDNA increases with increasing growth rate (activity). DGGE has been used to evaluate this ratio among different natural populations by comparing pattern and intensities of bands derived from using either rDNA or rRNA (using reverse transcriptase to generate cDNA) as the template (Teske et al., 1996). Song et al. (2005) assessed biodiversity and seasonal variation of the cyanobacterial assemblage in rice fields of China, applying the DGGE technique. Most of the time rRNA genes have been the main targets of microbial diversity studies using DGGE but some researchers have also used catabolic genes such as methane mono-oxygenase for DGGE analysis (Fjellbirkeland et al., 2001, Knief et al., 2003). Digitization of DGGE profile (Zhang and Fang, 2000) and statistical analysis of DGGE fingerprinting patterns (Fromin et al., 2002) have improved the molecular assessment of biodiversity. Microbial community of cyanobacteria mats in the intertidal zone of oil-polluted coast of Saudi Arabia (Al-Thukair et al., 2007) and soil microbial community analysis using DGGE (Nakatsu, 2007) are some of the recent reports of DGGE used in cyanobacterial biodiversity assessment. Kardinaal et al. (2007) have studied rRNA ITS region DGGE profile to judge the genotypic variation in *Microcystis* species in relation to microcystin concentrations in freshwater lakes. Thus, DGGE/TGGE has the advantage of being reliable, reproducible and rapid. These techniques provide cost effective means to examine and compare a large number of samples within a short time frame.

SSCP

Single strand conformational polymorphism is a technique that is quite similar to DGGE/TGGE. Like DGGE/TGGE, this was also originally developed to detect point mutations (Orita et al., 1989). SSCP analysis is the electrophoretic separation of single-stranded nucleic acids based on subtle differences in sequence (often a single base pair) that results in a different 3 dimensional folded secondary structures. This brings about measurable difference in mobility through a gel. In fact the mobility of double-stranded DNA in gel electrophoresis is dependent on strand size and length but is relatively independent of the particular nucleotide sequence. However, the mobility of single strands is noticeably affected by very small changes in sequence, because of the relatively unstable nature of single-stranded DNA. Actually in the absence of a complementary strand, the single strand experiences intra-strand base pairing, resulting in loops and folds that give the single strand a unique 3D structure, regardless of its length. Therefore, even a single nucleotide change could dramatically affect the strand's mobility through a gel by altering the intrastrand base pairing and its resulting 3D conformation. Single-strand conformation polymorphism analysis takes advantage of this quality of single-stranded DNA and is a means of detecting DNA polymorphisms or sequence variations. In this approach 16S rDNA sequences are amplified, denatured and separated on non-denaturing gel. Differences in mobility are detected by silver staining or automated DNA sequencers if primers used are fluorescently labeled. SSCP analysis offers an inexpensive, convenient, and sensitive method for determining genetic variation. Lee et al. (1996) first used this technique to assess the diversity of natural communities. There have been a small number of reports about the use of SSCP to document cyanobacterial diversity. The diversity of epilithic biofilms dominated by cyanobacteria along a subtropical rocky shore of Mexico was done by applying SSCP and dominance of thick-sheathed cyanobacteria like

Xenococcus and *Chroococcidiopsis* in those habitats was reported. PCR-SSCP has also been used to measure succession of bacterial communities (Peters et al., 2000). The detection of sequence variation using PCR-SSCP is good enough but the sensitivity declines if amplified fragment length increases.

T-RFLP

Terminal restriction fragment length polymorphism is a quantitative molecular technique that was developed by Liu et al. (1997) for rapid analysis of microbial community diversity in various environments. The technique employed PCR in which one of the 2 primers used was fluorescently labeled at the 5'end and was used to amplify a selected region of bacterial genes encoding 16S rRNA from total community DNA. The PCR product was digested with restriction enzymes, and the fluorescently labeled terminal restriction fragment was precisely measured by using an automated DNA sequencer. Computer simulated analysis of T-RFLP shows that with proper selection of PCR primers and restriction enzymes, DNA sequences could be PCR amplified and classified into unique terminal restriction fragment lengths or ribotypes. Terminal restriction fragment length polymorphism analysis is a commonly used fingerprinting technique that is basically an improvement of amplified fragment length polymorphism (AFLP) (Tiedje et al., 1999). This involves restriction digestion of double stranded fluorescently end labeled PCR fragments as one primer is labeled at 5'terminus with a fluorescent dye. Since a single species will produce single terminal fragment, each band represents a single operational taxonomic unit or ribotype; thus the banding pattern can be used to measure species richness or evenness as well as similarities between samples (Marsh et al., 1997). There are several reports on use of this technique for microbial diversity studies (Garcia-Martinez et al., 1999; Lukow et al., 2000). T- RFLP like any PCR based method may underestimate true diversity because only numerically dominant species are detected due to availability of large quantity of template DNA. Moreover, since different enzymes will produce different community fingerprints (Dunbar et al., 2000), it is important to use at least two to four different restriction enzymes. Thies (2007) has suggested that T-RFLP can be used to analyze communities of bacteria, archaea, fungi, other phylogenetic groups or subgroups, as well as functional genes. The method is rapid, highly reproducible and often yields a higher number of operational taxonomic units than other commonly used PCR-fingerprinting methods.

RISA/ARISA

Ribosomal intergenic spacer analysis/automated ribosomal intergenic spacer analysis are DNA fingerprinting techniques used to assess microbial diversity and are similar in principle to RFLP and T-RFLP. RISA was developed by Borneman et al. (1996) and was first applied to assess microbial diversity of soil (Zwart et al., 2002). The intergenic spacer region (IGS) located between the 16S and 23S ribosomal subunit in the rRNA operon is amplified by PCR using primers, denatured and separated on a polyacrylamide gel under denaturing conditions. Primers are designed to target the conserved regions in the 16S and 23S genes. Amplification products differing in length are separated on polyacryamide gels because of heterogeneity of the IGS length and sequence. This tool has been successfully used to assess community diversity considering each band corresponding to one organism. This method has also been used to compare the dynamics both between and within microbial populations (Belknap and Hasselkorn, 1987).

RFLP (Restriction fragment length polymorphism)

It is a powerful molecular tool in studies of biodiversity. RFLP is generated by the presence and absence of a recognition site for the same restriction endonuclease in the same region of a chromosome from different individuals of a species. As a result the concerned restriction enzyme produces fragments of different length representting the same chromosome region of different individuals. These differences are detected by gel electrophoresis combined with hybridization with a labeled (usually radio labeled) probe specific for that chromosome region. Thus, RFLP marker results from a combination of a specific restriction endonuclease and a specific DNA sequence used as probe. The lanes of the different strains/selected species are compared and RFLPs are detected due to differential movement of a band on their gel lanes; each of such band is regarded as a single RFLP locus. Lyra et al. (1997) demonstrated the significance of this technique in a study based on 7 enzymes, which separated a number of hepatotoxic heterocystous cyanobacterial strains. However, they were unable to separate *Aphanizomenon* sp. from the bloom forming neurotoxic *Anabaena* strains.

Furthermore, the heterogeneity of the band confirms the grouping obtained by RFLP analysis. Neilan et al., (2002) have also described the molecular evolution and DNA profiling of toxic cyanobacteria with the help of RFLP. The genus *Anabaenopsis* was subdivided into two groups by RFLP banding pattern analysis; the pattern also clearly separated all three isolates of *Anabaena flos-aquae* (Rippka et al., 1979). But, the judicious choice and number of restriction enzymes is essential to achieve the desired level of taxonomic resolution.

RAPD

Randomly amplified polymorphic DNA (RAPD) bands are

Table 1. List of unicellular marine cyanobacterial strains collected at Hiddens and Boiensdor station (BO), showing 16S rDNA sequences and common RAPD bands.

Organism	Strain	16S rDNA sequenced	Common RAPD bands
Synechocystis sp.	H1 19	305nt	5
Chroococcus turgidus	B01	305 nt	0
Synechococcus sp.	B045	305nt	N
Synechococcus elongatus	B052	305nt	0
Synechocystis sp.	B079	1479 nt	5
Microcystis sp.	B083	305nt	5

n = no data, nt = nucleotide.
Classification was performed by J Rethmeier, Untersuchungen zur OX kologie und zum Mechanismus der SulÆdadaption mariner Cyanobakterien der Ostsee PhD thesis University of Bremen Germany (1995)

produced by using PCR equipment or a thermal cycler. RAPD polymorphism is detected by using oligonucleotide usually 10 bases long of random sequences as primer in a PCR reaction. The strains, which have their genomic DNA sequences complementary to the primer oligonucleotide, will be amplified in PCR. The amplified products are detected in the agarose gel while in those strains which have no complementary sequence, no product will be detected. In practice, some bands do appear in each of the strains tested. Stringency of paring between primers and the template DNA is reduced to ensure amplification as some of these bands are present in some and absent in the other strains. These bands constitute the RAPD loci.

Genetic variability of the cosmopolitan, ubiquitous fresh water cyanobacterium *Phormidium retzii* was assessed using RAPD markers (Eskew et al., 1993). 6 unicellular marine cyanobacteria strains of the order, chroococcales isolated from the Baltic Sea were examined by RAPD PCR (Mikolajczak et al., 1999). RAPD-PCR was used to reveal DNA sequence polymorphism to a level beyond the taxonomic range approached by the 16S rRNA gene.

In the case of unicellular marine cyanobacteria from the Baltic Sea, a unique band obtained from the RAPD-pattern was cloned and finally used to compare the strains by dot-blot hybridization using this sequence as probe. Table 1 demonstrates that out of 6, 3 cyanobacterial strains (*Synechocystis* sp. H19, *Synechocystis* sp. BO79, *Microcystis* sp. BO83) showed almost 100% sequence homology as identified by 16S rRNA gene-RAPD map (Mikolajczak et al., 1999). No polymorphism was detected in the RAPD band pattern of these strains. In addition, positive dot blot signals were obtained using the cloned RAPD- probes with these strains. These results suggest that 3 of the 6 unicellular marine cyanobacteria strains examined should be considered as a single species. The investigation shows that the molecular techniques applied are useful tools to complement cyanobacterial taxonomy and to infer interspecies relationship. This technique is considered as superior to RFLP because it has large genomic abundance, automated and involves no restriction enzymes as well as probes and therefore, less expensive.

Molecular markers used to assess cyanobacterial biodiversity

Molecular assessment of cyanobacterial biodiversity has exploited lots of markers till date but the most frequently used ones are 16S rRNA gene, phycocyanin locus, RNA polymerase, *nif* gene, IGS regions etc. Some of these may be explained as highlighted below.

16S rRNA gene

16S rRNA gene sequencing is the most widely applied strategy for assessing cyanobacterial biodiversity in nature. For broad phylogenetic studies, sequence data from the 16S rRNA gene are the most commonly used due to their efficacy for distinguishing higher-level taxonomic groups, as well as traditional species. Diversity assessment of *Phormidium retzii* (Oscillatoriales) has been done with the help of 16S rRNA gene sequence analysis (Lee et al., 1996). This group traditionally includes the well-known genera *Oscillatoria, Phormidium* and *Lyngbya*, which are primarily differentiated by sheath properties. However, sheath production is only one of the typical diagnostic features subjected to the direct effects of both environmental and culture conditions and therefore not phylogenetically informative (Rippka et al., 1979; Sigler et al., 2003). The study of 16S rRNA gene is also helpful in understanding the phylogenetic relationship between *Synechocystis tridemni* and *Prochloron didemni*. The cytological similarities suggest that *Prochloron* may have evolved from an ancestral *Synechocystis* like alga. However, there are no data available to compare them phylogenetically. DNA sequence information for the small subunit rRNA gene obtained from cyanobacterial culture has been used to investigate the presence of cyano-bacteria and their abundance in natural habitat (Rudi et al., 2000).

However, there are some pitfalls in the use of 16S

rRNA gene for the studying of biodiversity. One of those is that the size of the genes for the 16S molecules is extremely constant (with the total variation of about 200bp for a mean length of 1500 bp (Linton et al., 1994; Rainey et al., 1996; Moreira and Philippe, 2000) and therefore different genes cannot be easily separated by size.

Additionally, the 16S rDNA sequence, in spite of having hyper variable and extremely informative regions for close relationship, is often not divergent enough to give good separation in close relation, e.g. species of the same genus (Normand et al., 1996). Both these problems are simplified if the spacer region located between the 16S and 23S rRNA gene is included in the PCR amplify-cation (Garcia-Martinez, 1999). This region is extremely variable in size and sequence even within closely related taxonomic groups. Therefore, the 16S-23S spacers can be a powerful tool to help researchers in their work to discern the relationships among closely related micro-organisms especially cyanobacteria.

Phycocyanin locus

Genetic diversity of cyanobacteria has been further characterized by determining the DNA polymorphism within the phycocyanin (PC) locus (Neilan et al., 1995). The distribution of PC in aquatic microorganisms especially in cyanobacteria makes the study of PC gene sequence heterogeneity ideal for the classification of fresh water cyanobacteria. The entire PC operon contains genes coding for 2 bilin subunits and 3 linker polypeptides (Whitton, 1992). The intergenic spacer (IGS) between the 2 bilin subunit genes, designated β (cpcB) and α (cpcA) of the PC operon is selected as a potentially highly variable region of DNA sequence useful for the identification of cyanobacteria to the strain level. With the help of this approach biodiversity among *Anabaena, Aphanizomenon, Cylindrospermopsis, Microcystis, Nostoc, Nodularia and Oscillatoria* have been assessed (Neilan et al., 2002).

RNA polymerase

DNA-dependent RNA polymerase is a potential molecule for examining organismal diversity because it exists in single copies in prokaryotic genome (Bergsland and Haselkorn, 1991). PCR is used to amplify DNA-dependent RNA polymerase gene sequences specifically from the cyanobacterial population to assess biodiversity (Palenik, 1994). The various polymerase subunits contain highly conserved regions that can be used to develop PCR primers. Two such regions have been used to develop primers that will amplify specifically a fragment of the *rpo*C1 subunit gene from the cyanobacterial lineage. The sequences obtained have been valuable for probing cyanobacterial evolution (Palenik and Haselkorn, 1992), with results similar to that for 16S rRNA (Urback et al., 1992). In addition, the nucleotide sequence divergence between closely related species is much greater for the *rpo*C1 gene fragment than for the 16S rRNA sequences. For example *Synechococcus* isolates, WH7805 and WH8103 are 83% identical for the whole *rpo*C1 fragment and 98.6% identical for 16S rRNA. In view of this, the use of *rpo*C1 fragment primers is extended for looking at cyanobacterial diversity in a natural environment (Palenik, 1994).

Nif gene

One of the most important and widely used molecular tools for N_2-fixing cyanobacterial biodiversity assessment is *nif* gene sequence analysis (Lee et al., 1996). The analysis of *nif* gene is widely used because it is highly conservative and encodes dinitrogenase reductase, a protein subunit in the nitrogenase complex, involved in N_2-fixation and common to all N_2-fixers. The 324-bp *nif* H fragment is useful in characterizing diazotrophic communities. *Nif* H gene has been used in genetic characterization of 11 isolates of *Cylindrospermopsis raciborskii* from diverse geographic origins and also in characterization of N_2-fixing cyanobacteria (Ben-Porath et al., 1993). Of the 102 randomly selected groups, 45 of *Nostoc* sp., 44 of *Anabaena* sp., 5 of *Anabaenopsis* sp., 3 isolates of *Nodularia* sp. and 5 isolates of an unidentified branching organism were identified by *nif*H gene amplification by PCR (Teaumroong et al., 2002). Phylogenetic analysis based on *nif* gene clearly distinguishes all cyanobacterial strains at intra-species level. This showed a wide range of diversity among the N_2-fixing cyanobacteria found in soil.

Biodiversity documentation using statistical packages

The huge amount of data in biodiversity assessment requires a statistical analysis. However, the increasing sophistication of techniques reveals the existence of an important number of structural and computational artifacts (Sneath and Sokal, 1973; Moreira and Philippe, 2000). The UPGMA (unweighted pair group method with arithmatic mean) is the simplest method of tree construction. It was originally developed for constructing taxonomic phenograms, that is, trees that reflect the phenotypic similarities between OTUs, but it can also be used to construct phylogenetic trees if the rates of evolution are approximately constant among the different lineages. For this purpose the number of observed nucleotide or amino acid substitutions can be used. UPGMA employs a sequential clustering algorithm, in which local topological relationships are identified in order

of similarity, and the phylogenetic tree is built in a stepwise manner. But it is not very common method employed to construct phylogenetic tree due to its less sensitivity. Nevertheless, the neighbor-joining method is proposed for reconstructing phylogenetic trees from evolutionary distance data (Saitou and Nei, 1987). The principle of this method is to find pairs of operational taxonomic units (OTUs = neighbours) that minimize the total branch length at each stage of clustering of OTUs starting with a star like tree. The branch lengths as well as the topology of a parsimonious tree can be quickly obtained by using this method. Using computer simulation, efficiency of this method in obtaining the correct unrooted tree in comparison of other tree-making methods such as UPGMA is superior. However, among the various statistical packages, PHYLIP (phylogeny inference package) is the most widely acclaimed and popular phylogeny package. This package includes programs to carry out parsimony, distance matrix methods, maximum likelihood, and other methods on a variety of data, including DNA and RNA sequences, protein sequences, restriction sites, 0/1 discrete characters data, gene frequencies, continuous characters and distance matrices. However, PAUP (phylogenetic analysis using parsimony) is the most sophisticated parsimony program, with many options. It includes parsimony, distance matrix, invariants, and maximum likelihood methods and many indices and statistical tests. Though PHYLIP and PAUP cover almost all needs of modern phylogenetic analysis, in many cases intensive study is required in order to learn the use of the command line features of these packages. The package, MEGA2 (molecular evolutionary genetic analysis 2) for the Microsoft windows platform, offers a convenient and easy-to-use graphical user interface. MEGA2 carries out parsimony, distance matrix and likelihood methods with branch-and-bound search available for parsimony and bootstrapping available for all methods and has a reasonably good set of incorporated statistical tests and sequence evolution models. More information related to these packages is available on the website-http:// evolution.genetics.washington.edu/phylip/ software.html.

BIODIVERSITY AMONG CYANOBACTERIAL COMMUNITIES FROM DIVERSE HABITATS

Fresh water cyanobacteria

The blue-green algae are found in a wide variety of habitats. Most of the species are fresh water inhabitants. They are unicellular (e.g. *Chroococcus*), colonial (e.g. *Gloeocapsa, Gloeotrichia*) or form chains of cells in trichomes (e.g. *Oscillatoria, Scytonema*). In colonial forms all cells of a colony occur in a common gelatinous matrix. The cells of the colony may divide to form definite (e.g. *Merismopedia*) or indefinite and irregular (*Aphanothece*) assemblage of cells. In filamentous form (a trichome surrounded by a sheath is called a filament), each trichome has its own gelatinous sheath. The filaments are usually unbranched and uniseriate (e.g. *Anabaena, Nostoc, Lyngbya*) or may sometimes show pseudobranching (e.g. *Scytonema, Tolypothrix*).

Due to the immense diversity among fresh water cyanobacteria one can use molecular techniques for genetic profiling, assessment of abundance and diversity. Based on the above-described molecular techniques, several reports are now available on the diversity of fresh water cyanobacteria, and systematics of *Phormidium retzii* using RAPD and 16S rDNA markers (Lee et al., 1996). Garcia-Pichel et al. (2001) used PCR in combination with DGGE to probe the diversity of oxygenic phototrophic cyanobacteria in cultures, complex microbial communities and those in association with lichens. Sequences of 16S rRNA genes are independent of growth conditions and can be retrieved by PCR from small amount of DNA extracted from laboratory cultures or natural environments. The detection of the slightly different 16S rRNA gene sequences is not sufficient to prove the presence of *Schizothrix, Phormidium, Scytonema, Nostoc* and *Chlorogloeopsis* in an envionmental sample. The *rpo*C1 gene encoding the γ subunit of RNA polymerase has been described as an alternative target for the analysis of cyanobacterial phylogeny (Bergsland and Heselkorn, 1991) and community structure. However, the sequence data available for these genes are rather limited, whereas the determination of 16S rRNA gene sequences is a routine procedure in prokaryotic taxonomy today. Gugger et al. (2002) reported that the planktonic *Anabaena* strains were not distinguishable from *Aphanizomenon* strains by morphological analysis. But sequencing of the 16S rRNA gene, the spacer region of the ribosomal operon (ITS1) and the *rbc*LX (RuBisCo) region performed on 26 *Anabaena* strains and 14 *Aphanizomenon* strains isolated from several lakes in Denmark, Finland and France revealed differences between them. DNA fingerprinting has been found to yield significant diversity among nitrogen-fixing cyanobacteria in soil samples collected from different ecosystems (Lowe, 1980). PCR methods using DNA dependent RNA polymerase (*rpo*C1) have been found quite useful in understanding molecular phylogeny of *Anabaena circinalis* (Fergusson and saint, 2000). Likewise 16S rRNA gene sequences have been used to study *Azolla-Anabaena* endosymbiont (Eskew et al., 1993).

DNA-DNA hybridization has been found to be very precise (Sigler et al., 2003) in delineating the 2 *Chroococcidiopsis* species isolated from hot and cold deserts. 16S rRNA gene analysis revealed that the cyanobacterial phylotypes observed in dolomite were related to known diazotrophs including *Anabaena, Calothrix, Scytonema* and *Nostoc.*

Svenning et al. (2005) studied the phylogeny of symbiotic cyanobacteria within the genus *Nostoc* based on 16S rDNA sequence analysis. Since the genera, *Nostoc*

and *Anabaena* exhibit very similar properties, the absence of hormogonia, the motile stage of the filaments in *Anabaena* has been used to distinguish them (Wilmotte and Hardman, 2001), although hormogone formation is dependent on growth conditions (Ward et al., 1998). Analysis of the genetic diversity of symbiotic *Nostoc* strains using molecular methods revealed heterogeneity reflecting high genetic diversity (West and Adams, 1997; Costa et al., 1999; Nilsson et al., 2000). Sequence heterogeneity between strains at a higher taxonomic level has also been demonstrated using 16S- RFLP and ITS-RFLP combined with DGGE analyses of the functional *het*R gene (Svenning et al., 2005). Phylogenetic analysis revealed that *Nostoc* strains are intermixed within the genus *Anabaena* and vice-versa.

Notwithstanding, Dyble et al. (2002) characterized *Cylindrospermopsis raciborski* isolates from diverse geographic origins based on *nif*H gene. Ten 16S rDNA probes to identify the cynobacteria genera, *Microcystis, Plankothrix, Anabaena* and *Aphanizomenon*; in addition a probe which corresponds to the *Nostoc* (which includes *Nostoc, Anabaena* and *Aphanizomenon* sp.) has been reported by Rudi et al. (2000).

Marine cyanobacteria

The range of habitats and conditions occupied by cyanobacteria as a group, however, is wider than the most eukaryotic phototrophs. Cyanobacteria proved themselves successful in occupying freshwater, brackish, marine and hypersaline environments. Marine forms employ both halophily and halotolerance as survival strategies. The open ocean, the largest and ecologically most stable marine environment is the home of picophytoplaktonic unicells (Waterbury et al., 1979), classified within the genera, *Synechocystis, Synechococcus* and *Prochlorococcus*. These tiny microorganisms contribute significantly to the primary production of lakes, oceans and lagoonal waters. *Anacystis nidulans* axenically cultured marine strain proved worth separating from freshwater counter parts on the basis of nucleotide sequencing (Giovannoni et al., 1998). Picophytoplaktonic phototrophic prokaryotes compete for and subdivide their niches by depth differentiating primarily according to their abilities to utilize light and nutrients. They have also diversified horizontally with species composition changing from the nutrient pelagic to the nutrient enriched neritic provinces (Waterbury et al., 1979).

Bloom forming nitrogen-fixing filamentous cyanobacteria, taxonomically clustering around the genera *Kathagnymene* and *Trichodesmium* are common in all tropical oceans. Species of *Trichodesmium* are characterized by distinctive mostly clonal colony formation with cell differentiation occurring along individual trichomes and thylakoid separation (Keratomization) intensified distal portions of trichomes (Souria, 1970, Carpenter et al., 1992). Function differentiating along multicellular trichomes has been shown recently by localizing the position of nitrogenase gene. Bloom forming planktonic heterocystous cyanobacteria, on the other hand, seem to be restricted to eutrophic, enclosed epicontinental seas and phosphorus-overloaded lakes. In the Baltic Sea, for example the heterocystous nostocalean *Nodularia spumigena* is a prominent bloom former (Gumpert et al., 1987, Komárek et al., 1993). Other bloom-forming heterocystous cyanobacteria such as *Aphonizomenon* and *Anabaena* replace *Nodularia* in the eastern part of the Baltic toward the lower salinity range. Use of molecular technique demonstrates much more diversity among marine cyanobacteria sequences retrieved directly from the Greazt Sippewissett Salt Mars, MA, USA (Dillon and Wilmotte, 1999). However, *Prochlorococcus* is the dominant phototroph in the temperate oceans. The molecular phylogeny of *Prochlorococcus* ecotypes has been assessed by Rocap et al. (1999). Further, Shimada et al. (1999) described the molecular phylogenetic relationship between *Synechocystis trididemni* and *Prochloron didemni* using 16S rRNA gene. 6 unicellular marine cyanobacteria strains of the order, chroococcales isolated from the Baltic Sea were examined by DNA sequence analysis and RAPD-PCR (Mikolajczak et al., 1999). Further, Palenik (1994) assessed the phylogeny of the cyanobacteria inhabiting Sargasso Sea using *rpo*C1 gene sequence. Sequencing and analysis of cloned fragment suggested that the population in the sample consisted of two distinct clusters of *Prochlorococcus* like cyanobacteria and four clusters of *Synechococcus* like cyanobacteria.

Extremophilic cyanobacteria

Molecular characterization of endolithic cyanobacteria inhabiting exposed dolomite has been demonstrated by Sigler et al. (2003) using 16S rRNA gene based DGGE analysis (Baudouin-Cornu et al., 2004). Communities of microorganisms that inhabit endolithic environment include heterotrophic bacteria, fungi (Hirsch et al., 1995), eukaryotic algae, cyanobacteria and fungal/cyanobacterial symbionts (lichens) (Gerrath et al., 1995). So far, the most wide spread of these groups are cyanobacteria and eukaryotic algae, which have been detected in significant abundance in almost every endolithic ecosystem (Bell et al., 1986). Most previous investigations of endolithic communities utilized culture dependent techniques through which standard morphological characteristics were used to identify community members (Castenholz and Waterbury, 1989). During the past decade culture independent molecular techniques have greatly facilitated community analysis of environmental samples. In each of the adverse/extreme environment, the predominance of similar organisms including the genera *Gloeocapsa, Chroococcidiopsis, Nostoc, Scyto-*

nema (Friedmann and Friedmann, 1984, Broady, 1986, 1989, Banerjee et al., 2000) suggests that stresses common to endolithic environments worldwide have selected for a niche specific assemblage of tolerant organisms. The precambrian microfossils suggest the occurrence of oxygenic phototrophs of phylum cyanophyta. There are several reports of occurrence of cyanobacteria under stromatolitic reefs (Saitou and Nei, 1987; Walter, 1987). However, Neilan et al. (2002) identified cyanobacteria associated with stromatolites from distinct geographical locations using 16S rRNA gene sequences. They found that this ecosytem was mainly dominated by bloom forming species of *Anabaena, Nodularia, Oscillatoria, Microcystis, Cylindrospermum, Synechocystis, Cyanophora, Gloeothece, Trichodesmium, Aphanizomenon* and *Microcoleus.*

Nevertheless, a large number of new thermophilic cyanobacterial isolates have been obtained in recent years (Casamatta et al., 2003, Stetter, 1996). However, the microbial biodiversity within hot spring cyanobacterial mat communities were analysed (Casamatta et al., 2003) using 16S rRNA gene sequences along with DGGE and various cyanobacteria like *Pleurocapsa, Phormidium, Anabaena, Synechocystis, Osillatoria, Microcoleus and Pseudanabaena* were found to inhabit Octopus hot spring. DGGE surveys have revealed that 16S rRNA gene distribution changes along the thermal gradient which exists over many meters where the mat occurs in the hot spring.

CONCLUSION AND FUTURE PERSPECTIVES

This review provides a considerable amount of information on the cyanobacterial biodiversity assessment using molecular approaches, the problems encountered in assessment of cyanobacterial diversity and describes the promising tools used for the generation of molecular information. The fingerprinting approach (DGGE, T-RFLP, SSCP, etc.) based on PCR amplification without any need to establish a clone library, is currently the most widely used tool in the assessment of biodiversity. The two new tools appear to have potential to provide interesting information in the next few years; they include the Real-time quantitative PCR and DNA microarray analysis. It is hoped that more work will be done on ribosomal RNA sequencing and other molecular techniques to determine cyanobacterial genotypic relationship. However, molecular information should be integrated with other characteristics of the strains for better understanding of cyanobacterial phylogeny. This will form the basis for a polyphyletic taxonomy that will not only be of practical use but will reflect the evolutionary relationship of the strains.

It is important to study microbial diversity not only for basic scientific research, but also to understand the link between diversity and community structure and function. Human influences such as pollution and chemical applications could adversely affect microbial diversity and perhaps also ecosystem functioning. For instance, Buckley and Schmidt (2001) found significantly higher amounts of 16S rRNA for all microbial groups analyzed in fields that have never been cultivated as compared to agricultural fields. This suggests a decrease in microbial biomass or activity in cultivated fields. Similarly, the diversity of microbes has been shown to increase from agricultural fields to natural systems (Daniell et al., 2001, Menendez et al., 2001). However, it is not known what these reductions in diversity mean to ecosystem functioning and if it is important for sustainability of ecosystems. There is disagreement within the scientific community of whether taxonomic or genetic diversity is important as long as functional diversity is maintained. Given the limitations of our ability to study diversity and how diversity relates to function, it would be prudent to assume that functional redundancy does not exist and taxonomic diversity is important to maintain. Knowledge of microbial diversity and function in soils is limited because of the taxonomic and methodological limitations associated with studying these organisms. Although methods to study diversity (numerical, taxonomic, structural, molecular) are improving day by day, there is still no clear evidence of microbial biodiversity and its correlation with sustainability of ecosystems. It is challenging to soil microbiologists to develop techniques to study soil microbial diversity especially cyanobacterial biodiversity when it is currently unclear as how accurate these techniques are. We do not know what is present in a gram of soil and therefore it is difficult to conclude whether one technique of studying diversity is better than others. Given the current state of knowledge, we feel that the best way to study soil microbial diversity would be to use a variety of tests with different endpoints and degrees of resolution to obtain the broadest picture as possible and maximum information regarding the microbial community.

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