Karyotype Diversity in Three Asparagus L. Species

Suma Akter, Kazi Nahida Begum, Syeda Sharmeen Sultana and Sheikh Shamimul Alam*

Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

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Summary Three Asparagus species viz. A. racemosus Willd., A. officinalis L., and A. setaceus (Kunth) Jessop were investigated by cytogenetical and molecular analysis for authentic characterization. The diploid chromosome number $2n=20$ was found in A. racemosus and A. setaceus, while, A. officinalis possesses $2n=22$ chromosomes. Diploid chromosome number $2n=22$ is the first report for A. officinalis. These three species differed in respect of other karyotypic features such as total length of $2n$ chromosome complements, number of satellites, range of relative lengths, centromeric indices, etc. Some unique CMA- and DAPI-banded chromosomes were found in three species which could be used as marker chromosomes for the respective species. A pair of satellites was observed in A. racemosus after CMA- and DAPI-staining but absent in orcein staining indicating the stain-specific nature of satellites. The chromosomes of three Asparagus species possessed different structural abnormalities such as deletions, tandem duplications, and dispersed distribution of GC- and AT-rich repetitive sequences. This organizational variation of chromosomes was one of the major reasons for karyotype diversification. Four primer combinations were applied for RAPD analysis in three species of Asparagus to find out their genomic relationship. The three species showed several unique bands useful as markers for each species. Therefore, the three Asparagus species could authentically be characterized by conventional and fluorescent karyotype together with RAPD fingerprinting.

Key words Fluorescent banding, Karyotype, RAPD, Asparagus L.

The genus Asparagus L. belongs to Liliaceae consisting of about 300 species (Kumar et al. 2016). The plants of this genus are herbaceous, perennial, tender woody shrubs and vines, distributed throughout the tropical and sub-tropical countries of the world (Kumar et al. 2016, Camadro 1994). According to Siddiqui et al. (2007), only four Asparagus L. species were reported from Bangladesh. These are i) A. adscendens Roxb., ii) A. racemosus Willd., iii) A. setaceus (Kunth) Jessop, and iv) A. acerosus Roxb. In Bangladesh, this genus is common in the Sal (Shorea robusta) forests of Gazipur, Mymensingh and Sherpur districts (Siddiqui et al. 2007).

In addition, another edible species namely A. officinalis L. was introduced from the USA by Professor (retired) Dr. Mahbubur Rahman Khan, Department of Botany, University of Dhaka to the Botanic Garden, Department of Botany, University of Dhaka. So far this species is not found in any place of Bangladesh (Prof. Dr. Abul Hasan, Department of Botany, University of Dhaka—personal communication).

The species of this genus have gained importance for several purposes such as ornamental plant, vegetables, and as a medicinal herb. The World Health Organization has estimated that 80% population of developing countries, being unable to afford pharmaceutical drugs, relies on traditional medicines, mainly plant based, to sustain their primary health care needs (Singh 2014). The root extracts of the medicinal Asparagus species were found to possess anti-ulcer (Sairam et al. 2003), antioxidant (Siddiqui et al. 2007, Kamat et al. 2000), anti-diarrhoeal (Venkatesan et al. 2005), anti-carcinogenic, immune stimulant and hepato protective activities and also used in nervous disorders, bronchitis, inflammation, dyspepsia (Garabadu and Krishnamurthy 2014).

The increasing demand of the Asparagus species available in Bangladesh for various purposes has caused a serious reduction in native populations due to over harvesting and deforestation. These plants are now considered endangered in its natural habitat and has also been recognized as ‘vulnerable’ (Siddiqui et al. 2007, Warrier et al. 2001). In this situation, analysis of genetic diversity becomes essential. In order to elucidate genetic diversity and proper conservation, authentic characterization of species are required. For genetic characterization, stable and reliable method must be followed.

Karyotype is a stable and reliable character, which is specific for each specimen. Only a few earlier workers tried to characterize different Asparagus L. species with classical karyotype analysis which were mostly confined to $2n$ chromosome counting. There are diploid, tetraploid and hexaploid species in the genus, with a basic chromosome number of $X=10$ (Camadro 1994). Diploid chromosome number $2n=20, 22, 24, 28, 30, 40,$

* Corresponding author, e-mail: ssalam81@yahoo.com

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and 60 chromosomes for different *Asparagus* L. species were reported earlier by different scientists (Osaka 1938, Sharma and Bhattacharyya 1957, La Cour 1952, Subramanian 1973). Moreover, there is no report even on 2n chromosome counts for the available *Asparagus* L. species from Bangladesh.

However, conventional karyotype analysis alone is unable to express critically the differences among related species when these may possess similar 2n chromosome number and even other karyotype parameters (Khatun and Alam 2010, Khatun et al. 2011). Minute deletion, inversion, tandem duplication, etc. could not be possible to detect by conventional karyotype analysis. In such a case, modern cytogenetical techniques are necessary for comparative study among related species.

Staining with DNA-base specific banding with fluorochromes such as chomomycin A₃ (CMA) and 4’-6 diamidino-2-phenylindole (DAPI) are the effective methods for critical karyotype study such as identification of individual chromosome, determination of amount and site of AT- and GC-rich base pairs in chromosomes, etc. (Schweizer 1976, Alam and Kondo 1995, Sultana et al. 2011).

Molecular marker analysis has become another important tool in studying genetic information and diversity (Bered et al. 2005). DNA fingerprinting by Random Amplified Polymorphic DNA (RAPD) is one of the molecular methods for characterizing different plant specimens. It includes characterization of genetic variability, genome fingerprinting, genome mapping, gene localization, analysis of genome evolution, population genetics, taxonomy, etc. The advantages of RAPD analysis over other methods are its low sample DNA requirement and the high frequency of polymorphic bands (Williams et al. 1990).

In the present study, a combination of cytogenetical and molecular analysis has been carried out for the first time to characterize the three *Asparagus* species available in Bangladesh viz. *A. racemosus* Willd., *A. officinalis* L., and *A. setaceus* (Kunth) Jessop with the following aims:

i. to make a full strength karyotype for each species.

ii. to compare the fluorescent banding pattern after staining with CMA- and DAPI-fluorochromes and find out the diversity in their karyotypes.

iii. to elucidate the phylogenetic relationship among three *Asparagus* species on the basis of RAPD banding patterns.

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

Materials and methods

Among three *Asparagus* species, *A. setaceus* (Kunth) Jessop was collected from a nursery in Agargaon, Dhaka, Bangladesh and the other two spp. namely *A. racemosus* Willd. and *A. officinalis* L. from the Botanical Garden, Department of Botany, University of Dhaka. These three species were identified by Professor Dr. Md. Abul Hassan, Prof. Salar Khan Herbarium, Department of Botany, University of Dhaka and maintained in the Botanic Garden, Department of Botany, University of Dhaka, Bangladesh.

**Cytogenetical study**

Healthy root tips (RTs) were collected and pretreated with 8-hydroxyquinoline (0.002M) for 2.25h at 18–20°C followed by 15min fixation in 45% acetic acid at 4°C. The pretreated RTs were hydrolyzed for 40–45s (depending on thickness of root) at 60°C in a mixture of 1N 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 24h before study. The air-dried slides were first pre-incubated in McIlvain’s buffer (pH 7.0) for 30min followed by Diamycin A (0.1mg mL⁻¹) treatment for 10min. The slides were rinsed mildly in McIlvain’s buffer supplemented with MgSO₄ (5mM) for 15min. One drop of CMA (0.1mg mL⁻¹) was added to the materials for 25min in a humid chamber and then rinsed with McIlvain’s buffer with MgSO₄ for 10min. Slides were mounted in 50% glycerol and kept at 4°C for overnight before observation. These were observed under Nikon (Eclipse 50i) fluorescent microscope with blue violet (BV) filter cassette. For DAPI-staining, after 48h of air drying, the slides were first pre-incubated in McIlvain’s buffer (pH 7.0) for 25min and treated in Actinomycin D (0.25mg mL⁻¹) for 10min in a humid chamber. The slides were immersed in DAPI solution (0.01mg mL⁻¹) for 20min 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 (Analylykijena, 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 25µL containing template DNA (25 ng) 2µL, de-ionized distilled water 18.8µL, Taq buffer A 10× (Tris with 15mM MgCl₂) 2.5µL, primer (10µM) 1.0µL, dNTPs (2.5mM) 0.5µL, and Taq DNA polymerase (5U µL⁻¹) 0.2µL. PCR amplification was done in an oil-free thermal cycler (Biometra...
UNOII, Germany) for 46 cycles after initial denaturing 94°C for 5 min, denaturing at 94°C for 1 min, annealing at 34–36°C for 30 s, extension at 72°C for 3 min, and final extension at 72°C for 5 min. Four random primers such as primer OPG-3 (5'-GAGCCCTCCA-A-3'), primer OPG-6 (5'-GTGCCTAACC-3'), primer OPG-9 (5'-CTGACGTCA-C-3'), and primer-3 (5'-TGCGAGCCTGTG-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 V 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 out for further investigation. The scores obtained using all primers in the RAPD analysis were then pooled out for further investigation. The scores obtained using all primers in the RAPD analysis were then pooled out for further investigation. The scores obtained using all primers in the RAPD analysis were then pooled out for further investigation.

Results and discussion

Karyotype

In the present study, A. racemosus and A. setaceus were found to possess 2n=20 chromosomes (Figs. 1 and 3). Similar 2n chromosome numbers of these two species were reported by earlier scientists (Osaka 1938, Sharma and Bhattacharyya 1957, Mehra and Malik 1961, Sastry 1981). Besides, different chromosome number for A. racemosus such as 2n=22 (Subramanian 1973), 2n=30 (Thombre 1959), 2n=40 (Venkateswarlu and Raju 1957), and 2n=48 (Sinha et al. 1972) were also reported. The presence of 2n=30 and 2n=40 chromosomes indicated triploid and tetraploid nature, respectively. On the other hand, 2n=22 and 2n=48 indicated aneuploid nature of the plants. B chromosomes were also reported for this species (Sheidai and Inamdar 1993). However, no B chromosomes were found in any cell in the present investigation. In this study, no numerical changes regarding chromosome number were found in any sample. Therefore, the samples of A. racemosus used in this experiment are diploid (2n=2x=20).

On the other hand, in this study 2n=22 chromosomes were observed in A. officinalis (Fig. 2). Different 2n chromosome numbers of this species were reported earlier such as 2n=20 (Ma et al. 1985, Deng et al. 2012), 2n=40 (Krasnikov and Shaulo 1990, Castro et al. 2013), and 2n=44 (Kondo et al. 2014). 2n=22 chromosomes of A. officinalis were not found after reviewing the literature. Therefore, the diploid chromosome number of 2n=22 for A. officinalis is probably the first record for this species. The present 2n chromosome number suggesting that the specimen used in this study may either be very close to A. officinalis or a different cytotype.

In this study, three Asparagus species were found to possess all metacentric chromosomes (Figs. 10–12) with chromosomal length ranging from 0.48–1.58 μm (Table 1). The range of chromosomal length for A. officinalis was 0.77 to 1.58 μm. A number of disagreements regarding the chromosomal length and centromeric position were reported earlier for A. officinalis. Mukhopadhyay and Ray (2013) reported the range of chromosomal length as 0.92–5.83 μm. Deng et al. (2012) classified the 2n=20 chromosomes into five long, one medium, and four short pairs. On the other hand, Melo and Guerra (2001) reported longer pairs with 2.4 μm (four pairs), medium 1.9 μm (two pairs), and short 1.5 μm (four pairs).

After comparing the results of Mukhopadhyay and Ray (2013) with Melo and Guerra (2001), it was found that the longer chromosome (5.8 μm) mentioned by Mukhopadhyay and Ray (2013) were absent in the karyotypes reported by Melo and Guerra (2001). The present result was compared to that of Melo and Guerra (2001). It was found that the longer chromosome (2.4 μm) and the medium chromosome (1.9 μm) mentioned by Melo and Guerra (2001) were absent in A. officinalis used in this study (Table 1). The chromosomal length around 1.5 μm was common in the specimens used in this study and by Melo and Guerra (2001). In addition to the chromosomes of 1.5 μm length, nine pairs of chromosomes with around 1 μm even less than 1 μm length were found in this study (Figs. 10–12, Table 1). The chromosomal length 1 μm or less was not reported earlier. Therefore, this category (1 μm or below) in respect of chromosomal length reported first time for A. officinalis used in this study.

Satellite

In this investigation, a pair of satellites was found in chromosome pair IV of A. officinalis (Figs. 2, 11, Table 1). Deng et al. (2012) reported one pair of satellites on the short arm of the chromosome pair V. In contrast, four pairs of satellites bearing chromosomes were reported by Rudall et al. (1998) of which three pairs on the long arms and one pair on the short arm. Therefore, the difference regarding the number of satellite bearing chromosomes was observed in different specimens of A. officinalis.
indicating the existence of different cytotypes. A pair of satellites was found in pair I and VI of *A. officinalis* after CMA- and DAPI-staining, respectively (Figs. 4, 7, 13, 16). However, in orcein staining no satellite was found in this species (Figs. 1, 10). On the other hand, a pair of satellites observed in pair IV of *A. officinalis* was not found after CMA- and DAPI-staining (Figs. 2, 5, 8, 11, 14, 17). This feature of satellite revealed stain specificity. Alam and Kondo (1995) described the stain specific nature of satellites and some chromosomes in *Drosera* species. Later different workers reported stain specificity of satellite in few plant species (Khatun and Alam 2010, Khatun et al. 2011, Sultana and Alam 2007, Sultana and Alam 2016). The reason for stain specificity was not clear, however, it may due to the presence of certain DNA sequences, which made the satellites stain specifically. Therefore, the stain specific nature of satellite is a remarkable feature of the *Asparagus* karyotype.

Fluorescent banding

The number, location, distribution, and intensities of CMA-bands varied in these species. Most of the CMA-bands were present at the terminal regions of respective chromosomes in these three *Asparagus* species (Figs. 4, 5, 6, 13, 14, 15, Table 1). In contrast, only one centromeric CMA-band was found in *A. racemosus* and *A. officinalis* (Figs. 4, 5, 13, 14). The presence of maximum terminal CMA-bands indicated a tendency of accumulating GC-rich repetitive sequences at the chromosomal ends (Zaman and Alam 2009).

In contrast, a member of chromosome pair X of *A. racemosus* Willd. and both the members of pairs II and VI of *A. setaceus* (Kunth) Jessop were entirely fluoresced with CMA (Figs. 4, 6, 13, 15). In these entirely fluoresced chromosomes, GC-rich repeats were not con-
Table 1. Comparative orcein-, CMA- and DAPI-karyotype analysis of three species of Asparagus L.

<table>
<thead>
<tr>
<th>Species</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>A. racemosus</td>
<td>20</td>
<td>0.53–1.23</td>
<td>18.18</td>
<td>20m</td>
<td>6</td>
<td>14.30</td>
<td>4</td>
<td>16.83</td>
<td>2 in DAPI</td>
</tr>
<tr>
<td>A. officinalis</td>
<td>22</td>
<td>0.77–1.58</td>
<td>24.66</td>
<td>22m</td>
<td>3</td>
<td>5.13</td>
<td>4</td>
<td>16.04</td>
<td>2 in DAPI</td>
</tr>
<tr>
<td>A. setaceus</td>
<td>20</td>
<td>0.48–1.47</td>
<td>18.50</td>
<td>20m</td>
<td>6</td>
<td>25.18</td>
<td>8</td>
<td>13.92</td>
<td>2 in Orcein and CMA</td>
</tr>
</tbody>
</table>

m=m=metacentric chromosomes

Table 2. Compilation of RAPD analysis in three species of Asparagus L.

<table>
<thead>
<tr>
<th>Primer codes</th>
<th>Size ranges (bp)</th>
<th>Total bands</th>
<th>Common bands (bp)</th>
<th>Polymorphic bands (bp)</th>
<th>Unique bands (bp)</th>
<th>Polymorphism (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPG-3</td>
<td>300–2500</td>
<td>14</td>
<td>—</td>
<td>14</td>
<td>750, 600 in A. racemosus, 2500, 1500, 1250, 1000, 700, 450 in A. officinalis, 1600, 1100 in A. setaceus</td>
<td>100</td>
</tr>
<tr>
<td>OPG-6</td>
<td>200–2900</td>
<td>16</td>
<td>—</td>
<td>16</td>
<td>2900, 2200, 1500, 1200, 950, 650, 550, 350, 200 in A. racemosus, 1600, 1400, 1300, 1100, 600, 500, 300 in A. setaceus</td>
<td>100</td>
</tr>
<tr>
<td>OPG-9</td>
<td>250–2000</td>
<td>8</td>
<td>—</td>
<td>8</td>
<td>1500, 500 in A. racemosus, 2000, 1200, 750, 250 in A. officinalis, 1400 in A. setaceus</td>
<td>100</td>
</tr>
<tr>
<td>Primer-3</td>
<td>600–4000</td>
<td>9</td>
<td>—</td>
<td>9</td>
<td>3000, 2500, 2000, 1500, 1300, 600 in A. officinalis, 2800, 1400 in A. setaceus</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>200–4000</td>
<td>47</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Fig. 23. UPGMA dendrogram based on Nei’s (1972) genetic distance summarizing the data on differentiation between three species of Asparagus by RAPD analysis.

Genetic relationships among three Asparagus species

The values of pair-wise Nei’s (1972) genetic distances were analyzed by using computer software “poptene32.” The data segregated the three species in two clusters, A. racemosus and A. setaceus were placed in cluster 1 with genetic distance 0.9598, whereas A. officinalis placed
alone in cluster 2 with genetic distance 1.2111 (Fig. 23).

The data clearly indicated the diverse genomic value of the three Asparagus species which correlated with the RAPD data (Table 2).

The foregoing discussion revealed that the three Asparagus species have diverse orcein-, CMA-, and DAPI-karyotype with different RAPD-banding pattern. Therefore, the three Asparagus species could be characterized authentically with the help of combined cytogenetical and molecular data.

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References


