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OPTIMIZATION OF DNA ISOLATION AND PCR PARAMETERS FOR RAPD ANALYSIS OF *GONYOSTOMUM SEMEN* (*RAPHIDOPHYCEAE*)

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Abstract

Servienė E., Kemežienė I., Kasperovičienė J., Čapukoitienė B., Rančelienė V., Koreivienė J., 2012: Optimization of DNA isolation and PCR parameters for RAPD analysis of *Gonyostomum semen* (*Raphidophyceae*) [DNR izoliavimas ir PGR parametrų optimizavimas *Gonyostomum semen* (*Raphidophyceae*) dumblių RAPD analizei]. – Bot. Lith., 18(1): 40–45.

The genomic DNA purification method for *Gonyostomum semen* algae was optimized by applying different DNA purification techniques and rational modifications. This method allowed to obtain high quality DNA preparations suitable for the phylogenetic analysis and genetic variability investigation of algae. DNA isolated by this method yielded strong and reliable amplification products showing their applicability for RAPD-PCR using random decamer primers. In the present study, the RAPD protocol was optimized for the evaluation of *Gonyostomum* biodiversity.

Keywords: DNA extraction, algae, invasive raphidophytes, RAPD-PCR analysis.

INTRODUCTION

The number of reports on the spread of invasive raphidophytes microalgae *Gonyostomum semen* (Ehrenberg) Diesing to new localities of Europe, Asia, Africa, North and South America has been increasing in the recent decades (Laugaste & Nõges, 2005; Figueroa & Rengefors, 2006; Peczula, 2007). In fresh water bodies it has become a nuisance algae forming intensive blooms and secreting toxic substances that can cause itching and allergic reactions (Cronberg et al., 1988; Findlay et al., 2005). The genetic diversity combined with different physiological characteristics of the strains provide for a higher capacity of the species to respond to gradual environmental changes. In order to perform extensive molecular studies of freshwater *Gonyostomum* species, especially for assessing their genetic diversity and performing the phylogenetic analysis, it is necessary to improve DNA isolation methods and optimize RAPD-PCR conditions.

The isolation of high-molecular-weight DNA suitable for digestion with restriction endonucleases, cloning, hybridization, PCR amplification can represent a serious problem in many organisms, including algae. The procedure that works with one algal group will often fail with others, probably because of the differences in cell wall structure, abundant polysaccharide content and the compositions of secondary compounds complicating the preparation of nucleic acids (Joubert & Fleurence, 2005; Varela-Alvarez et al., 2006). Thus, an efficient protocol for DNA isolation is required in each particular case. The problems encountered in the isolation and purification of DNA, specially from algae, include DNA degradation due to endonucleases, consolation of highly viscous polysaccharides, inhibitor compounds such as polyphenols and other secondary metabolites, proteins, which directly or indirectly interfere with the enzymatic reactions (Philips et al., 2001; Yilmaz et al., 2009). Moreover, the contaminating

RNA that precipitates along with DNA causes many problems, including suppression of PCR amplification, interference with DNA amplification involving random primers, e.g., RAPD or AFLP analysis, and improper priming of DNA templates during thermal cycle sequencing. Most of the published methods of DNA extraction from green algae require grinding tissues in liquid nitrogen (Fawley & Fawley, 2004). For the separation of abundant polysaccharides, cetyltrimethylammonium bromide (CTAB) treatments, cesium chloride gradient ultracentrifugation or lithium chloride methods have been applied during DNA extraction (Doyle & Doyle, 1987; Varela-Alvarez et al., 2006).

In the present study, different Genomic DNA Purification Kits and several DNA extraction methods suitable for fungi, bacteria, plants and algae were tested. We established a genomic DNA isolation protocol optimal for *Gonyostomum* microalgae. The extracted DNA was further tested for RAPD analysis.

MATERIALS AND METHODS

Collection of phytoplankton samples. Phytoplankton samples for *Gonyostomum* molecular study were collected from three lakes located in the eastern (Lake Slabada, 55° 08′ 16.01″ N, 25° 42′ 27.53″ E) and western (Lakes Natalka, 55° 44′3 9.77″ N, 22° 13′ 04.60″ E and Rešketis, 55° 48′ 24.23″ N, 22° 11′ 11.03″ E) parts of Lithuania. The algal biomass was concentrated with a plankton net (20 µm mesh) during the intensive blooming of *Gonyostomum* in August and September 2010. After transportation to the laboratory on ice in the dark, the collected biomass was pelleted by centrifugation at 5000 g for 10 min, washed with TE buffer pH 7.4, the supernatant was removed and the pellet was frozen at -20 °C until genomic DNA isolation.

Genomic DNA isolation. The *Gonyostomum* biomass was subjected to various genomic DNA extraction protocols developed: 1 – algae (Bolch et al., 1999), 2 – plants (Murray & Thopson, 1980), 3 – MoBio Plant Kit, 4 – MoBio Soil Kit; 5 – Zymo Research Plant/Seed Kit, 6 – Zymo Research Fungal/Bacterial DNA Kit, 7 – Zymo Research Quick Genomic DNA Extraction Kit, 8 – Fermentas Genomic DNA Kit, 9 – our modified Genomic DNA extraction protocol. Protocol 9: 100 mg of algal biomass was mixed with 200 µl TE and 400 µl of lysis solution (Fermentas, Genomic DNA Kit) and incubated at 65 °C for 60 min with occasional inversion of the tubes. Then 600 µl of chloroform was added, gently emulsified by inversion several times and centrifuged at 10000 rpm for 10 min at room temperature (RT). The supernatant was transferred to a new tube, and DNA was precipitated by mixing with 800 µl of a freshly prepared precipitation solution (Fermentas, Genomic DNA Kit), followed by 10 min of incubation at RT and harvesting a pellet at 10000 rpm for 15 min. The supernatant was removed, and the pellet was dissolved in 100 µl of 1.2 M NaCl solution by gentle vortexing; 1 µl of RNase A (final concentration 0.1 mg ml^{-1}) was added to each tube, the samples mixed and incubated for 40 min at 37 °C, followed by adding 2 µl of Proteinase K (0.5 mg ml^{-1}) and incubation for 40 min at 50 °C. After incubation, equal amounts of chloroform and isoamyl alcohol in ratio of 24 : 1 (v/v) were added with a gentle inversion for 10 min and centrifuged for 10 min at 10000 rpm at RT. The aqueous layer was carefully collected, mixed with 300 µl of cold 96 % ethanol and after incubation for 30 min at -20 °C DNA was harvested by centrifugation for 15 min at 10000 rpm at 4 °C, followed by pellet washing with 200 µl of 70 % ethanol. Finally, the DNA pellet was air-dried, dissolved in 100 µl of sterile deionized water by gentle vortexing and stored at -20 °C.

Yield and purity of DNA. The yield of extracted DNA was measured using a UV spectrophotometer (Genesys) at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm and 230 nm. The concentration and purity of DNA were determined also by running the samples on 0.8 % agarose gel based on the intensities of band was compared with the Gene Ruler™ DNA Ladder Mix (Fermentas) marker.

RAPD analysis. The extracted genomic DNA was tested for PCR amplification and RAPD parameters optimization using oligonucleotide decamer primers from the A, B and C series. The reactions were carried out in a DNA Thermocycler (Biometra). Reactions without DNA were used as negative controls. The reaction mixture (20 µl) for RAPD contained genomic DNA (20 ng), 10x Taq buffer, 0.2 mM of dNTP mix, 0.5 µM of single primer, ulta-pure distilled water and 0.5 U of Taq DNA polymerase. The thermocycler was programmed for the initial denaturation step of 3 min at 94 °C, followed by 40−45 cycles of denaturation for 45 s at 94 °C, annealing

for 45−60 s at 32−57 °C and extension for 75 s at 72 °C. The final extension was performed at 72 °C for 6 min, and the samples were kept at a temperature of 4 °C. The amplification products were electrophoresed using 2 % agarose gels in 1x TAE buffer at 70 V for 3 hours. The gel was visualized by 0.5 μg ml^{-1} ethidium bromide staining and photographed under UV light using a Gel Documentation System (MiniBisPro).

RESULTS AND DISCUSSION

The genomic DNA extraction method developed for *Dinophyceae* algae was used for *Gonyostomum semen* following Bolch et al. (1999). Cells were disrupted in SDS/STE solution, following phenol/chloroform extraction, DNA precipitation and removing polysaccharides in the presence of 2 M NaCl. The application of this method allowed obtaining 20 to 28 μg of genomic DNA from 100 mg of the biomass. Protein impurities were removed quite effectively, but we failed to completely eliminate polysaccharides (Table 1, Fig. 1) inhibiting the PCR reaction.

Following DNA purification method for plant cells (Murray & Thopson, 1980) (after incubation with SDS, extraction with phenol/chloroform, additional incubation with RNazeA and ProteinazeK, two steps of extraction

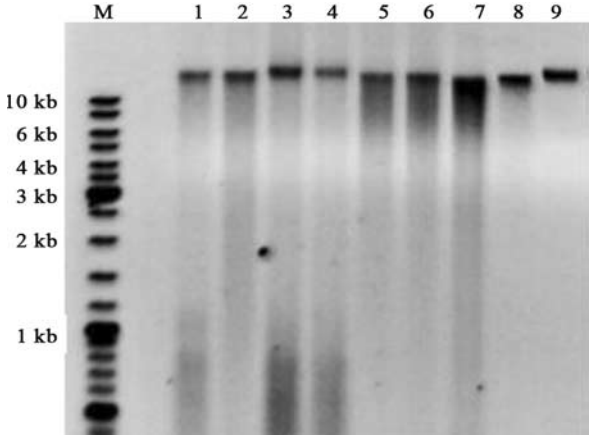

Fig. 1. Gel image of total genomic DNA isolated from *Gonyostomum* biomass. Genomic DNA extracted following the Protocols developed: 1 – for algae, 2 – for plants, 3 – using MoBio Plant Kit protocol, 4 – using MoBio Soil Kit; 5 – using Zymo Research Plant/Seed Kit, 6 – using Zymo Research Fungal/Bacterial DNA Kit, 7 – using Zymo Research Quick Genomic DNA Extraction Kit, 8 – using Fermentas Genomic DNA Purification Kit, 9 – using the modified Genomic DNA Extraction protocol, M- Gene Ruler DNA Ladder Mix (Fermentas)

Table 1. Yield and purity of DNA isolated from *Gonyostomum semen* by using different extraction methods

Method (No)	OD value (A 260 : 280 nm)	OD value (A 260 : 230 nm)	Yield (μg from 100 mg biomass)
1	1.6–1.8	0.9–1.1	20–25
2	1.5–1.7	0.4–0.6	30–35
3	1.2–1.5	1.1–1.4	30–40
4	1.3–1.4	1.3–1.5	15–20
5	1.5–1.7	0.9–1.1	30–80
6	1.4–1.6	1.0–1.2	30–80
7	1.3–1.5	1.1–1.2	30–80
8	1.4–1.6	1.4–1.5	50–60
9	1.7–2.0	1.8–2.0	45–50

with chloroform and final DNA precipitation in ethyl alcohol) up to 35 μg DNA from 100 mg of biomass was obtained, but the products were highly contaminated with polysaccharides (Table 1, Fig. 1). The introduction of additional incubation steps with 1.6 M NaCl − 13 % PEG6000 and 2 M NaCl (according to the Bolch et al., 1999) increased the purity of DNA, but the yield was very poor.

Application of the technology of genomic DNA extraction from plants, proposed by MoBio Company (cell disruption with glass beads and protein and polysaccharide removal by using several-column support) allowed us to obtain up to 40 μg of DNA. Employment of a MoBio Soil Kit gave a half as low DNA yield (only about 15−20 μg from 100 mg of biomass). According to electrophoretical and spectrophotometrical analysis data, the purity of the obtained DNA was tolerable, but not sufficient for performing PCR (Table 1). The gel images showed RNA contamination, which can cause many problems, including suppression of PCR amplification. However, the quality of DNA extracted using a Soil Kit was sufficient for the initial RAPD experiments (Fig. 2).

Zymo Research Plant/Seed, Fungal/Bacterial and Quick Genomic DNA Extraction Kits were used to obtain about 30 to 80 μg of DNA. DNA purity was similar to that obtained by Bolch et al. (1999) methodology (A 260/280 ratio was from 1.5 to 1.7, A 260/230 ratio – 1 to 1.2). Smears arranged from high to low molecular weight appeared on the gel images, indicating DNA degradation. The application of the column system allowed removing of the impurities inhibiting PCR reaction. However, RAPD spectra generated by a particular primer showed a high diversity among DNA samples

purified by different methods (Fig. 2). This demonstrates that such DNA is not applicable for RAPD analysis and genetic variability studies because of the misrepresentation of randomly amplified DNA fragments.

By applying the technology proposed by Fermentas for genomic DNA purification, 50 μg of DNA from 100 mg of algal biomass were obtained. The extracted DNA was analysed with a spectrophotometer and electrophoretically. We found that the standard method failed to completely remove the impurities (polysaccharides, RNA and proteins) (Table 1), but the obtained DNA was suitable for PCR amplification and RAPD analysis (Fig. 2). Therefore, some modifications of this protocol were introduced. At first, incubation time with extraction buffer at 65 °C was extended from 10 to 50 min to obtain a better cell lysis. Next, DNA dissolved in a 1.2 M NaCl solution was treated step by step, without changing the solution, with RNazeA (40 min at 37 °C) and ProteinazeK (40 min at 50 °C) for an effective removal of contaminants, saving the amount of extracted DNA and shortening the duration of the experiment. Then a traditional treatment with chlorophorm and DNA precipitation followed (see Materials and Methods for details). This optimization led to high-quality DNA preparations (A 260/280 ratio was about 1.8, A 260/230 ~ 2.0) suitable for the RAPD analysis. Protocol 9 registered better values for both parameters of genomic DNA than did the other eight protocols included in the study (Table 1). Also, the samples exhibited a single consolidated band without RNA contamination (Fig. 1) compared to the other protocols. Among the various protocols tried, the Fermentas genomic DNA Extraction Kit with modifications was found suitable for the extraction of a desired quantity and quality of genomic DNA from the *Gonyostomum* cells. Approximately 50−60 μg of DNA of 100 mg of *Gonyostomum* biomass was obtained employing this protocol, which is enough to carry out 2000−2500 typical RAPD reactions.

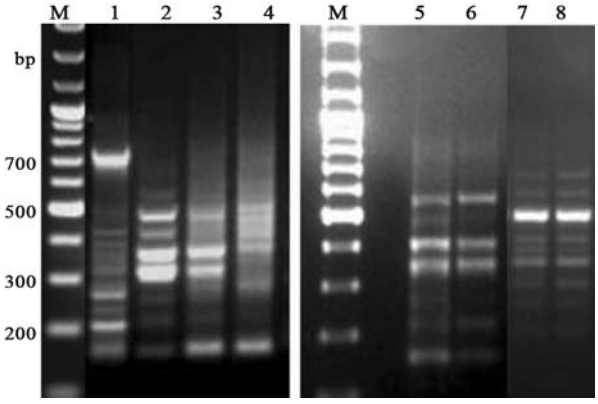

Fig. 2. RAPD-PCR assays conducted with primers OPB8-2 (lanes 1−6) and OPB14 (lanes 7, 8). DNA isolated with MoBio Soil Kit (lane 1), Zymo Research Plant/Seed Kit (lane 2), Zymo Research Fungal/Bacterial Kit (lane 3), Zymo Research Quick Genomic DNA Extraction Kit (lane 4), Fermentas Genomic DNA Kit (lanes 5, 7), using the modified Genomic DNA extraction protocol (lanes 6, 8), M- Gene Ruler DNA Ladder Mix (Fermentas)

The isolated DNA was tested in PCR amplification for RAPD profiling with several primers. The RAPD technology is a convenient, fast and efficient method for molecular typing. However, this assay is capable of reproducing the amplification of random fragments and generating a high degree of polymorphism only when used in well defined and optimized conditions. The RAPD-PCR technology is sensitive to changes in experimental parameters affecting amplification, banding pattern and reproducibility. Therefore, almost all the test parameters for RAPDs, such as the concentration of template DNA, primer, polymerase as well as tem-

Table 2. Optimization of RAPD-PCR reaction parameters for *Gonyostomum*

PCR parameter	Tested range	Optimal conditions
DNA concentration (ng)	1–150	20–40
Enzyme selected and concentration (U)	Taq pol (native & recombinant), Pfu pol, Hot start, Dream Taq pol 0.1, 0.5, 1, 2	Taq pol 0.5
Initial denaturation time (min)	1, 2, 3, 4, 5	3
Annealing temperature (°C)	30 to 60	50–52 for OPA primers 50 for OPB primers 46 for OPC primers
Annealing time (s)	30, 45, 60, 75, 90	45
Elongation time (s)	30, 60, 75, 90	75
Number of cycles	30, 35, 40, 45	40–45
Reaction volume (μl)	20, 50	20

perature and time intervals during the denaturation, annealing and elongation steps were optimized (Table 2). Variability in the DNA quality and quantity is the most common reason for differences in the RAPD profile. The standartization of DNA template concentration was also crucial to avoid artefacts on the band pattern. Therefore, by varying the concentrations of DNA within 1−125 ng, 20−40 ng was found to be the best. Lower concentrations of DNA template resulted in the absence of amplification, whereas higher concentrations gave smear and effect repeatability. The minimal-optimal primer concentration giving a proper RAPD pattern was 0.5 µM. An increased concentration leads to oligonucleotide dimer formation and influences RAPD generated products. The use of different DNA polymerases (Pfu, Dream Taq, Taq) also showed variations; hence, enzymes of appropriate quality and quantity should be used for reproducibility. The study demonstrated that 0.5 U of recombinant Taq DNA polymerase should be used for one reaction by performing 40−45 cycles of amplification. Considering the quality of RAPD products and the cost of PCR ingredients, 20 µl of the total reaction volume was selected. As RAPD variations may also be associated with the thermal cycle profile, the influence of the initial denaturation time, annealing temperature and time as well as of elongation time were evaluated (Table 2). An insufficient denaturation time leads to a reduced amplification and the lack of reproducibility. The optimal annealing temperatures for different groups of primers were as follows: OPA 50−52 °C, OPB 50 °C, OPC 46 °C. Higher or lower temperatures resulted in different specificity. These studies indicate a considerable possibility of increasing the efficiency of the RAPD-PCR by following the precise standartization protocol including the optimal concentrations of different components in combination with an appropriate thermal cycling. The present optimized protocol of *Gonyostomum semen* DNA isolation and RAPD techniques may serve as an efficient tool for the further molecular studies on microalgae.

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DNR IZOLIAVIMO OPTIMIZAVIMAS IR PGR PRADMENŲ ATRINKIMAS *GONYOSTOMUM SEMEN* (*RAPHIDOPHYCEAE*) TYRIMAMS RAPD METODU

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Santrauka

Ištyrus įvairias genominės DNR gryninimo technologijas ir pritaikius racionalias modifikacijas buvo optimizuotas *Gonyostomum* dumblių DNR gryninimo metodas, leidžiantis gauti aukštos kokybės DNR preparatus, tinkamus dumblių filogenetinei analizei ir genetinio kintamumo tyrimams. Gauti DNR produktai yra tinkami atlikti RAPD-PGR analizę, DNR pagausinimą, naudojant atsitiktinės sekos trumpus oligonukleotidinius pradmenis. Atlikus tyrimus buvo optimizuotas RAPD tyrimo protokolas, pritaikytas *Gonyostomum* dumblių biologinės įvairovės nustatymui.