Bangladesh J. Bot. **39**(2): 199-205, 2010 (December)

LOCALIZATION OF 5S rDNA AND 18S-5.8S-25S rDNA PROBES IN *CRINUM LATIFOLIUM* L. GENOME

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Key words: rDNA FISH, *Crinum latifolium*

Abstract

Two conserved repeats *viz.* 5S rDNA and 18S-5.8S-25S rDNA were used as probes for physical mapping in the genome of *Crinum latifolium* L. (2n = 3X = 33). Nine out of 11 5S rDNA signals were found in three chromosomes of group VI at metaphase. The 5S rDNA gene array were not AT-rich. Four 18S-5.8S-25S rDNA signals at interphase, prophase and metaphase showed their stable nature. The contraction and expansion of these signal regions proved the euchromatic nature of 18S-5.8S-25S rDNA. The 5S rDNA and the 18S-5.8S-25S rDNA signals provided useful cytogenetic markers for *C. latifolium* genome. Patterns of 5S rDNA signals suggested the possible structural aberration in the genome. Although the 5S rDNA sites suggested *C. latifolium* as autotriploid, 18S-5.8S-25S rDNA sites did not conclude anything about the nature of genome. Unequivocal determination of the nature of genome in *C. latifolium* remained unanswered.

Introduction

A number of species of the genus *Crinum* L. (Amaryllidaceae) collected from different regions of Bangladesh were considered in the past to study its cytogenetics (Akhter *et al.* 1992, Patwary and Zaman 1975, Patwary and Zaman 1978, Patwary and Zaman 1981, Zaman *et al*. 1977 a, b). Alam *et al*. (1991) reported that *Crinum defixum* and *C. pratense* have very few morphological differences with 2n = 22 chromosomes. They differentiated these two species by comparative karyotype analysis. Later Alam *et al*. (1998) carried out fluorescent banding technique with two commonly used fluorochrome dye *viz*. CMA and DAPI to distinguish the karyotypes of *Crinum defixum* and *C. pratense*. They reported that differential CMA- and DAPI-bands were species specific. Lubna *et al*. (2004) also found that fluorescent banding was quite suitable for distinguishing the karyotypes of some spp. of *Crinum*. But physical location of a particular DNA segment was not possible using this method. Molecular cytogenetics is a strong tool to study the organization of different repeats of the genomes. Repetitive sequence families are major component of plant genomes (Heslop-Harrison 2000). In genomic organizations, repeats are divided into tandemly arranged and dispersed sequences (Schmidt and Heslop-Harrison 1998). Tandem repeats are divided into satellite DNA, micro- and mini- satellites, telomeric repeats and ribosomal genes. Typical plant satellite DNA repeats range in size between 160 - 180 bp or 320 - 360 bp and are organized in tandem arrays with up to 10^5 copies per haploid genome (Hemleben *et al*. 2000). The most conserved tandemly arranged sequences are ribosomal RNA genes in eukaryotes comprising of 18S-5.8S-25S rDNA repeating units forming long arrays. Fluorescent *in situ* hybridization (FISH) has been widely used to localize rDNA gene arrays on plant chromosomes (Leitch and Heslop-Harrison 1992, Schmidt *et al*. 1994). Both the 5S rDNA and the 18S-5.8S-25S rDNA genes provide useful markers for chromosome identification and karyotyping (Doudrick *et al*. 1995, Brown *et al*. 1999, Begum *et al*. 2009).

A number of steps and chemicals are involved in FISH and thus, the procedure is expensive and time consuming. Hydrolyzing with enzyme mixture is one of the expensive steps in this technique. If this step could be replaced by another easier one, it would save money and time both.

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In the present study, an attempt was undertaken for the first time in Bangladesh to construct a physical map in the genome of *Crinum latifolium.* Here two different highly conserved repeats *viz.* 5S rDNA and 18S-5.8S-25S rDNA (complex) were used as probes. Moreover an alternative procedure for hydrolysis was tried. The aims of this molecular cytogenetic research were (i) to develop an alternative hydrolysis procedure, (ii) to construct the physical map with the above mentioned probes and (iii) characterize the karyotype of *Crinum latifolium* L.

Materials and Methods

Crinum latifolium L. grown in the Botanic Garden, Department of Botany, University of Dhaka was used. Roots were collected and washed in running tap water for 4 - 5 m. After removal of excess water, roots were pretreated with PDB for six h at room temperature (28-30°C) and fixed in 45% acetic acid for 15 m at 4° C. Both squash and dropping method were used for chromosome preparation. In case of squash method, the RTs were over hydrolyzed for 1 m and squashed with 45% acetic acid. The cover glasses were removed quickly and air dried for at least 48 h before study. For dropping method, procedure of Schwarzacher and Heslop-Harrison (2000) was followed with slight modification. Briefly, fixed roots were washed in enzyme buffer (0.01 M citric acid - sodium citrate, pH 4.6) to remove the fixative and digested at 37°C for 1 h 20 m in enzyme solution consisting of 2.5% pectinase, 2.5% cellulase, 2.5% pectolyase and 1.0% cytohelicase in enzyme buffer.

The clone pXV1 (Schmidt *et al*. 1994) containing the 5S rRNA gene from *Beta vulgaris* was labelled with biotin-16-dUTP using PCR while the clone pTa71 from *Triticum aestivum* (Gerlach and Bedbrook 1979) consisting of a large part of the 18S-5.8S-25S rRNA genes was labelled with digoxigenin-11-dUTP by nick translation.

FISH was performed according to Heslop-Harrison *et al*. (1991) with minor modification. Chromosome spreads were pre-treated with 100 μL^{-1} RNase A in 2× sodium saline citrate (2× SSC) for 1 h at 37°C and washed twice in 2× SSC. After incubation with 10 $\mu g\ mL^{-1}$ pepsin in 0.01 mM HCl for 20 min at 37°C, preparations were stabilized in freshly de-polymerized 4% (w/v) paraformaldehyde in water for 10 min, dehydrated in a graded ethanol series and air dried. The hybridization mixture consisting of 50 - 150 ng μL^{-1} of DNA probe, 50% (v/v) formamide, 10% (w/v) dextran sulfate, 0.1% sodium dodecyl sulfate (SDS) and 300 ng μL^{-1} of sheared salmon sperm DNA in 2× SSC, was incubated for 10 min at 70°C and chilled on ice. Then 30 µL of the hybridization mixture was added to the chromosome preparations and covered with a plastic coverslip. The hybridization mixture and the chromosomal DNA were denatured at 70°C for 5 m. The temperature was gradually decreased to 55, 50, 45 and finally 37°C using different water baths. Hybridization was carried out overnight at 37°C. Following hybridization, the slides were washed stringently in 20% (v/v) formamide in 0.1×SSC at 42°C to remove mismatched or unhybridized probe molecules. For the detection of digoxigenin- or biotin labelled probes, slides were equilibrated in 4× SSC/0.1% (v/ v) Tween 20 and blocked in 5% (w/v) bovine serum albumin in 4×SSC/0.1% (v/v) Tween 20 for 5 min. Slides were incubated with a final concentration of 2 $\mu g\ mL^{-1}$ of sheep antidigoxigenin antibody conjugated with fluorescein isothiocyanate (FITC) or streptavidin-Cy3 in a moist chamber at 37°C for 1 h. Excess antibody was removed by washing the slides in 4× SSC/0.1% (v/v) Tween 20 three times each for 5 min. After counterstaining with DAPI (4, 6-diamidino-2-phenylindole; 2$\mu g\ mL^{-1}$), the slides were mounted in antifade solution (AF1, Citifluor). Examination of slides was carried out with fluorescent microscope (HUND, Germany) equipped with filters for FITC, Cy3 and DAPI. Images were acquired directly with Applied Spectral Imaging v.3.3 software, coupled with a high-

resolution camera (Canon), and printed from Adobe Photoshop after contrast optimization using only functions affecting the whole image equally.

Results and Discussion

Squash method versus enzymatic method: Generally during FISH, chromosomes are hydrolyzed with an enzyme mixture which contains various enzymes in different proportions such as pectinase (5%), cellulase (4%), cytohelicase (2%) and pectolyase (0.5%). The enzyme mixture helps to digest the cell wall composed of pectin and cellulose. As a result, the chromosomes become free and the chemical can bind easily with it. This method is very effective however, requires a huge amount of materials for digestion and is therefore costly.

An alternative method for hydrolysis was tried in this present work. Here an individual root was over hydrolyzed (about 1m) in a mixture of 1N HCl and 45% acetic acid (2 : 1). After squashing, the cell wall bursts. As a consequence chromosomes become almost free from cytoplasm. This hydrolyzing method gave similar result to that of enzymatic hydrolysis method (Figs 2, 6). This method is quicker, less expensive and useful even with a single root. Therefore, this hydrolyzing method stands as a new technique for chromosome preparation during FISH.

5S rDNA FISH: In this work, 11 signals were frequently found in most of the cells. Out of 11 signals, 9 were present in 3 members of group VI (Fig. 4). The remaining two very small signals were found in a member of group I and X (Fig. 4). The occurrence of signals reveal that almost all 5s rDNA repeats are distributed in group VI.

The 11 signals were observed in almost every interphase nuclei (Fig. 1). However, in very rare cases less number of signals were found. The later signals were bigger than the earlier ones (Fig. 4). This finding indicates that 5S rDNA repeats were aggregated and thus, formed bigger and fewer numbers of signals at interphase.

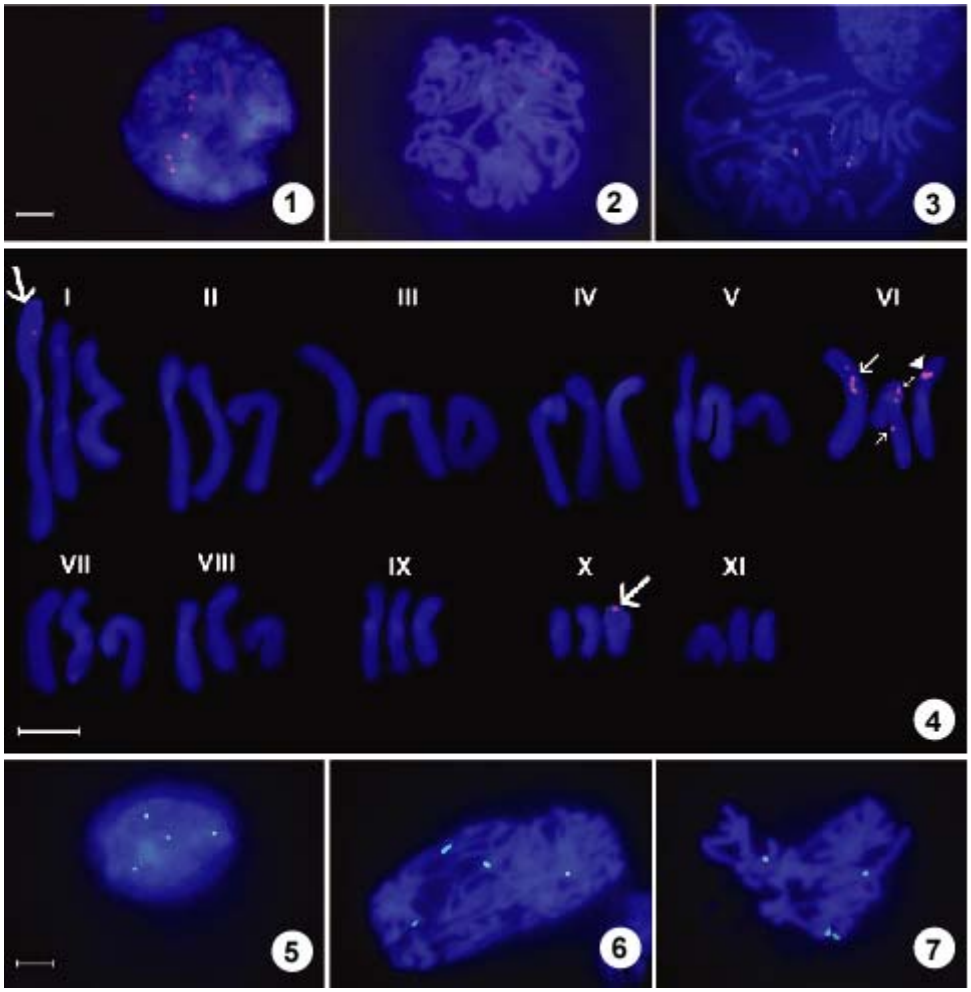
DAPI is a fluorochrome that binds to the AT-rich repeats of chromosomes fluorescing characteristic blue colour (Schweizer 1976, Alam and Kondo 1995). In the interphase nuclei, a number of blue fluorescing regions indicated the presence of AT-rich repeats. The 5S rDNA signals did not correlate with those blue fluorescing regions (Fig. 1). These signals were far apart from the blue fluorescing regions. This observation suggested that 5S rDNA repeats were at least not AT-rich if not rich in GC base pairs (Souza *et al.* 2008).

FISH has been widely used to localize rDNA gene arrays on plant chromosomes (Leitch and Heslop-Harrison 1992, Schmidt *et al*. 1994). The 5S rDNA gene provides useful markers for chromosome identification and karyotyping (Doudrick *et al.* 1995, Brown *et al*. 1999). Thus the 11 5S rDNA signals would be used as markers for the respective chromosomes.

18S-5.8S-25S rDNA FISH: Four green fluorescing signals were found in the metaphase chromosomes (Fig. 7). Due to much overlapping and lack of available materials (available only in the rainy season) it was not possible to prepare the karyotype. However, it was clear that the four signals were present in four different chromosomes. Four signals were also found in the interphase nuclei and prophase chromosomes (Figs 5, 6) indicating the stable position throughout the cell cycle.

The signals were small and spherical in the interphase nuclei and metaphase chromosomes but elongated in the prophase chromosomes (Figs 5-7). The chromatins are usually condensed at the interphase and contracted at metaphase, however, extended at prophase. The 18S-5.8S-25S rDNA probe is actually complementary sequence to the nucleolar organizing region (NOR) (Souza *et al*. 2008). NOR is composed of euchromatins and thus, transcribe to rRNA. The contraction and extension of signal regions at different stages of cell division clearly indicating the euchromatic nature of 18S-5.8S-25S rDNA.

The total length of the four signals was about 4.63 µm. This length was smaller than the total length of 5S rDNA signals (Table 1). 18S-5.8S-25S rDNA represents NOR region. The NOR regions are limited and localized in certain position of chromosomes in a genome (Schweizer 1976, Schmidt *et al*. 1994). On the other hand, 5S rDNAs are dispersed repeats and distributed all along the genome. As a result, the total length of 5S rDNA should be bigger than that of 18S-5.8S-25S rDNA.



Figs 1-7. FISH with 5S rDNA and 18S-5.8S-25S rDNA probes in *Crinum latifolium*: 1. 5S rDNA FISH in interphase nuclei, 2. 5S rDNA FISH in prophase chromosomes, 3. 5S rDNA FISH in metaphase chromosomes, 4. Karyotype prepared from 5S rDNA FISH of mitotic metaphase chromosomes, 5. 18S-5.8S-25S rDNA FISH in interphase nuclei, 6. 18S-5.8S-25S rDNA FISH in prophase chromosomes, 7. 18S-5.8S-25S rDNA FISH of mitotic metaphase chromosomes. Bars = 10 µm.

The chromosomes of *C. latifolium* have a characteristic number and position of rDNA sites. Therefore, the 5S- and 18S-5.8S-25S rDNA provide useful cytogenetic markers for unequivocal physical mapping in its karyotype.

Nature of genome in C. latifolium: The karyotype of *C. latifolium* clearly indicated that this species is a triploid (2n = 3X = 33, Lubna *et al*. 2004). A question was raised earlier as to whether this species was an auto- or allotriploid. Lubna *et al*. (2004) tried to determine the nature of genomes present in *C. latifolium* by differential fluorescent banding. On the basis of similar banding pattern in all three members a of certain group, they suggested this species to be autotriploid. However, different banding patterns among three members of another group, identify the species to be allo-triploid. Therefore, a confusion regarding the quality of genomes in this species still exist.

Table 1. Comparative analysis of 5S rDNA and 18S-5.8S-25S rDNA FISH on *Crinum latifolium* chromosomes.

Probes	No. of signals	No. of chromo-somes showed signals	No. of chromo-somes showed multiple signals	Total length of signals (μm)	% of repeats in the genome
5S rDNA	11	5	3	10.53	1.58
18S-5.8S-25S rDNA	4	4	-	4.63	0.696

In the present study, one member of group VI had four signals on the short arm (Fig. 4, big arrow). The second member of this group had in total three of which two signals were on the short arm and another on the long arm (Fig. 4, small arrow). A pair of signals was found on the short arm in the third member of this group (Fig. 4, arrow head). It indicated that the 3 chromosomes of this group possessed different numbers of signal i.e. one member of this group had four, another member three (in two different sites) and the remaining member had two signals. In addition to this group, one very low signal was found in only a member of group I and X (Fig. 4, thick arrow).

If *C. latifolium* is considered as auto-triploid, each member of group VI should have four signals (since it was the maximum number). Moreover, there should not be any signal in only a member of group I and X. The reason for different signal numbers on different chromosomes of group VI and presence of one signal in only a member of group I and X are not clear. However, the number and distribution of signals provided the following assumptions: (i) due to occurrence of translocation between a member of group VI with a member of group I and X two rDNA sites from a member of group VI translocated to a member of group I and X. Thus, only two signals were present in that member of group VI (Fig. 4, arrow head) and (ii) due to deletion of a rDNA site from another chromosome of group VI and occurrence of a paracentric inversion in the same chromosome, one rDNA site was absent and another shifted to another arm of the same chromosome (Fig. 4, arrow head). As a result this chromosome had three signals of which two in short arm and another in long arm. However, ignoring these structural changes (translocation, deletion and paracentric inversion) it could be suggested that the three chromosomes of group VI are homologous (except the aberrated regions) and in this regard *C. latifolium* may be considered as an autotriploid.

In contrast, the four 18S-5.8S-25S rDNA signals occurred on four different chromosomes (Fig.7). If this species is an autotriploid there must be three or multiple of three signals in homologous members in a group. Why instead of three, four equal signals were found in four different chromosomes is not clear with the data available. Therefore, the nature of genomes in *C. latifolium* remains unanswered.

Acknowledgements

The authors are grateful to Dr Rabeya Begum for her technical assistance. Sincere thank to Prof. Dr Thomas Schmidt, Technical University of Dresden, Germany for providing the labeled probes and other supports. Deep gratitude is due to Prof. Dr M Akhtaruzzaman (passed away recently), Department of Botany, University of Dhaka for going through the manuscript. They are grateful to the Alexander Von Humboldt Foundation for kindly providing Fluorescent microscope and other necessary instruments.

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(Manuscript received on 30 October, 2010; revised on 6 November, 2010)