Karyotype Diversity in Three *Asparagus* L. Species

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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, Warriar *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 $2n=20, 22, 24, 28, 30, 40,$

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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 chromomycin 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 (0.002 M) for 2.25 h at 18–20°C followed by 15 min fixation in 45% acetic acid at 4°C. The pretreated RTs were hydrolyzed for 40–45 s (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 pre-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 25 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 for overnight before observation. These were observed under Nikon (Eclipse 50i) fluorescent microscope with blue violet (BV) filter cassette. For DAPI-staining, after 48 h of air drying, the slides were first pre-incubated in McIlvaine's buffer (pH 7.0) for 25 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 (Analytikjena, 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 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-3'), primer OPG-6 (5'-GTGCCTAACC-3'), primer OPG-9 (5'-CTGACGTCAC-3'), and primer-3 (5'-TGCCGAGCTG-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 for constructing a single data matrix. This was used for estimating polymorphic loci, Nei (1972) gene diversity, genetic distance (D), and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among the specimens, using the computer program "POPGENE" (Version 1.31).

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.*

Figs. 1–22. Orcein-, CMA- and DAPI-stained mitotic metaphase and RAPD analysis of three species of *Asparagus* L. 1. Orcein-stained mitotic metaphase chromosomes of *A. racemosus*, 2. Orcein-stained mitotic metaphase chromosomes of *A. officinalis*, 3. Orcein-stained mitotic metaphase chromosomes of *A. setaceus*, 4. CMA-stained mitotic metaphase chromosomes of *A. racemosus*, 5. CMA-stained mitotic metaphase chromosomes of *A. officinalis*, 6. CMA-stained mitotic metaphase chromosomes of *A. setaceus*, 7. DAPI-stained mitotic metaphase chromosomes of *A. racemosus*, 8. DAPI-stained mitotic metaphase chromosomes of *A. officinalis*, 9. DAPI-stained mitotic metaphase chromosomes of *A. setaceus*, 10. Karyotype prepared from orcein-stained mitotic metaphase chromosomes of *A. racemosus*, 11. Karyotype prepared from orcein-stained mitotic metaphase chromosomes of *A. officinalis*, 12. Karyotype prepared from orcein-stained mitotic metaphase chromosomes of *A. setaceus*, 13. Karyotype prepared from CMA-stained mitotic metaphase chromosomes of *A. racemosus*, 14. Karyotype prepared from CMA-stained mitotic metaphase chromosomes of *A. officinalis*, 15. Karyotype prepared from CMA-stained mitotic metaphase chromosomes of *A. setaceus*, 16. Karyotype prepared from DAPI-stained mitotic metaphase chromosomes of *A. racemosus*, 17. Karyotype prepared from DAPI-stained mitotic metaphase chromosomes of *A. officinalis*, 18. Karyotype prepared from DAPI-stained mitotic metaphase chromosomes of *A. setaceus*, Bar=10 μ m, 19. RAPD with primer OPG-3, 20. RAPD with primer OPG-6, 21. RAPD with primer OPG-9 and 22. RAPD with primer-3.

officinalis indicating the existence of different cytotypes.

A pair of satellites was found in pair I and VI of *A. racemosus* 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 be 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.

Species	2n	Range of chromosomal length (μ m)	Total length of 2n chromosome complement (μ m)	Centromeric formulae	No. of CMA-bands	% of GC-rich repeats	No. of DAPI-bands	% of AT-rich repeats	No. of satellites
<i>A. racemosus</i>	20	0.53–1.23	18.18	20m	6	14.30	4	16.83	2 in DAPI
<i>A. officinalis</i>	22	0.77–1.58	24.66	22m	3	5.13	4	16.04	—
<i>A. setaceus</i>	20	0.48–1.47	18.50	20m	6	25.18	8	13.92	2 in Orcein and CMA

m=metacentric chromosomes

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

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

fined to the terminal region but rather distributed along the chromosomes. The possible reason for these entirely fluoresced chromosomes was tandem duplication of GC-rich repeats along the length (Khatun and Alam 2010, Sultana and Alam 2007, Mahbub *et al.* 2007).

In *A. officinalis*, the percentage of GC-rich repeats was 5.13 (Table 1). The GC-rich percentage is about three times more in *A. racemosus* and about five times more in *A. setaceus* (Table 1). Therefore, the percentage of GC-rich repeats is an important karyotypic criterion of these three species.

Different DAPI-banding patterns were found in three *Asparagus* species as well. In *A. racemosus*, two chromosomes of pair X were entirely fluoresced while members of pair III were almost entirely fluoresced (Figs. 7, 16). One member of pair II, V, and XI were entirely fluoresced in *A. officinalis* (Figs. 8, 17). However, no entirely DAPI-fluoresced chromosome was found in *A. setaceus* (Figs. 9, 18). This banding pattern revealed that the AT-rich areas of certain chromosomes in *A. racemosus* and *A. officinalis* had been tandemly duplicated, whereas such duplication was not happened in any chromosomes of *A. setaceus*.

RAPD analysis

DNA from three *Asparagus* species was isolated to study RAPD with four oligonucleotide primers. The four primers generated in total 47 distinct bands with 100% polymorphism. These three species did not share any common fragments of DNA in four RAPD primers (Figs. 19–22, Table 2). Several unique fragments were observed in three *Asparagus* species with four primers. Such as (i) in primer OPG-3, 750, 600 bp in *A. racemo-*

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

sus, 2500, 1500, 1250, 1000, 700, 450 bp in *A. officinalis* and 1600, 1100 bp in *A. setaceus*, (ii) in primer OPG-6, 2900, 2200, 1500, 1200, 950, 650, 550, 350, 200 bp in *A. racemosus* and 1600, 1400, 1300, 1100, 600, 500, 300 bp in *A. setaceus* (iii) in primer OPG-9, 1500, 500 bp in *A. racemosus*, 2000, 1200, 750, 250 bp in *A. officinalis* and 1400 bp in *A. setaceus* (iv) in primer-3, 3000, 2500, 2000, 1500, 1300, 600 bp in *A. officinalis* and 2800, 1400 bp in *A. setaceus* (Table 2). The term unique sequence means that the sequence found in a species with a certain primer was absent in other species (Table 2). The unique bands were stable, reproducible and specific, thus could be used as a tool for characterization. In the earlier literature, there was no information about unique RAPD band (Ii *et al.* 2012, Singh *et al.* 2013, Kumar *et al.* 2016). Therefore, the use of unique RAPD band as characterization tool for *Asparagus* spp. is a pioneer approach.

Genetic relationships among three *Asparagus* species

The values of pair-wise Nei's (1972) genetic distances were analyzed by using computer software "popgene32." 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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