MODIFICATION OF PROTCOL FOR ISOLATION AND PURIFICATION OF GENOMIC DNA

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Abstract

A simple and efficient modified protocol for the extraction of high quality of genomic DNA from roots of *Salacia oblonga* collected from the three different places (Agumbi, Jogimat, Badsii) in Kerala, has been developed. The important modification steps in this CTAB (hexa decacyl trimethyl ammonium bromide) protocol include (a) use of 3.5 M NaCl in extraction buffer, (b) 2M NaCl during precipitation (c) Tris – saturated phenol in place of phenol : choloroform : isoamyl alcohol at purification phase (d) 80% ice cold ethanol for DNA precipitation and at last (e) performing all the reactions at room temperature. The DNA thus extracted from roots have 1.36, 1.7, 1.26 OD at A 260 /280 and the yield was 68, 87, 143 µg/g material. The extracted DNA was found to be suitable for DNA fingerprinting technique, RAPD (Random amplified polymorphic DNA) for development of molecular markers studies on genetic diversity. Three different samples (Agumbi, Jogimat, Badsii) in Kerala were subjected to RAPD analysis using 15 different random decamer primer. Out of 15 primers, the amplification was obtained only in 3 primers producing 29 bands showing the greatest genetic polymorphism between the samples.

Key Words: ***Salacia oblonga*, DNA isolation, CTAB, RAPD, Genetic variation**

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INTRODUCTION

Understanding the distribution of genetic diversity among individuals, populations and gene pools is crucial to efficiently manage the variability in germplasm collections for breeding programs (Glazzman and D"Hont ., 2001). Molecular markers are commonly used by plant biologists to perform a number of tasks, including the genetic finger printing of plant varieties, determining similarities among inbred varieties, mapping of plant species (Parab *et al*.,2008). DNA markers can be used for genetic fingerprinting, estimating genetic diversity, marker assisted selection in plant and animal breeding, and facilitating the map – based cloning of genes.

New techniques for the extraction, purification and amplification of plant DNA are being developed on a regular basis, enabling researchers to decrease preparation time and obtain readily reproducible results. Morphological markers are routinely used for genetic diversity analysis, but recently many molecular marker techniques have developed into powerful tools to analyze *Salacia oblonga (ponkoranti)* genotypes (Ayana,2004).

Recent pharmacological studies have demonstrated that *S. oblonga* induced improvement of type 2 diabetes and obesity associated hyperglycemia, dyslipidemia and related cardiovascular complications seen in human and rodents (Huang et al., 2007). *Salacia oblonga* has been marketed for preventing type 2 diabetes, as well as for aiding in weight loss. However, there is no molecular evidence of these benefits of. *Salacia oblonga.* Further molecular research is necessary to determine whether S. *oblonga* is a most useful in the treatment of diabetes (Augusti *et al.*, 2007).

Ecological and geographical differentiations are important factors that influencing strategies of breeding and sampling. To access the genetic diversity of S.oblonga, samples from different zones in Kerala, were collected for the study. It *is* a woody plant found in the forests of India and Sri Lanka. A large woody climbing shrub, branch lets cylindrical, densely sprinkled with lenticels, hairless. Leaves are simple, opposite (or) elliptic oblanceolate 7-15 x 3-5 cm base acute, apex acule (or) acuminate, margin toothed with

rounded to saw like teeth hair less, lateral nerves 7-9 Pairs prominent beneath leaf stalks 5-10 mm long(Vaidyaratnam,1996).

Salacia oblonga contains two potent alpha – Glucosidase inhibitors, Salicinol and kotalanol (Wolever et al., 1998). Methanol extracts from the roots of *S. oblonga* exhibit an inhibitory effect on the increase of serum glucose level in sucrose and maltose – loaded rats (Frandsen et al.,2002). *S. oblonga* has also been found to show inhibitory activity on aldose reductase which is related to such chronic diabetic complications as peripheral neuropathy, retinopathy and cataracts (Heacock et al.,2005).Recently it has been found that alpha – glucosidase inhibitors are potent therapeutic agents in carbohydrate – metabolic disorders such as diabetes mellitus (Matsuda *et al.*, 1999). Salacinol, Kotalanol and kotalagenin 16 acetate were found in the root of *S. oblonga* (Matsuda *et al.*, 1999). The hot water extract of *S. oblonga* contained 1.4% or 0.74% of mangiferin (MA), which was identified and quantified by a HPLC method (Li *et al.*, 2001) and as mangiferin is a prominent component in various *Salacia* species (Li *et al.*, 2001). The root bark is used for treatment of gonorrhea rheumatism and skin diseases (Vaidyaratnam, 1996). The present study was undertaken to develope simple and efficient method and to standardize the protocol for extraction, isolation and purification of genomic DNA from roots of the *S. oblonga* which was further used for molecular marker studies.

MATERIALS AND METHODS

Plant Material

S. oblonga is an important medicinal plant, which is found in all over India and Sri Lanka.Plant materials were collected from three different localities, such as Agumbi, Jogimat and Badsii in Kerala. For the present investigation the root materials were separated and it was stored at – 20^0C. This study was carried out in Biozone Research Technologies, Chennai.

DNA extraction

Fresh young *r*oot materials plucked from genetic garden were rinsed with distilled water and blotted gently with soft tissue paper; 0.1g of plant tissue, pre-cooled using liquid nitrogen, was ground to a fine powder with a mortar and pestle along with 10 mg (2% of extraction buffer) of polyvinylpyrrolidone (PVP) (Sigma). The extraction buffer (pH 8.0) contained 2% CTAB, 100mM Tris-HCl, 3.5 M NaCl 20mM EDTA, 0.2 M β-mercaptoethanol and 2% PVP. The powdered tissue was scraped into a 2ml microcentrifuge tubes containing preheated (65^0C) extraction buffer in a 1:5 ratio (0.5ml). β-mercaptoethanol was then added to the final concentration of 0.2 M and mixed well.

The mixture was incubated in water bath at 65^0C for 90 min and cooled for 5 min. An equal volume of chloroform: isoamyl alcohol mixture (24:1) was added to the extract and mixed by gentle inversion for 5 to 10 min to form and uniform emulsion. The mixture was centrifuged at 8000 rpm for 8 min at room temperature. Chloroform: isoamyl alcohol extraction step was repeated again. The aqueous phase was pipetted out gently, avoiding the interface.

To the above solution, 5 M NaCl (to final concentration 2M) and 0.6 volume of iso-propanol of the total solution was added and incubated at room temperature for 1 hour. To the above solution, two volumes of 80% ethanol was added and incubated again for 10 min at room temperature for DNA precipitation. After incubation, the mixture was centrifuged at 10000 rpm for 15 min. The white translucent pellet was washed with 70%ethanol, dried and resuspended in 200 µL of TE buffer (Harevey *et al*., 1994).

The sample was then treated with 20 µL of 10 mg/ml of RNase and incubated at 37°C for 60 min. After incubation with RNase, one volume of Tris saturated phenol (pH 8.0) was added and mixed gently by inverting the microcentrifuge tube till it formed a milky white emulsion. The emulsion was centrifuged at 10000 rpm for 5 min at room temperature. The supernatant was pipetted out into a fresh tube. The sample was then extracted with equal volumes of chloroform: isoamyl alcohol (24:1) twice. The DNA was

reprecipitated with 0.6 volumes of isopropanol, 2.0M NaCl (final concentration) and incubated for 10 min. To the above, 20 μL of sodium acetate and 1 volume of 80% ethanol were added, incubated for 30 min, and centrifuged at 10000 rpm for 15 min to pellet the DNA. The pellet was then washed with 70% ethanol twice; air –dried and finally suspended in 40-50 μl of TE buffer (Roman *et al.*, 1994).

Quantification of DNA

The yield of the extracted DNA was quanitified by spectrophotometer at 260 nm and the purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm according to the procedure of (Sambrook *et al.*, 1989). DNA concentration and purity was also checked by running the samples on 0.8% agarose gels along with standard 3 Kb marker (Biogene, USA 2000).

Agarose gel electrophoresis was carried out to separate, identify and purify the DNA fragments. RAPD amplification was generally followed by the method (William *et al.*, 1990) in thermal cycler (Eppendorf, Germany) using 15 decamer random primer (Table-1). Each tube contained 25 μl of reaction mixture.

Results and Discussion

Different CTAB extraction methods (Khanuja *et al.*, 1993; Michiels *et al.*, 2003) gave very less quantity of DNA with loads of polysaccharide and protein contamination. The extracted sample upon electrophoresis, gave weak bands, uneven migration, and often remained in the wells during electrophoresis as reported in the literature (Adames, 1991).This is due to the presence of polysaccharides and proteins (Sharma *et al.*, (2002) . DNA was seen as sharp bands in the present study (Fig. 1. lane I – IV). In extraction and purification phases, salts got precipitated along with the DNA and quality of the DNA decreased. With the available protocols, attempts for extraction of good quality DNA from S.oblonga were unsuccessful which made us to search for the new extraction protocol. One of the most significant steps of our protocol was the use of only Tris – saturated phenol (pH 8), followed by chloroform: isoamyl alcohol extraction. By using

Tris- saturated phenol (pH 8) followed by chloroform: isoamyl alcohol (24:1) extraction, protein impurities could be successfully removed, without affecting DNA yield.

Three different primers used for this study and size of amplification products varied, which are shown in Fig. 2, 3, 4 and Tables 2, 3, 4.The maximum numbers of bands (10) were produced from S3 sample and 8 bands were produced from S1 and S2 samples by the primer OPA7. Whereas minimum number of band (only one) was produced from S1 sample and 4 bands were produced from S2 and S3 samples by the primer OPN12. The primer OPN14 produced maximum number of amplified products in the sample S2 and S3.

Some of the primers produced polymorphic bands that are specific to a set of genotypes. Minimum number of 50 different loci should be used to estimate the genetic distances. Fernandez *et al.* (1999) used 18 primers and 77 polymorphic bands were detected that showed 77.5% polymorphism in 42 varieties.

For the present work, root samples of *Salacia oblonga were* collected from three different sites: Agumbi (S1), Jogimat (S2) and ADsii (S3) in Kerala. The samples were analyzed for genetic diversity using 15 random decamer primers. In this study, out of fifteen, three (OPN14, OPA7 and OPN12) chosen for the analysis based on clear and well resolved RAPD pattern, which are shown in the figure 2, 3, 4 and table 5 respectively. Thus, the present investigation concluded that the extraction, isolation and purification of genomic DNA are most important for RAPD analysis, which suggested that RAPD is appropriate for analysis of genetic variability. Moreover, RAPD technique differentiates the plant *S. oblonga* collected from three different places (Agumbi, Jogimat, Badsii) in Kerala though belonging to the same agro-climatic sub zones. But higher genetic variability may exists in different zones of where *Salacia oblonga* grown. It could be worked out in future studies by using various techniques of molecular biology to find out the morphological and genetic diversity.

Table 1

List of primers used in the study along with their sequences and some characteristics of the amplification products.

S. No	Primer code	Sequence 5' to 3
1	OPA –05	AGGGGTCTTG
2	OPA – 07	GAAACGGGTG
3	OPA – 09	GGGTAACGCC
4	OPA- 10	GTGACGTAGG
5	OPA – 11	CAATCGCCGT
6	OPC – 07	GTCCCGACGA
7	OPC – 09	CTCACCGTCC
8	OPC – 10	TGTCTGGGTG
9	OPC – 11	TTCCCCCCAG
10	OPC – 20	ACTTCGCCAC
11	OPN – 11	TCGCCGCAAA
12	OPN – 12	CACAGACACC
13	OPN – 13	AGCGTCACTC
14	OPN – 14	TCGTGCGGG
15	OPN - 15	CAGCGACTGT

Table 2: Quality and Quantity of Genomic DNA extracted from *S. oblonga* root collected from three different places in Kerala State.

S.No.	Samples	Absorbance 260nm	Absorbance 280nm	Ratio 260/280nm	Yield (µg/g)
1	S1[Agumbi]	0.136	0.100	1.36	68
2	S2[Jogimat]	0.174	0.100	1.7	87
3	S3 [Badsii]	0.268	0.162	1.26	134

Table 3: Primer OPN 14 that produced specific bands with respect to different samples

Band Size (bp)	Samples		
	S1 (Agumbi)	S2 (Jogimat	S3 (Badsii)
250	0	1	1
200	1	0	0
300	1	1	1
500	0	1	1
600	1	1	1
700	0	1	1
750	1	0	0
900	0	1	1
1000	0	1	1
1400	0	1	1
1600	0	1	1

0 - absence of bands

1- Presence of bands

Table 4: Primers OPA-7 that produced specific bands with respect to different samples

Band Size (bp)	Sample		
	S1 (Agumbi)	S2 (Jogimat)	S3 (Badsii)
250	0	0	1
200	1	0	0
400	0	1	1
500	1	0	0
600	1	1	1
700	1	1	1
750	1	1	1
800	1	1	1
900	1	1	0
1300	1	1	1

1500	0	0	1
1300	0	1	1
1400	0	1	1

0- absence of bands

1 - presence of bands

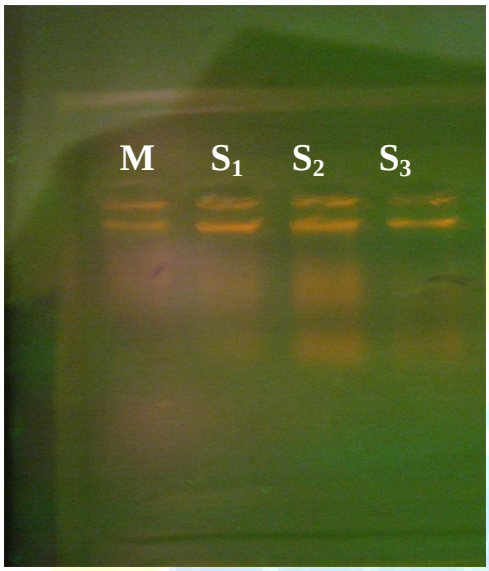
Table 5: Primers OPN-12 that produced specific bands with respect to different samples

Band Size (bp)	S1 (Agumbi)	S2 (Jogimat)	S3 (Badsii)
250	0	1	1
500	0	1	1
600	1	0	0
750	0	1	1
1000	0	1	1

Table 6: Primer amplification products of *S. oblonga*

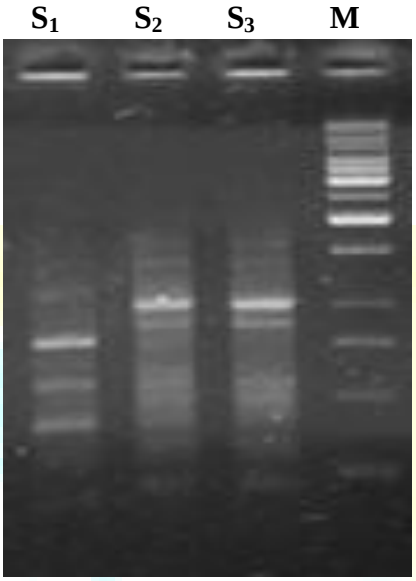
S. No.	Primer	S1 (Agumbi)	S2 (Jogimat)	S3 (Badsii)	Total
1	OPA-7	8	8	10	13
2	OPN-12	1	4	4	5
3	OPN-14	4	9	9	11
Total No. of Bands					**29**

Fig:1 Electrophorotic separation of genomic DNA extracted from *Salacia oblonga* using modified CTAB Protocols.



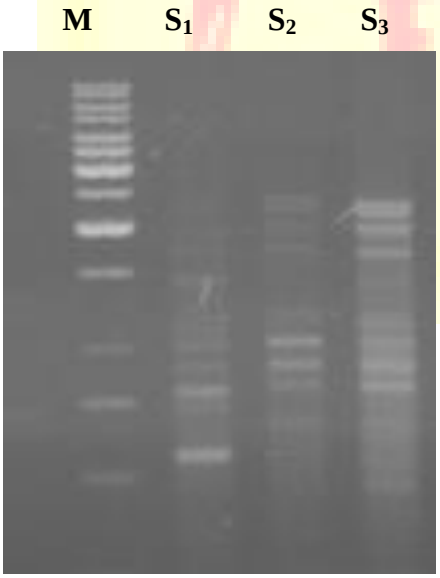
M - Marker DNA S_1 - Agumbi
S_2 - Jogimat S_3 - Badsii

Fig:2 Gel picture showing the RAPD pattern of *Salacia oblonga* from different places generated by RAPD primer OPN-14.



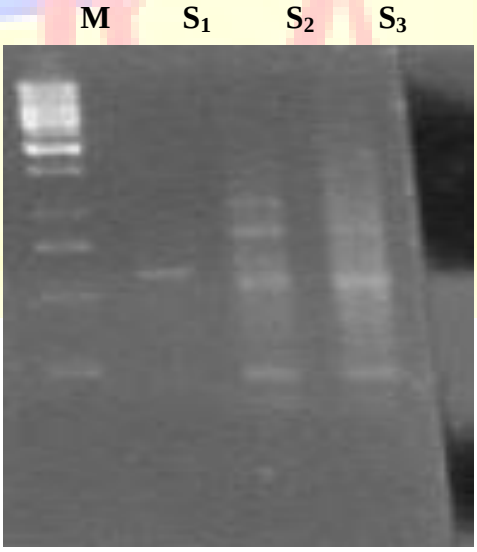
S_1 – Agumbi S_2 - Jogimat
S_3 - Badsii M - Marker DNA

Fig:3 Gel Picture showing the RAPD pattern of *Salacia oblonga* from different places generated by RAPD primer OPA-7.



M - Marker DNA S_1 - Agumbi
S_2 – Jogimat S_3 - Badsii

Fig:2 Gel picture showing the RAPD pattern of *Salacia oblonga* from different places generated by RAPD primer OPN-14.



M - Marker DNA S_1 - Agumbi
S_2 – Jogimat S_3 - Badsii

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