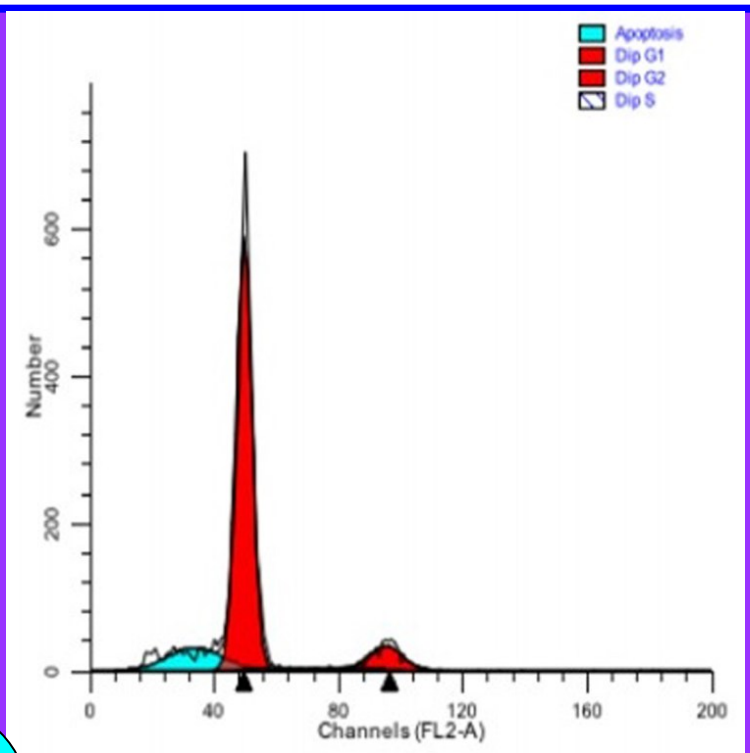

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Genome size determination of *Eclipta alba* and *Aloe barbadensis*

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Abstract

There is abundant genetic diversity of pharmacologically important plants around the globe and this pool of genetic variation serves as the base for selection as well as for plant improvement. The major cause of such genetic diversity is the variation in their genetic material, as called genome. In the present study, an attempt was made to determine the genome size of *Eclipta alba* and *Aloe barbadensis* by flow cytometry using *Pisum sativum* as a reference standard. The nuclear DNA was calculated as 8.7 pg for *E. alba* and 9.0 pg for *A. barbadensis*. The genome size of *E. alba* and *A. barbadensis* was estimated to be 4.27 x 10^9 bp and 4.42 x 10^9 bp, respectively. Information on genome size and DNA content of these two pharmacologically important plants would provide a useful tool for future molecular biological investigations.

Introduction

Genome size of an organism is an important biodiversity character with wide range of modern biological uses. Till date, genome size of only a fraction of plant species is known (Dolezel and Bartos, 2005). Knowledge of genome size and copy number of a gene in genome are essential for assessing the coverage of genomic library (Arumugunathan et al., 1999). Furthermore, knowing the genome size could be an additional parameter for species-specific phenology (Lysak et al., 2000). In recent years, the flow cytometry has become a preferred technique for estimating the nuclear DNA content because of its ease and accuracy (Bennett and Leitch, 2005a; Dolezel et al., 1998).

There is a huge demand of medicinal plants that are being depleted at an alarming rate (Gantait et al., 2014). Here, we carried out the genomic study of two traditionally important medicinal plants *Eclipta alba* and *Aloe barbadensis* (Ray et al., 2013; Jadhav et al., 2009). *A. barbadensis* has been used as folk medicine for over 2000 years for treating gastrointestinal problems, skin diseases, skin care, etc (Rajeswari et al., 2012; Kumar et al., 2010). Both plants have strong anti-alopecic effect (Ray et al., 2015; Datta et al., 2009). Alopecia has been observed as a major adverse effect of chemotherapy, immunosuppressant, anticancer and many others drugs (Roy et al., 2007). Being an important medicinal plant, genome size of both the plants was not reported till date which prompts us to select both the medicinal plants for the present study.

Materials and Methods

Collection of plants

E. alba and *A. barbadensis* plants were collected from the medicinal plants garden at Tezpur University, Tezpur, Assam, India. *A. barbadensis* plantlets were cultivated in pots with sand and less water-absorbing soil for a period of 30 days with regular pulverization. *E. alba* plantlets were grown in water absorbing soil for a period of 15 days till plants attained maturity and its seeds were also used for cultivation in higher quantity.

Morpho-phenological study of the plants

The important morphological characters such as plant height, leaf shape, arrangement, type of inflorescence, flower color and type of seeds were studied for both the selected plants and recorded. Height and length of the plant and size of the leaf were measured using measuring tape. Scale was used to measure length petiole, flowers diameter, etc. Color chart was used to describe the flower color. Data were collected from five sample plants. The phonological data such as flower initiation, flowering period, seed formation etc., were studied and recorded.

DNA isolation

Fresh young leaves were used for the isolation of DNA from the selected plant species. The leaves were collected in the morning, washed with distilled water repetitively, placed in between moist tissue papers and then stored in darkness at room temperature. The CTAB based DNA isolation protocol described by Doyle and Doyle (1990) was used and standardized with slight modification. The chloroform:isoamyl alcohol (24:1) washing was performed twice to clear the aqueous phase of the extract. Before addition of ice-cold isopropanol, 3 mL of 5M NaCl solution was added to the sample to precipitate the DNA.

Purity and yield of the isolated DNA

The concentration and the purity of the isolated DNA were measured by taking the reading at 260 nm and 280 nm in a UV/VIS spectrophotometer (Beckman DU® 530 Life Sciences) against blank and diluted sample. Isolated DNA sample (5 µL) was taken in a quartz cuvette and made up the volume to 1 mL by adding double distilled water. Since 1 OD (optical density) corresponds to 50 µg of double stranded (ds) DNA/mL, the following calculation was done to determine the concentration of DNA:

DNA concentration (µg/mL) = (OD_{260}) x (dilution factor) x (50 µg/mL).

The ratio of absorbance of DNA solution at 260 nm/280 nm is a measure of the purity of DNA sample and it should be in between 1.75 to 2.00.

Genome size determination

Genome size of the plants was determined by using flow cytometry according to the procedure described by Otto (1990) with minor modifications. The samples were analyzed in a FACS calibur flow cytometer (Becton Dickinson, USA) for relative DNA content of isolated nuclei. The instrument was calibrated using FACS COMP software. Garden pea (*Pisum sativum*) was used as the external reference standard. The use of an internal reference standard gave poor reading of results in peak quantities, probably resulting from interference between the staining solutions and the genome of pea and the selected species. For this reason external reference standard was used and controlled every 3 samples to check the calibration of the flow cytometer. The gain of the instrument was adjusted so that G_o/G_1 peak of pea (reference standard) was positioned at channel 200. The nuclear DNA content of the plant samples was estimated according to the equation:

2C nuclear DNA content of the sample = (9.09 x G_o/G_1 peak mean of the sample) / G_o/G_1 peak mean of pea.

The means of nuclear DNA content were calculated for each sample and analyzed as a single value.

Results and Discussion

Morpho-phenological character of the plants

E. alba belongs to the family *Asteraceae*, is a small herb with white flower heads. The species grows in moist and water-logged locality and is not usually found in dried areas and grows just after the first showers of rainy season. It is an annual, erect or prostate, much-branched herb and rooting at nodes. The mature plant attains an average height of 40.4 ± 3.4 cm and girth 4.1 ± 0.7 cm, covered with white hairs rising from the base. The root system consists of finely branched thin roots penetrating up to a depth of about 15-20 cm. Leaves of the plant are sessile, lanceolate or elliptic and oblong-lanceolate, distantly toothed, sharp, narrowed and pointed at both ends. Lengths of the leaves ranges from 2.6 ± 0.2 to 3.5 ± 0.8 cm and breadth varied in between 1.3 ± 0.2 to 1.7 ± 0.3 cm (Table I).

Table I

Morphological characteristics of *E. alba*

Plant No.	Plant height (cm)	Girth of the plant (cm)	Leaf length (cm)	Leaf breadth (cm)	Petiole length (cm)
1	42	3.4	2.7 ± 0.4	1.4 ± 0.3	4.4 ± 0.7
2	45	4.1	2.6 ± 0.2	1.3 ± 0.2	3.8 ± 0.5
3	36	3.7	3.4 ± 0.2	1.5 ± 0.1	4.7 ± 0.7
4	40	3.9	2.6 ± 0. 7	1.7 ± 0.3	5.2 ± 0.2
5	39	5.2	3.5 ± 0.8	1.6 ± 0.1	4.6 ± 0.4

Table II

Phenological characters of *E. alba*

Plant No.	Flower length (cm)	Flower diameter (cm)	Length of inflorescence (cm)	Dia. of the inflorescence (cm)	Fruit length (cm)	Seed weight (mg)
1	1.2 ± 0.1	0.9 ± 0.2	4.2 ± 1.2	0.5 ± 0.1	0.3 ± 0.0	2.1 ± 0.3
2	1.1 ± 0.1	0.7 ± 0.1	5.2 ± 0.7	0.5 ± 0.3	0.3 ± 0.1	1.4 ± 0.1
3	1.3 ± 0.1	1.0 ± 0.3	4.9 ± 1.4	0.7 ± 0.2	0.4 ± 0.2	1.2 ± 0.3
4	1.0 ± 0.1	0.6 ± 0.2	6.9 ± 2.4	0.7 ± 0.2	0.5 ± 0.1	1.1 ± 0.4
5	1.1 ± 0.1	0.8 ± 0.1	4.7 ± 3.0	0.7 ± 0.3	0.5 ± 0.0	1.1 ± 0.1

E. alba plants flower for 15 days and flowers are solitary. Flowers are axilliary or terminal, tubular, 6 sepals present in each calyx, occasionally very minor tooth on the top of the achene. Flower heads are sub-globose and small. The length of the flowers ranged from 1.0 ± 0.1 to 1.3 ± 0.1 cm and the diameter 0.6 ± 0.2 - 1.0 ± 0.3 cm (Table II). On the other hand, the length of the inflorescence ranges from 4.2 ± 1.2 - 6. 9 ± 2.4 cm and the diameter varies from 0.5 ± 0.3 - 0.7 ± 0.3 cm. Fruits are dark green, minute and length varies from 0.3 ± 0.0 - 0.5 ± 0.1 cm and width 1.5-2.0 cm. Seeds are minute, oval shaped, black in color and weight varies between 1.1 ± 0.1 and 2.1 ± 0.3 mg. The obtained morpho-phenological data of *E. alba,* is in agreement with the characters described by Kanjilal and Bor (1939).

A. barbadensis commonly referred to as *Aloe vera*, is one of the 420 species belonging to the family *Liliaceae*. It is a stemless, perennial, succulent plant arising directly from the stem. The average height of the plant at maturity is 56.4 ± 6.3 cm and girth at the base 10.8 ± 2.8 cm and at the top 44.2 ± 5.2 cm. Leaves grow in a spiral rosette around the stem in the ground level but the stem can grow up to 12-15 cm in older plants while in younger plants, it is 5-8 cm and the average width of the stem is 6-8 cm. There are 20-25 leaves per plant; older leaves are more erect as compared to the younger ones. In the young plants, leaves are bright green in color with whitish spots on both sides and on full maturity leaves become grey-green with the disappearance of the whitish spots. The average length of leaves varies from 41.8 ± 3.9 - 50.5 ± 6.1 cm and breadth ranges from 5.1 ± 1.1 - 9.7 ± 0.9 cm (Table III), tapering in the middle with saw-like teeth along the margin of the leaves. The main root grows vertically inside the soil from the rhizosphere base, up to a length of 40-45 cm, from where root hairs arise laterally. Petiole is absent as leaves grow directly from the stem. Panda (2003) reported similar data for the plants of *A. barbadensis*.

Genomic DNA isolation, purification and yield

The genomic DNA from *E. alba* and *A. barbadensis* was isolated using Doyle and Doyle (1990) protocol with slight modifications. In this study tender leaves were used having high cell density and less polysaccharides (Towner, 1991). The protocol involves repetitive washing with chloroform: isoamyl-alcohol (24:1) to remove the aqueous phase. The ice-cold isopropanol was added and it was kept overnight at room temperature to precipitate the DNA. 1.5M NaCl solution was used in the experiment to remove polysaccharides by increasing their solubility in isopropanol so as to bind and precipitate DNA. Warude et al. (2003) reported that 1.5 M NaCl was effective in removing polysaccharides. It also helps to modulate the cation concentration in the extraction buffer (Kawata et al., 2003). For better yield and purity, RNase was added in the reaction mixture and incubated at 37°C for 1 hour. Further to separate polysaccharides from the DNA, CTAB is used as a detergent in the extraction buffer. Richards et al. (1994) suggested that the polysaccharides present in the cell may interfere with biological enzymes such as polymerases, restriction endonucleases and also ligases. According to Kawata et al. (2003), CTAB was included in the extraction buffer as reagent for protein denaturation in the isolation process. Also to remove the magnesium ion, a necessary co-factor for nucleases, EDTA was included in the extraction buffer (Kawata et al., 2003; Puchooa et al., 2004). Oxidations of polyphenols present in the plant crude extracts reduce the purity of the isolated DNA. For the purpose, β-mercaptoethanol was used as a strong reducing reagent and used to prevent the oxidation of polyphenols (Kawata et al., 2003; Puchooa et al., 2004; Pirttilä et al., 2001).

The isolated DNA from both the plants was electrophoresed along side *Hind* III digested λ DNA marker (Figure 1). The purity of the isolated DNA from both the plants was calculated by taking OD at 260 and 280 nm in a UV/VIS spectrophotometer (Beckman DU® 530 Life Sciences). The purity of the isolated DNA samples was found to be 1.8 and 1.8, respectively suggesting high-quality and the yield was calculated using optical density at 260 nm. Yields of the DNA isolated from both the plants are presented in Table IV. The molecular weights of the isolated DNA were much

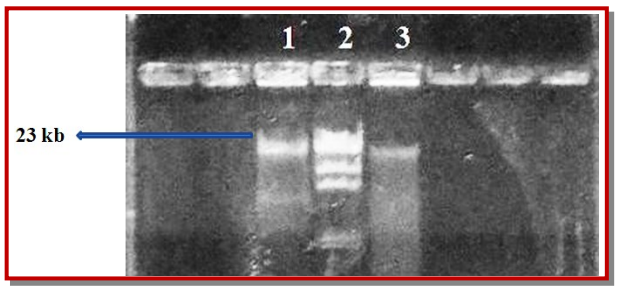

Figure 1: Isolation of genomic DNA from selected plants (lane 1: DNA of *E. alba*; lane 2: *Hind* III digested λ DNA marker; lane 3: DNA of *A. barbadensis*)

in DNA content of both the standard and the tested plants. The gain in the instrument was set so that the fluorescence peak of the external reference standard *P. sativum* could be placed in channel 202 of the 1023-channel scale. The fluorescence peak of DNA nuclei of *E. alba* was recorded at channel 195 (Figure 2a) and that of *A. barbadensis* at channel 201 depicted in Figure 2b. The peak ratio of *E. alba* and *A. barbadensis* were 1.0 and 1.0, respectively (Table V). The absolute 2C nuclear DNA content of *E. alba* and *A. barbadensis* was calculated to be 8.7 and 9.0 pg. The genome size of *E. alba* and *A. barbadensis* was estimated to be 4.3 x 10^9 bp

Table III

Morphological characters of *A. barbadensis*

Plant No.	Plant height (cm)	Girth of the plant (cm)	Leaf length (cm)	Leaf breadth (cm)
1	54	9	41.8 ± 3.9	5.1 ± 1.1
2	50	10	43.5 ± 2.7	6.2 ± 1.0
3	52	12	42.4 ± 4.0	8.1 ± 0.8
4	61	8	50.5 ± 6.1	9.3 ± 1. 7
5	65	15	48.7 ± 4.6	9.7 ± 0.9

above 23 kb. The DNA yield was calculated to be 16.5 and 12.3 µg/g in the case of *E. alba* and *A. barbadensis*, respectively.

Genome size determination

Flow cytometric analysis of the isolated nuclei resulted and 4.4 x 10^9 bp.

Estimation of DNA content in cell nuclei is an important application in plant sciences which has mostly been done with flow cytometry. It is a well-accepted method for the determination of genome size

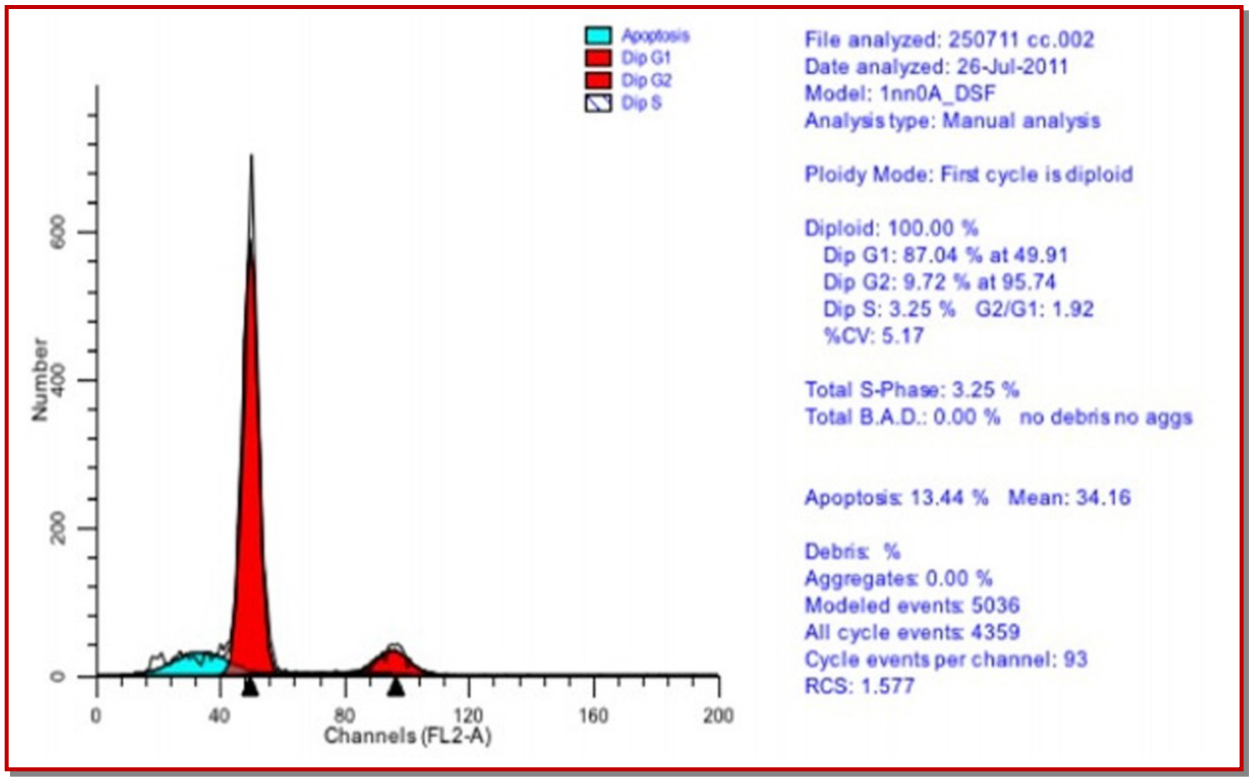

Figure 2a: Genome size of *E. alba*

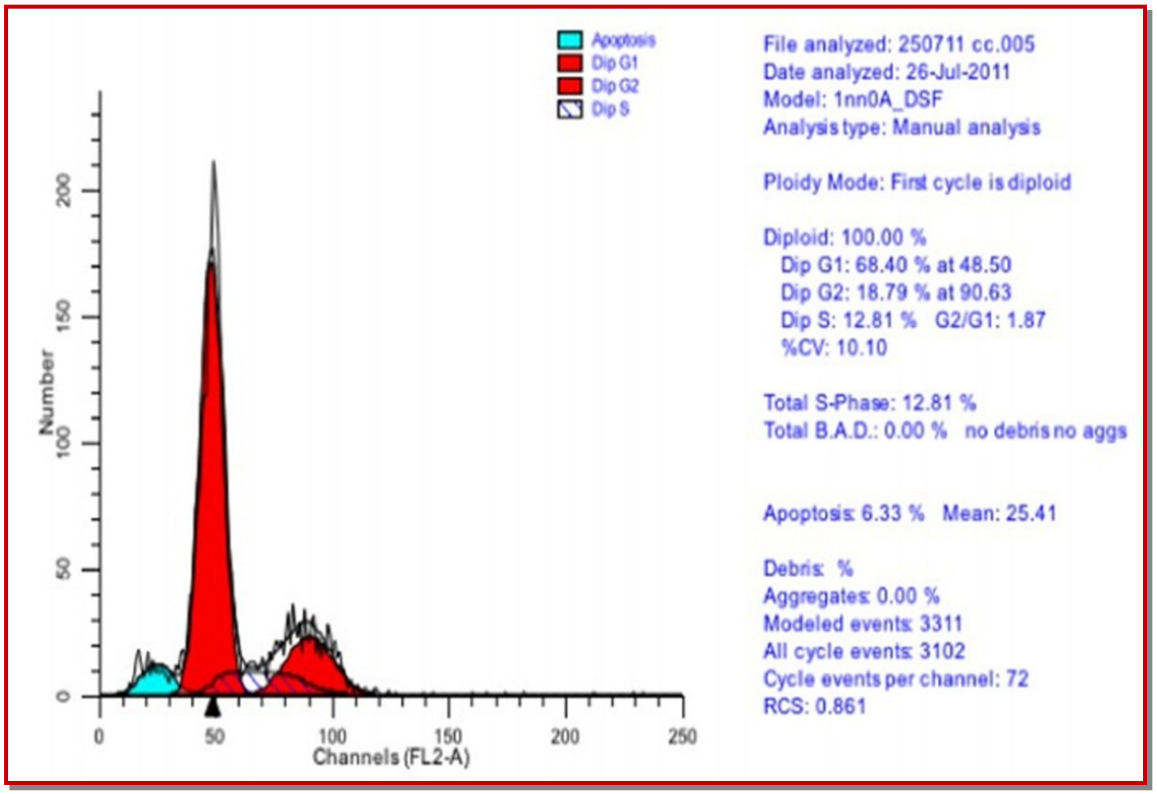

Figure 2b: Genome size of *A. barbadensis*

Table IV

Purity and yield of isolated DNA

Plant No.	*E. alba*	*A. barbadensis*
A260	0.2	0.1
A280	0.1	0.1
A260/A280	1.8	1.8
DNA yield (µg/g)	16.5	12.3

Table V

Genome size determination

Plant No.	*E. alba*	*A. barbadensis*
Fluorescence peaks	195	201
Peak ratio	1.0	1.0
2C DNA content (pg)	8.7	9.0
C-value (pg)	4.3	4.5
C-value (bp)	4.3×10^9	4.4×10^9

and estimation of nuclear DNA content because of its accuracy and ease. The instrument can measure a large number of nuclei content from a small amount of tissue and the relative DNA content. The result of the analysis is usually displayed in the form of a histogram of relative fluorescence intensity, representing relative DNA content. The genome size of an unknown sample can be determined only after comparison with the nuclei of a reference standard (Doležel and Bartoš, 2005). Flow cytometry has two key advantages, first a large number of cells/particles can be evaluated in a very short time, which makes the results statistically strong and representative of the whole population. Even at rates up to 1,00,000 cells/second, approximately 20 parameters from each cell/particle can be collected and analyzed. The second key advantage is the ability to physically separate single cells from mixed populations at rates up to 70,000 cells per second (Doležel et al., 2007). In the present study, propidium iodide (PI) was used as flurochrome for measuring the nuclear DNA content and the genome size of both plants. PI-based flow cytometry produced consistent result based on Feulgen micro-spectrophotometry (Johnston et al., 1999). For nucei isolation, Otto buffer was used which is phosphate/citric acid buffer, having pH 7.3 and it works well for separating nucleus and DNA. The isolated nuclei can be kept in Otto buffer at room temperature for prolonged time periods without negative influence on staining of DNA (Doležel and Bartoš, 2005).

The most important criterion in genome size determination is, the correct choice of reference standard, which has largely been neglected (Doležel et al., 1998). *P. sativum* was selected as the reference standard for the

flow cytometric analysis of the selected plants isolated cell nuclei. *P. sativum* is stable, easy to grow, and high quality nuclei suspensions can be prepared from leaves, which appear to be free from compounds interfering with PI staining (Baranyi and Greilhuber, 1995; Baranyi et al., 1996). The 2C value of the nuclear genome of *P. sativum* is 9.09 pg (Dolezel and Gohde, 1998) and is in the known range of genome sizes of plants which facilitates calibration of reference standards with higher or lower genome sizes (Doležel and Bartoš, 2005). For the present investigation, the genome size of *E. alba* was shorter as compared to the genome size of *A. barbadensis*. Although the result is preliminary, this is the first study attempted for determination of nuclear DNA content of both *E. alba* and *A. barbadensis*.

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