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Genome study of North-East Indian *Rubus alceifolius*

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ABSTRACT

An attempt was made to isolate genomic DNA along with the estimation of nuclear DNA content of North-East Indian Rubus alceifolius. A simple, efficient and reliable CTAB method is standardized for isolation of genomic DNA from fresh young leaves of R. alcifolius after modifying some of the key steps of earlier protocol described by Khanuja et al. (1999). The modified procedure yielded a high amount (38 µg g^{-1} fresh leaf tissue) of good quality DNA free from contaminants and found to be suitable for restriction digestion with Eco RI and Hind III enzymes. The nuclear DNA content of R. alceifolius was estimated by one step DNA flow cytometry procedure (Otto, 1990) with slight modifications. The 2C DNA content of this species was estimated to be 5.681 pg. This is the first study attempted for determination of nuclear DNA content of North-East Indian R. alceifolius.

Key words: *Rubus alceifolius*, Genomic DNA, nuclear DNA, CTAB protocol, Otto buffer, flow cytometry.

INTRODUCTION

Rubus alceifolius Poir (Syn. *R. moluccanus auct. non* Linn.) is a 2.5-3.0 m tall simple- leaved South-East Asian straggling or sub-scandent shrub with hooked prickles belonging to the family Rosaceae. The plant is distributed throughout India, Australia and Malayasia [1]. The plant is considered as a serious invasive weed in La Reunion Island [2]. Genetic diversity of *R. alceifolius* through AFLP markers and genome size through flow cytometry from large number of populations from Vietnam, Thailand and La Reunion Island was investigated [3, 4]. But the populations of *R. alceifolius* from North-East India have not been sampled yet. In North-East India, the plant is being used as traditional medicine. Ethnobotanical survey conducted in different parts of Assam revealed a number of therapeutic uses of this plant. The root extract of the plant is given in piles and dysmenorrhoea. Decoction of tender shoots is prescribed for cough and pneumonia while unripe fruit is rubbed over the tongue to cure fungal infection. Voucher specimen was deposited in the herbarium of the department of Botany, Namrup College, Assam.

The experimental objective was (i) to establish a reliable method for isolation of pure genomic DNA from *R. alceifolius* and digest the DNA with restriction enzymes to check the purity and suitability for further processing in recombinant DNA technology and (ii) to estimate the nuclear DNA content or genome size of R. *alceifolius*

MATERIALS AND METHODS

Genomic DNA isolation: The protocol used for isolation of DNA was the modification of earlier established CTAB protocol described by Khanuja *et al* [5]. The procedure worked well with isolation of quality DNA from fresh as well as dry tissues of many genera of medicinal and aromatic plants and the protocol does away with the use of phenol, which makes the protocol less hazardous.

Modification of the protocol: (i) Addition of 4% PVP in the extraction buffer to remove polyphenols during DNA isolation, (ii) additional chloroform: isoamyl alcohol (24:1 v/v) extraction to remove large amounts of precipitates from the plant extract and (iii) overnight isopropanol precipitation at room temperature to improve yield and quality of DNA

Restriction digestion: Restriction digestion was performed in a total volume of 25 µl and contained 10 µg DNA and 10 units of restriction enzymes with the recommended buffer. The incubation was done for 6 h at 37^{o}C using *Eco* RI, *Hin*d III (M/S Banglore Genei, Banglore, India) and mixture of both *Eco* RI and *Hin*d III. After digestion, the reaction mixture was electrophoresed on 0.8% (w/v) agarose gel prepared in 1 x TAE buffer (pH 8.0), stained with 0.5 µg ml^{-1} ethidium bromide and visualized under a UV transilluminator. The gel photographs were scanned through a Gel Doc system (BIO RAD Gel Doc 1000). After digestion, the reaction mixture was electrophoresed on 0.8% (w/v) agarose gel prepared in 1 x TAE buffer (pH 8.0), stained with 0.5 µg ml^{-1} ethidium bromide and visualized under a UV transilluminator. The gel photographs were scanned through a Gel Doc system (BIO RAD Gel Doc 1000).

Nuclear DNA content estimation: Nuclear DNA content was estimated according to DNA flow cytometry procedure of Otto [6] with slight modifications. Seeds of *R. alceifolius* were collected from Namrup (27.18^{0} N and 95.33^{0} E), Assam and seedlings were raised. Leaf tissues of mature leaves of 60 days old seedlings were taken for flow cytometric analysis. Briefly, about 20 mg of young leaves were chopped in a plastic Petridish with a new razor blade in 0.5 ml of ice cold Otto I buffer containing 0.1 M citric acid monohydrate and 0.5 % (v/v) Tween 20. Then added another 0.5 ml of ice cold Otto I buffer and mixed properly with the help of a micropipette. The suspension was filtered through a 42 µm nylon mesh to separate the nuclei from the debris and incubated the sample for 5 min. Each sample contained about 0.5 ml of filtrate. 2 ml of Otto II buffer (0.4 M $Na_2HPO_4.12H_2O$) was added to the filtrate and the suspension was supplemented with propidium iodide (PI) and RNase (both at 50 µg ml^{-1}). The sample was stored at room temperature for about 15 min and the prepared material was shaken in order to separate nuclei clusters. The sample was then analyzed in a FACS Calibur flow cytometer (Becton Dickinson, USA) for relative DNA content of isolated nuclei. *Pisum sativum* was used as external reference standard. Mean position of G_0/G_1 peak of the sample and reference standard were determined by analyzing the data with CellQuest software. The mean DNA content per plant was based on at least 5000 scanned nuclei. During analysis, after every three samples, the reference plant was

controlled to check the calibration of the flow cytometer. The gain of the instrument was adjusted so that G_0/G_1 peak of *P. sativum* (reference standard) was positioned at channel 200.
(iv) *Computation of nuclear DNA*: The nuclear DNA content of *R. alceifolius* was estimated according to the following equation:

R. alceifolius 2C nuclear DNA content (pg) =

$$\frac{\textit{R. alceifolius}\ G_o/G_1\ \text{peak mean}}{\textit{P. sativum}\ G_o/G_1\ \text{peak mean}} \quad \text{X } 9.09$$

One picogram of DNA equaling 980 megabase pairs (Mbp) was considered when converting picogram values to base pairs. *P. sativum* was used as reference standard whose 2C DNA content is 9.09 pg [7]. Samples were kept in ice between analyses. The mean nuclear DNA content was calculated for each sample and analyzed as single value.

RESULTS

Genomic DNA isolation: The modified DNA extraction procedure yielded 38 µg g-1 of DNA from fresh leaf tissues of *R. alceifolius*. This was more than two times higher than the yield obtained through the original protocol (Table 1). Fig. 1A shows conspicuous band of undigested DNA sample with no visible RNA contamination and nor any sign of DNA degradation during DNA preparation. Fig. 1B shows complete homogenous restriction of the DNA sample with *Eco* RI and *Hin*d III restriction enzymes. The *Eco* RI digestion was more complete. After double digestion DNA was well digested, indicating the absence of impurities and inhibitors. Spectrophotometric measurement of the isolated DNA samples at 260 nm and 280 nm gave an absorbance ratio A260/A280 of 1.84 indicating the absence of significant levels of contaminated proteins and polysaccharides.

Table 1 DNA yield and purity using the original protocol and modified steps

Original protocol		Modified steps											
Khanuja *et al.**		Addition of 4% PVP in the extraction buffer (a)		a + two washings with chloroform: isoamyl alcohol (24:1) (b)		a + b+ isopropanol precipitation for 1 h at 4°C		a + b + isopropanol precipitation for 1 h at 25-30°C		a + b + overnight isopropanol precipitation at 4°C		a + b + overnight isopropanol precipitation at 25-30°C	
Y	A	Y	A	Y	A	Y	A	Y	A	Y	A	Y	A
18	1.49	23	1.58	24	1.61	27	1.65	26	1.71	41	1.74	38	1.84

[Y - DNA yield (µg g^{-1} from fresh leaf tissue); A - A_{260}/A_{280} ratio (DNA purity)]
** DNA extraction was performed strictly in accordance with the protocol mentioned.*

Table 2. Nuclear DNA content of North-East Indian *R. alceifolius*

Sl. No.	Name of the species	Number of samples	2C DNA (pg)	SE	C DNA (pg)	C DNA (bp)
1	*R. alceifolius*	3	5.681	0.14	2.84	2.77 x 10^9

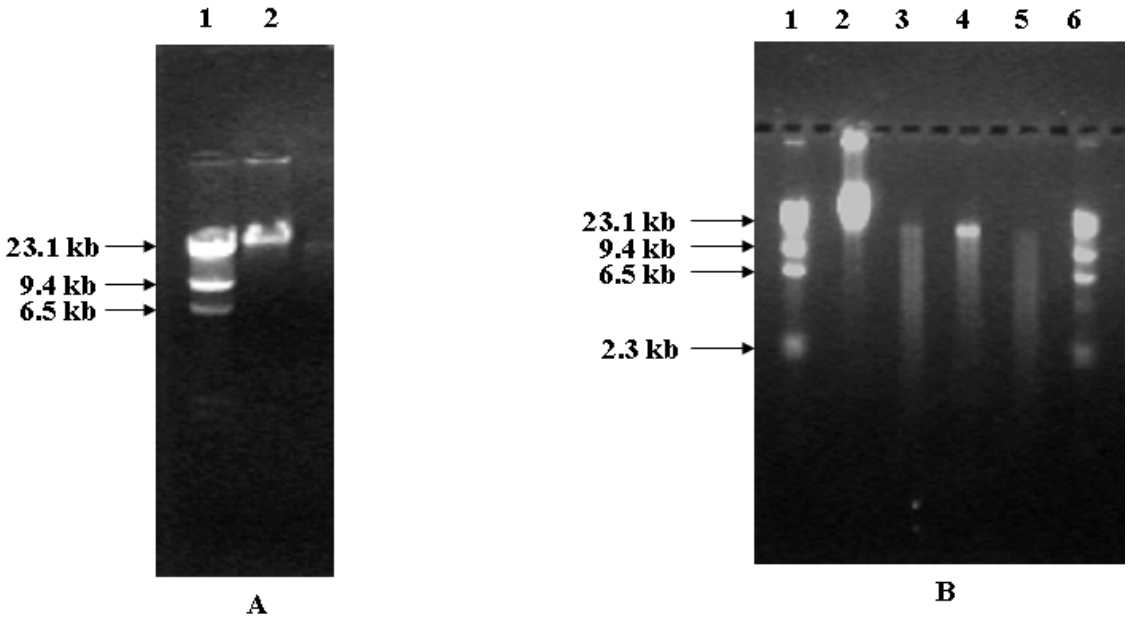

Fig 1. Genomic DNA and Restriction Digestion profile from *R. alceifolius*

(A) Lane 1, λ-DNA digested with Hind III (molecular size marker); Lane 2, Genomic DNA sample
(B) Lanes 1 & 6, λ-DNA digested with Hind III (molecular size marker); Lane 2, undigested sample; Lane 3, DNA digested with Eco RI; Lane 4, DNA digested with Hind III: Lane 5, DNA digested with Eco RI and Hind III.

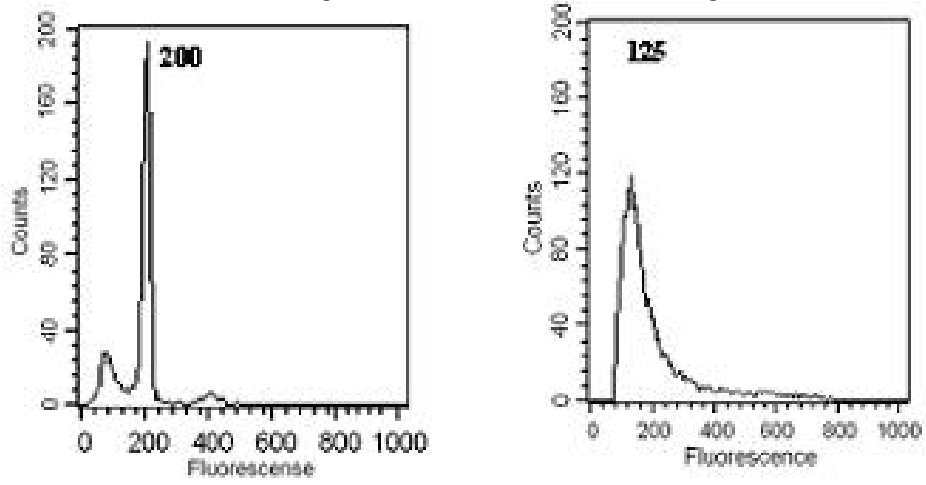

Fig 2. Flow cytometric histograms of PI stained nuclei isolated from *R. alceifolius*

A. The fluorescence peak of PI stained nuclei from P. sativum (2C = 9.09 pg DNA), which served as external reference standard. The gain of the cytometer was adjusted so that G_0/G_1 peak was positioned at channel 200. The 2C DNA content of the investigated plant species was determined after comparison with the fluorescence peak of the reference standard, whose 2C DNA content was known.
B. The fluorescence peak of PI stained nuclei from R. alceifolius. The ratio of G_0/G_1 peak means of R. alceifolius compared to that of P. sativum was equal to 0.625 (125/200) and hence the 2C DNA content was estimated to be 0.625 x 9.09 = 5.681 pg

Nuclear DNA estimation: Flow cytometric analysis of the isolated nuclei resulted in histograms of their DNA content as compared to the reference standard and represented one peak of the G_0/G_1 nuclei of *R. alceifolius*. The nuclear DNA content of the plant species was presented in the

Table 1 while the histogram of the fluorescence peak was presented in the Fig. 1. The fluorescence peak of PI stained nuclei from *P. sativum* was calibrated to the channel 200 (Fig. 2A). The result from the PI-stained nuclei isolated from *R. alceifolius* showed G_o/G_1 peak in channel 125 (Fig. 2B). The ratio of G_o/G_1 peak mean of *R. alceifolius* compared to that of *P. sativum* was found to be 0.625 (125/200) and the 2C DNA content estimated to be 0.625 x 9.09 = 5.681 pg. The DNA C-value or genome size for *R. alceifolius* was estimated to be 2.84 pg (Table 2). Using the conversion factor (1 pg = 0.978 x 10^9 bp) the genome size of *R. alceifolius* was found to be 2.77 x 10^9 bp, (Table 2).

DISCUSSION

Addition of 4% PVP in the extraction buffer purged the phenolic contents of the extract and facilitated better yield and quality of genomic DNA (Table 1). Additional chloroform: isoamyl alcohol (24:1 v/v) extraction step cleared the aqueous phase of the extract and removed large amounts of precipitates from the extract. Isopropanol precipitation overnight at room temperature (25^oC-30^oC) improved the yield and quality of the isolated DNA (Table 1). These modifications helped to increase the quality and quantity of genomic DNA isolated from *R. alceifolius*. Restriction endonuclease digestion requires fairly clean and large quantities of DNA [8, 9]. The purity of the isolated DNA obtained through the modified protocol was evident from the restriction digestion experiment (Fig. 1B). Thus the protocol modified was effective enough in obtaining high yield of quality DNA.

Otto buffer used in this study, comprised phosphate /citric acid buffer of pH 7.3 and consisted of separate nuclear isolation and staining steps. It is possible to keep isolated nuclei in the Otto I buffer at room temperature for prolonged periods of time without a negative influence on DNA staining [10]. The use of internal standard gave poor reading of the result in peak qualities. This is probably resulting from interference between the staining solutions and the genomes of two species. For this reason, external reference standard was preferred and controlled after every three samples to check the calibration of the flow cytometer by adjusting the gain of pea to channel 200. Earlier report also indicated that results obtained from both external and internal reference standard were found to be statistically similar DNA content in some plant species [11, 12]. In the earlier investigation the species gave poor reading in peak qualities when internal standard was used [3]. It is of interest that external standardization is only applicable to species/buffer combination used in this investigation and preferable until the issues with internal standardization of the species under study is fully understood.

The nuclear DNA content of *R. alceifolius* from Vietnam, Thailand and La Reunion Island was earlier determined to be 1.29 - 1.75 pg for triploid and tetraploid species respectively using flow cytometry [3]. The difference in nuclear DNA content of this North-East Indian *R. alceifolius* observed in the present investigation might be due to intraspecific variations in the genome size because of differences of ecological conditions or evolution of the species. In the genus *Rubus*, hybridization between closely related species is very frequent and gives viable and fecund offspring [4]. The North-East Indian *R. alceifolius* might be of hybrid and higher ploidy origin. Another explanation of this observed variation could be due to the proportion of nuclei in the G_2 stage of the cell cycle, with double DNA content as compared to nuclei in the G_0 and G_1 stages. Although the result is preliminary, this is the first study attempted for determination of nuclear

DNA content of North-East Indian *R. alceifolius*. Nevertheless, this preliminary study needs further information to evaluate the exact determination of genome size of the plant, such as taking samples from different localities and geographical regions of North-east India, use of different nuclei isolation buffers, use of different reference standards viz. *Hordeum vulgare, Allium cepa* etc. along with ploidy analyses. A comparative survey is therefore necessary to address the problem.

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