

DNA Analysis of Five Microalgae Species Using Molecular Markers

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ABSTRACT

Background: Microalgae are important for biotechnology and environmental monitoring, but species identification is difficult because many taxa share similar morphology and life cycles. This study aimed to assess genetic diversity and phylogenetic relationships of five microalgae species using ISSR markers.

Methods: *Anabaena* sp., *Nostoc* sp., *Microcystis* sp., *Chlorella* sp., and *Scenedesmus* sp. were cultivated under laboratory conditions. Genomic DNA was isolated using CTAB- based extraction, quantified by *Microcystis* NanoDrop spectrophotometry, and evaluated by agarose gel electrophoresis. ISSR-PCR was performed using the dinucleotide repeat primers (AG)9C and (GA)9C. Amplified fragments were separated on agarose gels, compared using VisionCapt software, and used to calculate Nei's genetic distance and construct a phylogenetic tree with POPGENE v.1.32.

Result: Gel electrophoresis confirmed that the isolated DNA was of sufficient quality for PCR analysis. The (AG)9C primer generated polymorphic profiles, with *Anabaena* sp. and *Nostoc* sp. sharing bands at 750, 950, 1000, and 1150 bp, indicating close genetic similarity. *Microcystis* sp., *Chlorella* sp., and *Scenedesmus* sp. showed distinct fragment patterns. The (GA)9C primer also revealed interspecific variation, producing species- specific bands, while no visible amplified fragment was detected in *Scenedesmus* sp. Phylogenetic analysis grouped *Anabaena* sp. and *Nostoc* sp. together, while the remaining species formed separate branches.

Conclusion: ISSR-PCR using (AG)9C and (GA)9C primers effectively detected genetic diversity among the studied microalgae. The generated DNA fragments are useful molecular markers for biodiversity assessment, taxonomic identification, and future DNA barcoding studies. Integrating ISSR-PCR with sequencing approaches may strengthen microalgae classification and support biotechnological applications.

INTRODUCTION

The study of the DNA of microalgae and related microbial communities is key in the development of biotechnology, and provides further opportunities for the production of biologically active compounds. Microalgae have been studied for many years due to their unique biological characteristics, such as high biomass productivity, photosynthesis efficiency, ease of cultivation, and the potential in biodiesel production, the last of which surpasses similar indicators in plants [1,2]. In biotechnology, particularly, microalgae are considered to be promising sources of lipids and secondary metabolites. The study of the DNA of microalgae has significant relevance, because they are important producers of biologically active compounds with antibacterial properties, which means that there is potential application in medicine and pharmaceuticals. Furthermore, studies of microbial communities associated with microalgae contribute to the optimization of cultivation processes and increase the efficiency of production of valuable bio-derived products. One of the main areas of physiological research is the identification of species. However, the lack of noticeable structural features, including of phenotypic differences between species and genera, as well as morphological variance in multiphase life cycles, significantly complicate the identification process [3,4]. Traditional morphological methods are insufficient to distinguish among species with similar external characteristics. Currently, DNA barcoding is recognized as an effective tool for fast and accurate identification of various species [5-6]. The unique advantage of the method lies in its application in the classification of all forms of life. Nonetheless, the lack of a universal molecular marker in the identification of both eukaryotes and prokaryotes remains a significant issue. For example, the mitochondrial COI/ COX1 gene encoding the cytochrome C oxidase subunit has been successfully used as a molecular barcode to identify most animals [7]. However, there is no agreed upon universal marker for plants yet. Instead, sections of chloroplast DNA (cDNA) are more often used, including the ribulose-1,5-bisphosphate carboxylase (rbcL) large subunit gene and the trnH-psbA intergenic spacer [8]. The carboxylase/ oxygenase (RuBisCO) (rbcL) and maturase K (matK) genes usually have a higher barcoding level compared to other genes. Their use makes possible more accurate distinction between interspecific and intraspecific genetic distances within a group of organisms. The rbcL gene is distributed among most eukaryotic phytoplankton, providing a higher resolution than the 18S rDNA gene [9-11]. Recently, some studies have shown that multiple DNA barcoding of various gene combinations provides a better definition than does a single DNA barcode. For example, sequence analysis of rbcL, tufA and internal transcribed spacers (ITSs) has been used to distinguish among green microalgae, such as *Chlorella* and *Scenedesmus* [12]. Some researchers of diatoms have utilized barcoding that applies four different types of genes, including ITSs and conservative sites (CSs), which demonstrates their significant differences, thus making them suitable for species identification [13].

Before conducting polymerase chain reaction (PCR) to identify microalgae, performing exacting DNA extraction and purification is especially key. In order to extract DNA from microalgae, a sufficient amount of biomass, of at least 0.1-0.5 g, should be collected from a single source only after cultivation [14]. Molecular analysis of microalgae solves many problems, including species identification, assessment of genetic diversity, and determination of environmental adaptations. The goals include: improving biotechnology applications, monitoring environmental impacts, and improving taxonomy through DNA barcoding techniques, inclusive of the rbcL, matK and ITS regions. The main challenges are the optimization of DNA extraction, selection of universal genetic markers, and application of advanced molecular tools alongside traditional methods. Particularly, molecular markers play a key role in species identification and biodiversity research. They make possible the study of genetic differences between and within species, which is especially important for complex groups of organisms such as microalgae. The use of markers, among which are ITS, rbcL, and matK, helps to improve the taxonomy, identify new species, and understand ecological adaptation. These methods integrate molecular data with ecological and morphological characteristics to achieve more accurate monitoring of biodiversity [14].

Microalgae are very diverse and can be found in both freshwater and marine environments. Freshwater microalgae include *Chlorella* and *Scenedesmus*, which are important for research in biofuel production and environmental monitoring, due to their high biomass productivity. Marine microalgae, including *Phaeodactylum* and *Symbiodinium*, play an important role in aquatic ecosystems and have potential applications in biotechnologies such as biofuels and pharmaceuticals. These genera represent only a small portion of microalgae, each of which plays a unique biochemical and ecological role. Understanding their chemical composition, such as the structure of cell walls, is crucial in optimizing DNA extraction methods and advancing the study of these microorganisms for various biotechnological purposes [15].

The chemical composition of the cell walls of microalgae significantly affects DNA extraction. Many species of microalgae, especially diatoms, have complex silicon-based or cellulose-rich cell walls, which can make DNA extraction difficult. The rigid and often thick nature of these walls may require special protocols in order to break down barriers and release intact DNA. For example, diatoms that possess a silicon-based shell often require more aggressive processing, inclusive of enzymatic cleavage or mechanical destruction to successfully extract DNA. Algae with simpler cellulose-based walls, such as some green algae, may be easier to process, but still need optimized methods for the purpose of minimizing degradation or contamination. The type of cell wall, along with other factors, including cell size and age, play a crucial role in the effectiveness of DNA extraction methods [16]. DNA extraction from microalgae is a fundamental step in various molecular biology studies, including genetic analysis, species identification and metabolic studies. There are several methods used to extract DNA from microalgae, each with its own advantages and limitations. Physical methods, such as cell wall destruction, are commonly used to destroy the protective structures of microalgae. Mechanical destruction of cells using a bead mill or mortar and pestle is effective in destroying thick, rigid cell walls. Application of high-frequency sound waves is also used to destroy cell walls and membranes, and is especially useful for smaller or softer cells [16].

Several forms of DNA extraction are available, including: chemical lysis, one of the most common methods, which uses buffer solutions, such as cetyltrimethylammonium bromide (CTAB), and is effective for plant and algae species with thick polysaccharide cell walls, as it destroys cell walls and removes polysaccharide contaminants, but can be toxic and difficult to handle [17]; sodium dodecyl sulfate (SDS), which is a detergent that destroys cell membranes, promoting protein denaturation and DNA release [18,19]; enzymatic treatments, which are used to break down cell walls with less mechanical effort, thus providing a softer alternative; natural enzymes, such as lysozyme, which are used mainly for bacterial-type cell walls, destroying peptidoglycan layers, but are not always effective for all types of microalgae; commercial enzymes, such as Proteinase K, which is an enzyme that breaks down proteins, preventing contamination of the DNA sample [20]; and, commercial kits, such as the Qiagen DNeasy Plant Mini Kit and Sigma-Aldrich GenElute Plant Genomic DNA Miniprep Kit, among others, which offer standardized protocols that simplify the extraction process, and usually include reagents for cell destruction and DNA binding, with the main advantages being reproducibility and time savings, though they can be expensive and are not optimized for all types of microalgae [21]. DNA extraction from microalgae is associated with unique difficulties. Polysaccharide contamination results in a high content of polysaccharides in many types of microalgae, which can alter the DNA sample, thus making difficult the extraction of pure DNA [22]. Inhibitors include secondary metabolites, such as phenolic compounds and proteins, which can inhibit PCR and other subsequent analyses, thus leading to additional purification steps [23].

Molecular markers are important tools in the study of microalgae, because they provide insight into taxonomy, biological diversity, and evolutionary relationships. These markers are widely used in various molecular techniques, inclusive of DNA barcoding, genetic diversity studies, and species identification. Due to its universal presence in all eukaryotic organisms, the 18S rRNA gene is one of the most commonly used molecular markers for microalgae, as it has a high degree of conservatism and provides valuable phylogenetic information. Similarly, the regions of the internal transcribed spacer (ITS) that are located between rRNA genes are variable, and are excellent in distinguishing among closely related species. These markers are necessary for the identification of various microalgae taxa, including both marine and freshwater species [24,25]. Chloroplast genes, such as *rbcL*, a large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, and *tufA*, a coding protein involved in the synthesis of chloroplast protein, are widely utilized in the study of photosynthetic microalgae, which are more specific to such algae and provide insight into their evolutionary relationships and functional adaptations related to photosynthesis. The *rbcL* gene, in particular, is a reliable marker for the identification of various algae species, because it participates in the critical carbon fixation process [26].

Furthermore, inter-simple sequence repeat (ISSR)-PCR markers are used to detect genetic variability based on the amplification of sites between simple repeats of sequences, and are highly polymorphic, so do not require prior sequence information, which makes them valuable in studying genetic diversity within and between microalgae populations. Additionally, they are used to assess intraspecific variability, such as in cloned cultures, and interspecific differences, thus providing information on the biological diversity among various microalgae [27,28]. Random amplified polymorphic DNA analysis (RAPD-PCR) is another molecular method used to

identify the genetic variability of microalgae, and involves the amplification of random DNA fragments using short primers. Although RAPD-PCR is fast and does not require prior knowledge of the genome, it has limitations in reproducibility and resolution in comparison to other methods, such as ISSR-PCR or sequence-based markers. Despite these limitations, RAPD-PCR can be useful for preliminary studies of genetic diversity and population structure [29,30].

Overall, the utilization of molecular markers allows for species identification, assessment of genetic diversity, and ecosystem monitoring. Those like 18S rRNA and *rbcL* are crucial for the identification of microalgae species, especially ones that are morphologically similar or lack distinctive physical characteristics, allowing for accurate taxonomic classification. For example, the ITS regions are commonly used to differentiate species in complex groups such as diatoms and dinoflagellates [31]. ISSR-PCR and RAPD-PCR are effective tools for the study of the genetic diversity of microalgae, both in natural populations and under controlled laboratory conditions, allowing assessment of intraspecific diversity, genetic variability, and the influence of environmental factors on microalgae populations. For example, ISSR-PCR markers have been used to assess the genetic diversity of *Chlorella* and other freshwater algae [32]. Similarly, RAPD-PCR has been applied to the assessment of genetic diversity among marine microalgae, helping in conservation and restoration efforts [33]. In the research with *Chlorella* species, RAPD-PCR with decamer random primers was used to assess the genetic variability in *Chlorella* spp [34]. RAPD markers were applied to the amplification of microalgal DNA extracted using the CTAB method. The results showed that primers OPB-01, OPB-11 and OPH-08 exhibited the highest intraspecific variability, with 100% polymorphism, whereas OPH-07 showed the lowest polymorphism, only 13%, at amplicon levels. Despite morphological similarities, a significant genetic polymorphism was observed among *Chlorella* species. The findings indicate that four RAPD bands are useful in studying the genetic diversity of microalgae, as they allow for differentiation of species. In another study, RAPD markers were used to assess genetic differences among species within three genera of unicellular microalgae: *Tetraselmis*, *Chlorella* and *Nannochloropsis* [35]. Although morphological identification was challenging, due to the similarities among the various strains, RAPD-PCR analysis revealed genetic diversity, the lowest of which was observed between *Tetraselmis* and *Chlorella*, at 0.36, and the highest was between *Chlorella* and *Nannochloropsis*, at 0.69. Additionally, significant genetic diversity, in the range of 0.58-0.83, was detected within cultured populations of *Nannochloropsis*. Limited genetic diversity in microalgae cultures may threaten their long-term stability and adaptability under laboratory conditions. Using the genetic fingerprinting method called amplified fragment length polymorphism (AFLP-PCR), genetic diversity among strains of *Chlorella vulgaris* was examined, along with the identification of cryptic species [36,37]. Different isolated strains of the same species were shown to be genetically distinct, emphasizing the need for careful strain identification in experiments. Further studies showed that AFLP-PCR could be utilized in differentiating pigment mutants of *ParaChlorella kessleri*, as well as in detecting viral infections in *Chlorella* sp. The research also addressed issues related to contamination, strain mislabeling, and cryopreservation, which highlights how AFLP-PCR can be used to ensure the genetic integrity of cryopreserved microalgae. Further, the study also examined the optimization of microalgae DNA isolation methods and the use of RAPD-PCR and ISSR-PCR in order to study the genetic diversity of *Chlorella* sp. and *Scenedesmus* sp. under laboratory conditions. Molecular methods, including the use of molecular markers, are increasingly being used in monitoring the health of aquatic ecosystems. These methods make possible detection of harmful algal blooms, species invasions, and changes in the composition of microbial communities. For example, DNA markers have been utilized in identifying those responsible for algal blooms, such as toxic dinoflagellates [38,39]. In addition, molecular instruments are used to monitor the effects of environmental stressors, such as pollution or temperature changes, on microalgae communities, providing early warnings of environmental shifts. Next-generation sequencing (NGS) and metabarcoding have revolutionized the study of microalgae, providing fast and accurate identification of species in complex communities. These technologies facilitate high-throughput sequencing of DNA sites, such as ITS and 18S rRNA, which allows researchers to assess biodiversity and understand environmental dynamics [40].

Finally, among the methods that have so far been used to study microalgae, metagenomics provides a comprehensive approach to understanding their biodiversity and functional roles in ecosystems, by analyzing genetic material directly from environmental samples, which makes possible differentiation among both dominant and less common species. The expansion of metagenomic research may provide insight into how microalgae respond to environmental changes, pollution and climatic stressors. Therefore, future studies may further integrate these tools in the analysis of interactions between microalgae and other microorganisms in the aquatic environment. The development of automated systems for DNA extraction and analysis

can significantly improve efficiency and reproducibility in microalgae research. Robotic platforms and automated workflows using commercial kits are increasingly being used to process large volumes of samples in environmental monitoring and biotechnological applications [41]. Researchers should focus on adapting these systems to complex samples, such as microalgae with rigid cell walls. Nonetheless, the diversity of microalgae poses challenges in selecting suitable molecular markers for identification and classification. Universal markers that work in broad taxonomic groups will simplify molecular study. Chloroplast genes such as *rbcL* and *psbA*, along with ribosomal markers such as 18S rRNA, are promising candidates for such purposes [31]. Additionally, studies should be aimed at increasing the resolution of these markers to distinguish closely related species.

Generally, significant improvements in physical, chemical and enzymatic DNA extraction methods, along with the development of reliable molecular markers, such as ribosomal genes (18S rRNA, ITS) and chloroplast genes (*rbcL*, *tufA*), have expanded the collective knowledge of the taxonomy, genetics and biodiversity of microalgae. The use of molecular markers and effective DNA extraction techniques has become transformative for both scientific research and practical applications. These tools make possible accurate identification of microalgae species, assessment of genetic diversity, and monitoring of aquatic ecosystems, in addition to furthering biotechnological applications, such as biofuel production and pharmaceutical development [42]. Such molecular approaches have made a significant contribution to the conservation of aquatic ecosystems by providing accurate monitoring of species composition and environmental health, and have contributed to the development of biotechnology by identifying new microalgae with industrial potential. In the future, researchers might wish to focus on the integration of advanced technologies such as NGS and metagenomics in order to further expand the field, identify sustainable practices for environmental management, and develop commercial products [43].

METHODS

Five (5) species of blue-green microalgae (*Anabaena* sp., *Nostoc* sp., *Microcystis* sp., *Chlorella* sp., and *Scenedesmus* sp.) were collected and grown under laboratory conditions. Samples of the microalgae grown on nutrient medium were then harvested in order to isolate their genomic DNA. Genomic DNA was isolated using a CTAB-based extraction procedure [37]. DNA concentration and purity were assessed using a NanoDrop OneC spectrophotometer (ThermoScientific, USA), and DNA integrity was evaluated by agarose gel electrophoresis [37].

PCR

Polymerase chain reaction (PCR) was performed using the standard method with approximately 50–60 ng of isolated DNA in a 20 µL reaction mixture. Each ISSR primer was used at a final concentration of 0.1 µM. The sequence of the first primer was (AG)₉C, i.e. 5'-AG AG AG AG AG AG AG AG AG C-3'. The sequence of the second primer was (GA)₉C: 5'-GA GA GA GA GA GA GA GA GA C-3'. The synthesis of these primers was carried out using an AFM-800 automatic synthesizer (Biosset, Russia). The reaction protocol for the two primers were as follows: 37 cycles at 95°C for 2 min to denature the template, then annealing temperatures/ times of 95°C/30 sec, 55°C/30 sec and 72°C/2 min, and a final extension period of 72°C for 7 min. The DNA sections obtained were visualized in 2% agarose gel using a Quantum ST5 1100 UV Table (Vilber Lourmat, France). GeneRuler 100 bp Plus DNA Ladder (ThermoScientific, USA), a commercial marker, was used to determine the molecular weight of sequences obtained as a result of amplification.

ISSR profile analysis

For the exploratory reanalysis presented in Figure 3, a binary matrix was reconstructed from the (AG)₉C fragment-size lists reported in the Results. Five size classes were included: 750, 950, 1000, 1150 and 1200 bp. Reported bands were coded as 1 and unlisted size classes as 0, assuming complete reporting for each culture. The reconstructed matrix has not yet been verified against the original band-scoring records.

Pairwise Jaccard dissimilarities were calculated using the *pdist* function in SciPy (Python). Clustering was performed by the unweighted pair-group method with arithmetic mean (UPGMA), using the linkage function with average linkage. The sample input order was *Anabaena* sp., *Nostoc* sp., *Microcystis* sp., *Chlorella* sp. and *Scenedesmus* sp. Only the (AG)₉C profiles were used; the (GA)₉C profiles and the DNA ladder were excluded. Figure 3 shows one clustering

solution under tied distances, with the horizontal axis indicating the dissimilarity levels at which clusters merge.

RESULTS

ISSR-PCR

Inter-simple sequence repeat (ISSR)-PCR analysis is considered to be one of the most basic and effective molecular genetic methods, and is widely used in breeding experiments, to assess the concentration and purity of different species, as well as to restore endangered species, and preserve genetic diversity. Indeed, this method has been widely used since 1994 in multi-focus genome mapping experiments. The ISSR-PCR method is based on the amplification of genomic regions located between microsatellite loci, where primers anneal to simple sequence repeat regions. In the present study, the dinucleotide repeat primers (AG)₉C and (GA)₉C, composed of repeated adenine–guanine motifs, were used to target these regions and generate reproducible DNA fragment profiles [8].

So as to perform analysis using ISSR markers, the extraction of a genomic DNA from the samples that were selected for research was carried out. Next, the quality and concentrations of the separated genomic DNA were checked. The image of the agarose gel electrophoresis showing the quality of the DNA is shown below in Figure 1.

As can be seen in Figure 1, no additional impurities are visible in the genomic DNA that had been isolated using an STA buffer, which means that the quality of the DNA is high and can be said to be suitable for PCR staging and genetic analysis.

Assessment of the genetic diversity of microalgae using ISSR-markers

PCR was carried out utilizing ISSR primers. The resulting samples that used the (AG)₉C primer were analyzed with gel electrophoresis (Figure 2). Electrophoresis was performed in agarose gel, along with the GeneRuler 100 bp Plus DNA Ladder, used to determine the size of the fragments. The fragment profiles obtained via electrophoresis were compared using VisionCapt software (Vilber Lourmat, France). The study took into account only clearly visible fragments on the agarose gel. Figure 2 shows the results of gel electrophoresis performed after PCR using the (AG)₉C primer. The highest number of amplified fragments obtained with the (AG)₉C primer was observed in *Anabaena* sp. and *Nostoc* sp. (Figure 2, lanes 1 and 2), whereas only one band was detected in the *Scenedesmus* sp. sample (Figure 2, lane 5).

For the (AG)₉C primer, the electrophoresis profiles showed the following amplified fragments: *Anabaena* sp. had bands at 750 bp, 950 bp, 1000 bp, and 1150 bp (Figure 2, lane 1); *Nostoc* sp. had bands at 750 bp, 950 bp, 1000 bp, and 1150 bp (Figure 2, lane 2); *Microcystis* sp. had a band at 1200 bp (Figure 2, lane 3); *Chlorella* sp. had bands at 750 bp and 1200 bp (Figure 2, lane 4); and *Scenedesmus* sp. had a band at 750 bp (Figure 2, lane 5). Gel bands show the differences in the length of amplified fragments, which allows assessment of their genetic diversity. For example, *Anabaena* sp. and *Nostoc* sp. both have common bands at 750, 950, 1000 and 1150 bp, which indicates their genetic similarity. *Microcystis* sp. and *Chlorella* sp. have unique bands, which highlight the differences in their DNA sequences. Differences in the length of fragments amplified by primer (AG)₉C show diversity of the genetic structures of the samples. A comprehensive assessment of these fragments shows that they can be used as molecular genetic markers in order to describe the relationships between different species of microalgae. Figure 3 below shows the phylogenetic distances of the microalgae in the study, as determined using POPGENE v.1.32 software.

Figure 3 shows the phylogenetic relationships among the five studied microalgae species based on ISSR-PCR profiles obtained with the (AG)₉C primer. The analysis was based on differences in amplified DNA fragments and was performed using Nei's genetic distance in POPGENE v.1.32. Only biological samples were included in the phylogenetic analysis; the DNA ladder was used solely as a molecular size marker during electrophoresis and was not included in the distance matrix or tree construction. According to the obtained dendrogram, *Anabaena* sp. and *Nostoc* sp. formed the closest cluster, which is consistent with their shared amplified fragments at 750, 950, 1000 and 1150 bp. The remaining species, *Microcystis* sp., *Chlorella* sp. and *Scenedesmus* sp., formed a separate cluster, with each species occupying an individual terminal branch. This indicates interspecific differences in their ISSR profiles and confirms the genetic variability

among the analyzed microalgae species. Thus, the ISSR-PCR fragments amplified with the (AG)9C primer provided informative molecular markers for assessing genetic similarity and differentiation among the studied samples.

Figure 4 shows the results of gel electrophoresis performed after PCR using the (GA)9C primer. The methodology applied was exactly the same as has been outlined above with regards to the (AG)9C primer. For the (GA)9C primer, the amplified fragment patterns differed among the studied samples: *Anabaena* sp. showed bands at 1050 bp, 1300 bp, and 1500 bp (Figure 4, lane 1); *Nostoc* sp. showed bands at 500 bp, 1000 bp, and 1400 bp (Figure 4, lane 2); *Microcystis* sp. showed faint bands at 900 bp and 1000 bp (Figure 4, lane 3); *Chlorella* sp. showed bands at 950 bp and 1200 bp (Figure 4, lane 4); whereas no visible amplified fragment was detected for *Scenedesmus* sp. (Figure 4, lane 5). In the case of primer (GA)9C, all samples had different bands. The use of the (GA)9C primer made possible isolation of DNA regions with GA repeats, which illustrates the variability in genomic sequences in different species or strains. ISSR-PCR analysis indicated a high level of genetic diversity among the samples studied, which provides a basis for further genetic research. The use of PCR with the (AG)9C and (GA)9C primers allowed data to be obtained on the phylogenetic relationships of microalgae and to identify unique DNA fragments that can serve as molecular genetic markers.

Figures

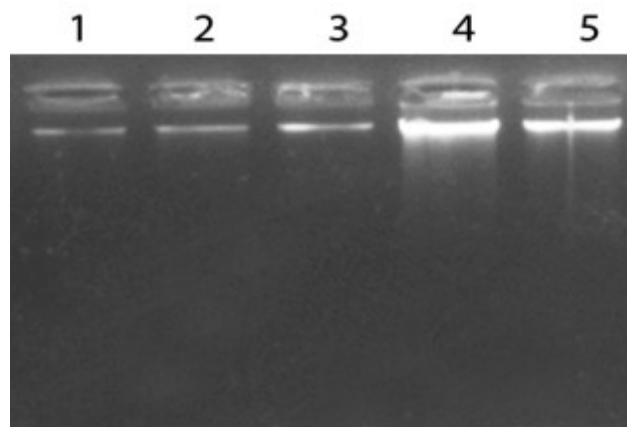


Figure 1: Genomic DNA extracted from microalgae: 1) *Anabaena* sp.; 2) *Nostoc* sp.; 3) *Microcystis* sp.; 4) *Chlorella* sp.; and, 5) *Scenedesmus* sp.

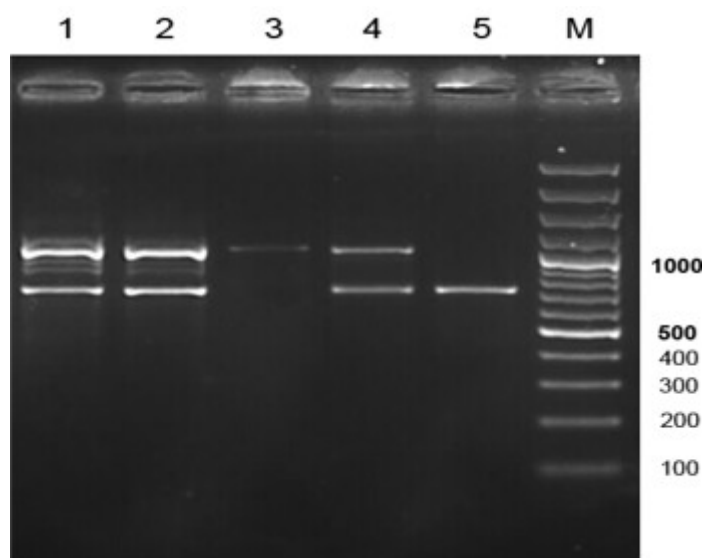


Figure 2: ISSR electrophoresis obtained as a result of PCR on samples with the (AG)9C primer: 1) *Anabaena* sp.; 2) *Nostoc* sp.; 3) *Microcystis* sp.; 4) *Chlorella* sp.; 5) *Scenedesmus* sp.; and, M) GeneRuler 100 bp Plus DNA Ladder.

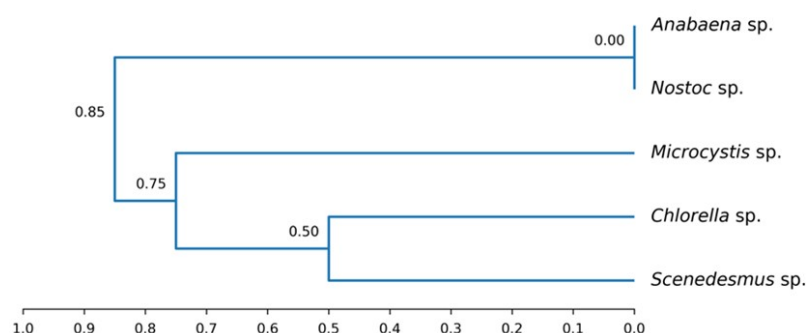


Figure 3: Phylogenetic tree of the studied microalgae species constructed from ISSR-PCR banding patterns generated with the (AG)9C primer and analyzed using Nei's genetic distance: *Anabaena* sp., *Nostoc* sp., *Microcystis* sp., *Chlorella* sp., and *Scenedesmus* sp.

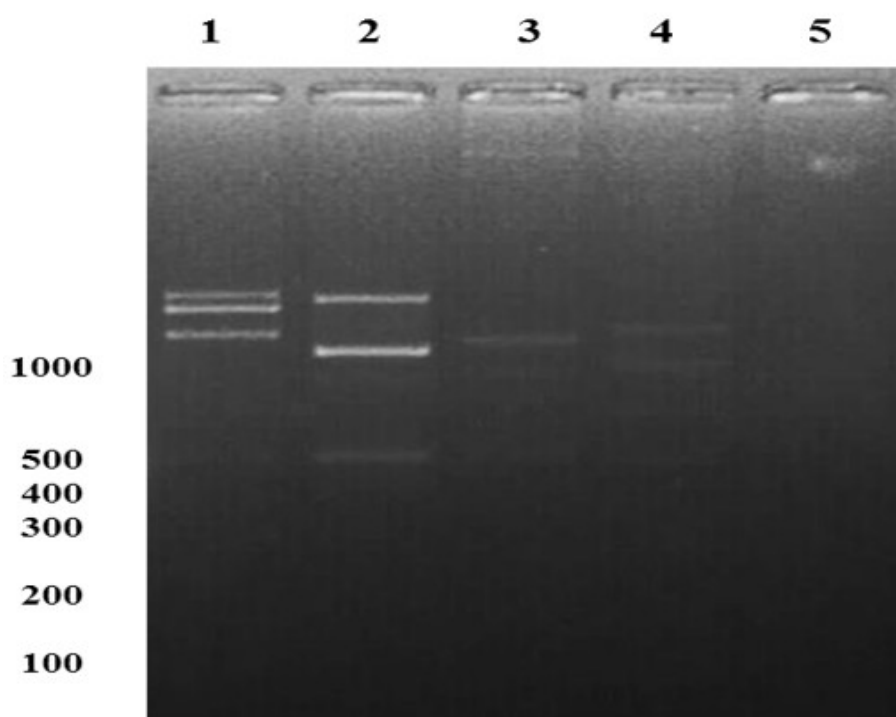


Figure 4: ISSR electrophoresis obtained as a result of PCR on samples with the (GA)9C primer: 1) *Anabaena* sp.; 2) *Nostoc* sp.; 3) *Microcystis* sp.; 4) *Chlorella* sp.; 5) *Scenedesmus* sp.

DISCUSSION

ISSR-PCR using the dinucleotide primers (AG)9C and (GA)9C revealed differences among the fragment profiles of the five studied cultures. *Anabaena* sp. and *Nostoc* sp. had identical reported (AG)9C profiles, whereas their reported (GA)9C profiles differed. No visible amplification product was detected in *Scenedesmus* sp. with (GA)9C under the conditions used. These results of the study align with recent research trends and highlights the theory that ISSR markers can be effective tools for preliminary genetic fingerprinting of cyanobacteria and green microalgae, complementing sequence-based barcoding approaches [44-46].

The performance of the dinucleotide ISSR primers in this study aligns with recent reports demonstrating the utility of such markers for microalgae characterization. A study on *Chlorella* sp. strains from Southern Vietnam utilized 14 ISSR primers and generated 184 amplified products, of which 180 were polymorphic, yielding a high polymorphism rate of 97.8% [47]. In comparison, our more restricted primer set produced clear, reproducible bands but captured a narrower spectrum of genetic variation. The (AG)₉C primer alone produced multiple discrete fragments in *Anabaena*, *Nostoc*, *Chlorella*, and *Scenedesmus*, indicating that even a limited ISSR panel can provide meaningful differentiation [48, 49]. Recent advances have also extended to strain-specific marker development, for instance [45].

The exploratory dendrogram in Figure 3 summarizes similarities among the reported (AG)9C ISSR profiles. *Anabaena* sp. and *Nostoc* sp. clustered together because their reported profiles for this primer were identical. This similarity concerns the fragment-size classes examined and does not establish evolutionary relationships among the studied cultures. Interpretation of the dendrogram remains conditional on the completeness of the reported fragment-size lists used to reconstruct the binary matrix. Our study supports this claim by suggesting that *Anabaena* sp. and *Nostoc* sp. may require more testing at the molecular level.

Looking at the broader view of DNA barcoding and the lack of standardization. We believe that ISSR-PCR offers a complementary approach to sequence-based barcoding [50]. Our results demonstrate that ISSR profiles generated with dinucleotide primers can indeed function as such phenotypic barcodes, distinguishing taxa that are morphologically cryptic [51]. However, to fully realize this potential, future work should validate ISSR-based identifications against sequence-based barcodes from the same samples, thereby building a bridge between fingerprinting and sequence databases.

Irrespective of these results in the study, certain limitations must be highlighted, such as the study only evaluating one strain per species, thereby not accounting for intraspecific genetic diversity, as studies have shown that strains of the same species isolated from various geographic regions or biological niches can have significant genetic divergence. Second, we utilized only two ISSR primers, whereas comparable research generally uses 10-14 primers to get reliable estimates of genetic diversity. The restricted primer set may exaggerate the apparent similarity between *Anabaena* and *Nostoc*, perhaps underestimating genuine genetic distances. Third, our research is based on banding patterns rather than sequence data; without sequencing the amplified fragments, we cannot determine the genetic origins of the bands or compare them to public databases.

For future research, we recommend increasing the range of the ISSR primer panel to at least 12 primers to increase the probability of higher-resolution genetic profiles and more reliable phylogenetic inference. We propose that the combination of ISSR-PCR and next-generation sequencing (NGS) or metabarcoding methods would enable the simultaneous characterization of microalgae and their associated microbial communities. We also suggest using verified ISSR markers on environmental samples rather than pure cultures so as to evaluate their efficacy for quick, field-based biomonitoring of hazardous algal blooms and variations in aquatic community structure.

The present study has demonstrated the application of two specific dinucleotide repeat sequences, (AG)₉C and (GA)₉C, as primers for amplification in inter-simple sequence repeat (ISSR)-PCR, for the purpose of determining the phylogenetic relationships among five (5) species of microalgae: *Anabaena* sp., *Nostoc* sp., *Microcystis* sp., *Chlorella* sp., and *Scenedesmus* sp. Furthermore, the use of Nei's genetic distance calculation and VisionCapt software allowed the interspecific genetic distances to be calculated, which permitted the construction of a phylogenetic tree. The PCR results and mathematical analysis showed that *Anabaena* sp. and *Nostoc* sp., *Microcystis* sp. share close genetic similarities, as do *Microcystis* sp., *Chlorella* sp., and *Scenedesmus* sp.; although, *Scenedesmus* sp. is different enough to require a separate subbranch. DNA barcoding of plants is thus far less advanced than for animals, for which the mitochondrial COI/COX1 gene encoding the cytochrome C oxidase subunit has been successfully applied. Therefore, the present results add to the research that is underway to find a plant equivalent, so as to allow for easier identification of microalgae, particularly in complex, mixed and polluted samples.

The further study of microalgae is necessary in the quest to shift away from traditional chemicals, medicines and fuels to more eco-friendly alternatives. Adding to the database of microalgae species and strains will provide a more complete understanding of their genetic diversity, evolutionary relationships, and suitability for industrial application. To this end, integration of ISSR-PCR with next-generation sequencing (NGS), in order to study complex microbial communities associated with microalgae, may lead to the development of new biotechnological products. Additionally, investigation of the influence of external factors on the genetic structure of microalgae, which requires ease of identification, will improve the management of their natural ecosystems and allow for proper cultivation. The present research team plan to further the work done thus far by studying additional sequences in the pursuit of improving the DNA barcoding of microalgae.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Conceptualization, Data curation, Formal analysis, Project administration, Supervision- Gulzira Yernazarova, Aliya Ramazanova, Sana Kabdrakhmanova;

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Declaration of Generative AI Use

During the preparation of this manuscript, the authors used generative artificial intelligence solely to proofread the English text and correct spelling, grammar, and punctuation. AI was not used for any other purpose, including study design, data collection or generation, calculations, statistical analysis, interpretation of results, formulation of conclusions, or preparation of figures. The authors reviewed and approved the final text and take full responsibility for the accuracy and integrity of the data and all content presented in this manuscript.

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