Cytogenetical and Molecular Characterization of *S. violaceum*, *S. sisymbriifolium* and a Putative Hybrid between ♀ *S. violaceum* and ♂ *S. melongena*

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Received June 19, 2012; accepted January 7, 2013

Summary Two wild *Solanum* species, viz. *S. violaceum*, *S. sisymbriifolium*, and a putative hybrid between ♀ *S. violaceum* and ♂ *S. melongena* were investigated to elucidate their genomic information. For this reason, the orcein and CMA-stained karyotypes of these three specimens were compared. In addition, RAPD with five primer combinations were conducted to make comparative DNA finger printing. The three specimens were found to possess $2n=24$ chromosomes. The karyotypes of the three specimens consisted of mostly metacentric chromosomes with no gradual decrease in chromosomal length suggesting symmetric karyotypes. A pair of CMA-positive satellites was found in both the members of pair II of *S. violaceum* and the putative hybrid. The CMA-karyotype of *S. sisymbriifolium* was different from the other two specimens. *Solanum violaceum* and the putative hybrid showed exactly the same DNA finger printing in two primers (Batch-7736-037 and Batch-7736-039) and almost the same in the other three primer combinations. On the other hand, the RAPD finger printing of *S. sisymbriifolium* was quite different from the other two specimens. The comparative karyotype and RAPD analysis clearly indicated that the putative hybrid was not actually a hybrid between *S. violaceum* and *S. melongena*, but rather showed a close resemblance to the mother plant.

Key words Karyotype, CMA-banding, RAPD, *Solanum*.

The family Solanaceae consists of about 850 genera with 2800 species (spp). *Solanum* is the representative genus of this family having about 1400 spp. (Cronquist 1981, Mabberley 1987). In Bangladesh, there are 17 spp. belong to *Solanum* distributed throughout the country. The three most economically important species of this genus are *Solanum tuberosum*, *S. lycopersicon* and *S. melongena*. In addition, the 2 wild relatives of *S. melongena* Lamk., namely *S. violaceum* Ortega and *S. sisymbriifolium* L., are also medicinally important.

Recently, a breeding program has been undertaken between *S. melongena* with its two wild species, viz. *S. violaceum* and *S. sisymbriifolium*, to detect the nature of gene flow (Das 2010). The cross between ♀ *S. sisymbriifolium* and ♂ *S. melongena* was unsuccessful whereas hybridization between ♀ *S. violaceum* and ♂ *S. melongena* seemed to be successful. However, this success has not been confirmed.

A major constraint for cytological study is that most of the *Solanum* species possess $2n=24$ chromosomes (Fawzia and Alam 2011, Sultana and Alam 2007). Moreover, all the chromosomes are almost identical in shape and size. Therefore, conventional karyotype analysis is not enough to distinguish among *Solanum* species. In such a situation, some modern methods may be used.

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DOI: 10.1508/cytologia.78.35

Chromosome staining with fluorochromes (CMA and DAPI) is one such method. Schweizer (1976) initiated this technique. CMA binds with GC-rich repetitive sequences of the genome and gives characteristic yellow color bands. With the help of fluorescent staining, it was possible to solve taxonomic problems in several species. Moreover, different categories, such as form, variety, and sub-species, may also be characterized with fluorescent banding technique (Sultana and Alam 2007, Huq *et al.* 2007, Hiron *et al.* 2006, Sultana *et al.* 2006, Akter and Alam 2005, Jessy *et al.* 2005, Lubna *et al.* 2004, Alam and Deen 2002, Hizume and Tanaka 1998, Alam *et al.* 1998, Alam and Kondo 1995, Kondo and Hizume 1982).

In recent years, RAPD analysis through the Polymerase Chain Reaction (PCR) has been used widely to characterize and trace the phylogeny of diverse plant and animal species. The main advantages of RAPD analysis over other methods are its low sample DNA requirements and the high frequency of detectable polymorphic bands (Williams *et al.* 1990). DNA based molecular markers have contributed widely in the studies of genetic diversity and phylogenetic analysis of plants (Savolainen and Chase 2003, Nybom 2004). These were used as versatile tools to provide the correct estimate of genetic diversity as they are independent from the effects of environmental factors (Powell *et al.* 1995, Garcia *et al.* 1998, Kuras *et al.* 2004) and are a source of informative polymorphism. DNA based molecular markers have been successfully used to discriminate between individual genotypes in a wide range of plant and animal species.

A combined research program of karyotype analysis and RAPD should be carried out to determine the nature of genome of two wild relatives of *S. melongena*. For any breeding programme of *S. melongena* to be successful, genomic information about wild relatives will be extremely useful. It is also essential to elucidate the nature of hybrids since true hybrids will indicate free gene flow between *S. melongena* and *S. violaceum*.

In the present study, karyotypes of *Solanum violaceum* and *Solanum sisymbriifolium* and the putative hybrid were compared. The aims of this study were:

- i. to compare the karyotypes of *Solanum violaceum*, *S. sisymbriifolium* and the putative hybrid plant of *S. violaceum* Ortega and *S. melongena* L. after staining with orcein and CMA;
- ii. to make DNA finger printing by RAPD analysis; and
- iii. to ascertain the hybrid status of the specimen.

Materials and methods

The following species of *Solanum*, viz. *Solanum violaceum* Ortega, *Solanum sisymbriifolium* Lamk., and a hybrid plant of ♀ *S. violaceum* Ortega and ♂ *S. melongena* L., were used in this study. These specimens were collected from different parts of Dhaka district and maintained in the Botanic garden, Department of Botany, University of Dhaka, Bangladesh.

Cytogenetical study

Healthy roots were collected and pretreated with 0.002 M 8-hydroxyquinoline for 1 h at room temperature (25–30°C) followed by 15 m in 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 7 s. The root tips were stained and squashed in 1% aceto orcein. For fluorescent 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 48 h before study. The air-dried slides were first pre-incubated in McIlvaine's buffer (pH 7.0) for 30 m in followed by Distamycin A (0.1 mg/ml) treatment for 10 m in. The slides were rinsed mildly in McIlvaine's buffer supplemented with MgSO₄ (5 mM) for 15 m in. One drop of CMA (0.1 mg/ml) was added to the materials for 15 m in and rinsed with McIlvaine's buffer with

Mg²⁺ for 10 min. Slides were mounted in 50% glycerol and kept at 4°C for overnight before observation. These were observed under Hund fluorescent microscope with Blue Violet (BV) filter cassette.

Molecular study (RAPD)

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 a spectrophotometer (Analytikjena, Specord 50, Germany). The A260/280 readings for DNA samples were 1.6–1.8.

PCR amplification and primer survey

The PCR reaction mixture for 25 µl contained template DNA (25 ng) 2 µl, de-ionized distilled water 18.8 µl, Taq buffer A 10X (Tris with 15 mM MgCl₂) 2.5 µl, primer (10 µM) 1.0 µl, dNTPs (2.5 mM) 0.5 µl, Taq DNA polymerase (5U/µl), 0.2 µl. PCR amplification was done in an oil-free thermal cycler (Biometra UNOII, Germany) for 46 cycles after initial denature 94°C for 5 min, denature at 94°C for 1 min, annealing at 36°C for 30 s, extension at 72°C for 3 min and final extension at 72°C for 5 min. Five primers were used from Operon Technologies, USA such as Batch-7736-030 (GAA ACG GGT G), Batch-7736-034 (TCA CGT CCA C), Batch-7736-036 (CCC GCC TTC C), Batch-7736-037 (GTC CTC GTA G) and Batch-7736-039 (GCC ACG GAG A) series.

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 8 µl and 99 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. RAPD analysis was then combined to create a single data matrix. This was used for estimating linkage distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) Dendrogram among the varieties using computer program "Statistica." Linkage distances were computed from frequencies of polymorphic markers to estimate genetic relationship among the studied specimens using UPGMA (Sneath and Sokal 1973). The dendrogram tree was constructed using the computer software Statistica.

Results and discussion

Orcein karyotype

Although the three specimens were found to possess 2n=24 chromosomes, *Solanum violaceum* and the putative hybrid (♀ *Solanum violaceum* × ♂ *Solanum melongena*) differed from *Solanum sisymbirifolium* in other karyotype features (Figs. 1–3, 7–9). The individual chromosomal length of *Solanum violaceum* and the putative hybrid were 0.84–1.50 µm and 1.48–0.84 µm, respectively (Table 1). The individual chromosomal length of *Solanum sisymbirifolium* was 1.90–0.95 µm (Table 1). Therefore, the chromosomes of *Solanum sisymbirifolium* are a little larger than the other 2 specimens. Since the ranges of individual chromosomes of the three specimens were less than 1 µm, no gradual decrease in chromosomal length was observed (Table 1). A pair of satellites was found on the short arms of pair II in ♀ *Solanum violaceum* and the putative hybrid (Figs. 1, 2, 7, 8), whereas

Table 1. Comparative orcein and CMA-karyotype analysis in *S. violaceum*, *S. sisymbriifolium* and a putative hybrid between ♀ *S. violaceum* and ♂ *S. melongena*

Specimens	2n	No. of satellites	Range of chromosomal length (μm)	Total length of 2n chromosome complements (μm)	Centromeric formulae	No. of CMA bands	Total length of CMA banded portion (μm)	% of CMA bands	CMA-banded formulae
<i>S. violaceum</i>	24	2	0.84–1.50	27.17	20 m+4 sm	2	0.63	2.31	2β+22δ
♀ <i>S. violaceum</i> × ♂ <i>S. melongena</i>	24	2	0.84–1.48	28.32	20 m+4 sm	2	1.26	4.45	2β+22δ
<i>S. sisymbriifolium</i>	24	—	0.95–1.90	33.80	22 m+2 sm	3	2.50	7.40	1α+2θ+21δ

m=metacentric chromosome, sm=sub-metacentric chromosome, α=Band in whole chromosome, β=Band in satellite, θ=Band in upper terminal region and δ=No band.

no such satellite was observed in ♂ *Solanum sisymbriifolium* (Figs. 3, 4). The three specimens thus differed in this features. Both ♀ *Solanum violaceum* and the putative hybrid possessed similar centromeric formulae, of 20 m+4 sm. On the other hand, a centromeric formula of 22 m+2 sm was determined in ♂ *Solanum sisymbriifolium* (Table 1). The three specimens showed two similarities, viz. i. presence of maximum metacentric chromosomes, and ii. no gradual decrease in chromosomal length. These features revealed that the three specimens had symmetric karyotypes. According to Stebbins (1971), symmetrical karyotypes have a primitive character. Therefore, *Solanum violaceum*, *Solanum sisymbriifolium* and the putative hybrid might be considered as primitive.

CMA karyotype

A pair of dot-like CMA-positive bands was found in ♀ *Solanum violaceum* and the putative hybrid in interphase nuclei and prophase chromosomes (data not shown). In metaphase, a pair of CMA positive bands was found on the short arm of pair II (satellite portion) in these 2 specimens (Figs. 4, 5 10, 11). CMA bands indicated the occurrence of GC-rich repeats. Presence of the similar bands in each stage of cell division revealed the stable nature of GC-rich domains. The satellites are the stable domains of a karyotype. Therefore, these bands were actually satellite bands of pair II (Figs. 10, 11). Since, these satellited portions were fluoresced with CMA, this indicates they are rich in GC-rich base pairs. Usually, the satellites are GC-rich (Sultana and Alam 2007, Khatun and Alam 2010).

In *Solanum sisymbriifolium*, 2 bands in the upper terminal regions of pair V were observed (Figs. 6, 12). In addition, a member of pair VI fluoresced entirely. No such band was found in its homologue. Why the two members of pair VI showed heteromorphism in respect of CMA banding was not clear. However, it may be due to unequal crossing over (Stebbins 1971) of GC-rich segments that makes one chromosome rich in GC-repeats. On the basis of CMA-banded karyotype and the percentage of GC rich repeats, *Solanum sisymbriifolium* is different from the other two specimens.

RAPD analysis

Five primer combinations were attempted on 3 specimens of *Solanum*. In the primer batches no. 39 and 37, *Solanum violaceum* and the hybrid showed exactly the same finger printing pattern (Figs. 13, 14). In the other three primers, these two specimens showed almost identical banding patterns (Figs. 15, 16, 17). In contrast, *Solanum sisymbriifolium* showed different finger printing patterns (Figs. 13–17). The DNA finger printing patterns revealed that *Solanum violaceum* and the putative hybrid are very closely related whereas *Solanum sisymbriifolium* is genomically different from these two specimens. This has been confirmed by cluster analysis (Fig. 18). The cluster dendrogram showed that *Solanum violaceum* and the hybrid were placed in the same cluster with very

Figs. 1–17. Comparative cytogenetical and molecular analysis of *S. violaceum*, *S. sisymbirifolium* and a putative hybrid between ♀ *S. violaceum* and ♂ *S. melongena*. **1.** Orcein-stained mitotic metaphase chromosomes of *S. violaceum*, **2.** Orcein-stained mitotic metaphase chromosomes of hybrid, **3.** Orcein-stained mitotic metaphase chromosomes of *S. sisymbirifolium*, **4.** CMA-stained mitotic metaphase chromosomes of *S. violaceum*, **5.** CMA-stained mitotic metaphase chromosomes of hybrid, **6.** CMA-stained mitotic metaphase chromosomes of *S. sisymbirifolium*, **7.** Orcein karyotype of *S. violaceum*, **8.** Orcein karyotype of hybrid, **9.** Orcein karyotype of *S. sisymbirifolium*, **10.** CMA karyotype of *S. violaceum*, **11.** CMA karyotype of hybrid, **12.** CMA karyotype of *S. sisymbirifolium*, Bar=10µm. **13–17.** RAPD analysis with five primers. **13.** Batch-7736-037, **14.** Primer Batch-7736-039, **15.** Primer Batch-7736-030, **16.** Primer Batch-7736-034 and **17.** Primer Batch-7736-036.

low genetic distance (Fig. 18). On the other hand, *Solanum sisymbirifolium* placed in a totally different cluster with high genetic distance (Fig. 18).

Status of the putative hybrid plant

Recently, an interspecific hybridization program has been carried out between *Solanum melongena* (BARI Begun 5-Nayantara) and its wild relative *Solanum violaceum* (Das 2010). The aim of

Fig. 18. Cluster analysis by unweighted pair group method of arithmetic means (UPGMA) of three plants in *Solanum* based on five RAPD markers.

the hybridization programme was to detect the nature of gene flow between these two species. A plant has been developed from such a cross. This plant was suspected to be a hybrid between these two species on the basis of a few morphological parameters. However, no cytological or molecular confirmation was made in favor of the hybrid status. An extensive karyotype study of BARI Begun-5-Nayantara was carried out (Fawzia and Alam 2011). In the present study, a detailed karyotype and RAPD study of *Solanum violaceum*, *S. sisymbirifolium* and the putative hybrid was conducted to elucidate the status of the putative hybrid plant. The comparative analysis of *Solanum melongena* (BARI Begun 5-Nayantara), *Solanum violaceum* and the putative hybrid plant is given below:

- i. the range of individual chromosomal length in *Solanum violaceum* and the putative hybrid was $0.84\text{--}1.50\mu\text{m}$ and $1.48\text{--}0.84\mu\text{m}$ respectively (Table 1), whereas it was $2.13\text{--}1.11\mu\text{m}$ in *Solanum melongena* (BARI Begun 5-Nayantara, Fawzia and Alam 2011). The hybrid should have had chromosomes of about $2.13\mu\text{m}$ in length but it did not have such chromosomes.
- ii. the centromeric formula of *Solanum violaceum* and the putative hybrid was same, *i.e.* $20\text{m}+4\text{sm}$ (Table 1). In contrast, it was $15\text{m}+9\text{sm}$ in *Solanum melongena* (BARI Begun 5-Nayantara, Fawzia and Alam 2011). The putative hybrid should have had an intermediate centromeric formula.
- iii. the total length of $2n$ chromosome complement was $27.17\mu\text{m}$ in *Solanum violaceum* and $28.32\mu\text{m}$ in the putative hybrids, whereas it was $38.29\mu\text{m}$ in *Solanum melongena* (BARI Begun 5-Nayantara, Fawzia and Alam 2011).
- iv. *Solanum violaceum* and the putative hybrid possessed two CMA positive satellites (Figs. 4, 5, 10, 11). On the other hand, *Solanum melongena* (BARI Begun 5-Nayantara) had four different CMA positive bands (Fawzia and Alam 2011). Were it a true hybrid, it should have one satellited chromosome and an intermediate CMA banding pattern.
- v. *Solanum violaceum* and the putative hybrid showed exactly the same RAPD finger printing patterns in primers 39 and 37 (Figs. 13, 14). These two specimens showed an almost identical banding pattern in the other 3 RAPD primers (Figs. 15, 16, 17).
- vi. DNA finger printing pattern revealed that *Solanum violaceum* and the putative hybrid are very closely related whereas *Solanum sisymbirifolium* is genomically different from these two specimens. This has been confirmed by cluster analysis (Fig. 18). The cluster dendrogram showed that *Solanum violaceum* and the hybrid were placed in the same cluster with very low genetic distance (Fig. 18). On the other hand, *Solanum sisymbirifolium* was placed in a totally different cluster with high genetic distance (Fig. 18).

The foregoing discussion clearly indicates that the putative hybrid is not actually a hybrid between ♀ *Solanum violaceum* × ♂ *Solanum melongena*, but rather it has a close resemblance to its female parent.

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