

ORIGINAL RESEARCH ARTICLE

Development of RAPD (Random Amplified Polymorphic DNA) Markers for Authentication of *Limnophila heterophylla* (Roxb.) Benth.

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ABSTRACT

Limnophila heterophylla (Roxb.) Benth., an ethno medicinal plant, locally known as ‘Ambakasia’ by tribal people of Odisha is used for treatment of various diseases. The use of highly discriminatory methods for the identification and characterization of genotypes is essential for plant protection and appropriate use. Current study deals with molecular characterization through RAPD (Random Amplified Polymorphic DNA) markers study details and their role in laying down standardization for the genetic fingerprinting of *Limnophila heterophylla*. Two primers used were able to amplify the DNA from the plant species. The amplified products of the RAPD profiles ranged from 72 to 1351 bp. DNA were 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. Obtained results may serve as complementary tool for quality control of *Limnophila heterophylla*.

Key words: *Limnophila heterophylla* (Roxb.) Benth., Molecular characterization, DNA - RAPD, *Ambakasia*, Odisha

INTRODUCTION

The knowledge of beneficial properties of different herbs is generally found to be transmitted from generation to generation among parents to their offspring of the tribal people. One of such plant, identified as *Limnophila heterophylla* (Roxb.) Benth. of the family Scrophulariaceae^[1], is a small, annual, diffuse herb growing in places along streams, under shade across India, especially state like Odisha^[2]. During the last few decades there has been an increasing interest in the study of medicinal plants and their traditional use in different parts of India and there are many reports on the use of plants in traditional healing by either tribal people or indigenous communities of India^[3-9]. Apart from the ethnic groups, various forest dwellers and urban people also possess unique knowledge regarding plants. Proper identification is crucial for the preservation of plants growing in extreme arid regions. Traditionally, subjective methods based on the morphological features such as shape, color, texture, and odour are used for the discrimination of herbal medicines. However, these methods are difficult to apply accurately for discrimination and authentication. The use of chromatographic techniques and marker compounds to standardize botanical preparations is

also limited because the medicines have variable sources and chemical complexity, which is affected by growth, storage conditions and harvest times^[10-11]. Among the polymerase chain reaction (PCR)-based molecular techniques, random amplified polymorphic DNA (RAPD) is convenient in performance and does not require any information about the DNA sequence to be amplified^[12]. Due to its procedural simplicity, the use of RAPD as molecular markers for taxonomic and systematic analysis of plants^[13], as well as in plant breeding and the study of genetic relationships have considerably increased^[14]. In spite of its reputation, *Limnophila heterophylla* leaves not yet been investigated scientifically and hence it was thought worth to study it in detail. In this study, we successfully utilized the RAPD technique for rapid characterization of *Limnophila heterophylla*. The present paper highlights macroscopic characters and molecular level studies i.e. DNA fingerprints of the leaves for its authentication and standardization.

MATERIALS AND METHODS

Collection and preservation

Collection of the plants were done in the month of January when it was fully in matured state from its

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natural habitat of Gandhamardan hills, (21°0`-21°45`N and 82°45`-83°5`E) which are located in Bolangir and Sambalpur district of Odisha, India.

The plant, commonly known by tribal people as 'Ambakasia' is growing in different parts of Odisha^[15]. It was identified as *Limnophila heterophylla* (Roxb.) Benth. of family Scrophulariaceae by studying the morphological characters of various parts of the plant and comparing them with the various characters mentioned in various floras and herbarium^[15-18]. The plants were shaken to remove adherent soil, dirt etc. and washed with water, wherever required and herbarium specimen was prepared (Herbarium No. 6064) and was stored in Pharmacognosy department, for further documentation. Few were stored in solution of AAF (70% Ethyl alcohol: Glacial acetic acid: Formalin) in the ratio of (90:5:5)^[19].

Molecular characterization (DNA fingerprints)

Fresh leaves are used in Molecular characterization (DNA) fingerprints obtained by standard and most convenient RAPD method.

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

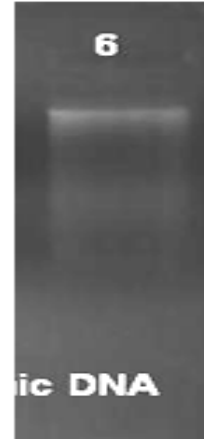
Macroscopy of leaf *Limnophila heterophylla* Leaves

Thin, ovate to oblong, 1.5 – 1.8cm long, margin - serrate or crenate serrate, apex - acute to sub acute, base - symmetrical, surface - glabrous, venation - reticulate, Veins – obscure on upper surface, prominent on lower surface, petiole winged. Taste – Astringent bitter, Odour – characteristic. Flowers purplish, sessile or sub-

sessile, 1- 1.2 cm long, axillary solitary often spicate.



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:

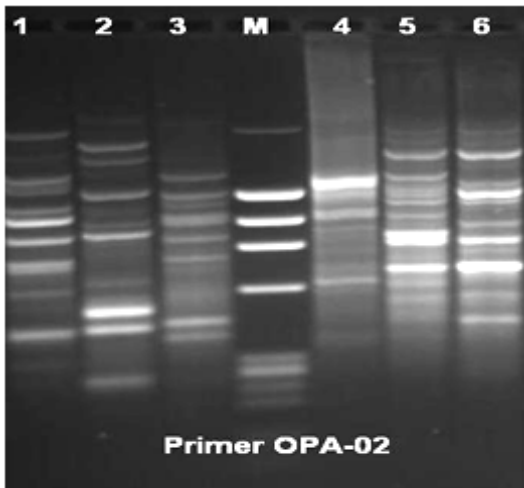
- **Marker (M):** PhiX/Hae III
- **Fragment sizes:** 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078 and 1351bp.

Primer sequences:

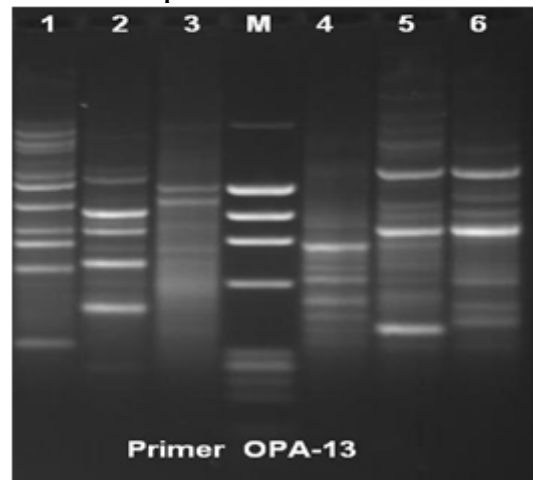
OPA-02	TGCCGAGCTG
OPA-13	CAGCACCCAC

Sample ID: 6 - *Limnophila heterophylla*

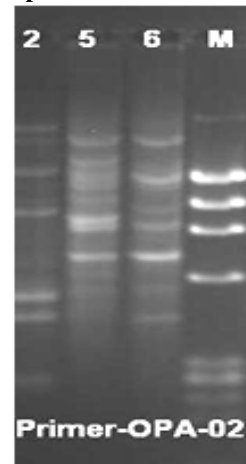
RAPD with Random primer OPA-2



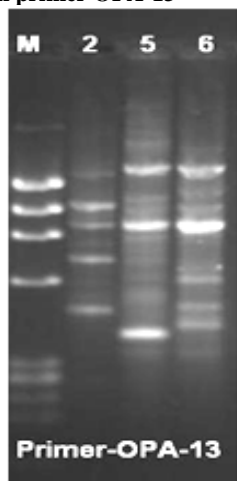
RAPD with Random primer OPA-13



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 samples 1 showed distinct pattern. This shows that these plants are closely related, yet there are some variations.

CONCLUSION

Establishing the standards is an fundamental part of establishing the correct recognition and class of a crude drug. 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. The present work is under taken to produce some useful gauze in standardization.

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