Standardization of Genomic DNA Isolation Method from mature leaves and SSR-PCR conditions for Flemingia semialata an Alternative Lac Host Plant

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Abstract

Flemingia is an important multipurpose leguminous perennial shrub, grows from 0.5 to 2.5 meters and sometimes upto 3 meters. It is one of the most suitable plant species for kusumi lac strain which produces best quality lac in the world. To develop improved and productive varieties of *Flemingia spp.*, it was needed to know the genetic diversity present in the germplasm and breeding material. Molecular markers are best tool to study the genetic diversity present in breeding population and to utilize molecular markers, genomic DNA of high quality and quantity was required. To obtain high quality and quantity of genomic DNA at lesser cost, it was needed to standardized a robust genomic DNA isolation protocol. Therefore, in the present study, the DNA isolation protocol of Murray and Thompson (1980) was modified and optimized. The standardized protocol yielded 1200–1500 ng/µl genomic DNA with an average of 1359 ng/µl DNA from 40 samples of *F. semialata* and *F. macrophylla*. A very low level of RNA, protein, phenolics and polysaccharide contaminants were recorded (A_{260}/A_{280} ratio ranges from 1.8–1.85) from the isolated DNA. The isolated DNA was utilized for molecular characterization and genetic diversity assessment of *Flemingia semialata* and *Flemingia macrophylla* by using microsatellite markers. The standardized protocol was yielded genomic DNA of good quality and quantity from both, juvenile leaf and matured leaf samples. The optimized protocol was also utilized for the genomic DNA isolation of *Shorea robusta* and *Buchanania cochinchinensis*.

Introduction

Flemingia is one of the most important multipurpose perennial shrubs, belongs to the family fabaceae. It is considered to be originated in tropical and subtropical region of the old world especially in Indian subcontinent. It is globally distributed from Africa through Indo-Malaysia to Australia (Kumar and Kumar, 2013). *Flemingia* Roxb. ex Ait was reported for the first time in 1812 and since then several species have been included in the genus. There are about 39 species included in the genus *Flemingia* out of which, 15 species are found in India (Kumar and Kumar, 2013). These species are naturally distributed and are being used for different purposes but, two species namely *Flemingia semialata* and *Flemingia macrophylla* plays significant role in lac cultivation due to its short stature, perennial nature, easy to manage, wide adaptability and most importantly capacity to produce premium grade kusumi lac (Kumar and Kumar, 2013, Singh et al., 2015, Kumar et al., 2017). These two species are perennial woody shrubs and grown successfully as lac host plant in Jharkhand, west Bengal, Odisha and Madhya Pradesh. Despite these species are extensively utilized for lac cultivation and distributed naturally in the region, the genetic diversity harbored in the germplasm is very less known (Kumar et al., 2013). The molecular markers are extensively utilized by researchers for estimating genetic diversity present in germplasm of plant species (Mahajan and Gupta, 2012). For estimating molecular genetic diversity, the pre-requisite is genomic DNA of high quality and quantity. To obtain the high quality and quantity of genomic DNA repeatedly, it is needed to standardize the genomic DNA isolation protocol.

The genomic DNA isolation protocol development is a continuous process and researchers continuously work either to discover easy and effective method to isolate DNA or to modify the existing protocols.

Most of the available DNA extraction protocols are modifications of cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987) and utilize liquid nitrogen (-196^{o}C), enzymes and lyophilization (freeze-drying) for grinding and rupturing the cell and nuclear walls (Agbagwa et al., 2012). The handling of liquid nitrogen is very difficult and hazardous. The DNA isolation kits offered by various firms viz. Qiagen, Promega corporation, Nucleopore etc. are expensive and their utilization makes molecular work very costly. The method to extract DNA should be simple, cost-effective and non-hazardous and should give good quantity and pure DNA. The presence of good quality DNA is a key factor of success of most of the PCR- based molecular markers study such as Random amplified polymorphic DNA (RAPD), Simple sequence repeats (SSR), Single nucleotide polymorphisms (SNP), Amplified fragment length polymorphisms (AFLP) etc (Sambrook et al., 2001).

We were not able to find the genomic DNA isolation protocol in literature specific to *Flemingia semialata*. The DNA isolation protocols devised and utilized for other leguminous crops were also utilized but no protocol isolated pure DNA in high quantity. Therefore, genomic DNA isolation protocol for *Flemingia semialata* has been standardized through the present work. The effectiveness of the optimized protocol was validated by utilizing the isolated genomic DNA for PCR with SSR markers after optimization of PCR reaction, which yielded polymorphic bands.

Materials And Methods

The genomic DNA was isolated from the young and old leaves of *Flemingia semialata* and *F. macrophylla* planted in the research plot of Institute of Forest Productivity, Ranchi (Jharkhand), India. The leaf samples were collected in juvenile stage i.e., after 10 days of its emergence and at mature stage i.e., after eight months of its emergence.

The DNA isolation protocols developed for leguminous plants (Agbagwa *et al*., 2012), chick pea (Joshi *et al*., 2010) and *Cajanus cajan* (Singh *et al*., 2012) were utilized to isolate the genomic DNA of Flemingia. The DNA obtained by using these protocols were not in high quantity and quality so, it was needed to standardize the protocol to yield high quality and quantity of genomic DNA of flemingia at lower cost. Therefore, the DNA isolation procedure suggested by Murray and Thompson (1980) was modified and standardized for *Flemingia* species to extract the high molecular weight genomic DNA.

Reagents, Chemicals and Laboratory materials

The DNA extraction buffer consists of 2% Cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 100 Mm Tris-HCL, 20 Mm EDTA, 3% PVP and 0.3% β-mercaptoethanol. Stock solutions of different chemicals of DNA extraction buffer were prepared as per the standard method. Other reagents and materials were chloroform: isoamyl alcohol (24:1), 10µl of RNase (20mg/ml), Ice chilled isopropanol (100%), ethanol (70 %) and TE buffer (10 mM Tris base and 1mM EDTA). Laboratory material and equipment includes, mortar-pestle, 1.5 ml and 2 ml microcentrifuge tubes, micropipettes, microtips, Eppendorf tube holding racks, Centrifuge, refrigerator etc.

Sample collection and preparation

Fresh juvenile and matured leaves were collected in zip bags from the experimental field and kept in ice box. The leaves were stored in refrigerator at -20^{o}C in the laboratory to maintain the freshness of the leaves. The collected samples were washed with distilled water to remove particulate contaminants and unwanted materials from the leaf surfaces. To facilitate the grinding of samples, the mid ribs and veins of leaves were removed and leaf lamina were chopped into pieces.

Extraction and purification protocol

In the standardized protocol, 200 mg leaf sample was taken, washed, mid ribs and veins were removed, leaf lamina were chopped and grinded in a fine paste with 1 ml pre-heated DNA extraction buffer (2% CTAB, 1.4 M NaCl, 100 Mm Tris-HCL, 20 Mm EDTA, 3% PVP, 0.3% β-mercaptoethanol) in mortar-pestle. The paste was transferred to 2 ml microcentrifuge tubes and incubated at 65°C for 60 minutes in hot water bath. After incubation, microcentrifuge tubes were cooled down to room temperature and an equal volume of chloroform: isoamyl alcohol (24:1) was added to the microcentrifuge tubes, followed by gentle inversion of tubes for 10 minutes. Then, tubes were centrifuged at 15,000 rpm for 10 minutes at room temperature. Supernatant was transferred into a fresh tube into which, 10µl of RNase (20mg/ml) was added and incubated at 37°C for an hour. Again chloroform: isoamyl alcohol (24:1) separation was done to obtained clear supernatant. Upper aqueous phase was taken into another fresh 1.5 ml microcentrifuge tube and equal volume of ice chilled isopropanol was added. Mixture was gently mixed by inverting the tubes and incubated at -20°C for 60 minutes (or at 4^{o}C for overnight). Centrifugation was done at 10,000 rpm for 10 minutes at 4°C in order to obtain DNA pellet. Supernatant was discarded and DNA pellet was washed with 70% ethanol two times. The washing of DNA pellet was done with 200 µL 70% ethanol with centrifugation at 5000 rpm for 5 minutes at 4^{o}C. The ethanol was discarded and pellet was air dried until ethanol was evaporated completely. The dried pellet was dissolved in TE buffer (10mM Tris base and 1mM EDTA) and stored for further use.

Quality assessment and quantification of extracted DNA

The quantity and purity of extracted genomic DNA was estimated in Bio-photometer Plus (Eppendorf), where 10 µl DNA sample and 90 µl TE buffer was taken in the cuvette and OD was measured at 260 nm. Purity of DNA was determined by calculating the ratio of absorbance at 260 nm and 280 nm wavelength. The extracted genomic DNA was electrophoresed in 0.8% agarose gel containing 10 mg/ml EtBr submerged in 0.5X TBE buffer for 1 hr at 80V and image of the gel was captured using gel documentation system. Through the absorbance and transmittance value the DNA quantity estimated which was subjected to make a dilution for PCR analysis.

PCR based amplification of extracted DNA through SSR marker

The extracted genomic DNA was subjected to PCR amplification by using SSR markers. For SSR-PCR reaction, a 20µl reaction mixture was prepared that contains 1X buffer with 2.5 mM MgCl2, 0.25 mM

dNTPs, 0.25 µM of each forward and reverse primer, 1U Taq polymerase, 50 ng/µl template and molecular biology grade water (nuclease free) for maintaining volume (Table 1). The reaction mixture was mixed properly and put into the thermal cycler (CG Palm Cycler, Genetix Biotech Asia Pvt. Ltd.) for amplification. The thermal profile utilized for PCR is given in Table 2.

Table 1: Chemicals and their concentration utilized for Polymerase Chain reaction

Sl. No.	Components	Concentration utilized
1	10X Taq DNA polymerase buffer	1X
2	Taq DNA polymerase	1U/reaction
3	dNTPs	0.25 mM
4	Forward primer	0.25 µM
5	Reverse primer	0.25 µM
6	Template (DNA)	50 ng

Table 2: Thermal profile utilized for Polymerase Chain reaction (PCR)

PCR Steps	Temperature	Time	Cycle
Initial denaturation	95ºC	5 min	
Denaturation	95ºC	60 s	Repeat 30 times
Annealing	55ºC-60ºC	30 s	
Extension	72ºC	60 s	
Final extension	72ºC	10 min	
Storage	4ºC	α	

Note: Annealing temperature varied for different SSR primers

Results And Discussion

The genomic DNA of flemingia was isolated by using DNA isolation protocols developed for leguminous plants (Agbagwa *et al*., 2012), chick pea (Joshi *et al*., 2010) and *Cajanus cajan* (Singh *et al*., 2012) and compared with the genomic DNA isolated by using modified and optimized protocol. The utilized three protocols yielded comparatively lesser quantity of DNA with impurities as compared to the optimized protocol (Table 3).

Table 3: Genomic DNA quantity and purity estimated through Bio-photometer

S.N.	Protocols used for Flemingia DNA extraction	Concentration of DNA (ng/µl)	Purity of DNA (A_{260}/A_{280})
1.	Leguminous plant (Agbagwa *et al*., 2012)	200-300	1.40 to 1.60
2.	*Cajanus cajan* (Singh *et al*., 2012)	600-700	1.68 to 1.70
3.	Chick Pea (Joshi *et al*., 2010)	-	-
4.	Modified and optimized protocol	1200-1500	1.80 to 1.85

The genomic DNA obtained by using protocol of Agbagwa *et al.* (2012) was brownish in color which showed the presence of polyphenolics in the isolated DNA as impurity. Also, the quantity of DNA obtained was very low i.e., ranged from 200 ng/µl to 300 ng/µl. The A260/A280 ratio was also ranged from 1.40 to 1.60 which showed presence of protein as contaminant in the isolated DNA. The protocol of Singh *et al.* (2012) was also yielded low quantity of genomic DNA varied from 600 ng/µl to 700 ng/µl. The A260/A280 ratio was also varied from 1.68 to 1.70 which shows the presence of protein as impurity in the isolated DNA (Table 3). The similar result was also obtained through the 0.8 % agarose gel electrophoresis (Figure 1). The protocol of Joshi *et al.* (2010) failed to isolate genomic DNA of flemingia.

The standardized protocol yielded 1200-1500 ng/µl high molecular weight genomic DNA with an average of 1359 ng/µl DNA from 40 samples of *F. semialata* and *F. macrophylla* (Table 3). The A260/A280 ratio was also ranged from 1.80 to 1.85 which showed the purity of isolated DNA and indicated the presence of insignificant level of RNA in the extracted DNA (Agbagwa *et al*., 2012). As protocol involves utilization of RNase which ensured the removal of RNA and extraneous nucleic acid from the sample. The color of obtained DNA was very clear which resulted due to removal of polyphenolics from the sample by PVP added in DEB buffer. The protocol was also not utilized any lyophilization or liquid nitrogen or enzymatic digestion as reported by various authors (Manen *et al*., 2005, Chakraborti *et al*., 2006, Arbi *et al*., 2009) for grinding/rupturing the cell wall and nuclear wall which reduces the cost of per sample DNA isolation and risk of health hazard. The 0.8% agarose gel electrophoresis was also done to verify the quality of DNA isolated by using various protocols and standardized protocol. The Gel image confirmed the purity and intactness of isolated genomic DNA through optimized protocol compared to DNA isolated by other protocols (Figure 1).

Amplification of extracted DNA by SSR marker

The SSR primer (c46278_g2_i6) was used to amplify the isolated genomic DNA of *F. macrophylla* (FM) and *F. semialata* (FS) and obtained good amplification with polymorphic bands (Figure 3). The PCR product was run on 2% agarose gel to visualize the amplicon and polymorphic bands and found clear and intense bands of 80bp, 100bp and 130 bp size.

Declarations

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Competing Interest: The authors declare no competing interest.

Authors Contribution

Aditya Kumar (Experimental design, Standardization of DNA isolation protocol, manuscript writing)

Khushboo Kumari (Contributed in standardization of protocol)

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Figures

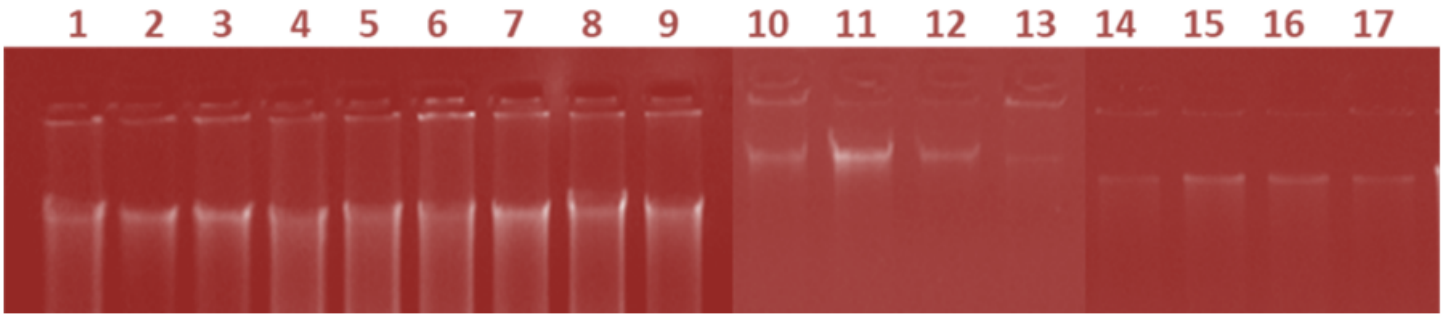

Figure 1

0.8% agarose gel image of extracted genomic DNA of Flemingia

Lane 1-9: Genomic DNA isolated by using optimized protocol.

Lane 10-13: Genomic DNA isolated by using protocol of Agbagwa *et al.* (2012)

Lane 14-17: Genomic DNA isolated by using protocol of Singh *et al.* (2012)

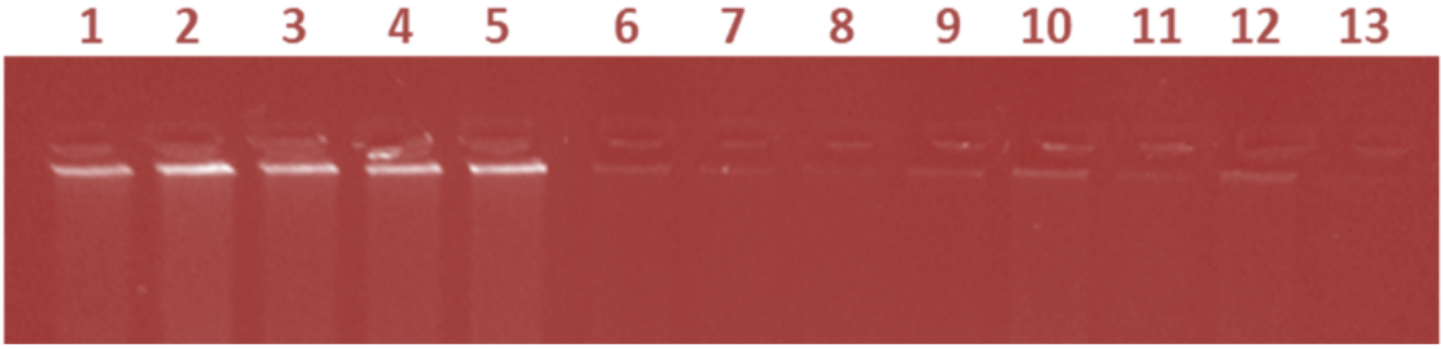

Figure 2

0.8% agarose gel image of extracted genomic DNA from *Buchanania cochinchinensis* and *Shorea robusta* plant by using standardized protocol

Lane 1-5: Extracted genomic DNA of *B. cochinchinensis*

Lane 6-13: Extracted genomic DNA *Shorea robusta*

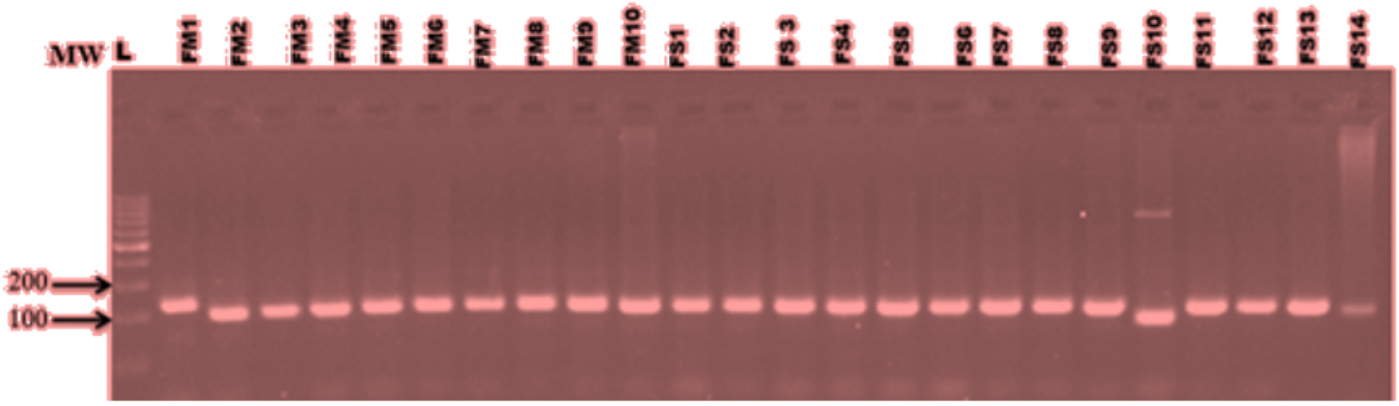

Figure 3

2% agarose gel image of amplicons of *F. macrophylla* (FM) and *F. semialata* (FS) DNA with SSR primer (c46278_g2_i6)

Lane L: 100bp DNA step ladder.

Lane 1-10: amplicons of *F. macrophylla* genomic DNA

Lane 11-24: amplicons of *F. semialata* genomic DNA