

ORIGINAL RESEARCH ARTICLE

Development of RAPD (Random Amplified Polymorphic DNA) Markers for Authentication of *Croton roxburghii* Balak.

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ABSTRACT

Plants are the local heritage with global importance. World is endowed with a rich wealth of medicinal plants. *Croton roxburghii* Balak. of family Euphorbiaceae, is considered as the source plant of 'Nagadanti' in Ayurveda. It is a medium sized tree being distributed all over India. Its bark and root is prescribed by tribal people and traditional practitioners of Odisha, for snake bite and many other diseases. Certain species of croton are used as a source drug of other Ayurvedic drugs also. The morphological identities of many croton species are similar. Current study deals with molecular characterization (DNA) RAPD (Random Amplified Polymorphic DNA) markers study details and their role in laying down standardization, by amplifying the genomic DNA isolated from the fresh leaves. The investigative characters are the unique bands obtained in PCR amplification clearly discriminated having many bright and light bands indicating the genuinity of the plant. RAPD may serve as a complementary tool for quality control.

Key words: *Croton roxburghii* Balak., Molecular characterization, DNA - RAPD, *Nagadanti*, Gandhamardan hill ranges, Odissa.

INTRODUCTION

Croton roxburghii Balak. (Euphorbiaceae) is considered as the botanical source of 'Nagadanti' of various classical texts^[1-5]. It is a medium sized tree. Leaves are large; greenish in color, crenate or serrate, bark is hard and greyish brown in colour. The medicinal potency of *C. roxburghii* has been known to traditional healer and widely used in folklore medicine, in Odisha. The bark and root is used in the treatment of snake bite, insect bites, cholera, skin diseases and stomach disorders^[1].

Establishing the standards, is a fundamental part of crude drug identification. Before any drug integrated in pharmacopoeia, these standards must be established. The majority of the information on the identity, purity and quality of the plant material can be obtained from its macroscopy and DNA fingerprints. Molecular markers based on DNA sequences offer means of identification with much greater reliability than the morphological traits. RAPD remains one of the most extensively used molecular techniques due to its simplicity, low cost and high speed. Thus, RAPD markers have been successfully used in many crops in providing a convenient and rapid assessment of

genetic diversity among different genotypes^[6-8]. RAPD markers have been already successfully used on other medicinal and aromatic crops^[9-13].

In spite of its reputation, *C. roxburghii* leaves not yet been investigated scientifically and hence it was thought worth to study it in detail. The present study was undertaken to establish the macroscopic characters and molecular level studies i.e. DNA fingerprints of the leaves, for its authentication and standardization, which might serve as a valuable tool for effective screening of genetic resources for future research and to improve and sustain genetic diversity of different varieties.

MATERIALS AND METHODS

Collection and preservation

Leaves of *C. roxburghii* were collected, during the month of January, from its natural habitat of Gandhamardna hills, (21°0`-21°45`N and 82°45`83°5`E) located in Bargarh district of Odisha, India.

The plant was identified by local herbal experts and by referring various floras^[3-5,14-16] and also with the help of botanist of Gujarat Ayurved

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University, IPGT & RA, Jamnagar. The collected plant parts were washed properly under running water and herbarium specimens were prepared for further documentation. The herbarium specimens were stored in Pharmacognosy museum of IPGT & RA, Gujarat Ayurved University, Jamnagar. Herbarium No. 6047 for future reference.

Molecular characterization (DNA fingerprints)

Fresh leaves are used in Molecular characterization (DNA) fingerprints obtained by standard and most convenient RAPD method. The RAPD reaction was performed according to the method developed by McClelland *et al.* (1995).

RAPD profile generated from genomic DNA isolated from fresh leaves found identical with 2 random primers. The number of unique bands specific to genuine as well as other samples with different primers.

DNA extraction protocol:

DNA was extracted from the sample using CTAB Buffer.

- Tender leaves were selected and crushed to powder using dry ice.
- 200mg of this powdered leaves was taken in a centrifuge tube.
- 6 ml of CTAB extraction buffer was added and incubated at 60°C for 30 min.
- Centrifuged at 10000 rpm for 10 minutes.
- Supernatant was transferred to a fresh centrifuge tube carefully.
- Equal volumes of Chloroform was added and mixed well.
- Centrifuged at 10000 rpm for 10 minutes.
- The aqueous layer was pipetted out into the fresh centrifuge tube without taking the Interface.
- Equal volumes of Isopropanol and 1/10th volumes of 3M Sodium acetate were added and mixed well. Left at room temperature to stand for 5-10 minutes.
- Centrifuged at 10000 rpm for 10-15 minutes. The supernatant was discarded. The pellet was washed with 1ml of 70% ethanol.
- The pellet air dried and suspended in 400 µl of 1X Tris- EDTA buffer. Since DNA sample had inhibitors for PCR, the samples were column purified.

Column purification

- The column was placed in collection tube, 400µl of equilibration buffer was added to the column and centrifuged at 10000rpm for 1min. Collected buffer was discarded.
- 400µl of equilibration buffer was added to the DNA samples, mixed and loaded into the column (This step was repeated till the DNA sample was completed).Flow through was collected.
- 500µl of wash buffer 1 was added, centrifuge at 10000rpm for 1min and buffer was collected.
- 500µl of wash buffer 2 was added, centrifuge at 10000rpm for 1min and buffer was collected.
- The column was centrifuged empty for 2 min with collection tube to completely remove the wash buffer.
- 50µl of elution buffer was added to the column placed in new collection tube. Incubated at room temperature for 2 min and centrifuged at 10000rpm for 1min and eluted sample was saved (elution 1).
- Previous step was repeated.(DNA eluted in this fraction also) (elution2)

Quantification of eluted DNA samples was done by loading into the agarose gel.

Lysis buffer: 100 mM Tris-HCl (pH8.0), 1.2 M NaCl, 20 mM EDTA, 2% CTAB. 2µl loaded from both the elutions. DNA was seen Conc 50ng/µl. 1µl of sample was used for PCR.

Amplification: Two random primers were used.

Random primer OPA-02 &
Random primer OPA-13.

RESULTS AND DISCUSSION

Macroscopy of *Croton roxburghii* Leaves

Shape - oblong, lanceolate; size - large, 12.5 -25 cm in length, 5.7 -11.5 cm in breadth; surface - thick, coriaceous, glabrous; venation - midrib more prominent at lower side, secondary nerves 12, slender. Midrib and lateral nerves arising at an angle of 65° are distinct, more prominent at lower surface and anastomosing near the margin. Margin - Irregularly crenate, exhibiting a small blackish, circular, sessile gland in the groove. Apex –

subacute; base – symmetrical; petiole - 1-5cm in length, 0.8-0.15cm in diameter. cylindrical with a faint long longitudinal groove in the centre of the ventral surface.

Organoleptic characters:



Colour of leaf upper green, lower pale green, Odour slightly aromatic and taste slightly bitter



Quantitation of column purified Genomic DNA



2 µl of DNA loaded was about 100ng

AMPLIFICATION: A cocktail was made with PCR master mix and respective Random primer

	For 1 reaction	For 12 reactions	Notes
Double Distilled water	19 µl	2280 µl	
2X PCR master mix	20 µl	240µl	1X Contains 100µM each of dATP, dGTP, dCTP and dTTP. Assay buffer with 15mM MgCl ₂ , 3U/reaction Taq Polymerase.
Random Primer	1 µl	12 µl	10pM used for each reaction
Total Volume	40 µl x 12	480µl	

38µl of this was aliquoted into 12 different labeled PCR vials and to this 2 µl of respective template DNA was added. The PCR was set.

PCR Conditions:

Temperature	Time	No. of cycles
94°C	2 minutes	1
94°C	30 seconds	40
45°C	1 minute	
72°C	1min 30 sec	1
72°C	7 minutes	

NOTE:

- **Marker (M):** PhiX/Hae III
- **Fragment sizes:** 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078 and 1351bp.

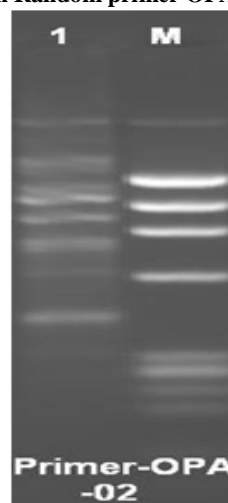
Primer sequences:

OPA-02	TGCCGAGCTG
OPA-13	CAGCACCCAC

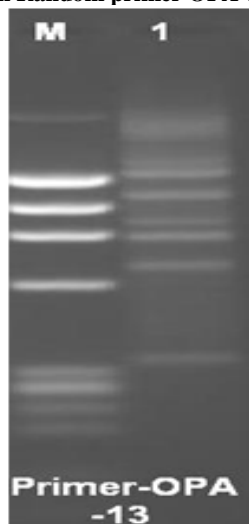
Sample ID: *Croton roxburghii*

Quantitation of column purified genomic DNA

RAPD with Random primer OPA-2



RAPD with Random primer OPA-13



DNA extracted by regular CTAB method did not show amplification. Plant extracts had to be column purified to get the DNA amplified. RAPD analysis shows that OPA-02 and OPA-13 primers amplified the DNA to produce good polymorphic bands. Differentiation or the DNA fingerprint got is very clear with these primers. While sample showed distinct pattern.

CONCLUSION

The majority of the information on the identity, purity and quality of the plant material can be obtained from its macroscopy and DNA fingerprints. The observations of the present will produce some useful gauge in standardization of the plant *Croton roxburghii*.

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