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RESEARCH ARTICLE

ISOLATION OF GENOMIC DNA FROM *PORTIERIA HORNEMANNII*

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ABSTRACT

The Protocols followed for the isolation of genomic DNA from *Portieriahornemannii* have certain limitations due to the presence of polysaccharides and polyphenols in the sample. DNA isolated by these protocols is contaminated with a sticky and viscous matrix. In our modified DNA isolation method polysaccharides and polyphenols are removed prior to the precipitation of the DNA. Then the genomic DNA was precipitated using isopropanol. This protocol yielded a high molecular weight DNA isolated from fresh as well as dry leaves of *Portieriahornemannii*, which was free from contamination. Isolated DNA can be used for restriction digestion and PCR amplification. The main objective of the present protocol is to provide a simple method of isolation of DNA, using in house prepared reagents.

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INTRODUCTION

Portieriahornemannii is a species of red algae (Fuller *et al.,* 1992). Thalli are orange-red coloured, gelatinous, 3- 12 cm in height, overlapping flattened branches with discoid holdfast, irregularly tpinnate-alternate branching in one plane. The terminal branches at the distal porion of the thallus have slightly expanded curved or in-rolled tops. Gland cells are scattered in both nemathecial and normal cortical tissue (Andrews JH 1979). Growing on lower intertidal coral reefs to upper sub tidal zone exposed to strong wave action (Engels *et al.,* 2009). In this investigation an attempt was made to obtain a good quantity of DNA and good quality of DNA which can be helpful in successful construction of Phylogenetic tree.

MATERIALS AND METHODS

rBcl Gene Sequencing and Phylogenetic Studies

Purified genomic DNA was required for performing studies on genetic identification of *Portieria hornemannii.* It was extracted by using a modified method of (Moller *et al.,* 1992). This procedure was suitable for the extraction of good quality of DNA from Portieria *hornemannii.* The principle steps are as follows.

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Homogenization or Disruption of cells: The cells were lysed to release the nucleus by grinding using extraction buffer and Mercaptoethanol.

Inhibition of DNAase: Degradation of DNA by nucleases was by the addition of chelating agent, EDTA.

Dissociation of nucleoprotein complexes: DNA protein interactions was disrupted with Mercaptoethanol. Alkaline pH and high salt concentrations improved the efficiency of the process.

Removal of Contaminants: Proteins were removed with the treatment with Phenol, Chloroform and Isoamyl Alcohol.

Precipitation of DNA: The released DNA was precipitated with alcohol. The DNA in the aqueous phase was precipitated with cold ethanol. The precipitate was re-dissolved in buffer and treated with phenol to remove the last traces of protein followed by the re-precipitation in cold ethanol.

Reagents

Preparation of Solutions

Isolation buffer (1 litre)

- 0.35 M Sorbitol : 63.8 g
- 50 mMTris-HCl pH 8.0: 50 ml of 1 M Tris-HCI
- 5 mM EDTA : 10 ml of 0.5 M EDTA

- 0.1% BSA : 1 g
- Distilled water : to a final volume of 1000 ml
- Add 1 ml 2— mercaptoethanol just prior to use.

Wash buffer (100 ml)

- 0.35 M Sorbitol : 6.38 g
- 50 mMTris-HCl pH 8.0 : 5 ml of 1 M Tris-HCl
- 25 mM EDTA : 5 ml of 0.5 M EDTA
- Distilled water : to make a final volume of 100 ml

Lysis buffer (100 ml)

- 5% sodium sarcosinate (W/v): 50 ml of 10% Sodium sarcosinate
- 50 mMTris-HCL pH 8.0 : 5 ml of 1 M Tris-HCl
- 25 mM EDTA : 5 ml of 0.5 M EDTA
- Distilled water : 40 ml

DNase-I buffer (100ml)

- 0.35 M Sorbitol : 63.5 g
- 50 mMTris-HCl pH 8.0 : 50 ml of 1 M stock
- 15mM MgCl2 :3g
- DNase-I : 300 mg

Isopropanol (NaCl saturated)

- Saturate isopropanol with 10 mMNaCl and water.

1 M Tris-HCL

- Trizma base; 121.1 g
- Distilled water : 800 ml
- Concentrated HCL : 40-70 ml
- Adjust the pH to 7.0-8.8 with concentrated HCI.
- Add distilled water to make a volume of 1 litre.
- Depends into aliquots and sterilize by autoclaving.

0.5 M EDTA pH 8.0 (1 litre)

- (EDTA.2H2O)Na2: 186.1g
- (Ethylene diaminetetraacetatedihydrate-disodium salt)
- Add distilled water to make a final volume of 1 litre.
- Adjust he pH to 8.0 with NaOH (about 20 g of NaOH pellets).
- Depends into aliquots and sterilize by autoclaving.
- 5 M NaCl (1 liter)
- NaCl :92.2 g
- Add distilled water to make a final volume of 1 litre.
- Depends into aliquots and sterilize by autoclaving.

Pronase (20 mg/ml)

- Pronase :0.2 g
- Distilled water : 10 ml
- Self-digest or 2 hours at 37C.
- Depends into aliquots and store at -20 C.

Procedure

The following procedure was adopted for extracting good quality DNA from *Portieria hornemannii*.

Chloroplast isolation

- Keep the plants in the dark for 48 hours to reduce chloroplast starch levels.
- Collect young and healthy leaves.
- Wash leaves thoroughly in tap water and cool them to 0°C.
- Place 1 g of leaves (cut into small pieces) in 4 ml of ice-cold isolation buffer.
- Homogenize in a pre-chilled mortar and pestle for few minutes.
- Filter the homogenate through dense nylon mesh (50 uM).
- Centrifuge at 1000 rpm for 15 minutes at 4°C.
- Collect the supernatant and re-centrifuge for 20 minutes at 4000 rpm.
- R e-suspend the pellet in 400 uL wash buffer (for 1 g starting material) by vigorous swirling of the tubes.
- Centrifuge at 4000 rpm for 20 minutes. Collect the chloroplast pellet.

Isolation of Chloroplast DNA by DNase-l treatment method

- Re-suspend the chloroplast pellet in 1 ml of DNase-I butter (for 10 g starting material).
- Incubate on ice for 1 hour.
- Add three volumes of wash buffer and centrifuge at 2500 rpm for 15 minutes at 4°C.
- Re-suspend pellet in wash buffer. Repeat washing and centrifuging for two times.
- After final wash re-suspend pellet in 0.1 to 2.0 ml of wash butter.
- Add one-tenth volume of pronase (10 mg/ml self-digested for 2 hours at 37°C) and incubate for 2 minutes at room temperature.
- Gently add one-fifth volume of lysis buffer and mix by slowly inverting the tube several times over a period of 10-15 minutes at room temperature.
- Remove residual starch and cell wall debris from the chloroplast lysate by centrifuging at 10,000 rpm for 10 minutes at room temperature.
- Collect the supernatant. Mix intensely for 5 minutes with an equal volume of buffer equilibrated phenol.
- Centrifuge for 10 minutes at 12,000 rpm. Collect the upper aqueous phase.
- Add equal volume of phenol-chloroform (1:1) and repeat step 10.
- Collect the upper aqueous phase. Add equal volume of chloroform-isoamyl alcohol (24:1) and repeat the centrifugation step. »
- Precipitate the DNA with 1/10 volume of 5 M ammonium acetate and 1 volume of isopropanol at -20°C for 2-3 hours or overnight.
- Centrifuge for 3-5 minutes at 12,000 rpm.
- Wash the pellet with 70% ethanol, air dry and dissolve in 20 ul of TE buffer.

Quality check of DNA by AGE

The quantified DNA was assessed for quality by agarose electrophoresis using AGE. Agarose submersible gel electrophoresis was carried out on 1.0 percentagarose gel to

resolve the genomic DNA isolated for its quantity and quality check. The technique consisted of separation on 1.0% agarose gel electrophoresis of DNA fragments and visualization of DNA fragments.

Preparation of 1.0% Agarose gel:

Agarose is a linear polymer extracted from seaweed (Red Algae – *Graccillaria* seen in marine water). Purified agarose is a powder insoluble in water or any buffer at room temperature, but dissolves on boiling, then is poured into a mould and allowed to solidify on cooling. Agarose undergoes polymerization that is sugar polymers cross link with each other and converts it to the gel state. The density or the pore size of the gel is determined by the concentration of agarose. The reagents used and the procedure for the preparation of agarose is as follows.

Reagents

1. TAE Buffer (50X): 2M Tris Acetate + 0.05M EDTA (Set pH 8.3)
2. Agarose gel: Weigh 0.5 g of agarose to 50ml of 1X TAE buffer.

Procedure

1X TAE buffer was prepared by diluting approximate amount of 50X TAE buffer. 0.5g of agarose was added to 50ml of 1X TAE buffer. The above mixture was heated to boiling until the agarose dissolves to get a clear solution. Once the temperature of the agarose solution reached 60ºC, 10µl of Ethidium Bromide was added and mixed gently using micropipette.

Electrophoresis of DNA fragments

Electrophoresis is a technique used to separate charged molecules. The matrix of the agarose gel acts as a molecular sieve through the negatively charged DNA (at neutral pH) fragments move on application of electric current and when the electric field is applied across the gel it migrates towards the anode. The comb of an electrophoresis set was placed in the gel apparatus, approximately 2cm away from the cathode. The prepared agarose was poured into the central part of the tank with care to avoid generation of air bubbles in the process. The gel was then allowed to solidify. 1X TAE buffer was poured into the gel tank to a height of 0.5 – 0.8 cm above the surface of the gel.

The comb was gently lifted ensuring that the gel remained intact. Power cord was connected to the electrophoretic power supply and the samples were loaded into the wells. The power supply was switched on to run the gel at 50volts. The sample was allowed to run till the tracking dye-bromophenol blue reached the base of the gel nearer to the anode.

Visualisation of DNA fragments

DNA is not naturally coloured, so it is not visible in the gel. The inter-calating agent – Ethidium Bromide helps in the visualisation of the fragments of DNA when the gel is placed in an UV transilluminator. The gel was carefully taken and placed on the Gel Doc Alphaimager HP and banding pattern was analysed using Alphaease software.

Amplification of DNA

PCR was used to amplify a specific region of DNA in order to produce a large number of identical copies (William *et al.,* 1990). The method uses a heat stable DNA replication enzyme called a DNA polymerase, the four deoxy nucleotide building blocks of DNA and two small single stranded DNA segments called primers, which flank the "target" region of DNA, are amplified and are complementary to each strand. There are 3 basic steps in the PCR that are carried out at different temperature to create conditions optimal for:

- DNA Denaturation: it is used to separate the double stranded DNA into single strands.
- Annealing: Primer binding or hybridization to each of the single strands of DNA at either the beginning or the end of the target sequence, depending upon the single strand of DNA. Hybridization combines complimentary single stranded DNA into a single molecule.
- DNA polymerase elongation: The enzyme attaches to the primer single stranded DNA duplex and synthesizes the complimentary strand of DNA, using the existing single strand as a template. Newly synthesized DNA strands can serve as additional template for complimentary strand synthesis. PCR rapidly amplifies DNA, because both strands are copied, there is an exponential increase in the number of copies.

Assuming there is only a single copy of the target gene before cycling starts, the following could be inferred.

Cycle→Single strand copy number
Cycle 1→4 copies
Cycle 2 →8 copies
Cycle 3 →16 copies
Cycle 35→68.7 billion copies

After 35 cycles of PCR there will be over 68 billion copies. In reality, PCR starts with many copies of the target gene, so the end result is typically higher. Each cycle takes a few minutes. Factoring in the time to change temperatures, the entire process can be done in several hours.

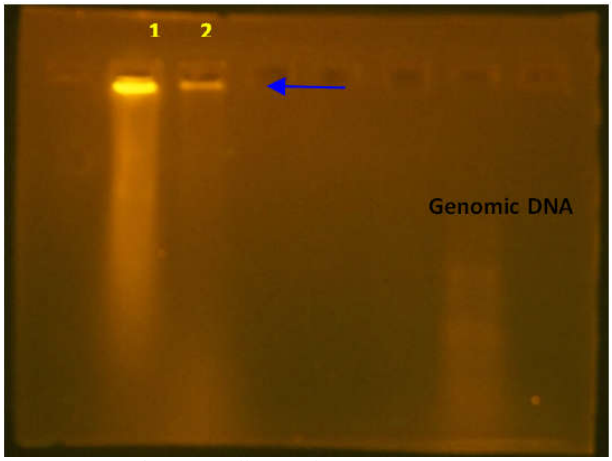
Visualization of amplified DNA

After the completion of PCR amplification, the amplified samples were resolved on agarose gel. Agarose gel (1.0%) was casted with1x TAE buffer and the samples were loaded on the wells. The samples were loaded with 2µl of loading dye containing TBE buffer, Glycerol and Bromophenol blue. Electrophoresis was carried out at 60volts for 4 hours. After the gel were documented in gel documentation system GEL DOC - Alphaimager HP using Alphaease software.

RESULTS AND DISCUSSION

Genetic identification of *Portieria hornemannii* was performed using CTAB (CetylTrimethyl Ammonium Bromide) method. The genomics DNA was isolated by following the modified method of Moller *et al.,* 1992. The good quantity of DNA was obtained through is method with a good molecular weight. The DNA yield obtained was about 6 - 10µg. Polymerase chain reaction was performed in a thermo cycler to produce multi copies of a specified DNA using the reaction mixture. Nearly 700 base pairs was present in the sequence obtained from the

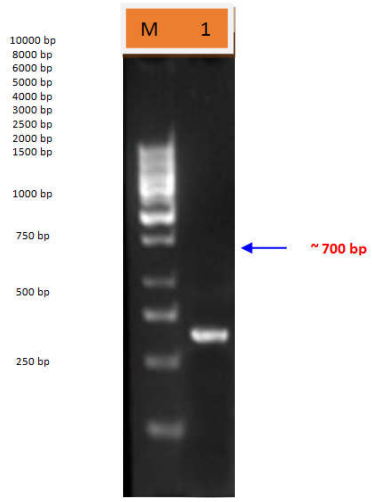
PCR amplification through the *rBcl* primer (Figure 1 and 2).The sequences of these 18S rRNA regions were compared against the sequences available from GenBank using the BLASTN program (Altschul*et al.*, 1990) and were aligned using CLUSTAL W software (Thompson *et al.,* 1994). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei and Kumar, 2004) and are in the units of the number of base substitutions per site. Phylogenetic trees were constructed using the Neighbor-Joining method (Saitou and Nei, 1987) (Figure:3).



(Lane 1 & 2: P. hornemannii genomic DNA)

Figure 1. Genomic DNA from given algal sample

Bootstrap analysis was done based on 1000 replications. The MEGA4 package (Tamura *et al.,* 2007) was used for all analyses. This evolutionary history was inferred using the UPGMA method (Sneath *et al*., 1973). The optimal tree with the sum of branch length = 0.46500602 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 679 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.,* 2007).



(Lane M: 1 KB DNA Ladder; Lane 1 : Sample)

Figure 2. PCR amplification of algal sample using 18S rRNA gene

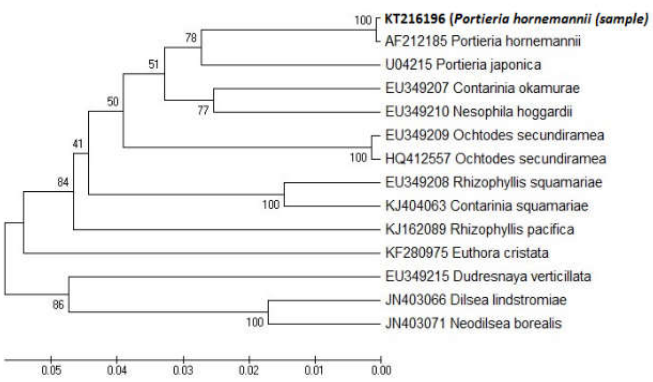


Figure 3. Evolutionary relationships of 14 taxa

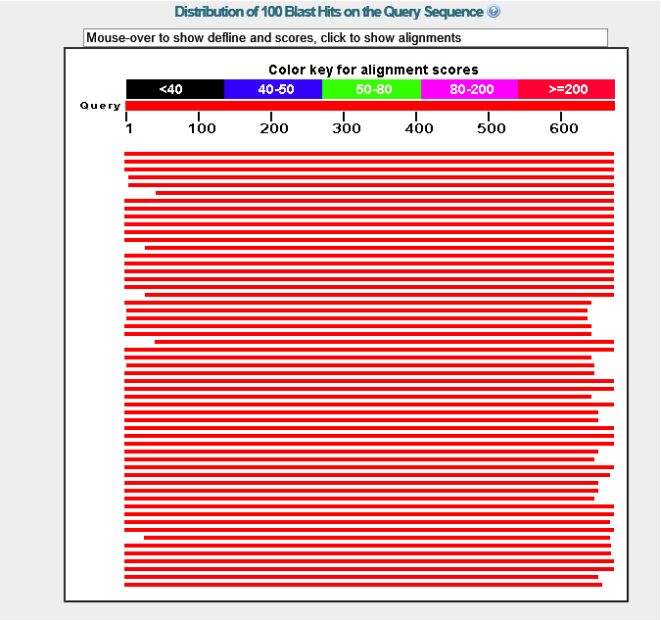


Figure 4. BLAST Analysis of *Portieriahornemannii*

Based on the BLAST analysis (Figure:4) and phylogeny analysis clearly revealed that the given algae sample was belongs to the taxa *Portieria hornemanii*. The sequence derived was succesfully deposited in NCBI database with the Acession Number KT216196.

>gi|927029234|gb|KT216196.1| Portieriahornemanii ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene, partial cds; chloroplast

GATCCTGATTATGTAGTAAAGGATACGGATATACTAGCATTATT
CCGTGTAAGTCCACAACCAGGTGTTG
ATCCAATAGAAGCTTCTGCTGCTGTTGCGGGTGAGTCATCTACA
GCTACTTGGACTGTAGTTTGGACAGA
TCTTTTGACAGCATGTGATTTATATAGAGCTAAAGCTTATAAAG
TAGATTCCGTACCTAATGCAACAGGT
CAGTATTTTGCTTATATTGCATATGATATTGATTTATTTGAAGA
GGGATCTATTGCTAACCTAACAGCAT
CAATTATTGGTAACGTTTTTGGTTTCAAAGCTGTTAAAGCTCTA
AGATTAGAAGATATGCGTATACCTTT
TGCTTATTTAAAGACTTTCCAAGGTCCTGCAACAGGAGTTATTG
TAGAACGTGAACGCATGGACAAGTTT
GGTCGCCCATTTCTAGGTGCAACTGTAAAACCTAAACTAGGTTT
ATCAGGGAAAAACTATGGTCGAGTAG
TATATGAGGGTCTTAAAGGTGGATTAGATTTCCTAAAAGATGAT
GAAAATATTAACTCTCAACCTTTTAT
GCGCTGGAAAGAGAGATTTTTATATTCAATAGAAGGTGTAAATC
GTTCCATTGCTGCCTCAGGTGAAGTA
AAAGGTCATTATATGAATATTACAGCAGCCACTATTGAAGATAT
G

Several studies have been already published on the biogeography and phylogrnetic studies for various seaweeds (Verlaque *et al*. 2003, Meusnier *et al*. 2004). The DNA obtained from the CTAB method was good enough in quantity for PCR amplification but the quality of the DNA is not so good for the construction of the phylogenetic tree. Even though there are several methods available for the isolation of the DNA from the algal sample, some alga yield DNA which cannot be used for the PCR amplification or for other restriction enzyme (Hong *et al.,* 1992). In some seaweed species, DNA obtained will not show good results in the PCR amplification due to the presence of inhibitors for DNA polymerase. These cytosolic inhibitory activity in the PCR reaction is associated with the anti-viral and anti-tumor effect of the seaweeds from which the DNA extraction was done. (Kim *et al*. 1999, Cann *et al*., 2000, Eitsuka and Nakagawa, 2004). But in this investigation a good quality and a good quantity of DNA was obtained which was helpful for both PCR Amplification and Construction of Phylogenetic tree.

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