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Rapid Molecular Identification of *Tetraselmis* Using Enzymatic Digestion of the *18S rDNA* Gene

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Abstract: Tetraselmis is a genus of quadriflagellated single-celled green algae belonging to the Phylum Chlorophyta, commonly used in aquaculture with very promising biotechnological potential. The varied morphological characteristics, in some cases, have led to confusion in taxonomic identification. To solve this problem, new techniques based on molecular markers and restriction enzymes can ensure the identification of microalgae without sequencing. This study aimed to compare *in silico* modeling with an experimental restriction pattern based on the *18S rDNA* gene for the identification of a microalgae strain. The strain grew in a culture medium, based on organic fertilizer. Theoretical analyses allowed the design of three primers based on the alignment of eight sequences obtained from NCBI, applying the Geneious Prime® 2019 and V1.3 and Oligo Calculator version 3.2. programs. The *in silico* restriction patterns was obtained with the NEBcutter v2.0 program. Experimental analyses began with the extraction of the DNA using the TENS protocol, then PCR amplification using PM-016F/PM-016R and PM-001F/PM-016R primers of *18S rDNA* and finally the product was digested with *BbvCI* and *Eco53kI; BstUI, RsaI* and *MspI* enzymes. The DNA concentration extraction reached 3200 ng μl^{-1} and a purity of 2.0. The PCR amplified two products: 950 bp and 1400 bp, which brought us closer to identifying the microalgae. The *in silico* modeling and experimental restriction patterns showed similar fragments. In this way, the efficient response of restriction enzymes was demonstrated by confirming that the PM013 strain corresponds to the Tetraselmis genus. This method can be considered as a fast and safe alternative to identify wild microalgae in a basic molecular biology laboratory.

Keywords:

1. Introduction

Tetraselmis (Class Chlorodendrophyceae) is a genus of microalgae that has been considered an industrial source, due to the content of proteins, vitamins, lipids, carbohydrates, fatty acids and bioactive compounds. Tetraselmis can influence the growth and survival of fish and crustaceans because of its antioxidant properties [1]. It also accumulates many attractive oils and starches for the production of biofuels [2].

Morphologically, Tetraselmis is an ovoid green cell covered by a solid wall called teak. It has a single large chloroplast and four identical flagella in two different pairs; they can form colonies during some stage of their life cycle [3]. Species of this genus have been found in marine and freshwater ecosystems worldwide [4]. These morphological characteristics observed through light and electron microscopy have been the basis for taxonomic studies of microalgae [5, 6].

During their life cycle, the genus Tetraselmis, acquires complex cellular characteristics that lead to confusion between taxa, but it has been insufficient for its identification [7-9]. Important results improved since the 90's, when the taxonomy of algae introduced the application of phylogenetic analyzes with molecular markers [5, 10].

Several molecular markers have been utilized to identify

microalgae such as: *rcbL, ITS1, ITS2, 18S rDNA, 23S rDNA* [6, 11, 12]. The *18S rDNA* gene is the most widely used, because its sequence has highly conserved flanking regions and repetitive arrangements which establish the phylogenetic relationship within distant organisms and between lineages and Phylum [4,13,10,14]. In this way, *Chlorella sorokiniana* [15], *Tetraselmis indica* [4], *Dunaliella salina* [8] and diatoms [16] were identified.

Commonly, the identification of genes is realized with DNA extraction, the amplification of a specific product and its sequencing [17, 18, 14]. However, mapping genes, using restriction enzymes, generates fast and reliable results instead of sequencing [19]. Bioinformatics programs can predict restriction patterns through *in silico* modeling, which is based on the sequence of the molecular marker and the relative location of the restriction sites for different enzymes [20-21]. *In silico* modeling provides the size of the DNA fragments and can subsequently be validated experimentally [22, 19].

This work is being proposed to compare *in silico* modeling with experimental restriction patterns based on the *18S rDNA* gene for the identification of a microalgae strain isolated from the Santa Elena Peninsula - Ecuador, morphologically identified as Tetraselmis.

2. Materials and Methods

2.1. Microalgae Strain and Cultivation

The sample was obtained from the Santa Elena Peninsula, Ecuador (2 ° 11'36.7 "S 80 ° 59'10.0" W) and registered with the code PM013 corresponding to the project INCYT-PNF-2017M3121. The strain was cultivated following a previous methodology [23]. The culture medium was prepared in 200 ml bottles based on an organic fertilizer (2% NPK) (Marchfoltm), containing sodium metasilicate as an inorganic source and enriched with vitamins (1 ml l^{-1}). The cultures were installed in triplicate for six days under the following conditions: temperature of 24 ± 0.5°C, a photon flux density of 60 µmol m^{-2} s^{-1}, provided by white light fluorescent lamps (Sylvania 18W), photoperiod 12/12 hours (light/dark), pH 7, shake manually twice daily, following a previous protocol [24]. Biomass was obtained by centrifugation at 13000 rpm.

2.2. Theoretical Molecular Analysis

2.2.1. Aligments and Primers Design

Eight sequences of the *18S rDNA* gene were selected from GenBank and aligned with the Clustal method using the ClustalW Alignment v2.0.12 program to obtain a conserved region and from this alignment, three Tetraselmis specific primers were designed: PM-001F *Forwards* (5'-GAGTGTTCAAAGCAAGCCTACG -3'), PM-016F *Forwards* (5´- AGAAACGGCTACCACATCCA -3´) and PM-016R *Reverse* (5´-TGTACAAAGGGCAGGGACGTAATC-3 '), using Geneious Prime® 2019 software. v1.3 and Oligo Calculator v3.27 [25].

2.2.2. In Silico Modeling of the 18S rDNA Gene

To obtain the Tetraselmis *in silico* restriction pattern, we used the consensus sequence from the *18S rDNA* and it was analyzed by the BioEdit program Map restriction and NEBcutter v2.0. The enzymes *BstUI, MspI*, *Rsal*, *BbvCI* and *Eco53kI* were selected.

2.3. Experimental Molecular Analysis

2.3.1. Total DNA Extraction

The extraction of the total DNA was carried out using a modifying TENS protocol [26]. In an eppendorf tube, 50 mg of Tetraselmis, 600 µl of TENS buffer and 2 µl of proteinase K were added and the solution was incubated at 37°C for 15 min. Then 200 µl of phenol and 200 µl of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 10000 rpm for 10 minutes, the supernatant was transferred to a new tube containing 500 µl of 95% ethanol frozen, the sample was left to rest at -20°C for 30 min and subsequently centrifuged. The pellet was washed with 800 µl of 75% ethanol frozen and finally resuspended in 100 µl of UltraPure ™ Water. The extracted DNA went through a purification process with sodium acetate (3M, pH 5.2) [27]. Then it was measured by spectrophotometry at an A_{260}/A_{280} wavelength with a 2000cc Nanodrop.

2.3.2. PCR Amplification

PCR experiments were carried out using primers PM-016F and PM-016R; other with PM-001F and PM-016R. The PCR reactions were performed in a total volume of 15 µl, containing 25ng of genomic DNA, 0.125 mM of each primer, 0.2 mM of dNTPs, 3mM of $MgCl_2$, 1 U Taq DNA Polymerase and Buffer 1X (All products from Invitrogen™) [28]. The PCR conditions were as follows: an initial denaturation of the template DNA at 94°C for 10 min, followed by 35 cycles of 94°C for 40s, 61°C for 30s and 72°C for 1 min 30s, and finished with a final extension at 72°C for 5 minutes using a thermal cycler (Bio-Rad T100). The PCR fragments were separated by 1% agarose gel electrophoresis with 0.5X GelRed (Biotium).

2.3.3. Digestion of the PCR Product

The PCR product was digested with the enzymes *BbvCI* and *Eco53kI* for the first reaction; *BstUI, MspI* and *Rsal* for the second. All enzymes were from Invitrogen™ and the performance was according to a previous protocol [19]. 10 µl of the PCR product was used for the enzyme reaction (1U/enzyme) in a final volume of 50 µl. The sample was incubated at 37°C for 2 hours. The product was run on a 1% agarose gel electrophoresis, with Sybr® Green staining and visualized in an UV Transilluminator.

3. Results

3.1. Sequence Alignment and Primer Design

In order to determine the conserved region of the *18S rDNA* gene, we selected eight pre-established sequences related to Tetraselmis, and we analyzed the genetic

relationship. The alignment showed that the species share a high percentage of similarity (99%) (Table 1). The conserved region was evident from 380 bp to 1680 bp.

Based on the conserved region, we designed three primers (PM-016F, PM-016R and PM-004F) to identify the Tetraselmis genus. The Geneious Prime® 2019 program V1.3 and Oligo Calculator v3.27 provided the optimal characteristics of the primers (Table 2).

Table 1. *Sequences of the genus Tetraselmis from GenBank used in this study.*

Sequences	Nº (bp)	Similarity data (%)	Nº Access (Genbank)
Tetraselmis sp.	1674	100	GQ917221.1
Tetraselmis chuii	1751	99.94	JN903999.1
Tetraselmis sp.	1701	100	AJ431370.2
Tetraselmis carteriiformis	1641	99.24	FJ559384.1
Tetraselmis striata	1674	99.26	JQ315739.1
Tetraselmis suecica	1674	100	JF489949.1
Tetraselmis subcordiformis	1719	99.01	KU561107.1
Tetraselmis marina	1669	99.77	KY045847.1

Table 2. *Characteristics of the primers designed for the amplification of the Tetraselmis 18S rDNA gene.*

Reaction	Primer	Sequences (5`→3`)	Lenght (bp)	G+C (%)	Tm[1] (°C)	Ta[2] (°C)	Product length (bp)
1	PM-016F	AGAAACGGCTACCACATCCA	20	50	59	61	1400
	PM-016R	TGTACAAAGGGCAGGGACGTAATC	24	50	62		
2	PM-001F	GAGTGTTCAAAGCAAGCCTACG	22	50	61	59	950
	PM-016R	TGTACAAAGGGCAGGGACGTAATC	24	50	62		

[1]Tm: temperature medium theoretical
[2]Ta: temperature hybridization used in this study

3.2. Quality of DNA Extracted and Amplification by PCR

The quality of the extracted DNA was 3200 ng μl^{-1} and its purity was 2.0 under the absorbance ratio A_{260}/A_{280}. Due to high concentration of DNA, it was diluted (1:25) to be used in PCR amplification.

From the theoretical analysis, the size of the first PCR product was 1400 bp with PM-016F/PM-016R and the second product was 950 bp with PM-001F/PM-016R. This would allow the amplification of much of the length of the *18S rDNA* in Tetraselmis species. The experimental results displayed the same desired bands (Figures 1 and 2), demonstrating that the PM013 strain would correspond to the Tetraselmis genus.

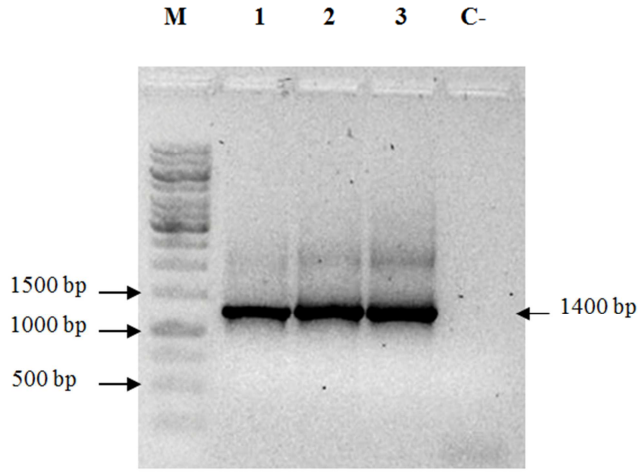

Figure 1. *PCR of 18S rDNAs amplified with PM-016F/PM-016R primers. Lanes 1 to 3: different samples of Tetraselmis (PM013). C-: negative control M: molecular weight marker (1kb Invitrogen™). Right arrow indicates the size of the fragment.*

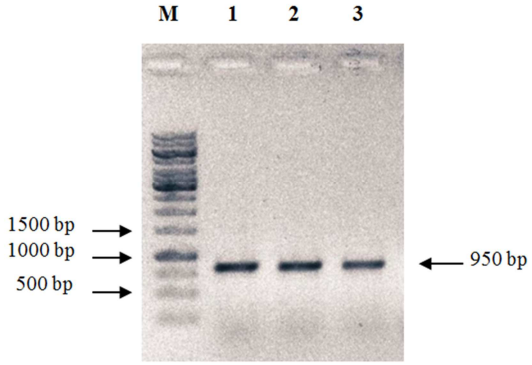

Figure 2. *PCR of 18S rDNAs amplified with PM-001F/PM-016R primers. Lanes 1 to 3: different samples of Tetraselmis (PM013). M: molecular weight marker (1kb Invitrogen™). Right arrow indicates the size of the fragment.*

3.3. Comparison of the in Silico Versus Experimental Restriction Pattern

The enzymes *BbvCI* and *Eco53kI* digested a PCR product of the first reaction and the enzymes *BstUI, RsaI* and *MspI* digested the second reaction. The *in silico* modeling predicted restriction patterns where each enzyme made more than two cuttings along it (Figure 3A and 4A).

The experimental restriction patterns showed fragments of similar sizes to the *in silico* modeling. The *BbyCI* performed two cuttings: 900 bp and 500 bp, while *Eco53kI* produced a fragment of 1000 bp and the other of 400 bp. The *BstUI* revealed three cuttings: a 700 bp fragment and another of approximately 200 bp; the 50 bp fragment was unseen. The *RsaI* enzyme produced two cuttings, the largest fragment was 900 bp and the small fragment was unseen in the 1% agarose gel. The *MspI* generated three cuttings, a 400 bp fragment and two overlapping fragments of approximately 250 bp,

while the smallest fragment was impossible to show (Figure 3B and 4B).

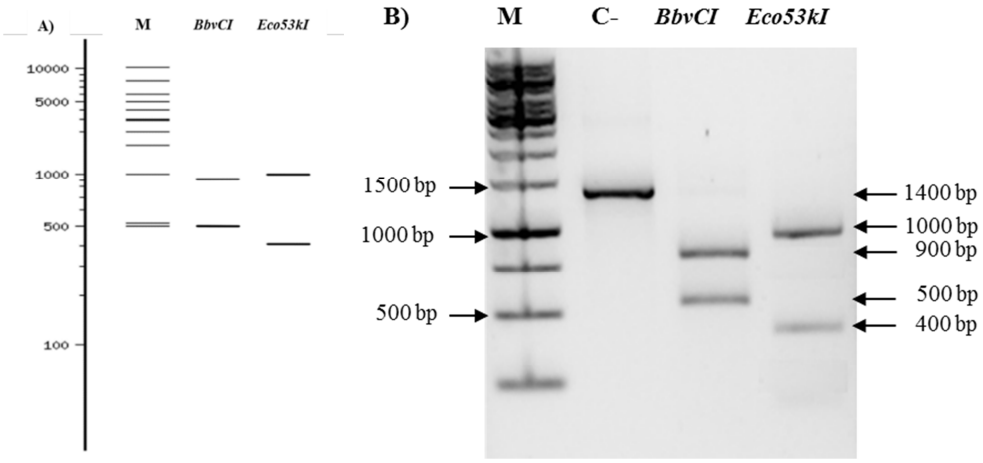

Figure 3. *A) In silico modeling of the restriction pattern of the 18S rDNA gene Tetraselmis digested with BbvCI and Eco53kI enzymes. B) Experimental restriction pattern of the 18S rDNA gene digested with BbvCI and Eco53kI enzymes. C-: undigested PCR product; M: molecular weight marker (1Kb Invitrogen™) 1% agarose gel. The arrows indicate the size of the fragments.*

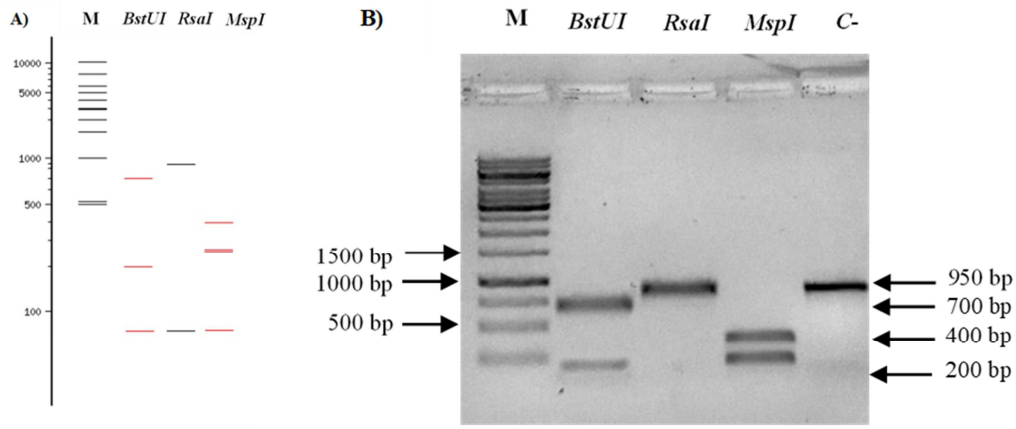

Figure 4. *A) In silico modeling of the restriction pattern of the 18S rDNA gene Tetraselmis digested with BstUI, RsaI and MspI enzymes. B) Experimental restriction pattern of the 18S rDNA gene digested with BstUI, RsaI and MspI enzymes. C-: undigested PCR product; M:–molecular weight marker (1Kb Invitrogen™) 1% agarose gel. The arrows indicate the size of the fragments.*

4. Discussion

We identified a *Tetraselmis* sp. microalgae strain isolated from the Santa Elena Peninsula, Ecuador, and compared it with *in silico* modeling and experimental restriction patterns based on the *18S rDNA* gene.

The sequence comparison allows us to infer that the similarity and evolution grade of a new molecule can be based on a database such as NCBI [29, 30]. Taking advantage of the existing information, we performed the alignment of eight sequences of the *18S rDNA* gene from various Tetraselmis species, to find the conserved region; this allowed us to obtain a more reliable result with real data. Furthermore, according to a previous work [13], sequence conservation is one of the most direct ways of estimating the precision of an alignment in computational biological models and the use of a bioinformatic program that provides us with accurate data such as Clustal W Alignment v2.0.12.

The conserved region of the Tetraselmis *18S rDNA* gene facilitated the design of the three primers (PM-016F, PM-016R and PM-001F) used in this study. According to a previous work, primer design should start with sequence alignment to ensure specificity [34].

The size of the *18S rDNA* gene in Tetraselmis has been described previously, and it oscillates around 2500 bp [32]. However, other studies found PCR products between 1000 bp and 1500 bp [33, 34, 28]. This evidence agrees with our results, because we amplified two products, one of 950 bp and the other of 1400 bp, this fact allowed us to take steps toward the molecular identification of the PM013 strain as a *Tetraselmis* sp.

A strong PCR amplification also depends on the quality of the template DNA. The TENS protocol has been efficient for the extraction of total DNA in microorganisms, especially in microalgae, because it contributes to the proteins denaturation of the cell wall that causes a total lysis of the cell [35], high concentration and purity [31, 26, 34]. We obtained DNA of high quality in concentration and purity which we recommend for molecular studies of microalgae.

In this era of new knowledge, the molecular identification

has contributed to deciphering species at any stage of their life cycle [9, 17, 16]. The PCR product has been generally sequenced and evaluated using bioinformatic programs that ensure the functional or phylogenetic relationship [13, 16, 14]. However, the benefit of using restriction enzymes could be an alternative technique based on the PCR product and is faster and reliable [21, 28, 19]. For example, Olmos, *et al.* (2000) implemented this technique in the molecular identification of *Dunaliella* sp [8]; while Chin, *et al.* (2018) and Pillacela, *et al.* (2020) performed an *in silico* analysis to obtain the restriction pattern before being experimentally evaluated *Pyrodinium bahamense* [34] and *Haematococcus* sp [19], respectively.

In our study, we predicted the *in silico* restriction patterns for two PCR products, and then we digested experimentally using the enzymes *BbvCI* and *Eco53kI; BstUI, RsaI,* and *MspI*. The results were satisfactory, the experimental restriction patterns were highly related to the *in silico* modeling, although small fragments (<100 bp) were unseen because they correspond to the ends of the sequence, left by the restriction enzymes cuttings, and are generally imperceptible and lack biological validity. The same variations were presented by Pillacela, *et al*. (2020) and Cienfuegos, *et al*. (2008) [19, 39].

Currently, *in silico* modeling is important in molecular biological studies [36, 18] because they predict reliable results and save time and money in the experimental stage [37, 38]. The high similarity between the restriction patterns obtained *in silico* and experimentally demonstrated the veracity of the results. This suggests the PM013 strain, isolated from the Santa Elena Peninsula Ecuador, is related to *Tetraselmis* sp. In addition, laboratories can work in basic molecular biology to identify organisms at the genus level.

5. Conclusions

Theoretical analyses allowed to find the conserved region of the Tetraselmis *18S rDNA* gene, thus it facilitated the design of primers PM-016F/PM-016R and PM-001F/PM-016R, which provided PCR products of 1400 bp and 950 bp, respectively. We could show similar fragments between the *in silico* modeling and experimental restriction patterns after the enzymes *BbvCI* and *Eco53kI; BstUI, RsaI* and *MspI* digested each product, which confirms that the PM013 strain corresponds to the *Tetraselmis* sp. Those results were supported by high quality DNA obtained with the TENS protocol, which allows us to suggest its usefulness in molecular biology studies of the Tetraselmis. Finally, we infer that bioinformatics applications and *in silico* modeling are essential before experimental tests of biological processes, in this work they allowed us a fast and effective response of restriction enzymes when carrying out molecular identification in a basic laboratory where sequencing work is difficult.

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