Fluorescent Banding and RAPD Analysis of Four Varieties of *Lablab purpureus* L.

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Summary Four released varieties of *Lablab purpureus* L. from Bangladesh Agriculture Research Institute (BARI), viz. BARI Sheem-1, BARI Sheem-2, BARI Sheem-5, and BARI Sheem-6, were characterized by differential karyotype and RAPD analysis. $2n=22$ metacentric chromosomes were found for all except BARI Sheem-1 ($20m+2sm$). The four varieties were found to possess symmetric karyotypes. Each variety had a different fluorescent banding pattern. BARI Sheem-2 showed a maximum of 10 CMA bands. All terminal CMA bands supported the equilocal distribution of heterochromatin. The four varieties showed different DAPI banding patterns. DAPI bands in different locations suggested the random distribution of AT-rich repeats. Few chromosomes of these varieties could be used as markers. Every variety had its own RAPD fingerprinting pattern with different primer combinations. In spite of similar conventional karyotype features, the four varieties could be differentiated by their CMA, DAPI, and RAPD banding patterns. This information may be helpful for the correct breeding of BARI.

Key words Karyotype, CMA- and DAPI-banding, RAPD, *Lablab purpureus* L.

Lablab purpureus L. (Country Bean) belongs to Fabaceae (Rai *et al.* 2010). It is one of the most diverse domesticated legume species having multiple uses. It has been widely distributed in many tropical and subtropical countries covering South and Central America, the East and West Indies, Africa, Asia, China, India, and Bangladesh as an annual or a short-lived perennial (Whyte *et al.* 1953).

This species is rich in minerals and vitamins (Basu *et al.* 2002). The dried seeds of *Lablab purpureus* are important as pulse. Immature pods and seeds are eaten as vegetables (Ahmed *et al.* 2009). Plants of this species have some medicinal and ethno-botanical importance. The plant is anticholesterolemic, antidotal, antivenous, carminative, and hypoglycaemic (Ahmed *et al.* 2009).

In Bangladesh, a number of local varieties with variable characteristics are available. This species has wide polymorphism in respect of fruit and seed color, size, and weight. These germplasms open a wide possibility for developing new characters through breeding programmes (Choudhury *et al.* 1975). In spite of diverse germplasms, the total production of this species was low. The lack of high-yielding varieties is one of the reasons for the low yield and variability of this crop in Bangladesh.

The scientists of Bangladesh Agriculture Research Institute (BARI) have been trying to develop a high-yielding country bean variety (locally called “Sheem”) for the last two decades. They have been able to produce several recommended varieties, such as BARI Sheem-1, BARI Sheem-2, BARI Sheem-3, BARI Sheem-4, BARI Sheem-5, BARI Sheem-6, *etc.* These varieties are identified solely on the basis of morphological and agronomical traits. Sometimes this type of identification

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was not found to be reliable because the same germplasm may show different morphologies in different environments due to phenotypic plasticity. Characterization on the genomic level is an authentic one. Unfortunately, no such characterization is available for these BARI bean varieties. An authentic characterization of the varieties released from BARI from another point of view is essential. Nowadays, a number of seed companies are selling different so-called "high-yielding seeds" of various species. For this reason, BARI varieties should be patented to prevent trade piracy (Fawzia and Alam 2011, Zaman and Alam 2009).

For this purpose, a comparative karyotype analysis has been conducted. Karyotype usually plays an important role for characterizing a germplasm since it is very stable and specific (Stebbins 1971, Begum and Alam 2009, Zaman and Alam 2009). Differential fluorescent staining has been used for karyotype analysis. The two most common fluorochromes, *viz.* chromomycine A₃ (CMA) and 4',6-diamidino-2-phenyl indole (DAPI), were able to identify individual chromosomes, chromosomal aberrations, the nature of satellites, and the position and amount of GC- and AT-rich repeats in the chromosomes, thereby characterizing each karyotype (Schweizer 1976, Kondo and Hizume 1982, Alam *et al.* 1998).

DNA fingerprinting by random amplified polymorphic DNA (RAPD) has been widely using since the late 80's of the last century to assess intra-specific genetic variation on a molecular level. It includes characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy, *etc.* Genetic analysis using molecular marker technologies can provide a powerful approach to understanding the organization and distribution of genetic resources in nature and to managing populations (Welsh and Clelland 1990, Williams *et al.* 1990, Jahan *et al.* 2012).

In this investigation, an attempt was undertaken to characterize four BARI bean varieties, *viz.* BARI Sheem-1, BARI Sheem-2, BARI Sheem-5, and BARI Sheem-6 by fluorescent banding-and RAPD analysis.

The aim of the present study was:

- i. to compare the Orcein, CMA, and DAPI karyotypes of each variety, and
- ii. to obtain DNA fingerprints of each variety by RAPD analysis.

Materials and methods

The seeds of four varieties of *Lablab purpureus* L., *viz.* BARI Sheem-1, BARI Sheem-2, BARI Sheem-5, and BARI Sheem-6 were collected from BARI. These seeds were germinated and maintained in the Botanic Garden of the Department of Botany, University of Dhaka.

Cytogenetical study

Healthy and young root tips were collected and pretreated with 0.002 M 8-hydroxyquinoline for 10m at room temperature (18–20°C), followed by 15m fixation in 45% acetic acid at 4°C. These were then hydrolysed in a mixture of 1 N HCl and 45% acetic acid (2 : 1) at 60°C for 10–12 s. The hydrolysed root tips were stained and squashed in 1% aceto orcein. For CMA staining, Alam and Kondo's (1995) method was followed with slight modification. After hydrolysing and dissecting, the materials were squashed with 45% acetic acid. The cover glasses were removed quickly onto dry ice and allowed to air dry for at least 48 h before study. The air-dried slides were first pre-incubated in McIlvaine's buffer (pH 7.0) for 30m followed by Distamycin A (0.1 mg/ml) treatment for 10m. The slides were rinsed mildly in McIlvaine's buffer supplemented with MgSO₄ (5mM) for 15m. One drop of CMA (0.1 mg/ml) was added to the materials for 15m and rinsed with McIlvaine's buffer with MgSO₄ for 10m. Slides were mounted in 50% glycerol and kept at 4°C overnight before observation. These were observed under a Hund fluorescent microscope with a blue violet (BV) filter cassette. For DAPI-staining, the method proposed by Alam and Kondo

(1995) was followed after slight modifications. For DAPI-staining, after 48 h of air-drying, the slide was first pre-incubated in McIlvaine's buffer (pH 7.0) for 10 m. The slide was treated in 0.25 mg/ml actinomycin D for 10 m in a humid chamber. After antibiotic treatment, the slide was washed with distilled water in such a way that the cover glass was removed. The slide was immersed again in McIlvaine's buffer (pH 7.0) for 15 m followed by treating in DAPI solution (0.1 mg/ml) for 20 m. After rinsing in McIlvaine's buffer (pH 7.0) for 10 m, the slide was mounted with 50% glycerol and kept at 4°C. These were observed under a Nikon (UFX-IIA) fluorescent microscope with an ultra violet (UV) filter cassette.

DNA isolation

Tender leaves were harvested and the total genomic DNA was extracted by using a modified CTAB method (Doyle and Doyle 1987). The DNA concentration was quantified with a spectrophotometer (Analytikjena, Specord 50, Germany). The A_{260}/A_{280} readings for the DNA samples were 1.6–1.8.

PCR amplification and primer survey

The PCR reaction mixture (25 μ l) contained 2 μ l of template DNA (25 ng), 18.8 μ l of de-ionized distilled water, 2.5 μ l of Taq buffer A 10 $\times$ (Tris with 15 mM $MgCl_2$), 1.0 μ l of primer (10 μ M), 0.5 μ l of dNTPs (2.5 mM), and 0.2 μ l of Taq DNA polymerase (5 U/ μ l). PCR amplification was done in an oil-free thermal cycler (Biometra UNOII, Germany) for 46 cycles after initial denature at 94°C for 5 m, followed by denature at 94°C for 1 m, annealing at 36°C for 30 s, extension at 72°C for 3 m, and final extension at 72°C for 5 m. Four random primers were used in the present study for screening: OPA-1 (5'-CAG GCC CTT C-3'), Primer-8 (5'-GTC CTC GTA G-3'), Primer-9 (5'-AAC GCG TAG A-3'), and Primer-15 (5'-CCT TCC CTC T-3').

Gel electrophoresis

The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing 8 μ l of ethidium bromide and 99 ml of 1 $\times$ TAE buffer. Agarose gel electrophoresis was conducted in 1 $\times$ TAE buffer at 50 V and 100 mA for 1.5 h. A DNA ladder (1 kb) was electrophoresed alongside the RAPD reactions as a marker. DNA bands were observed on a 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 four primers in the RAPD analysis were then pooled for constructing a single data matrix. This was used for estimating polymorphic loci, Nei's (1972) gene diversity, genetic distance (D), and for constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among the specimen using the computer program "POPGENE" (Version 1.31).

Results and discussion

The four varieties of *Lablab purpureus* were found to possess $2n=22$ chromosomes. Except for two sub-metacentric chromosomes of BARI Sheem-1, all chromosomes of the four varieties were metacentric. This result showed that the four varieties had strict symmetric karyotypes that were primitive in nature (Stebbins 1971).

Fluorescent banding

Different CMA banding patterns were found in different varieties. The number of CMA bands

Table 1. Comparative orcein, CMA-, and DAPI-karyotype analysis in four morphological forms of *Lablab purpureus* L.

Morphological forms	$2n$	Karyotype formulae	No. of CMA-positive bands	% of GC-rich repeats	CMA-banded karyotype formulae	No. of DAPI-positive bands	% of AT-rich repeats	DAPI-banded karyotype formulae
BARI Sheem-1	22	20 m+2 sm	2	3.02	$2\alpha+20\delta$	18	30	$10\alpha+2\beta+6\theta+4\delta$
BARI Sheem-2	22	22 m	10	27.84	$8\alpha+2\beta+12\delta$	16	31.16	$11\alpha+1\beta+4\theta+6\delta$
BARI Sheem-5	22	22 m	6	17.80	$4\alpha+2\beta+16\delta$	4	13.44	$1\alpha+2\beta+1\theta+18\delta$
BARI Sheem-6	22	22 m	3	6.38	$3\alpha+19\delta$	18	28.46	$5\alpha+2\beta+11\theta+4\delta$

m=metacentric chromosomes, sm=sub-metacentric chromosomes, α =band in terminal region, β =band in entire length of chromosome, θ =band in centromeric region, δ =no band

ranged from 2 to 10 (Table 1). The lowest numbers of bands (two bands) were observed in BARI Sheem-1 and the highest (10 bands) in BARI Sheem-2 (Figs. 5, 6, Table 1). In the variety BARI Sheem-1, two CMA-positive bright bands were observed (Fig. 5) — one on each short arm of each member of pair X (Fig. 13). In total, 10 chromosomes showed CMA bands in the variety BARI Sheem-2 (Fig. 6). Among these 10 chromosomes, both the members of pair VII fluoresced entirely. A thick CMA positive band was found on the short arm of each member in pairs II and III. These bands were so thick that they almost entirely occupied the respective short arms. A smaller CMA positive band was observed on the upper terminal regions of both the members in pairs IV and VI in this variety (Fig. 14). In variety BARI Sheem-5, a total of six chromosomes showed CMA-positive bands (Fig. 7). Both the members of pair XI fluoresced along their entire length (Fig. 15). A big and bright band was found on the short arm of each member of pairs II and VI (Fig. 15). In BARI Sheem-6, the entire short arm of a member in pair II fluoresced. However, the short arm of the homologue did not fluoresce at all (Fig. 16). One bright band was found at the upper terminal region in each member of pair VIII (Fig. 16).

The CMA bands of the four varieties were thick and prominent, indicating the presence of much GC-rich repeats (Schweizer 1976). All the GC-rich repeats of these varieties were located at the terminal regions. GC-rich portions were reported sometimes as heterochromatin (Sultana and Alam 2007, Khatun and Alam 2010). Sumner (1990) used the term “Equilocal Distribution of Heterochromatins”, and explained that the heterochromatin had a tendency to localize on a same location of non-homologous chromosomes. Therefore, the CMA positive bands of these four varieties might be heterochromatic in nature.

The four varieties showed unique DAPI banding patterns (Figs. 9–12). Unlike CMA banding, the DAPI bands were observed in the upper terminal region, lower terminal region, intercalary region, and centromeric region of the chromosomes. The DAPI banding pattern suggested the random distribution of AT-rich repeats among the genomes of these four varieties. In variety BARI Sheem-1, 18 chromosomes showed DAPI positive bands (Fig. 9). Members of pair IX fluoresced entirely. A very thick band occupying almost the entire short arm was observed in each member of pairs I and II. One band was found at the intercalary region of the short arm in both the members of pair III. Members of pair VIII showed centromeric bands. Bands were observed on the long arm in each member of pair V of which one was thicker. One band was found in the lower terminal region in pair XI (Fig. 17). Heteromorphism in respect of position of bands was observed in pairs IV and X. A centromeric and terminal band was found in only one member of pairs IV and X, respectively; no such bands were found in their homologue members (Fig. 17).

In BARI Sheem-2, 16 chromosomes showed DAPI positive bands (Fig. 10). Upper terminal bands were found in each homologue member of pairs II, IV, and VII. In contrast, each member of pairs III and V had a lower terminal band. A pair of centromeric bands was found in each member

Figs. 1–24. Orcein-, CMA-, and DAPI- stained mitotic metaphase and RAPD analysis of four varieties of *Lablab purpureus* L. 1. Orcein-stained mitotic metaphase chromosomes of BARI Sheem-1, 2. Orcein-stained mitotic metaphase chromosomes of BARI Sheem-2, 3. Orcein-stained mitotic metaphase chromosomes of BARI Sheem-5, 4. Orcein-stained mitotic metaphase chromosomes of BARI Sheem-6, 5. CMA-stained mitotic metaphase chromosomes of BARI Sheem-1, 6. CMA-stained mitotic metaphase chromosomes of BARI Sheem-2, 7. CMA-stained mitotic metaphase chromosomes of BARI Sheem-5, 8. CMA-stained mitotic metaphase chromosomes of BARI Sheem-6, 9. DAPI-stained mitotic metaphase chromosomes of BARI Sheem-1, 10. DAPI-stained mitotic metaphase chromosomes of BARI Sheem-2, 11. DAPI-stained mitotic metaphase chromosomes of BARI Sheem-5, 12. DAPI-stained mitotic metaphase chromosomes of BARI Sheem-6, 13. CMA-stained karyotype of BARI Sheem-1, 14. CMA-stained karyotype of BARI Sheem-2, 15. CMA-stained karyotype of BARI Sheem-5, 16. CMA-stained karyotype of BARI Sheem-6, 17. DAPI-stained karyotype of BARI Sheem-1, 18. DAPI-stained karyotype of BARI Sheem-2, 19. DAPI-stained karyotype of BARI Sheem-5, 20. DAPI-stained karyotype of BARI Sheem-6 (Bar=5 μ m for Figs. 1–20), 21. RAPD analysis with OPA-1, 22. RAPD analysis with Primer-8, 23. RAPD analysis with Primer-9, 24. RAPD analysis with Primer-15. M=1 kb DNA ladder, BS-1=BARI Sheem-1, BS-2=BARI Sheem-2, BS-5=BARI Sheem-5, BS-6=BARI Sheem-6.

Fig. 25. UPGMA Dendrogram based on Nei's (1972) genetic distance summarizing the data on differentiation between four varieties of *Lablab purpureus* L. according to RAPD analysis based on four primer combinations.

of pair IX. Heteromorphism in respect of position of DAPI band was found in pairs I, VI, and XI. A member of pair I had a centromeric band, whereas an upper terminal band was found in its homologue. A member of pair VI fluoresced entirely, while no such fluorescence was found in its homologue. A centromeric band was found in only one member of pair XI (Fig. 18).

In BARI Sheem-5, four chromosomes showed the DAPI bands (Fig. 11). One member of pairs I and VI fluoresced entirely, whereas no band was found in its homologue. One member of pair II and pair VIII had a centromeric band and a terminal band, respectively. In contrast, no band was found in their homologue members (Fig. 19).

In BARI Sheem-6, both the members of pairs II, III, IV, V, and IX had centromeric DAPI bands. The members of pair VII fluoresced more than half of their length. A DAPI band was found in the lower terminal region in both members of pair I. Only one member of pair VI fluoresced entirely. One centromeric band was found in a member of pair VIII, whereas no such bands were found in its homologue. One member of pair X entirely fluoresced. On the other hand, the other member had a lower terminal band (Fig. 20).

Marker chromosomes

Two chromosomes in BARI Sheem-2 (pair VII) and in BARI Sheem-5 (pair XI) entirely fluoresced with CMA (Figs. 14, 15). On the other hand, 2 chromosomes in BARI Sheem-1 (pair IX), 1 in BARI Sheem-2 (pair VI), 2 in BARI Sheem-5 (pair I, VI) and 2 in BARI Sheem-6 (pair VI and X) fluoresced entirely with the DAPI stain (Figs. 17–20). These chromosomes were so unique that they could be used as marker chromosomes of the respective variety.

Structural aberration

Several chromosomes showed heteromorphism in respect of the presence or absence of CMA and DAPI bands (Figs. 13–20). The absence of a band in the respective locus of homologous chromosomes indicated deletion of that portion.

In BARI Sheem-2, one member of pair I had an interstitial DAPI band, whereas a terminal DAPI band was found in its homologue member (Fig. 18). This feature suggested the occurrence of pericentric inversion.

Brightly DAPI fluorescing chromosomes were found in a member of pair VI of BARI Sheem-2, a member of pair I and VI of variety BARI Sheem-5, and a member of pair VI in variety BARI Sheem-6 (Figs. 18–20). No such staining was found in their respective homologue. The probable reasons might be (i) unequal crossing over between the homologous chromosomes or (ii) non-reciprocal translocation of AT-rich repeats.

RAPD analysis

Different RAPD fingerprinting patterns were observed in the four varieties (Figs. 21–24). On the basis of RAPD banding patterns, the phylogenetic dendrogram showed similarity between BARI Sheem-1 and BARI Sheem-6, which were thus placed in one cluster. Additionally, BARI Sheem-2 and BARI Sheem-5 showed similarity and were placed in another cluster (Fig. 25). The RAPD patterns revealed the diversification in the genome of the four varieties.

Varietal characterization

Although the four varieties showed narrow differences in respect of conventional karyomorphological features, each had distinct CMA, DAPI, and RAPD banding patterns. They possess some marker chromosomes specific to their respective variety, which allowed the four BARI bean varieties to be characterized. This information will be helpful to patent the germplasms.

References

- Ahmed, Z. U., Hasan, M. A., Begum, Z. N. T., Khondker, M., Kabir S. M. H., Ahmed, M., Ahmed, A. T. A., Rahman A. K. A., and Haque, E. U. (eds.) 2009. Encyclopedia of Flora and Fauna of Bangladesh. Angiosperms: Dicotyledons (Fabaceae-Lythraceae), Vol. 8. Asiat. Soc. Bangladesh, Dhaka. pp. 120–121.
- Alam, Sk. S., Azhar, S. S., Sarker, R. H., and Zaman, M. A. 1998. Karyotype analysis with differential banding in *Crinum pratense* and *Crinum defixum*. *Cytologia* **63**: 223–227.
- Alam, Sk. S., and Kondo, K. 1995. Differential staining with Orcein, Giemsa, CMA and DAPI for comparative chromosome study of 12 species of Australian *Drosera* (Droseraceae). *Am. J. Bot.* **82**: 1278–1286.
- Basu, A. K., Samantha, S. K., and Sasmala, S. K. 2002. Genetic analysis for some seed parameters in *Lablab* bean. *Veg. Sci.* **29**: 17–19.
- Begum, K. N., and Alam, Sk. S. 2009. Karyotype analysis in three morphological forms of *Colocasia fallax* Schott. *Cytologia* **74**: 209–214.
- Choudhury, B. D., Bhat, P. N., and Singh, V. P. 1975. Genetic diversity in cluster beans. *Indian J. Agric. Sci.* **45**: 530–535.
- Doyle, J. J., and Doyle, J. L. 1987. A rapid DNA isolation procedure from small quantities of fresh leaf tissues. *Phytochem. Bull.* **19**: 11–15.
- Fawzia, R., and Alam, Sk. S. 2011. Fluorescent karyotype analysis in four varieties of *Solanum melongena* L. *Cytologia* **76**: 1–7.
- Jahan, I., Habib, M. A., Alam, N., and Alam, Sk. S. 2012. Karyotype and RAPD analysis in three commercial varieties of *Cucurbita maxima* Duch. *Cytologia* **77**: 149–154.
- Khatun, M., and Alam, Sk. S. 2010. Conformation of species status of *Corchorus trilocularis* by differential chromosome banding and isozyme assay. *Cytologia* **75**: 83–88.
- Kondo, T., and Hizume, M. 1982. Banding for the chromosomes of *Cryptomeria japonica* D. Don. *Japan J. Forestry Soc.* **64**: 356–358.
- Nei, M. 1972. Genetic distance between populations. *Am. Nat.* **106**: 283–292.
- Rai, N., Ashish, K., Singh, P. K., Singh, M., Datta, D., and Rai, M. 2010. Genetic relationship among Hyacinth bean (*Lablab purpureus*) genotypes cultivars from different races based on quantitative traits and random amplified polymorphic DNA marker. *Afr. J. Biotechnol.* **9**: 137–144.
- Schweizer, D. 1976. Reverse fluorescent chromosome banding with Chromomycin and DAPI. *Chromosoma* **58**: 307–324.
- Stebbins, G. L. 1971. Chromosomal Evolution in Higher Plants. Addison-Wesley Publishing Company, California. pp. 97.
- Sultana, S. S., and Alam, Sk. S. 2007. Differential fluorescent chromosome banding of *Solanum nigrum* L. and *Solanum villosum* L. from Bangladesh. *Cytologia* **72**: 213–219.
- Sumner, A. T. 1990. Chromosome banding. Unwin Hyman, London. p. 317.
- Welsh, J., and Clelland, M. Mc. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18**: 7213–7218.
- Whyte, R. O., Nilsson-Leissner, G., and Trumble, H. C. 1953. Legumes in Agriculture. FAO, Rome. pp. 272–273.
- Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6531–6535.
- Zaman, Y. M., and Alam, Sk. S. 2009. Karyotype diversity in three cultivars of *Momordica charantia* L. (Cucurbitaceae). *Cytologia* **74**: 473–478.