Karyotype and RAPD Diversity in Four Varieties of Gossypium hirsutum L.

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Received June 24, 2017; accepted August 5, 2017

Summary Four recently released varieties of Gossypium hirsutum L. by Bangladesh Cotton Development Board (CDB) viz. CB-12, CB-13, CB-14, and Rupali-1 were studied cytogenetically and at the molecular level to elucidate the genomic diversity among the varieties. Although the four varieties were found to possess 2n=52 chromosomes, they differed in respect of karyotype formulae such as 48m+4sm in CB-12, 52m in CB-13, and 50m+2sm in CB-14 and in Rupali-1. Moreover, these varieties differed with respect to other karyotypic features such as total length of 2n chromosome complements, number of satellites, range of relative length, centromeric index, etc. Different number of satellites viz. two in CB-12 and Rupali-1 and four in CB-13 and CB-14, were found after only CMA-staining. CMA-fluoresced satellites of four cotton varieties suggest stain specificity. A wide range of CMA-positive bands were found in these four varieties. Out of four varieties no DAPI-positive band was found in CB-13 and Rupali-1. Fluorescence banding revealed genomic alteration and karyotype diversification within the four varieties. The five RAPD primers generated 53 bands with 90.56% polymorphism indicating diverse genomic nature. Two unique RAPD bands were found in Rupali-1 which could be used as markers. Therefore, each variety could be characterized by diverse karyotypes and molecular data.

Key words Fluorescent banding, Karyotype, RAPD, Gossypium hirsutum L.

Cotton (Gossypium sp.) is the important cash crop in Bangladesh after Jute. Cotton fibre obtained from the epidermis of the cotton seeds is often called the king of fibers. Cotton, unique among agricultural crops, provides food and fibre. Cotton is a major natural fibre crop and also provides edible oil and seed by-products for livestock food.

Commercial cotton fibres are mainly obtained from different Gossypium species. The genus Gossypium L. belongs to the family Malvaceae possessing 50 different species (Chaudhary et al. 2010) of which 45 species are diploid (2n=2x=26) and five allotetraploids (2n=4x=52) (Brubaker et al. 1999). Out of 50 Gossypium species, only four are cultivated: Gossypium herbaceum L., G. arboreum L., G. barbadense L., and G. hirsutum L. Among the cultivated species, G. hirsutum has the highest yield potential and is the largest globally cultivated species with about 90% contribution to the world cotton market.

Being an important cash crop, breeders of different national agencies are attracted to this crop. As a consequence, Bangladesh Cotton Development Board (CDB) has been conducting research since 1991 and was able to release initially eleven germplasms (Upland Cotton) namely, CB-1, CB-2, CB-3, CB-4, CB-5, CB-6, CB-7, CB-8, CB-9, CB-10, and CB-11. Recently CDB has released four more varieties of G. hirsutum viz. CB-12, CB-13, CB-14, and Rupali-1 through a series of selections based on morphological and physiological features (yield, fiber quality, resistance against certain pests and diseases, etc.) (Iqbal et al. 1997).

A successful breeding program depends on the complete knowledge and understanding of the genetic diversity within and among genetic resources of the available germplasms. Though various breeding procedures like hybridization, mutation, and polyploidy breeding, etc. can be used for enlarging the available variability, it is always essential to quantify and qualify the genetic variability.

Karyotype analysis provides a preliminary idea about the genome of a specimen. It is stable and specific for each individual. Only a few earlier workers tried to characterize cotton with classical karyotype analysis (Beasly 1942, Mehetre et al. 1980, Nei and Li 1985, Wang et al. 1996, Mehetre and Thombre 1977, Davie 1933, Mehetre and Thombre 1980, Sanamyan et al. 2011, Farzaneh et al. 2010).

Probably due to the large number and small size of chromosomes, it was not possible to determine detailed karyotype by the earlier researchers. Actually conventional karyotype analysis alone is unable to critically express the differences among different species when they may possess similar 2n chromosome numbers or other

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DOI: 10.1508/cytologia.82.535
karyotype parameters (Khatun and Alam 2010, Khatun et al. 2011). In such a case, a combination of modern cytogenetical and molecular techniques is necessary for comparative study among different species.

Staining with DNA-base specific fluorochromes such as chromomycin A3 (CMA) and 4′-6-diamidino-2-phenylindole (DAPI) is a method for karyotype study that is quite satisfactory for detailed and critical chromosome analysis such as identification of individual chromosomes, determination of amount and site of AT- and GC-rich base pairs in chromosomes, etc. (Schweitzer 1976, Alam and Kondo 1995, Kondo and Hizume 1982, Jessy et al. 2005, Islam and Alam 2011, Sultana et al. 2011).

Moreover, DNA fingerprinting by RAPD is one of the molecular methods for characterizing species. This method has been used as versatile tool for investigating various genomic aspects of organisms (Williams et al. 1990).

In 2013, an attempt was undertaken in the molecular cytogenetics laboratory, Department of Botany, University of Dhaka, to characterize 11 varieties of *G. hirsutum* (CB-1, CB-2, CB-3, CB-4, CB-5, CB-6, CB-7, CB-8, CB-9, CB-10, CB-11) released by CDB (Sultana and Alam 2016a).

As a continuation of the previous work, the present investigation was carried out to characterize and compare the recently released four varieties of *G. hirsutum* by CDB with the following aims:

i. to construct the conventional orcein-stained karyotype for each variety,

ii. to compare the fluorescent banding pattern after staining with CMA- and DAPI-fluorochromes,

iii. to elucidate the phylogenetic relationship among four varieties of *G. hirsutum* on the basis of RAPD banding patterns, and

iv. to characterize each variety with cytogenetical and molecular markers.

Materials and methods

Four varieties of *Gossypium hirsutum* L viz. CB-12, CB-13, CB-14, and Rupali-1 were collected from Bangladesh Cotton Development Board (CDB) and maintained in the Botanic Garden, Department of Botany, University of Dhaka, Bangladesh.

**Cytogenetic study**

Healthy roots were collected and pretreated with 8-hydroxyquinoline (0.002 M) for 3 h at 20–25°C followed by 15 min fixation in 45% acetic acid at 4°C. The pretreated RTs were hydrolyzed for 1–1.20 min (depending on thickness of root) at 60°C in a mixture of 1 N HCl and 45% acetic acid (2:1). The root tips were stained and squashed in 1% aceto-orcein. For CMA- and DAPI banding, Alam and Kondo’s (1995) method was used with slight modification. After hydrolysing and dissecting, the materials were squashed with 45% acetic acid. The cover glasses were removed quickly on dry ice and allowed to air dry for at least 24 h before study. The air-dried slides were first incubated in McIlvaine’s buffer (pH 7.0) for 30 min followed by Distamycin A (0.1 mg mL⁻¹) treatment for 10 min. The slides were rinsed mildly in McIlvaine’s buffer supplemented with MgSO₄ (5 mM) for 15 min. One drop of CMA (0.1 mg mL⁻¹) was added to the materials for 15 min in a humid chamber and then rinsed with McIlvaine’s buffer with MgSO₄ for 10 min. Slides were mounted in 50% glycerol and kept at 4°C overnight before observation. These were observed under Nikon (Eclipse 50i) fluorescent microscope with blue violet (BV) filter cassette. For DAPI-staining, after 24 h of air drying, the slides were first incubated in McIlvaine’s buffer (pH 7.0) for 27 min and treated in Actinomycin D (0.25 mg mL⁻¹) for 10 min in a humid chamber. The slides were immersed in DAPI solution (0.01 mg mL⁻¹) for 20 min and mounted with 50% glycerol. These were observed under a Nikon (Eclipse 50i) fluorescent microscope with ultra violet (UV) filter cassette.

**DNA isolation**

Leaves were harvested and total genomic DNA was extracted by using modified CTAB method (Doyle and Doyle 1987). DNA concentration was quantified through spectrophotometer (Analylijkjena, Specord 50, Germany). The A 260/280 readings for DNA samples were 1.6–1.8.

**PCR amplification and primer survey**

The PCR reaction mixture for 25 μL containing template DNA (25 ng) 2 μL, de-ionized distilled water 18.8 μL, Taq buffer A 10× (Tris with 15 mM MgCl₂) 2.5 μL, primer (10 μM) 1.0 μL, dNTPs (2.5 mM) 0.5 μL and Taq DNA polymerase (5 U μL⁻¹) 0.2 μL. PCR amplification was done in a 2720 thermal cycler (Applied Biosystems by Life Technologies) for 45 cycles after initial denaturing 94°C for 5 min, denaturing at 94°C for 45 sec, annealing at 34°C for 30s, extension at 72°C for 3 min and final extension at 72°C for 5 min. Five random primers such as Primer-21 (5′-GGC ACT GAG G-3′), Primer-23 (5′-GTC AGG GCA A-3′), OPC-26 (5′-CAC GTT ATC GCA-3′), OPC-96 (5′-ACC AAG AAA GGG-3′), and OPF-22 (5′-AAG ATC AAA GAC-3′) were used in the present study for screening.

**Gel electrophoresis**

The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing ethidium bromide (10 mg mL⁻¹) 8 μL and 100 mL 1×TAE buffer. Agarose gel electrophoresis was conducted in 1×TAE buffer at 50 volts and 100 mA for 1.5 h. DNA ladder (1 kb) was electrophoresed alongside the RAPD reactions as marker.
DNA bands were observed on UV-transilluminator and photographed by a gel documentation system.

**Scoring and data analysis**

The PCR products were analyzed after gel electrophoresis. The photographs were critically discussed on the basis of presence (1) or absence (0), size of bands and overall polymorphism of bands. These were carried out for further investigation. The scores obtained using all primers in the RAPD analysis were then pooled for constructing a single data matrix. This was used for estimating polymorphic loci, Nei (1972) gene diversity, genetic distance (D), and constructing a UPGMA (Un-weighted Pair Group Method of Arithmetic Means) dendrogram among the specimen using computer program "POPGENE" (Version 1.31).

Results and discussion

**Conventional karyotype**

In this study, four varieties of *Gossypium hirsutum* L. viz. CB-12, CB-13, CB-14, and Rupali-1 were found to possess 2n=52 chromosomes (Figs. 1–4, Table 1). A similar chromosome number for *G. hirsutum* L. was reported earlier by different scientists (Beasley 1942, Mehetre et al. 1980, Nei and Li 1985, Wang et al. 1996, Sultana and Alam 2016a). Thus the present report on 2n chromosome number of four varieties of *Gossypium hirsutum* L. correlates with the earlier reports.

Out of four varieties of *G. hirsutum* L., CB-13 was found to possess all metacentric chromosomes (52m) (Fig. 2, Table 1). In contrast, the other three varieties (CB-12, CB-14, and Rupali-1) had different numbers of sub-metacentric chromosomes (sm) viz. 4sm in CB-12 and 2sm in CB-14 and Rupali-1 (Figs. 3, 4, Table 1). Nie and Li (1985) observed 16 sub-metacentric chromosomes and 2 sub-telocentric chromosomes in *G. hirsutum*. Moreover, Sultana and Alam (2016) found a few sub-metacentric chromosomes in some varieties of *G. hirsutum* released by CDB earlier. According to Stebbins (1971), karyotypes consisting of only metacentric chromosomes are primitive in nature. The presence of a few sub-metacentric chromosomes indicated genetically more advanced karyotype for CB-12, CB-14, and Rupali-1 varieties that may help for wider ecological adaptation.

**Satellite**

The satellites of *G. hirsutum* have several strange features. After CMA-staining, satellites were found in the four cotton varieties (Figs. 5–8, 13–16, Table 1). The satellites were confined to chromosome pair XII in every variety. Sultana and Alam (2016a) reported the presence of a pair of satellites in chromosome pair XII in eleven germplasms of *G. hirsutum*. The above data suggested that satellites on chromosome pair XII was common and stable for all varieties of *G. hirsutum*. On the other hand, in this study, another pair of satellites was found in chromosome pair XIX of CB-13 and in pair XIV of CB-14 (Figs. 6, 7, 14, 15). Other than pair XII, no satellite was found in CB-12 and Rupali-1. Moreover, Sultana and Alam (2016a) did not report about extra satellites except pair XII. Therefore, these satellites were not stable for different varieties of *G. hirsutum* released by CDB. Another feature is that after DAPI-staining no satellite was found on pair XII in the four varieties of *G. hirsutum* (Figs. 9–12, 17–20, Table 1). The CMA-positive and the DAPI-negative feature, i.e., the reversible staining pattern of the satellites revealed that the satellites were made fully of GC-rich base sequences (Alam and Kondo 1995, Lubna et al. 2004, Ruma et al. 2006, Sumner 1990, Schweizer 1976).

Finally after orcein staining, no satellite was found in any chromosomes of four cotton varieties (Figs. 1–4). Therefore, the satellites showed stain specificity. Stain specific satellites were reported earlier. Alam and Kondo (1995) found a satellite in *Drosophila echinoblasta* at metaphase in orcein staining. It was however, absent in C-, CMA- and DAPI-banding. Alam and Kondo (1995) also reported on the nature of a small chromosome in *Drosophila ericksonae*. They carried out sequential staining of the same metaphase plate with Giemsa, CMA, and DAPI and found a small chromosome in Giemsa and DAPI, whereas it was not observed in CMA. From these observations, they suggested that there were stain specific satellites and chromosomes. Sultana and Alam (2016a) reported about stain specific satellites in 11 varieties of *G. hirsutum*. In the present study, the pair of satellites that were not observed after staining with orcein and DAPI might possess some kind of DNA sequence which made these satellites stain-specific and thus confirmed the presence of stain specific satellites in *G. hirsutum*.

**Fluorescent banding**

The number of CMA-bands varied from four (CB-12) to 13 (Rupali-1) (Figs. 5, 8, Table 1) and percentage of GC-rich repeats ranged from 1.33 (CB-12) to 6.74 (Rupali-1) (Table 1). Most of the CMA-bands were present at the terminal regions of respective chromosomes in the four cotton varieties (Figs. 5–8, Table 1). The presence of terminal CMA bands indicated a tendency of accumulating GC-rich repetitive sequences at the chromosomal ends (Zaman and Alam 2009). In addition to terminal bands, two centromeric CMA-bands were found in Rupali-1 revealing the presence of GC repeats in those regions (Fig. 8). A few chromosomes of CB-14 and Rupali-1 were entirely fluoresced with CMA. In these entirely fluoresced chromosomes, GC-rich repeats were not confined to the terminal or centromeric region but rather distributed along the chromosomes. The possible reason for these entirely fluoresced chromosomes was tandem...
Table 1. Comparative orcein-, CMA- and DAPI-karyotype analysis of four varieties of *Gossypium hirsutum* L.

<table>
<thead>
<tr>
<th>Varieties</th>
<th>2n</th>
<th>Range of chromosomal length (µm)</th>
<th>Total length of 2n chromosome complement (µm)</th>
<th>Centromeric formulae</th>
<th>No. of CMA-bands</th>
<th>% of GC-rich repeats</th>
<th>No. of DAPI-bands</th>
<th>% of AT-rich repeats</th>
<th>No. of satellites</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB-12</td>
<td>52</td>
<td>1.89–3.43</td>
<td>128.88</td>
<td>48m+4sm</td>
<td>4</td>
<td>1.03</td>
<td>14</td>
<td>9.82</td>
<td>2 in CMA</td>
</tr>
<tr>
<td>CB-13</td>
<td>52</td>
<td>1.71–3.82</td>
<td>142.31</td>
<td>52m</td>
<td>8</td>
<td>2.54</td>
<td>—</td>
<td>—</td>
<td>4 in CMA</td>
</tr>
<tr>
<td>CB-14</td>
<td>52</td>
<td>1.38–4.14</td>
<td>138.56</td>
<td>50m+2sm</td>
<td>5</td>
<td>2.08</td>
<td>11</td>
<td>4.24</td>
<td>4 in CMA</td>
</tr>
<tr>
<td>Rupali-1</td>
<td>52</td>
<td>1.94–3.45</td>
<td>136.27</td>
<td>50m+2sm</td>
<td>13</td>
<td>4.95</td>
<td>—</td>
<td>—</td>
<td>2 in CMA</td>
</tr>
</tbody>
</table>

* m = metacentric chromosomes, sm = submetacentric chromosome.


In CB-12, unlike CMA-staining, most of the banded chromosomes fluoresced entirely with DAPI fluo-

chromes. Therefore, the AT-rich repeats were less con-

fined rather distributed throughout the length of banded chromosomes. This banding nature makes these chromosomes different from the other. The probable reason for entirely fluoresced chromosomes was i) either these chromosomes were completely AT-rich by nature or ii) due to successive duplication of AT-rich repeats (Hossain et al. 2016, Hossen et al. 2016). Entirely DAPI-
banded chromosomes were also reported earlier by Sultana and Alam (2016a). Thus the present findings correlate with the earlier reports. In contrast, no DAPI-
positive band was observed in CB-13 and Rupali-1 (Figs.

10, 12, 18, 20, Table 1) revealing the lack of successive duplication of AT-rich repeats.

The four cotton varieties used in this study have dist-

inct CMA- and DAPI-banding patterns. On the basis of number, location, intensity, and percentage of GC- and AT-rich repeats, it was possible to construct character-

istic CMA- and DAPI-karyotypes for each variety of G. hirsutum.

Heteromorphicity

Heteromorphicity in respect of CMA-banding pat-

tern was found in chromosome pair XV and XXVI of CB-13, in pair XV, XXIII of CB-14, and in pair XIV of Rupali-1. In these pairs, one chromosome showed CMA-positive bands while its homologue member did not show any band. Probably the most accepted explana-
tion of this heteromorphicity was deletion of the banded region from the respective chromosomes (Figs.

6–8, 14–16).

Marker chromosomes

In CB-14, both the members of pair IV and XII were found to possess DAPI-positive bands in the two ter-

minal regions (Figs. 11, 19). This type of banding was not found in any chromosome of the four varieties. This banding pattern makes these chromosomes unique and thus could be used as marker chromosomes for CB-14.

Moreover, in CB-13, a lower terminal DAPI-negative

band was found in two members of pair VIII (Figs. 10, 18). The DAPI-negative band was not found in others varieties and these two chromosomes of CB-13 could also be used as marker chromosomes. Sultana and Alam (2016a) worked on 11 varieties of G. hirsutum, and did not report DAPI-negative band in any variety. Therefore, our observation of DAPI-negative chromosomes was the first report for G. hirsutum.

RAPD analysis

DNA from the four cotton varieties were studied with five oligonucleotide primers. The five primers generated 53 distinct bands. These four varieties shared five common fragments of DNA in five RAPD primers (Figs.

21–25, Table 2). Light and bright bands were produced in the RAPD reactions. Light bands produced from low homology between the primer and the pairing site on the DNA strand (Thormann et al. 1994).

Sultana and Alam (2016a) reported 100% polymor-

phism in 11 germplasms of Gossypium hirsutum reveal-
ing maximum genomic difference. In this study, 90.56% polymorphism was found in four varieties indicating that the newly released four varieties of G. hirsutum from CDB possessing relatively low polymorphism to that of Sultana and Alam (2016b).

In this research, two unique fragments (250 bp and 800 bp in primer-21 and primer-OPF-22, respectively) were observed only in Rupali-1. The term unique sequence means that the sequence found in a germplasm with a certain primer was absent in other germplasms (Table 2). The unique bands were stable, reproducible and specific, and thus could be used as a tool for characterization of Rupali-1. In the earlier literature, there was no information about unique bands (Esmail et al. 2008, Maleia et al. 2010, Saravanan et al. 2006) except Sultana and Alam (2016b). Therefore, the report for RAPD unique band is the pioneer for G. hirsutum.

Genetic relationships among four cotton varieties

The values of pair-wise Nei’s (1972) genetic dis-
tances analyzed by using computer software “pogene32” among four varieties of cotton were computed from combined data obtained from five RAPD primers. The values ranged from 0.2719 to 1.4351 (Fig. 26). The highest genetic distance (1.4351) was found between CB-13

<table>
<thead>
<tr>
<th>Primer codes</th>
<th>Total bands</th>
<th>Size ranges (bp)</th>
<th>Number of common band</th>
<th>Number of polymorphic band</th>
<th>Number and size (bp) of germplasms specific unique bands</th>
<th>Polymorphisms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer-21</td>
<td>08</td>
<td>200–600</td>
<td>0</td>
<td>8</td>
<td>Rupali-1 (250)</td>
<td>100.00</td>
</tr>
<tr>
<td>Primer-23</td>
<td>6</td>
<td>100–1000</td>
<td>1</td>
<td>5</td>
<td>—</td>
<td>83.33</td>
</tr>
<tr>
<td>OPC-26</td>
<td>15</td>
<td>300–2500</td>
<td>1</td>
<td>14</td>
<td>—</td>
<td>93.33</td>
</tr>
<tr>
<td>OPC-96</td>
<td>10</td>
<td>450–900</td>
<td>2</td>
<td>8</td>
<td>—</td>
<td>80.00</td>
</tr>
<tr>
<td>OPF-22</td>
<td>14</td>
<td>500–1500</td>
<td>1</td>
<td>13</td>
<td>Rupali-1 (800)</td>
<td>92.86</td>
</tr>
<tr>
<td>Grand Total:</td>
<td>53</td>
<td>100–2500</td>
<td>05</td>
<td>48</td>
<td>02</td>
<td>90.56</td>
</tr>
</tbody>
</table>
with Rupali-1. On the other hand, the lowest (0.2719) genetic distance was observed between CB-14 and Rupali-1. The difference between the highest and the lowest value of genetic distance revealed the wide range of variability persisting among the four cotton varieties. High genetic distance values between germplasm pairs were found due to differences in genetic constituents. The germplasms of lowest genetic distance can be used as parental source of a breeding line to improve cotton germplasm.

Acknowledgements

This research was partly supported by a grant from the Ministry of Science and Technology, People’s Republic of Bangladesh and Chromosome Research Centre, University of Dhaka, Bangladesh.

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