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GENOME SIZE DETERMINATION AND RAPD ANALYSIS OF FOUR EDIBLE AROIDS OF NORTH EAST INDIA

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ABSTRACT

Four edible aroid species were selected for the study. The genomic DNA of the plants was isolated and estimated. A part of the genomic DNA was used for analysis using six different primers from Operon Technologies, USA. The genome size determined for the aroids is in the order of Colocasia esculenta> Xanthosoma caracu> Xanthosoma sagittifolium > Amorphophallus paeonifolius. Amorphophallus species was found to be 50% similar to both Xanthosoma caracu and Colocasia esculenta. The analysis will provide a ground for exploring the vast diversified aroid population of the region.

Keywords: ***RAPD; aroid; genome size***

[I] NTRODUCTION

Corms were important root crops of Asia [1]. India-Myanmer area is considered as the center of origin for *C. esculenta* [2]. Assessment of genetic diversity of aroids needs immediate attention for the improvement of these crops. Few reports are available on the use of molecular markers to study genetic diversity in taro such as restriction site variation in rDNA, mitochondrial DNA [3] and RAPD markers [4]. Reports are available on unpredictable changes in the chromosomes during cell division resulting in the lack of uniformity in this crop [5]. They reported chromosome number of aroids to be 2n= 22,26,28,38 and 42 in the plants collected from various locations. RAPD profiling of *Xanthosoma caracu* was reported by Schnell *et al.* (1999) [6].

The amount of nuclear DNA and size of the genome (C-value) are two important biodiversity characters, the study provides a strong unifying element in biology with practical and predictive uses. The RAPD analyses of aroid species were important for their identifications and similarity assessment. The letter 'C' of C-value stands for 'constant'; the constant amount of DNA which could be a characteristic of a particular genotype [7]. Hinegardner (1976) described genome size as an important biodiversity parameter [8]. Till date, flow cytometry and fuelgen densiometry (densitometry) are the standard methods for the determination of genome size. But, these established techniques need sophisticated instruments, which is not possible in all situations. Although, the flow cytometry yields almost accurate and reproducible amount of 2C DNA content of plant species, the problem starts with expeditions to more distant areas, when the transport and maintenance of the material become an issue. Moreover, the cost of establishing a flow cytometry laboratory may be prohibitive in certain areas [9]. The north eastern region like many other region of the country, regarded to be the biodiversity hotspot is yet to establish a flow cytometry laboratory. On the basis of the above facts, the present investigation has been undertaken to estimate the genome size using the method described by Konwar *et al.* (2007) [10].

[II] MATERIALS AND METHODS

2.1. Plant materials

Four aroid species *A. paeonifolius*, *X. caracu*, *X. sagittifolium* and *C. esculenta* were grown in Tezpur University campus. The young tender shoots of the plants were used for the isolation of genomic DNA. The primers were obtained from Operon technol. Pvt. Ltd. (USA) with sequences as follows: OPW-04: 5-CAGAAGCGGA-3; OPW-05: 5-GGCGGATAAG-3; OPW-08: 5-GACTGCCTCT-3; OPW-10: 5-TCGCATCCCT-3; OPW-15: 5-ACACCGGAAC-3 and OPW-16: 5-CAGCCTACCA-3.

2.2. Isolation of Genomic DNA

Genomic DNA was isolated using the method described by Ronning et al. (1995) [11]. One gram leaf frozen with liquid nitrogen was ground using mortar and pestle. The extraction buffer 10 ml [100mM Tris-HCl, pH 8.0; 250mM NaCl; 25 mM ethylenediaminetetraacetic acid (EDTA); 0.1% (v/v) 2-mercaptoethanol; 100 mM diethyldithiocarbamic acid

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(DEDTC); 2% (w/v) polyvinylpyrrolindone (PVPP)] was added and grinding continued. The tissue homogenate was filtered through four layers of cheesecloth. Sodium dodecyl sulfate (SDS) was added to a final concentration of 1.25% the mixture was shaken vigorously and incubated at 65OC for 10 min. Potassium acetate was added to the final concentration of 1.2 M, again shaken vigorously and incubated at 0OC for 20 min, followed by centrifugation at 25,000 g for 20 min at 4OC. The supernatant was filtered through two layers of miracloth, combined with 2/3 volume ice cold isopropanol and precipitated at -20OC for overnight. The DNA was pelleted at 20,000 g for 15 min at 4OC. The pellet was dried and resuspended in 400µl Tris-EDTA (TE) pH 7.4. Each DNA sample was treated with ribonuclease A (RNase A, Bangalore Gene, India) with a concentration of 50 mg ml-1 and incubated at 37OC for 30 min. Organic extraction was performed adding an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) shaked vigorously and then centrifuged at 12,000 g for 10 min. The process was repeated with chloroform: isoamylalcohol. The DNA was then precipitated by adding NaCl to a final concentration of 0.2 M and two volumes of ice cold 95% ethanol, incubated at -20OC for 30 min and centrifuged for 15 min at 12,000 g to pellet the DNA. The pellet was dried and resuspended in 100 µl TE (pH 7.4).

2.3. Determination of yield and quality of DNA

The yield and quality of the DNA was determined using the method described by Gallagher (1987) [12]. The isolated DNA 50 µl was evaporated to dryness using Maxi Dry Plus under 1mbar atm pressure at 35^{O}C. The dried DNA was dissolved in 1 ml 1X TNE buffer containing 0.01 M Tris base, 1 mM EDTA and 0.2 M NaCl (pH adjusted to 7.4 with concentrated HCl). Both blank and samples were analyzed at 325nm for confirming clean cuvettes. Absorption at 230 nm for detecting any phenol contamination was also measured. The purity level of the DNA isolated was estimated from absorption at 260:280 nm. The quantification of the DNA was done putting the absorption value at 260 nm in the following formulae:

Concentration of dsDNA (µg/ml)=A_{260}/0.020

Where, A_{260}- absorption value of DNA in TNE after subtracting TNE blank absorption

value; ds, double stranded; 0.020- molar extinction coefficient with a unit of M^{-1}

cm^{-1}.

2.4. DNA amplification

PCR amplification was carried out using RAPD decamer primers (Operon Technologies, USA). The reaction mixture (25 µl) consists of 100 ng template DNA, 1X reaction buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl and 1.5 mM MgCl2), 100 µM of each of the dATP, dTTP, dCTP and dGTP, 5 pM primer and 0.5 U of Taq DNA polymerase (Bangalore gene, India). Amplification was performed using the thermal cycler Gene Amp PCR system 9700 (Applied biosystems) with a heated lid to reduce the evaporation under the conditions of an initial 1 min denaturation at 94OC, followed by 45 cycles of 1 min at 94OC, 1 min at 35OC, and 2 min at 72OC, with a final 5 min extension step at 72OC. Approximately, 15 µl of the amplified products were loaded on a 2% agarose gel and separated by electrophoresis in TAE (Tris 1.6 M, acetic acid 0.8 M, EDTA 40 mM) at 100 volts. After electrophoretic separation for 90 min, the gel was stained with ethidium bromide and visualized in UV light. A 100 bp ladder (Bangalore gene, India) was loaded in all gels used as the molecular marker. Following electrophoresis, the gel was photographed using Geldoc 1000 (Biorad).The primers used in the experiment were OPW-04, OPW-05, OPW-08, OPW-10, OPW-15 and OPW-16.

2.5. Genome size determination

Genome size determination was done using the method of Konwar *et al.* (2007) [10]. The method can be divided into two broad categories, (a) determination of leaf tissue and cell volume and (b) genome size determination.

(a) *Determination of leaf tissue and cell volume:* Fine transverse and longitudinal cross sections of the pre-weighted leaf tissue of 1 cm2 size were obtained with a sharp sterile blade and observed under a microscope (Carl Zeiss microlmaging, Germany) at 10X40x magnification. The volume of the whole tissue section (1 cm2) was determined (length×breath×thickness). The length, breath and thickness of rectangular cells; length and radius of the cylindrical cells and radius of spherical cells were measured with a micro scale having 400x magnifications. Data generated from five randomly selected cells of five random sections as well as cell volumes were recorded with the specific formulae. The intercellular space of the leaf tissue of the aroid was measured in five small leaf sections of known dimensions by measuring with a micro scale at 400x magnification.

(b) Genome size determination: The genome size of the plant species was determined using the following calculations:

Average volume of a single cell (l×b×t) = ×µ3 where,

l = length

b= breath

t = thickness

Volume of a tissue (l×b×t) = t µ3

Volume of the intercellular space = s µ3

Actual cell mass = (t-s) = v µ3

Total number of cells in the cell mass = v µ3/×µ3 = y

Now, weight of the tissue section = wg

wg tissue contains = y cells

So, 1g tissue will contain cells = y/w

DNA yield per gram of leaf tissue = d µg

= d×106 pg

So, one cell contains = (d×106)/(y/w) pg

= (d×106)/(y/w) × 978 Mbp

2.6. Data analysis for RAPD

The images were analyzed using Biored software for the estimation of molecular weight of the bands. The pair wise genetic similarity matrix between the genotypes was generated using Jaccard's coefficients.

[III] RESULTS

3.1. Yield and quality of the DNA

The absorption readings obtained of the purified genomic DNA of the four aroids were presented in **Table–1.** From the absorption values it can be concluded that the cuvettes were clean and there is no phenol contamination in the purified DNA [12]. The absorption in 325nm and 230 were taken to confirm phenol free DNA sample and clean cuvette **[Table–1]**. All four DNA samples show 260/280 ratio above 1.7 **[Table–1]**, which signifies the high purity of the DNA. The yield follows the order of X. sagittifolium > A. paeonifolius > C. esculenta > X. caracu. The X. sagittifolium shows not only high yield but also highly pure DNA **[Table–1]**.

3.2. RAPD analysis and dendrogram

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As shown in the Figure1 the primer OPW-15 sequences are present only in the C. esculenta. Figure 1 also depicts the absence of OPW-15 and OPW-16 sequences in the Amorphophallus. paeoniifolius. Amplification of genomic DNA using six primers yielded 25 reproducible RAPD loci for an average of 4.16 bands per primer; 21 (84%) of them were polymorphic and 4 (16%) were monomorphic. Among Xanthosoma sagittifolium and Xanthosoma caracu only four bands (16%) were polymorphic with the rest of polymorphism existing between Colocasia esculenta and Amorphophallus paeonifolius (figure 1) **[Table 2]**. As seen in the figure 2 the two species of Xanthosoma genus were more similar as compared to the Colocasia esculenta and Amorphophallus paeoniifolius. The Amorphophallus paeoniifolius was more similar to the two Xanthosoma species as compared to the Colocasi a esculenta.

The primers were selected in view of their polymorphism in Indian Colocasia esculenta cultivars [5]. The results showed that the both Xanthosoma sagittifolium and Xanthosoma caracu species are closely related to each other (66%) as compared to their similarity to others **[Table 3]**. Amorphophallus paeoniifolius species was found to be 50% similar to both X. caracu and C. esculenta but dissimilar to X. sagittifolium. Hence, it could be concluded that X. caracu and C. esculenta species are equally distant from A. paeonifolius species **[Table 3]**. C. esculenta used in this study might be the same morphotype M-5 having ID no. TC5 used for RAPD analysis by Lakhanpaul et al. (2003) [5].

3.3. Genome size analysis

The genome size determined for the aroids are in the order of C. esculenta> X. caracu> X. sagittifolium > A. paeonifolius. The genome size of C. esculenta was found to be 14.10 pg and it was in between 2C and 3C value as given by Bennett and Leitch (2005b) for C. antiquorum [13]. They also provided 1C value of X. sagittifolium to be 8.8 pg, which is 0.76 pg higher than that of the present investigation **[Table 4]**.

[IV] DISCUSSION

The study is a preliminary investigation to estimate the genetic similarity and dissimilarity between the edible species of aroids. The primers selected for the study were well established for the *C. esculenta* of India. In the present investigation it was observed that primers are also providing reliable polymorphism in *A. paeonifolius*. These primers selected for the study showed 88.9-100.0% polymorphism in the case of Indian *C. esculenta* morphotypes, which are also reported by Lakhanpaul *et al.* (2003) [5]. The genome diversity analyses of aroids with a limited number of genotype are not enough to prepare a dendrogram. A large scale diverse morphotypes using more number of suitable primers could be needed for the same.

The genome size of the edible species was determined using a comparatively easy and established method than prevalent expensive methods like fuelgen densiometry and flow cytometry. The genome size determination was already established by Konwar *et al.* (2007) [10]. At present, flow cytometry is becoming a popular method in determination of genome size. The method needs the establishment of the sophisticated costly equipment, flow cytometer. The cost is indeed prohibitive for most of the organizations and handling the equipment needs expert man power. On the other hand, discrepancies are observed in flow cytometry data of a single sample analysed in different laboratories. Hence, an effort was made to develop a novel but simple and less expensive method for the determination of genome size of plants without compromising the quality of the work. With the collection of suitable tender leaves from the raised corms of the selected edible aroids for genomic DNA isolation we could obtain most of the genomic DNA. The protocol followed in the investigation could yield quality DNA as evident from the UV-VIS spectrophotometric absorbencies ratio at 260:280 nm being 1.735-1.916. Two critical points were taken into account in this method; firstly, the method for the isolation of genomic DNA was such that it could isolate almost all the DNA from the nuclei, and secondly the accurate determination of the intercellular space in the concerned edible aroids species. The developing countries on one hand have a vast resource of plant biodiversity while on the other hand they face the problem of acquisition and maintenance of flow cytometers [14]. Hence, present method could be great help for the researcher of developing country having limited excess to the facilities like flow-cytometry.

Aroid species	260/280	DNA µg/gm leaf
Amorphophallus paeoniifolius	1.735	183.5
Xanthosoma caracu	1.814	159.21
Colocasia esculenta	1.754	175.21
Xanthosoma sagittifolium	1.916	207.24

Table: 1. DNA purity and content of the aroids

Primers	Base pair length (bp)
OPW-04	327a, 571b, 636a, 1264a.
OPW-05	471c, 542c, 610a, 812c.
OPW-08	903a, 924c, 1244a, 1992c
OPW-10	454a, 460c, 1027c, 1047a, 1153a, 473b.
OPW-15	1224c.
OPW-16	297d, 386c, 461d, 505c, 1149b, 2043b.

NB: a, *A. paeonifolius*; b, *X. caracu*; c, *C. esculenta* and d, *X. sagittifolium*.

Table: 2. Primers and base pair lengths of RAPD generated markers

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Aroids	*A. paeonifolius*	*X. caracu*	*C. esculenta*	*X. sagittifolium*
Amorphophallus paeoniifolius	1			
Xanthosoma caracu	0.5	1		
Colocasia esculenta	0.5	0.33	1	
Xanthosoma sagittifolium	0	0.66	0.33	1

Table: 3. Pair-wise genetic similarity matrix between 4 aroids based on Jaccard's coefficient

Aroid species	Genome size in pg (C)	Genome size in Mbp (C)
A. paeonifolius	5.04	4929.12
X. caracu	8.21	8029.38
C. esculenta	14.1	13789.8
X. sagittifolium	8.04	7863.12

Table: 4. Genome size of the aroid species

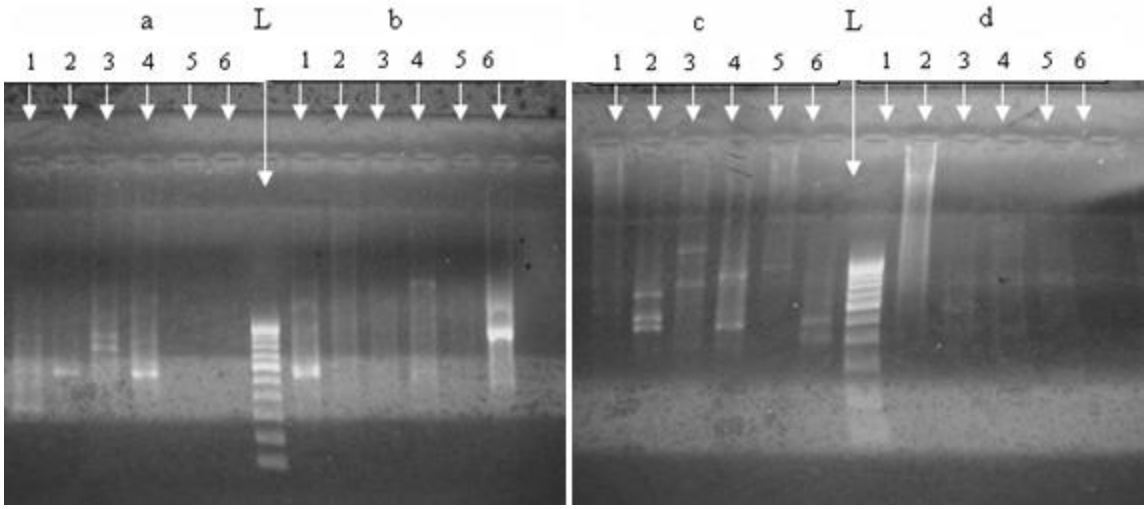

Fig: 1. RAPD profile of aroid species. 1-6: OPW-04, OPW-05, OPW-08, OPW-10, OPW-15 and OPW-16, respectively. (a) *A. paeonifolius*, (b) *X. caracu*, (c) *C. esculenta* and (d) *X. sagittifolium*. L represent kilobase ladder, 100-1000 bp were developed.

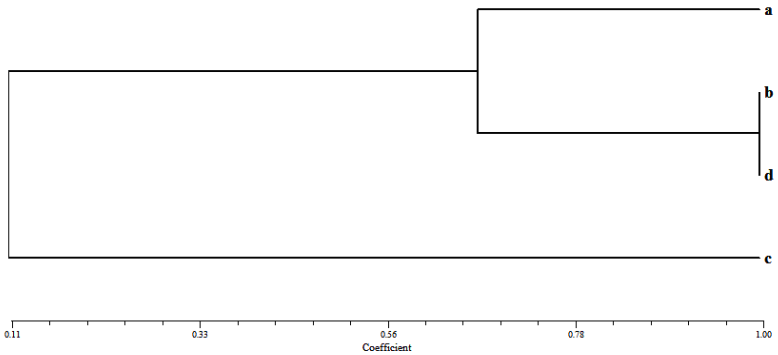

Fig: 2. The dendrogram based on RAPD data (a, *Amorphophallus paeoniifolius*; b, *Xanthosoma caracu*; c, *Colocasia esculenta* and d, *Xanthosoma sagittifolium*).

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[V] CONCLUSION

The genome size of the species was determined using a new method. The method did not require costly instruments like flowcytometry. As mentioned in the discussion the method provides comparable results with flow cytometry. Reports are available regarding variations among the flowcytometry results [7].The amount of DNA in the nucleous is directly proportional to the chance of survival of a species by not becoming extinct [7]. The contribution to the genome size research from a country like India is very little/not representative [7]. In the light of the discussion above, it can be suggested that popularizing this type of genome size estimating method will allow the developing country researcher to contribute more to the field of genome size. The RAPD analysis of the aroid species provides information on the genetic similarity and phylogenetic relation.

FINANCIAL DISCLOSURE

The present method is suitable especially for comparisons of genome sizes of different species and genus of living forms. The method needs up gradation and future research to establish it in the field of genome side estimation.

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