

Cyanobacterial Diversity Assessment Under Diverse Environments: A Molecular Approach

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Cyanobacteria are photosynthetic microorganisms that inhabit diverse environmental conditions worldwide, ranging from freshwater and soil to extreme conditions. Apart from their photosynthesis and nitrogen-fixing abilities, they also produce a large variety of molecules with high potential in pharmaceutical and industrial applications. Recent investigations have elucidated that most of the cyanobacteria that belong to orders Oscillatoriales, Nostocales, Chroococcales, and Synechococcales have been extensively characterized. In contrast, those that belong to Pleurocapsales, Chroococcidiopsales, and Gloeobacterales remain unexplored in terms of their molecular diversity and relative bioactivity. In recent decades, molecular biology techniques have revealed valuable insights into the role and functions of various prokaryotes, including cyanobacteria, by precisely mapping their diversity in their natural environment. This review provides an overview of molecular techniques and their advantages and limitations for studying cyanobacterial diversity.

Key Words: Diversity, Molecular tools, Microarray, Sequencing

Introduction

Cyanobacteria are a distinctive phylogenetic group of gram-negative prokaryotes capable of performing oxygenic photosynthesis (Kumari *et al.*, 2012; Soo *et al.*, 2017). Approximately 3.6 billion years ago, they played a remarkable role in the oxygenation of the atmosphere by generating molecular O₂, thereby accentuating life on earth (Soo *et al.*, 2017). Cyanobacteria are also considered the progenitor of chloroplasts and are involved in the fixation of atmospheric N₂, thus playing a substantial role in maintaining the nitrogen economy (Kumari and Rai, 2019). Cyanobacteria are ubiquitous in diverse ecological niches and conceal enormous diversity in their habits, morphology, physiology, and metabolism. They are present in freshwater, marine, hot, and cold springs and symbiotic associations (Gaysina *et al.*, 2019). Furthermore, cyanobacteria produce a wide range of toxins and secondary metabolites; therefore, they have attracted broad interest in biofuel and biotechnological/pharmaceutical applications (Demay *et al.*, 2019).

The quantification of numbers and types of cyanobacteria within a community is imperative to get functional insight into their role in regulating the fundamental structure of the ecosystem (Gaysina *et al.*, 2019). Since cyanobacteria exhibit a high degree of

morphological diversity compared to other prokaryotes, therefore, until recently, they were identified and categorized based on their morphological traits per-se., cell size, shape, colour, branching pattern, and cell contents (Demay *et al.*, 2019). With the technological advancements and their unicellular, colonial and filamentous nature, researchers have managed to assess their diversity at morphological, physiological, biochemical, and molecular levels to reveal the hidden potential of cyanobacteria for industrial/agricultural applications (Kumari and Rai, 2019). The morphological and physiological diversity assessment methods have several limitations, like the low resolution of the light microscope failing to provide a clear view of spores, akinetes, and other taxonomic traits (Anand *et al.*, 2019).

Biochemical diversity analysis involves assessing fatty acid composition, polar/nonpolar glycolipids, and interestingly lipid profiling of various cyanobacteria such as *Synechocystis* sp. strain PCC 6308, *Prochloron* sp. and *Synechococcus* strain revealed valuable insight into their phylogenetic distributions (Sallal *et al.*, 1990). However, the biochemical approach also has its limitations in assessing cyanobacterial diversity due to their growth limitations and nutrient composition variations (Sallal *et al.*, 1990). Therefore, to overcome

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these constraints, present-day cyanobacterial diversity analysis is being made at the molecular level by using various molecular biology tools based on DNA and RNA, such as RT-PCR, microarray, and fluorescence in-situ hybridization, and metagenomics (Anand *et al.*, 2019). Several researchers have exploited the above-mentioned molecular techniques to get novel insights into the structure and functions of microbial communities at genomic, proteomic, and metabolomic levels (Rott *et al.*, 2018). This article will summarize the state-of-the-art molecular techniques currently used for cyanobacterial diversity assessment. In addition, a comprehensive understanding of their mechanisms, functions, and limitations will also be discussed.

Molecular tools for assessing cyanobacterial diversity

Several molecular biology tools have been employed in recent decades to study the diversity and function of cyanobacteria and other prokaryotes (Anand *et al.*, 2019). DNA or RNA profiling/fingerprinting is one of the robust molecular tools readily exploited to unveil microbial diversity in a specific environment by ascertaining variation at the gene level (Madigan *et al.*, 2009). A wide variety of microbial communities has been categorized phylogenetically and functionally using this approach (Fig. 1). However, the application of this technique becomes limiting wherever it was difficult to establish microbial cultures in the laboratory (Madigan

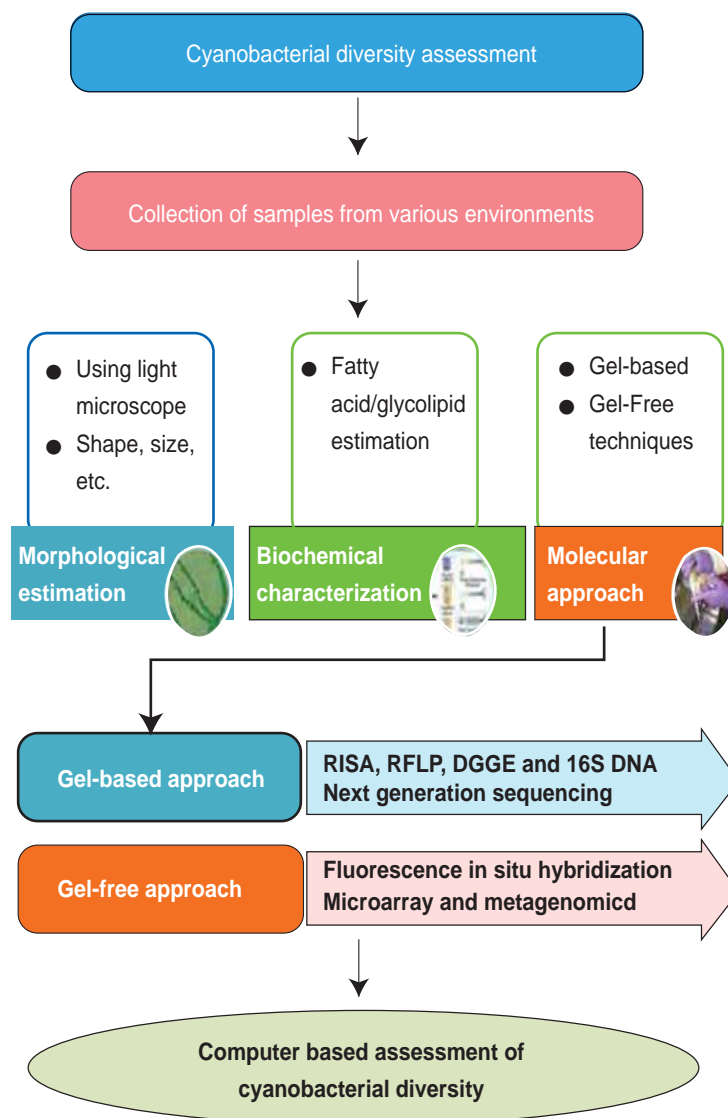


Fig. 1. A schematic overview of molecular techniques used for cyanobacterial diversity assessment

et al., 2009). Therefore, the above method has been revamped by integrating ultra-throughput sequencing technologies (NGS), metagenomics, DNA microarray for analysing gene expression patterns, and 16S rRNA gene library construction. Overall, the molecular techniques employed for cyanobacterial diversity and functional assessment have been grouped into two broad categories (i) gel-based and (ii) gel-free techniques.

Gel-based Techniques

Gel-based techniques mainly involving ribosomal intergenic spacer analysis (RISA), restriction fragment length polymorphism-PCR (RFLP-PCR), and denaturing gradient gel electrophoresis (DGGE) have been widely used to study cyanobacterial community (Kumari and Rai, 2019). RISA assesses microbial diversity using oligonucleotide designed using an intergenic spacer region between the 16S and 23S rRNA gene (Rottet et al., 2018). The functional mechanisms of RISA involve PCR amplification of DNA from different microbial samples with the specifically designed primer combinations, and the difference between banding patterns is visualized. Similarly, RFLP has also been extensively used to reveal the phylogenetic profile of several dominant microbial genera, including cyanobacteria, namely *Scytonema* and *Leptolyngbya* (Srivastava et al., 2016). RFLP involves restriction digestion of microbial DNA samples using specific restriction enzymes and amplifying the digested DNA fragments using particular primers (Lukow et al., 2000). The difference between amplicon size is evaluated, which is the basis of diversity assessment. Several studies have confirmed the applicability of both RISA and RFLP for ascertaining microbial diversity in *Anabaena* and other cyanobacteria in a precise, efficient, timely, and cost-effective way (Neilan et al., 1995).

DGGE is also a molecular tool that offers an extra edge for analysing microbial diversity, which involves the separation of different DNA samples based on increasing denaturing conditions (Kumari and Rai, 2019). DGGE technique offers high-resolution microbial diversity assessment by analysing the DNA molecules that differ by a single nucleotide (Song et al., 2005). Further, its robustness relies on the fact that it could work with lengthy DNA sequence analysis without compromising its specificity and efficiency. The DGGE technique work on a sequence-dependent separation mechanism where PCR products are visualized on polyacrylamide gel electrophoresis after denaturing the DNA samples either with formamide or urea (Jungblut

et al., 2005). As the denatured DNA fragment migrates on the gel, the shape of the DNA fragments changes dramatically, resulting in the slow migration of DNA along the established denaturing gradient, thus facilitating the visualization of the slightest change in the base pairs (Anand et al., 2019). A large body of literature has confirmed the efficiency and robustness of this technique in evaluating diversity analysis of toxin-producing cyanobacteria e.g. *Microcystis* and other prokaryotes (Kardinaal et al., 2007).

The 16S rRNA gene technique of library construction and diversity analysis involves the construction of primer sets from the variable region of the 16S rRNA gene, followed by the sequencing of the amplified products (Semenova et al., 2001). The 16S rRNA gene is ubiquitous to all microbes except viruses therefore used as the signature sequence for taxonomic characterization of microorganisms (Svenning et al., 2005). Various online tools and software packages such as *SILVIA*, *GreenGenes*, and *EzBioCloud* serve as the repository of 16S rRNA gene sequences of most of the microbes, which can be exploited for designing primers for diversity analysis (Yoon et al., 2017). In general, the fingerprinting of the microbial community relies on the PCR amplification of 16S rRNA gene fragments and evaluating the banding patterns on gel electrophoresis (Kumari and Rai, 2019). Further, researchers have employed quantitative RealTime PCR (qPCR) using a specific set of 16S rDNA-designed primers (Anand et al., 2019). The qPCR is a sensitive technique that evaluates diversity based on fold change in the expression of the concerned in the heterogeneous collection of microbial RNA samples (Palinska et al., 2018). Both the techniques are now being extensively used to analyse diversity in various prokaryotes and cyanobacteria such as *Nostoc* and *Synechococcus* (Palinska et al., 2018). However, amplification efficiency of both the techniques depends upon the quality, quantity, and purity of DNA/RNA samples and specific primers.

Gel-free techniques

Fluorescence *in situ* hybridization (FISH) is a molecular cytogenetic tool that detects specific DNA positions on the chromosome using sequence-specific fluorescent probes (Kumari and Rai, 2019). Several researchers have exploited FISH as a tool for species identification using a specifically designed oligonucleotide or polynucleotide probes, which are fluorescently labelled at 5' end using fluorescein and rhodamine (Threon and Cloete, 2000).

The FISH technique has been successfully used for the taxonomic classification of *Nodularia* strains from brackish water and several other cyanobacterial species using DNA-DNA and DNA-RNA hybridization (Suda *et al.*, 2002). Microarray is a high-throughput technique that has been extensively employed for microbial diversity assessment using a specific antibody against the gene of interest (Anand *et al.*, 2019). Microarray is gene-chip technology that can test many biological samples by performing DNA-DNA, DNA-RNA, and DNA-protein hybridization (Baudart *et al.*, 2017). This technique has contributed significantly to revealing the diversity of freshwater pathogens, including cyanobacteria, based on the hybridization of toxin encoding genes (Medlin, 2018). However, the efficiency of microarray techniques' depends on the specificity of the probe designed for the molecular assessment of the diversity.

Metagenomics is DNA sequencing-based technology and a culture-independent tool for analysing DNA samples isolated directly from the environmental samples (Lu *et al.*, 2016). This technique performs taxonomic and diversity analysis in detail and provides valuable insight into their physiology by studying the expression pattern of genes under distinct environments (Lu *et al.*, 2016). Compared to other molecular diversity assessment tools such as RISA, RFLP, DGGE, and 16S rRNA gene, metagenomics provides comprehensive information about the biodiversity and physiological behaviour of the concerned microbes (Laver *et al.*, 2015). Metagenomics techniques involve DNA/RNA library preparations followed by their assembly, sequencing, and data interpretations. The metagenomics technique is a complex molecular diversity analysis tool that is less biased than PCR and offers in-depth information related to the physiology and diversity of microbial communities.

Researchers have also identified and exploited various PCR-based molecular markers for cyanobacterial diversity analysis in conjunction with the earlier techniques. For example, 16SrRNA-based molecular markers are extensively used to decipher phylogenetic relationships (Komarek, 2016). Likewise, molecular identification based on the RNA polymerase β subunit (*rpoB*) gene has also been used to study cyanobacterial diversity under different environmental conditions (Lyra *et al.*, 2005). The *rpoB* gene is highly conserved and ubiquitously distributed, offering a more significant advantage over the 16S rRNA gene for diversity analysis

(Kumari and Rai, 2019). Microbiologists have also designed molecular markers based on nitrogen-fixing (*nif*) genes and have used them to analyse diversity among different microbial communities (Anand *et al.*, 2019). A marker based on the *nifH* gene has also been identified in cyanobacteria such as *Anabaena variabilis*, and other photosynthetic cyanobacteria to gain valuable insight into their phylogenetic relationship by correlating the data with morphological and biochemical characters (Thiel *et al.*, 1995).

Conclusion

Cyanobacteria represent immense diversity, as exhibited by the high degree of morphological variations. Quantifying their number and types within a community is central to understanding an ecosystem's structure and function. Recent advancements in molecular biology tools, especially in genomics, metagenomics, and proteomics, are imperative for the taxonomic assessment of cyanobacteria by outlining more significant differences among the lineages. These molecular techniques can precisely resolve species relationships by decoding their genome and can provide an in-depth understanding of cyanobacterial diversity under extreme environments.

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References

- Anand N, N Thajuddin, PK Dadheech (2019) Cyanobacterial taxonomy: morphometry to molecular studies. In: Mishra AK, DN Tiwari, AN Rai, (eds.) *Cyanobacteria—From Basic Science to Applications*. Academic Press, Elsevier, pp. 43–64.
- Baudart J, D Guillebault, E Mielke, T Meyer, N Tandon, S Fischer *et al* (2017) Microarray (phylochip) analysis of freshwater pathogens at several sites along the Northern German coast transecting both estuarine and freshwaters. *Appl. Microbiol. Biotechnol.* **101**: 871–886.
- Demay J, C Bernard, A Reinhardt, B Marie (2019) Natural products from cyanobacteria: Focus on beneficial activities. *Mar. Drugs* **17**: 320.

- Gaysina LA, A Saraf, P Singh (2019) Cyanobacteria in diverse habitats. In: Mishra AK, DN Tiwari, AN Rai (Eds.) *Cyanobacteria—From Basic Science to Applications*. Academic Press, Elsevier, pp. 1–28.
- Jungblut AD, I Hawes, D Mountfort, B Hitzfeld, DR Dietrich, BP Burns, BA Neilan (2005) Diversity within cyanobacterial mat communities in variable salinity meltwater ponds of McMurdo Ice Shelf, Antarctica. *Environ. Microbiol.* **7**: 519–529.
- Kardinaal WEA, I Janse, M Kamst-van Agterveld, M Meima, J Snoek, LR Mur, J Huisman, G Zwart, PM Visser (2007) Microcystis genotype succession in relation to microcystin concentrations in freshwater lakes. *Aquat. Microb. Ecol.* **48**: 1–12.
- Komárek J (2016) A polyphasic approach for the taxonomy of cyanobacteria: principles and applications. *Eur. J. Phycol.* **51**: 346–353.
- Kumari N, LC Rai (2020) Cyanobacterial diversity: molecular insights under multifarious environmental conditions. In PK Singh (ed.) *Advances in cyanobacterial biology*. Academic Press, pp. 17–33.
- Kumari N, OP Narayan, LC Rai (2012) Cyanobacterial diversity-shifts induced by butachlor in selected Indian rice fields in eastern Uttar Pradesh and western Bihar analyzed with PCR and DGGE. *J. Microbiol. Biotechnol.* **22**: 1–12.
- Laver T, J Harrison, PA O'Neill, K Moore, A Farbos, K Paszkiewicz (2015) Assessing the performance of the Oxford Nanopore Technologies MinION. *Biomol. Detec. Quantif.* **3**: 1–8
- Lu H, F Giordano, Z Ning (2016) Oxford Nanopore MinION sequencing and genome assembly. *Genom. Proteom. Bioinform.* **14**: 265–279.
- Lukow T, PF Dunfield, W Liesack (2000) Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. *FEMS Microbiol. Ecol.* **32**: 241–247.
- Lyra C, M Laamanen, JM Lehtimäki, A Surakka, K Sivonen (2005) Benthic cyanobacteria of the genus *Nodularia* are non-toxic, without gas vacuoles, able to glide and genetically more diverse than planktonic *Nodularia*. *Int. J. Syst. Evol. Microbiol.* **55**: 555–568.
- Madigan MT, DP Clark, D Stahl, JM Martinko (2010) Brock biology of microorganisms. 13th edition. Benjamin Cummings.
- Medlin LK (2018) CYANO RT-Microarray: A Novel Tool to Detect Gene Expression in Cyanobacteria. *Environ. Microbiol.* **1**: 17–21.
- Neilan BA, D Jacobs, AE Goodman (1995) Genetic diversity and phylogeny of toxic cyanobacteria determined by DNA polymorphisms within the phycocyanin locus. *Appl. Environ. Microbiol.* **61**: 3875–3883.
- Palinska KA, JC Vogt, W Surosz (2018) Biodiversity analysis of the unique geothermal microbial ecosystem of the Blue Lagoon (Iceland) using next-generation sequencing (NGS). *Hydrobiologia* **811**: 93–102.
- Rott E, A Pentecost, J Mareš (2018) Introduction: Recent developments in cyanobacterial research with special reference to aquatic habitats, molecular ecology and phylogenetic taxonomy. *Hydrobiologia* **811**: 1–6.
- Sallal AK, NA Nimer, SS Radwan (1990) Lipid and fatty acid composition of freshwater cyanobacteria. *Microbiology* **136**: 2043–2048.
- Semenova EA, KD Kuznedelov, MA Grachev (2001) Nucleotide sequences of fragments of 16S rRNA of the Baikal natural populations and laboratory cultures of cyanobacteria. *Mol. Biol.* **35**: 405–410.
- Song T, L Mårtensson, T Eriksson, W Zheng, U Rasmussen (2005) Biodiversity and seasonal variation of the cyanobacterial assemblage in a rice paddy field in Fujian, China. *FEMS Microbiol. Ecol.* **54**: 131–140
- Soo RM, J Hemp, DH Parks, WW Fischer, P Hugenholtz (2017) On the origins of oxygenic photosynthesis and aerobic respiration in cyanobacteria. *Science* **355**: 1436–1440.
- Srivastava M, MS Kaushik, AK Mishra (2016) Linking the physicochemical properties with the abundance and diversity of rhizospheric bacterial population inhabiting paddy soil based on a concerted multivariate analysis of PCR-DGGE and RISA. *Geomicrobiol. J.* **33**: 894–905.
- Suda S, MM Watanabe, S Otsuka, A Mahakahant, W Yongmanitchai, N Nopartnaraporn, Y Liu, JG Day (2002) Taxonomic revision of water-bloom-forming species of oscillatoroid cyanobacteria. *Int. J. Syst. Evol. Microbiol.* **52**: 1577–1595.
- Svenning MM, T Eriksson, U Rasmussen (2005) Phylogeny of symbiotic cyanobacteria within the genus *Nostoc* based on 16S rDNA sequence analyses. *Arch. Microbiol.* **183**: 19–26.
- Theron J, TE Cloete (2000) Molecular techniques for determining microbial diversity and community structure in natural environments. *Crit. Rev. Microbiol.* **26**: 37–57.
- Thiel T, EM Lyons, JC Erker, A Ernst (1995) A second nitrogenase in vegetative cells of a heterocyst-forming cyanobacterium. *Proc. Natl. Acad. Sci.* **92**: 9358–9362.
- Yoon SH, SM Ha, S Kwon, J Lim, Y Kim, H Seo et al (2017) Introducing EzBioCloud: A taxonomically united database of 16S rRNA and whole genome assemblies. *Int. J. Syst. Evol. Microbiol.* **67**: 1613–1617.