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A REVIEW ON METAGENOMIC APPROACHES TO ASSESS MICROALGAL DIVERSITY: OPTIONS & CHALLENGES

Microalgae represent large and diverse group of unicellular photosynthetic microorganisms that, being most abundant and efficient unicellular producer of a rich and complex biomass in all aquatic systems. Microalgae have gained global importance in recent years as potential source of renewable energy as well as bioindicators to manage an aquatic ecosystem. The accurate identification of diversity is a key challenge in microalgal ecology research. Moreover, the increased knowledge of the scope, structure, and dynamics of microalgal biodiversity is urgently needed. A rapid and reliable identification of Microalgae in general, and at lower taxonomic levels in particular, cannot be accomplished by morphological methods alone. Amplicon-based metagenome analysis using multiple primers approach can provide novel insight into the biological and ecological inferences, and address many general questions with a special focus on phylogenetics and taxonomy, to cast the widest possible taxonomic net for microalgae and yet reduce sequencing of non-microalgal eukaryotes. In this review, we highlight main advances taken place in the field of metagenomics over the last two decades, present statistics of the main metagenomic techniques and databases, and discuss opportunities, challenges and perspectives in metagenomics with special reference to analyzing microalgal diversity in different ecosystems.

Key words: Microalgae, metagenomics, diversity, amplicon, phylogenetics, profiling.

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Микробалдырлардың әртүрлілігін бағалаудағы метагеномдық тәсілдерге шолу: әдістер мен проблемалар

Микробалдырлар барлық су экожүйелерінде бай және күрделі биомассаның ең көп таралған және тиімді бір жасушалы продуцент болып табылатын бір жасушалы фотосинтездеуші микроорганизмдердің әртүрлі және үлкен тобын құрайды. Соңғы жылдары микробалдырлар жаңартылатын энергияның әлеуетті көзі, сондай-ақ сулы экожүйені басқару үшін биоиндикаторлар ретінде жаһандық мәнге ие болды. Микробалдырлардың алуантүрлілігін идентификациялау – олардың экологиясын зерттеудегі негізгі мәселе. Сондай-ақ, микробалдырлардың биоалуантүрлілігінің ауқымы, құрылымы және динамикасы туралы білім саласын кеңейту қажет. Микробалдырлардың төменгі таксономиялық деңгейін, тұтастай алғанда тек талдаудың морфологиялық әдістерімен ғана тез және сенімді идентификациялауға қол жеткізу мүмкін емес. Көптеген праймерлерді пайдалана отырып ампликондар негізінде биоалуантүрлілікті зерттеудегі метагеномдық тәсілдің дамуы биологиялық және экологиялық қорытындылардың жаңа түсінігін береді және микробалдырлар үшін барынша мүмкін болатын таксономиялық жүйе құру үшін филогенетика мен таксономияға ерекше назар аудара отырып, көптеген жалпы мәселелерді шешуге мүмкіндік береді. Бұл шолуда біз соңғы екі онжылдықтағы метагеномика

саласындағы негізгі жетістіктерге тоқталамыз, негізгі метагеномика әдістері мен деректер қорының статистикасын ұсынамыз, сондай-ақ метагеномика саласындағы мүмкіндіктерді, проблемалары мен перспективаларын талқылап, әр түрлі экожүйелерде микробалдырлардың әртүрлілігін талдауға ерекше назар аударамыз.

Түйін сөздер: микробалдырлар, метагеномика, биоалуантүрлілік, ампликон, филогенетика, профильдеу.

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Обзор метагеномных подходов
в оценке разнообразия микроводорослей: методы и проблемы

Микроводоросли представляют собой большую и разнообразную группу одноклеточных фотосинтезирующих микроорганизмов, которые являются наиболее распространенным и эффективным одноклеточным продуцентом богатой и сложной биомассы во всех водных экосистемах. В последние годы микроводоросли приобрели глобальное значение в качестве потенциального источника возобновляемой энергии, а также биоиндикаторов для управления водной экосистемой. Точная идентификация разнообразия является ключевой проблемой в исследованиях экологии микроводорослей. Кроме того, существует необходимость расширения области знаний о масштабах, структуре и динамике биоразнообразия микроводорослей. Быстрая и надежная идентификация микроводорослей в целом и в частности более низкого таксономического уровня не может быть достигнута только морфологическими методами анализа. Развитие метагеномного подхода в исследованиях биоразнообразия на основе ампликонов с использованием множественных праймеров дает новое понимание биологических и экологических выводов и предоставляет возможность решить многие общие вопросы с особым акцентом на филогенетику и таксономию для создания максимально возможной таксономической сети для микроводорослей. В этом обзоре мы освещаем основные достижения в области метагеномики за последние два десятилетия, представляем статистику основных метагеномных методов и баз данных, а также обсуждаем возможности, проблемы и перспективы в области метагеномики с особым акцентом на анализ разнообразия микроводорослей в различных экосистемах.

Ключевые слова: микроводоросли, метагеномика, биоразнообразие, ампликон, филогенетика, профилирование.

Abbreviations

μm – micrometer; ITS – Internal transcribed spacer (ITS); 18S rDNA – small subunit ribosomal DNA; bp – base pair; ABI – applied biosystems; LSU – larger subunit; SSU – smaller subunit; OTU – operational taxonomic unit.

Introduction

Microalgae are large and diverse group of unicellular photosynthetic microorganisms that, being most abundant and efficient unicellular producer of a rich and complex biomass in all aquatic systems, have gained global importance in recent years. The term 'Microalgae' is being broadly defined for the long time as those algae where the individual organisms generally require a microscope to be recognized. However, given the enormous diversity of taxonomically unrelated microbial eukaryotes existing in unicellular, colonial and filamentous forms [1-2], possessing higher-level taxonomic placement in three kingdoms including Bacteria, Chromista, and protozoa [3], it requires significant rethinking to provide an absolute and acceptable definition which may differentiate microalgae from the macroalgae.

Sustaining natural biological structural and functional attributes of aquatic ecosystems is of great concern for the last few decades. Currently, the monitoring and assessment of pollutants of the aquatic environment are mainly based on the determination of some chemical parameters. However, due to high costs of complex chemical analyses, nature, sources, distribution and level of emissions of pollutants, chemical analysis is not the only feasible way to obtain information for effective environmental monitoring. More recently, much attention has been given to use of algal flora biodiversity as bioindicators to manage an aquatic ecosystem according to the habitat requirements [4-6].

By biological diversity, it is often understood as number of microalgal species in a particular habitat, and how relatively abundant each of the species is. It is believed that the more number of species (greater variety) present in a particular ecosystem, they will be more likely to be naturally resilient. Some species are much more vulnerable than others to the change in environment (for example, as a result of anthropogenic impact), that leads to the increased richness for the dominating species, occupying the newly created ecological niches [7]. Biodiversity thus not only affected by change in the number of species, but it also takes into the account all aspects relevant to species dominance and rarity. Moreover, the biodiversity illustrates the uniqueness of the community, thus can serve as a bio-indicator to assess ongoing ecological or environmental changes. Microalgae play the most vital role in the sustaining and formation of aquatic ecosystems, because they form the first level of aquatic trophic chains and foundation of interspecific relationships.

Microalgae can survive across broad range of environmental conditions, which enabled it to occupy almost all ecological niches from freshwater, seawater, salt lakes to soil, rocks, and trees [8-9]. Microalgae constitute the dominant organism group that contribute to the function of sustainable ecosystem in their role as key primary producer for aquatic food webs [10], tiny aeration devices fixing inorganic carbon in aquatic habitats, contributing to the global nitrogen cycles, assimilating contaminating nutrients (nitrogen and phosphorous) for domestic and agricultural wastewater treatment [11-13], and creating environment friendly renewable biofuels [14-15].

Being primary producers, microalgae are most directly affected by variation of environmental and natural disturbances and exhibit high sensitivity to certain pollutant, which may lead to the quantitative change in microalgal community. Their total biomass is utilized as indicators of aquatic habitat qualifications, as some pollutant cause their death and decrease in species diversity [16], and in others – contribute to mass reproduction [17].

A great deal of progress has been made from technical point of view to increase the amount of biological and ecological data relevant to biodiversity measurement and to improve its accurate and consistent utility. Given the large number of indices to measure the biodiversity of algal community, considerable effort and background information is still required for observational, comparative and experimental biodiversity research.

Despite their importance, much of the information on clear description, characterization of microalgal communities and strains designation is somewhat inaccessible. The number of species of algae is very large, estimates figures in excess of over a million species [18], of which between 40,000 to 60,000 have been identified to date ([19]. Since, according to some estimates, hundreds of thousands to millions of microalgal species are still unknown, thus the role of many species for processes and functions of ecosystems is still not understood [20]. A better knowledge of microlgal biodiversity and its interrelation with the environment is crucially important.

The lack of basic information on microalgal species diversity at different taxonomic levels has significant implications for many aspects of ecosystem monitoring, conservation biology, and evolutionary biology [21-23]. The multilevel analysis of microlagal biodiversity will provide a system to understand the mechanism contributing to generate diversity, assess the way diversity is organized, and confer the value it may have to the structure and function of entire community in a given area [24]. This is particularly important for microalgal species and strains of economic value or environmental concern. The isolation, identification of indigenous microalgal strains with promising properties is a key to improving the feasibility of bio-prospecting for microalgal-derived high value products [25-27].

Recent advances in molecular biological techniques and bioinformatics have undoubtedly enabled the discovery and comprehensive assessment of thus-far-undiscovered forms of microbial life, including microalgae, in situ, without isolation into pure cultures. New species of microalgae are now being described and characterized combining morphological traits with molecular sequence data, utilizing either DNA sequence data and/or secondary structure of ribosomal DNA for phylogenetic applications. In particular, high-throughput amplicon sequencing of environmental DNA and/or RNA proved to be far more powerful and robust technique, when applied to characterize microbial diversity [28-31]. Gene-based biodiversity discovery has become an important application for biomonitoring diagnostic development and majority of the biodiversity studies have used this approach to not only improve the efficiency of biomonitoring, but also to expands its relevance for habitats and biota groups which have not been fully studied due to insufficient taxonomic knowledge or technical competency [32-35].

The DNA-based approach for the comprehensive assessment of microalgal communities at genus-, species- and strain-level utilizes various sequencing technologies to identify species provided as individual specimens or in environmental samples such as water, sediment or soil [36-38].

Metagenomics is increasingly being considered as promising technique, revealing the entire gene repertoire of the community recovered directly from environmental samples. This direct genetic analysis of genomes, by using high-throughput sequencing (HTS) of unpurified template DNA, has become the dominant source of publicly available sequence data. Marker gene metagenomics is a novel viable method to determine a taxonomic distribution or fingerprint profile through PCR amplification and sequencing of evolutionarily conserved and variable regions in 18S rRNA or 18S rDNA [39-42]. The DNA of individual specimens is typically analyzed using the Sanger sequencing platform, whereas the amplicon-based metagenome from environmental samples is analyzed using high-throughput next-generation DNA sequencing platforms such as the Illumina Miseq sequencing and 454-Roche pyrosequencing platform.

In the present paper, we review the currently available molecular techniques, tools, and methodologies for assessing microalgal diversity. A critical assessment of the criteria from a practicable and applicable viewpoint will be made, and a review and comparison of the molecular techniques being currently employed for phytoplankton will be presented.

I. Metagenomic Approaches for Microalgae Diversity

Metagenomics has been applied to study microalgae diversity in a variety of ecosystems, from ocean, and soil to the acid mine drainages, generating novel genomic data from otherwise uncultivated species and strains, broadening the framework of existing metagenomic-specific methods available for comparison and study. In the following sections we will review the current state of understanding in the study of microalgal diversity in the natural environment (i.e. water and soil) with the help of collection of published research results.

A. Water

It is estimated that marine waters and freshwater account for more than 90% habitable space on earth. These ecosystems are regarded as the mean for vast array of bio-productive resources, and most of the primary productivity are result of tremendous microbial activity by the microalgae, in particular the pico-phytoplankton (< 2.0 μm), nano-phytoplankton (< 20 μm) and micro-phytoplankton (20 – 200 μm). The microalgal abundance and diversity in the aquatic systems can vary greatly from one aquatic ecosystem to another because of the variation in environmental variables. Therefore, the type and the increasing level of microalgae are used as indicators of the ecological conditions and water quality of their ecosystems.

A study conducted in Cornwallis Island (Canadian high Arctic) using shotgun metagenomics and amplicon-based 16S rRNA gene sequencing compared the metagenomes of seawater and the overlying sea ice to relate the genomic data to identify the potential environmental drivers [43]. Among the metagenomic datasets obtained, it was found that microalgae, mainly the diatoms, significantly more abundant in sea ice, is likely a key driver in shaping the noted differences in microbial communities and nutrient availability. Another comparative metagenomics study based on 18S rRNA and ITS sequencing, conducted in three different watersheds, representing further three land use type (protected, agricultural, and urban), in southwestern British Columbia, found out that microalgae belonging to Streptophyta represented 16% of the total sequences obtained from environmental samples across all sites, and agricultural impacted sites were dominated by the Chlorophyta [44].

Another important effort was made for understanding the dynamics, ecology and environmental distribution of microalgae belonging to Chlorophyta in marine ecosystems [45]. The authors reviewed and summarized current knowledge on the phylogenetic, morphological and ecological diversity of unicellular marine and halo-tolerant Chlorophyta. Around 9,000 Chlorophyta 18S rRNA gene sequences from culture and environmental samples deposited in public databases were examined with the aim of assessing the extent of diversity and exploring their oceanic distribution based on a subset of 2,400 sequences for which geographical information is available. The study also evaluated the utility of using of the large subunit ribosomal rRNA (LSU) or ITS as potential suitable marker for explore microdiversity at the species level or below.

Clone libraries (100-200 bp) constructed for RNA (cDNA) and DNA sequencing using an ABI 3730xl DNA Analyzer were used [46] to generate a robust dataset characterizing the genetic diversity of Chlorophyceae from waters of the Persian Gulf. The two above-mentioned methodologies

were selected simultaneously considering the bias each methodology carries by itself. Based on the results acquired for both approaches, eleven Chlorophycean of environmental clones were recorded. The isolated and sequenced clones were found to be 100% homologous with Neochloris aquatic (D) and Picochlorum sp. and exhibited 99% homology with other environmental clones including Chlorella sorokiniana, Chlamydomonas sp., Picochlorum sp. and Nannochloris atomus. The data obtained from the study recommends the methodology as an efficient approach to analyze phylogenetic of microalgae in marine environments, and also indicates a significant increase in sense of relatedness between taxon abundance distribution and bias of the method when a single approach is used to estimate diversity.

B. Soil

Soil is one of nature's most complex ecosystems and comprise the most diverse microbial habitats on earth, harboring myriads of niches for microalgae with high taxonomic richness and functional diversity. Several studies performed on phototrophic microbial communities have provided the evidence that microalgal diversity are greatly influenced by a variety of biotic and abiotic factors such as vegetation types, altitude and soil physico-chemical composition [47-48].

Earlier studies [49-51] reported that change in soil physico-chemical parameters (pH, organic carbon, nitrogen) has least influence on the occurrence of green algae than other microalgae. Few studies have reported that the abundance of green algae, blue-green algae, yellow-green algae, and diatoms is expected to be high in alkaline and nutrient rich soils of temperate forests and grasslands whereas only green algae were commonly found in acidic and nutrient depleted soils [52-53].

A camparative study using combined approach based on cloning and sequencing of culture-independent rRNA genes and culture-dependent sequencing from the same samples, investigated the extensive microalgal diversity in soils from forest sites of the Schwäbische Alb Exploratory [54]. Among a total of 17 clones libraries, represented by 575 sequences of various green algae obtained, the majority of recovered true microalgal sequences (325) belonged to the Trebouxiophyceae (90% of the clones comprising 32 OTUs) or to Chlorophyceae (10% of the clones comprising 12 OTUs). The number of OTUs significantly varied between sampling sites of different forest management types. Three of the most abundant OTUs (OTUs 26, 28 and 29), taxonomically assigned to the Prototheca, represented more than 47 % of all clones retrieved from soil.

Another study [55] evaluated the polymerase chain reaction (PCR) approach based on rapid direct-extraction, rDNA fingerprinting and sequences analysis, providing unambiguous identification of soil microalgal communities. Upon clustering 18S rDNA sequences recovered from sampled sites with significant similarity, clusters represented two taxonomic groups of Chlorophyceae, Ulotrichales, genus Stichococcus; and Chlorococcales, genera Dimorphococcus and Coelastrum. The study results will certainly contribute in future to represent novel and uncultivated microalgal species that still remains to be described.

Analysis of biological soil crust from Ny-Ålesund, Svalbard, Norway, and the Juan Carlos I Antarctic Base, Livingston Island, Antarctica, was performed using two different methodologies: basic morphological identification using light microscopy and the metatranscriptome ribosomal sequence annotations [56]. Combining both approaches to study phytoplanktons in Arctic and Antartic samples resulted in identification of 143 and 103 genera of microalgae belonging to five taxonomic groups including Klebsormidiophyceae, Chlorophyceae, Trebouxiophyceae, Xanthophyceae and Cyanobacteria. The study findings illustrated the efficacy of the combined use of morphological and molecular methods, in comparison with classical single-method approach, to reveal accurate taxa richness for complex communities.

II. Metagenomic Strategies

As exemplified throughout this review, the sequenced-based metagenomics analysis of microalgae can be accomplished by one of the following methodological strategies: (i) High-throughput DNA sequencing of a clone library developed from PCR products of environmental DNA generated with a phylogenetic marker indicating the potential taxonomic origin (ii) sequenced based screening of random fragments to find a particular sequence or gene of interest, followed by sequencing of the adjacent regions to locate markers with improved taxonomic specificity. Currently, the distinction of microalgal individuals below the species level employ either shotgun metagenome sequencing or by focusing on intragenomic heterogeneity within phylogenetic (e.g., 18S rRNA or 18S rDNA) or functional gene targets.

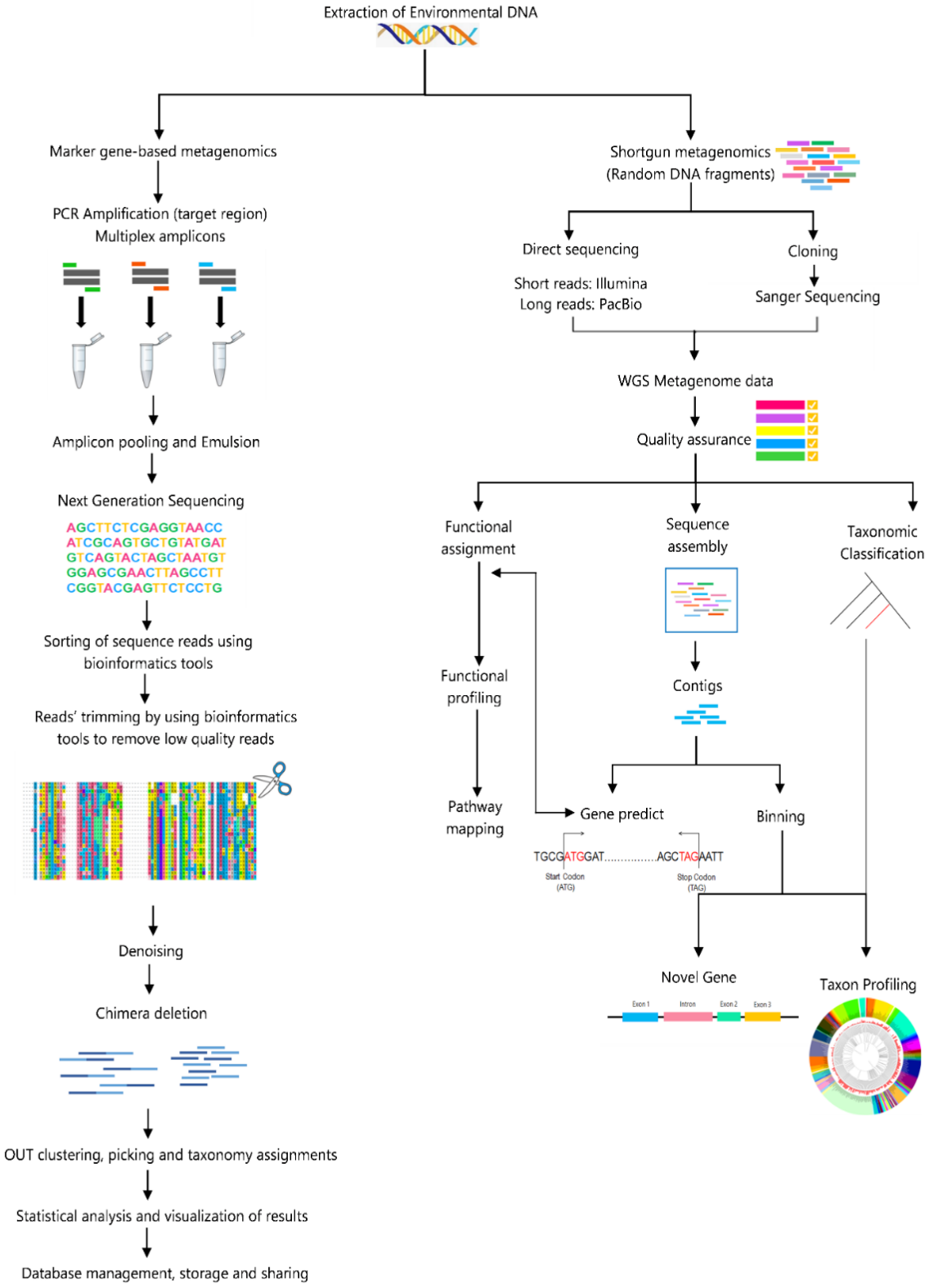

Figure 1 – Methodological Workflow of Metagenomics

The first approach refers to reconstructing large fragments or even entire genome without selecting any particular gene, alleviating biases from primer choice and enables the characterization of coding as well as non-coding components that can be used as phylogenetic markers [57-58]**.** In the later approach, specific internal conserved regions of DNA can be retrieved using taxonomical informative primer targets such as intergenic transcribed spacers (ITS) or the large ribosomal subunit (LSU) gene [59-61].

The research question enables the researchers to compare and determine the appropriate approach between assembly-based analyses and direct taxonomic classification of reads. The sequence conservation of regions of few genes has become an unprecedented resource for taxonomy in addition to being a phylogenetic anchor that requires no prior knowledge of full gene sequence [62-63]. In some cases, the sequence homology of most genes of practical importance is often most difficult to be identified by PCR or hybridization due to their far too divergent nature. However, the nucleotide sequence for a few classes of genes is well-conserved to facilitate their identification by sequence instead of function.

Several recent studies have addressed the use of the 18S-ribosomal-DNA (rDNA), 18S-ribosomal-RNA (rRNA), 28S-ribosomal-DNA and its variable regions as taxonomic markers for the classification of Eukaryotes [64-66] and the validity and limitations of using them in the taxonomic profiling of metagenomes have already been discussed [67-68]. An extensive effort is being put to establish similar universal molecular markers for microalgal taxa [69].

Since the early 1990s, there was a progressive shift towards molecular taxonomic studies for microalgae [70-71]. Beside genes and spacer sequences, the past studies relied heavily on exploring ribosomal operon (e.g., actin, *psb*a, *rbc*L, *tuf*A, RUBISCO spacer, and other chloroplast genes [72-74]. However, while the SSU and LSU has proven efficient for delineation at high taxonomic levels, they are not considered applicable for intraspecific differentiation [75]. The suitability of marker based on ITS regions were increasingly recognized for microalgal phylogenetic and taxonomic studies due to their high degree of interspecific variability, conserved primer sites, and multicopy nature in the genome [76-77]. The former study proposed 5.8S + ITS-2 fragment as ideal candidate marker for microalgae owing to its broad taxonomic range.

The utilization of multiple markers based on four gene loci and their combined data was formalized [78]. The study tested the efficiency of multiple markers based on four gene loci and their combined data (rbcL + tufA + ITS + 16S, rbcL + tufA and ITS + 16S), with three combined data having better resolution than single genes for higher intraspecific and interspecific divergence. Few studies reported *tuf*A gene applicability most suitable for DNA barcoding and phylogenetic reconstruction based on its wide coverage and sequencing success [79-83]. A comparison of marker gene-based and metagenomics techniques to estimate a microbial community's taxonomic composition is shown in Table 1, which implied that an efficient taxonomic resolution is more achievable by metagenomics profiling.

Table 1 – Comparison of metataxonomics (marker gene-based) and metagenomics profiling

Technique	Method principle	Advantage and Challenges	Main applications
Metataxonomics	Using amplicon sequencing of 18S-ribosomal-DNA (rDNA); 18S-ribosomal-RNA (rRNA); 28S-ribosomal-DNA; or ITS or rbcL or tufA or 23S universal plastid amplicon (UPA)	+ Faster, cost-effective and more reliable identification to species level + accessible to non-specialists - inability to quantify taxon abundance - Amplification bias - more than one primer sets needed for maximizing diversity coverage and to offset primer biases - lack of comprehensively cured reference databases for assigning taxon to the OTUs	Biodiversity monitoring Molecular phylogeny Microbial ecology
Metagenomics	Random shotgun sequencing of DNA or RNA (Sanger and 454/Roche sequencing) or long-read sequencing (Illumina/Solexa, SOLiD, PacBio SMRT System)	+ investigate uncultivable complete microbial communities in situ + No amplification bias + generated sequence reads does not require homology to known sequences (de novo profiling) - requires reference database of genes to classify sequence reads - requires high-quality DNA - requires more reads count for higher sensitivity	Structural and functional genomic screening contributing to discovery of novel genes Phylogenetic profiling Monitoring the biodiversity and the ecological status

A combination of good yield and high purity of metagenomics DNA is a prerequisite for the success of both targeted-amplicon and shotgun studies [84-85]. Much has been done for optimizing extraction protocol for efficient recovery of pure metagenomic DNA [86-89], including the utilization of commercial kits as tailor-made solutions for a particular application or sample [90]. Current evidence [91-92], suggest that no method or combination of methods exceeds ~80% accuracy indicating there is still significant room for improvement in this area.

In spite of the given importance of microalgae genome data regarded as priority research area for fundamental and applied aspects such as raw material for biofuels and bio-products [93], Hydrogen production [94], and supporting the mariculture industry [95], the microalgal representatives remained under-explored in the main metagenomics databases.

III. Databases

Significant advances in next-generation sequencing technology have facilitated genome sequencing with high throughput at low costs. NGS technologies hold great potential to have profound impact in various areas of research, including several that, so far, have mainly used approaches based on de novo sequencing i.e., sequencing novel genomes where no reference sequence is available for alignment, and resequencing i.e., genomes sequencing from a species for which a reference genome is already available.

Although the technological advances in nucleotides sequencing has led to a substantial increase in the release rate of sequenced genomes at unprecedented scales and rates but it is computationally challenging. All these complex and comprehensive raw data are useless without utilization of correct tools for analysis, annotation, storage, integration and translation. Resource integration and standardizing annotations are relevant for better understanding genetic diversity and deciphering complex mechanisms associated with microbial ecology, evolution, and diversity [96].

Since the publication of the first microalgal genome, red extremophile Cyanidioschyzon merolae in 2004 [97], over 100 microalgal genome projects have been launched and complete genome sequences of over 60 microalgal species been brought publicly available including green, and red microalgae as well as diatoms, dino-flagellates, nano-flagellates, and some uncommon species from underrepresented evolutionary branches (DOE Joint Genome Institute, http://genome.jgi-psf.org/ [98-100]; GOLD database, http://www.genomesonline.org/ [101]; Cyanobase, http://genome.kazusa.or.jp/cyanobase). During the last two decades, next-generation sequencing technology have greatly contributed to increasing number of sequenced microalgal genomes in public databases along with EST (expressed sequence tag) and transcriptome data sets and the breadth and depth of sequence assemblies and annotations are continuing to expand, with projects dedicated to filling in less characterized microalgal taxonomic groups [102-104].

The rapid expansion of genomic sequence data available and accessible in the afore-mentioned public repositories, and advances in databases analytic tools, makes it a daunting task for researchers to access, integrate, sort out and compare the best sequencing, specialized annotation and analysis strategies for microalgae. Fortunately, development of various customizable web-based genome browsers, model organism databases (MODs), molecule- or process-specific databases, and others has helped the researcher to find the needles in the haystack. A number of online databases are available for information on algal diversity and taxonomic studies, each with their own focus and limitations. Several published studies have described the gaps and sequence uncertainties in microalgal genome sequences and this provides an opportunity to review what we have learned so far from sequencing the genomes of microalgae. At present, multiple data sets are available for ongoing more than 60 algae genome projects at the Department of Energy Joint Genome Institute (JGI), including status. Assembles, and annotations of sequenced genomes (http://www.algaeu.com/strains-of-algae-publications.html).

EST data from many microalgal species are available at the EST sequence databases of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genbank/dbEST/), a special division of Genbank, and the Taxonomically Broad EST Database (TBestDB), http://tbestdb.bcm.umontreal.ca/searches/welcome.php). Sequencing of mitochondrial and chloroplast genomes has been performed with even more microalgal species than in the EST or genome sequencing projects [105], which are available at the NCBI organelle database (http://www.ncbi.nlm.nih.gov/genome/ organelle/) and the Organelle Genome Database (GOBASE, http://www.bch.umontreal.ca/gobase/gobase.html).

Significant progress have been made to sequence complete organelle genomes at a massive scale through the Organelle Genome Megasequencing Program (OGMP, http://gobase.bcm.umontreal.ca/). Furthermore, the genomic sequence information of various microalgal species has been updated in the Phytozome, a hub for genomic data from a few green microalgae (phytozome.jgi.doe.gov/pz/portal.html); the Greenhouse, largest eukaryotic algal genome collection available online (https://greenhouse.lanl.gov/greenhouse/); realDB, a genome resource for red algae (http://realDB.algaegenome.org); pico-Plaza 2.0 (http://bioinformatics.psb.ugent.be/plaza/versions/pico-plaza/); CoGe database, which utilize the genomics comparison tools to analyze algal genomes of interest (https://genomevolution.org/coge/); Ensembl Plant database (http://plants.ensembl.org); EnergyAlgaeDB, functional genomics database for energy microalgae (http://www.bioenergychina.org:8989/); and EUKREF, reference database of 18S sequence barcodes that correctly represent algal lineage (http://eukref.org/).

Table 2 – Characteristics and frequency of used sequencing platforms among genomes of microalgae published until 2018

Platforms	Sequencing Principle	Read Length	Accuracy Reads %	Time run	Output data/ run	No of genomes	% of total number of genomes
Sanger	Dideoxy sequencing	400~900 bp	99.999	20 mins~3 hrs	1.9~84 Kb	10	18.86%
454 GS FLX+/Roche	Pyrosequencing	600~800 bp	99.9%	24 hrs	0.7 Gb	4	7.54%
Solexa GAIIx/Illumina	Sequencing by synthesis	36~100 bp	98%	3~10 Days	600 Gb	18	33.96%
SOLiD4/Life Technologies	Sequencing by ligation	75 bp	99.94%	7 days	120 Gb	-	-
Ion Torrent (316 chip)/ Life Technologies	synthesis	200~400 bp	98~99%	2 hrs	1 Gb	1	1.88%
PacBio/Pacific Biosciences	synthesis	Up to 60 kb	90%	10 hrs	1-10 Gb	3	5.66%
Sequencing platforms used in combination							
Combination of Sanger Sequencing and Roche/454	-	-	-	-	-	2	3.77%
Combination of Sanger Sequencing and Illumina/Solexa	-	-	-	-	-	2	3.77%
Combination of Sanger Sequencing and PacBio	-	-	-	-	-	1	1.88%
Combination of Roche/454 and Illumina/Solexa	-	-	-	-	-	8	15.09%
Combination of Illumina/ Solexa and PacBio	-	-	-	-	-	4	7.54%

Figure 2 shows the proportion of sequences from different phyla of microalgae in some of the available international databases. Chlorophyta (green microalgae) and Ochrophyta (heterokonts) are the most represented in all databases, combinely constitute more than 65% of the publicly available sequences. In the future, mass of unculturable genomes likely to be generated from metagenomic samples and next-generation sequencing in the next few years continue to expand the international databases, the distribution of the phyla and number of species will likely change.

A number of online databases are available for information on algal diversity and taxonomic studies, each with their own focus and limitations, such as Barcode of Life Data Systems (BOLD),

a taxonomically curated database (http://www.barcodinglife.org); ITSoneDB (http://itsonedb.cloud.ba.infn.it/); and ITS2 Database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/); and R-Syst::diatom (http://www.rsyst.inra.fr/en). The range of software tools being used in major steps of metagenomic data processing, Sequence Mapping; taxonomic profiling, sequence assembly and gene prediction has already been provided in detail by [106-108].

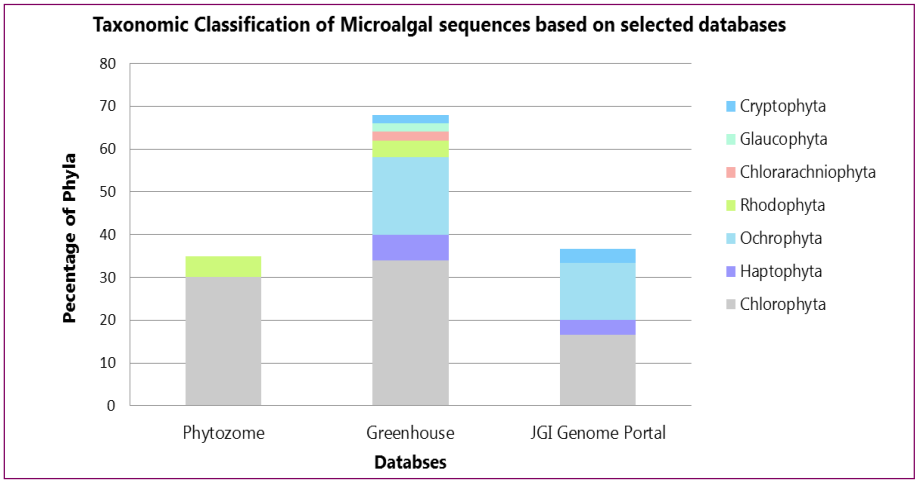

Figure 2 – Taxonomic classification of microalgal sequences based on selected databases

A number of recent published studies has resulted in exponential rise in the microalgal genome datasets, providing an opportunity to review at various level what we have learned so far from microalgae genome sequencing. In the future, mass of unculturable genomes likely to be generated from metagenomic samples and next-generation sequencing in the next few years continue to expand the international databases interrelating genomic datasets to ecological data, the distribution of the phyla and number of microalgal species will likely change.

To validate its use for bioassessment purposes requires the researcher to place greater emphasis on data mining tools and statistical analysis and interpretation and illustrate the biologically significant patterns in the datasets. While it may be possible to align traditional morphological taxonomy-based approaches with DNA-based biomonitoring approaches, the metagenomic methods and reference genome libraries need further validation to be complementary source of information for biomonitoring programs on a large scale.

IV. Conclusion

By shifting the realms of genomics from using model-organism as research tool towards studies of untapped resources of biodiversity in environmental samples, NGS has paved the way for researchers to carry out the fundamental and applied research on microalgal communities on a scale and precision that was unrealistic only a few years ago. The possibility of generating massive and disparate genomic datasets from both culturable and unculturable microorganisms using combination of deep sequencing and bioinformatics approaches has allowed the access to the collective data of mixed microalgae consortia in a less biased way, which enable us to deduce answer for important ecological and evolutionary questions.

To date, sequencing strategies used in the metagenomics study of microalgal diversity are currently dominated by short, high-throughput sequencing technologies, such as the Illumina NextSeq and HiSeq. Billion sequence reads of 100-300 bp can be generated via these technologies in a matter of days and are cost-effective for most large-scale microalgal related research [109-110]. Further advances in sequencing methods and data generation, such as single-molecule sequencing, synthetic long reads and Hi-C along with new assembly and scaffolding algorithms have made it possible to minimize the errors and misinterpretations. The high-quality genome assembly of microalgae has undergone a renaissance since the availability of single-molecule sequences, such as the PacBio

RSII/Sequel [111] and Oxford Nanopore MinION [112], which has allowed the researchers to run in-depth genome sequencing with reduced time and cost, requiring low DNA sample input and simple, rapid library preparation. Due to their promising potential application, improving the contiguity and quality of metagenome assemblies will positively affect the microalgal diversity and ecology research studies. To date, however, the widespread adoption of afore-mentioned technologies in metagenomics analysis has been limited.

Here, we have highlighted significant contributions and considerable developments in using metagenomic for studying microalgae diversity in recent decades, from a technological and computational perspective. Future technological advances are likely to reframe biological and ecological research questions that will have a significant impact on metagenomics application. We also discussed some of the challenges presented by comparability of different sequencing platforms. New transformative technologies hold the promise for researchers to confront these challenges, but as the enormous volume of data is unfolding, the researchers need to remain aware of these potential pitfalls and challenges while analyzing large and complex metagenomics datasets. Thus, the combination of modern methods of high-throughput sequencing with the classical bioindication approach is a promising way of solving global issues of biomonitoring of various aquatic ecosystems concerning the relationship between the structure of microalgae communities and water properties.

Our research group is using a targeted muti-marker based approach to investigate the genetic diversity of microalgae in Almaty region, the Republic of Kazakhstan [manuscript in preparation]. With ever-increasing worldwide collection of environmental samples along with the continued progression in metagenome sequence datasets, we might be able to explore deeper into the molecular novelties of this remarkably diverse eukaryotic group. At the same time, the types of microalgae communities identified by metagenomics associated with the ecological state of water can be used as bioindicators of the state of aquatic ecosystems. Metagenomic characteristics of aquatic ecosystems can be used to assess the sustainability of aquatic ecosystems when exposed to natural and anthropogenic factors.

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