Chromosome and Marker-Based Genome Analysis of Different Species of *Asparagus*

Sandip Mukhopadhyay^{1*}, and Suranjana Ray²

¹Centre of Advanced Study, Department of Botany, University of Calcutta,
35 Ballygunge Circular Road, Kolkata–700 019, India

²Department of Genetics, University of Calcutta,
35 Ballygunge Circular Road, Kolkata–700 019, India

Received April 5, 2013; accepted October 12, 2013

Summary The present investigation was carried out on six economically important species of *Asparagus* using chromosomal characteristics, 4C nuclear DNA contents, SDS PAGE of total leaf proteins, isozyme polymorphism, and molecular markers to assess their relationship both at inter- and intraspecific levels. Karyotype analysis revealed diploid ($2n=2x=20$) status in most of the species except tetraploidy ($2n=4x=40$) in *A. cooperi* and hexaploidy ($2n=6x=60$) in *A. densiflorus*. Karyotype asymmetry existed among the species as well as in same ploidy level of different species. The inter-chromosomal and intra-chromosomal asymmetry ranged from values of 0.902–0.977 and 0.197–0.486, respectively. Again, considering chromosome number, length, volume, and median chromosome number, the primitive nature has been observed in *A. densiflorus*. The difference in chromosome parameters in all the species suggested the occurrence of structural changes at the chromosomal level. However, a higher degree of correlations has been found between chromosome volume and 4C nuclear DNA content in the six species studied so far. The dendrogram obtained from SDS-PAGE and isozyme analysis clearly revealed a close relationship between *A. racemosus* and *A. pyramidalis*, but the dendrogram obtained from RAPD and ISSR revealed a close relationship among *A. pyramidalis*, *A. densiflorus*, and *A. racemosus*. *Asparagus plumosus* and *A. cooperi* also showed a close relationship in all aspects except in ISSR study. All these data clearly indicated that a better phylogenetic relationship can be drawn from the combination of cytological and molecular marker-based analysis, which may assist the breeding pattern or germplasm conservation of the gene pool of specified crops.

Key words *Asparagus*, Chromosome, ISSR, Nuclear DNA, SDS-PAGE, RAPD.

The genus *Asparagus* has about 150 species distributed throughout the world, out of which 17 species have been reported in different regions of India (up to 5,300 ft) (Manjunath 1948). The species of *Asparagus* have important medicinal, ornamental, and horticultural values. For example, *A. officinalis* is an important vegetable crop used worldwide, while *A. plumosus*, *A. densiflorus*, *A. pyramidalis*, *A. cooperi*, and *A. racemosus* have horticultural values. *Asparagus racemosus* has both horticultural and medicinal importance.

The study of genetic diversity within and among different species and populations of different plant species is necessary for crop improvement, conservation, and management. Karyotype analysis is an important, essential method based on chromosome structural parameters to study the origin, evolution, and classification of different genomes. The chromosome number of several different species of *Asparagus* has been reported previously; *A. densiflorus* Kunth *sprengeri* has $2n=60$ chromosomes, *A. cooperi* has $2n=40$ chromosomes, and *A. pyramidalis*, *A. plumosus* Baker, and *A. racemosus* Wild have $2n=20$ chromosomes (Kar and Sen 1985). In angiosperms, genome

* Corresponding author, e-mail: sandip135@yahoo.com

DOI:10.1508/cytologia.78.425

size varies between species at least up to 800-fold (Bennett and Smith 1976). A widely used method for genome size determination is Feulgen densitometry (Bennett and Leitch 1997). Genome size data is available for few *Asparagus* species (Jena *et al.* 2007). Nuclear DNA content data of *A. densiflorus* Kunth *sprengeri* and *A. officinalis* L. have been reported previously (Nag 2006).

Different species and populations can be characterized by their protein profile and specific isozyme activities. Polyacrylamide gel electrophoresis (PAGE) has been an extremely useful and important analytical tool for separation, estimation, and quantification of different polypeptides, and is the method of choice for locating any qualitative changes in protein metabolism at the cellular level (Everett and Wach 1985). Isozyme markers have contributed immensely to assess the genetic diversity among plant species. These markers, unlike morphological markers, are stable and have been useful in population studies (Aitkin *et al.* 1994). The major disadvantages of these markers lie in the limited number of informative markers and its proneness to environmental and developmental variation. DNA markers fulfill the requirements for cultivar identification. Among them, the widely used are RAPD, RFLP, and AFLP markers. RAPD is the most widely used marker because of its simplicity, speed, and low cost. Another useful marker is ISSR (Inter Simple Sequence Repeat), which involves the amplification of the DNA region located between two microsatellites loci (Zietkiewicz *et al.* 1994). Independent molecular marker-based linkage maps based on RFLP and RAPD analyses have been created for *A. officinalis* (Caporali *et al.* 1996, Jiang *et al.* 1997). RAPD and ISSR studies have been widely used for population genetic studies in both wild (Khasa and Dancik 1996) and cultivated species (Souframanien and Gopalakrishna 2004). RAPD analysis has mostly been performed on *A. officinalis* only (Khandka *et al.* 1996).

The objective of our present investigation is to determine the phylogenetic relationship between six economically important species of *Asparagus* using chromosome, protein, isozyme, RAPD, and ISSR markers, and also to check the compatibility of these markers for assessment of genetic diversity as well as to examine the level of genetic variation utilizing different chromosome parameters.

Materials and methods

Plant materials

Asparagus officinalis L. and population I of *A. densiflorus* Kunth *sprengeri* were collected from Tamil Nadu, India. *A. plumosus* Baker, *A. cooperi* Baker, and both populations of *A. racemosus* Wild were collected from a local nursery of Kolkata, India. *Asparagus pyramidalis* Baker and population II of *A. densiflorus* Kunth *sprengeri* were obtained from Kalimpong, West Bengal, India.

Chromosome analysis

For chromosome analysis, the acetic-orcein staining schedule was adopted (Mukhopadhyay and Sharma 1987). Karyotype asymmetry was calculated according to the indexes suggested by Romero Zarco (1986). In order to determine the difference, the analysis of variance (ANOVA) was performed on the total size of chromosomes, average size of the chromosomes, size of the long arms, and size of the short arms among the species and populations studied (Sheidai and Jalilian 2008) using the S plus 2000 software.

Cytophotometric analysis for estimating *in situ* nuclear DNA content

Cytophotometric analysis was carried out following the Feulgen staining schedule (Sharma and Mukhopadhyay 1984, Sharma and Sharma 1980). The 4C nuclear DNA value of *Allium cepa* var. *rosette* (67.1 pg) (Van't Hoff 1965) was taken as a standard.

SDS-PAGE of total protein and isozyme analysis

Qualitative estimation of the total protein profile was carried out using fresh leaf tissue sam-

ples (Lowry *et al.* 1951). Esterase and peroxidase analyses were performed using previous methods (Weeden 1983, Wendel 1989).

RAPD and ISSR analysis

Genomic DNA was isolated utilizing Doyle and Doyle's method (1987). RAPD and ISSR amplification reactions were performed based on a protocol previously reported (Williams *et al.* 1990, Goldman 2008). The amplification reaction products were size separated in 1.2% Agarose gel (Sigma) using $0.5 \times$ TBE buffer (0.45 M Tris borate, 1 mM Na₂EDTA), followed by ethidium bromide staining for visualization under UV illumination.

Data analysis

SDS-PAGE of total protein and isozyme analysis, as well as both RAPD and ISSR analyses for each primer sequence, were performed in duplicate and only those bands obtained twice clearly were scored either 1 as present or 0 as absent. The unweighted pair group method with arithmetic averages (UPGMA) and percent disagreement values were analyzed using Statistica version 6 to construct the matrix and the phylogenetic tree (Mirali *et al.* 2007).

Results and discussion

Chromosome analysis

Karyotype analysis revealed the presence of different chromosome types in the species of *Asparagus*. Karyotypic details of six different species of *Asparagus* are presented in Table 1 and Figs. 1.1a–x and 1.2a–b. The species *A. officinalis*, *A. plumosus*, *A. pyramidalis*, and *A. racemosus* possessed $2n=2x=20$ chromosomes as diploids, while *A. cooperi* was tetraploid with $2n=4x=40$ chromosomes, and *A. densiflorus* was hexaploid with $2n=6x=60$ chromosomes. In other words, the species possessed the chromosome numbers $2n=20$, $2n=40$, and $2n=60$, comprising of a distinct polyploid series. This indicates the fact that polyploidy has played a major role in the evolution of the different species of the genus *Asparagus*. In plants, polyploidy occurs most commonly through endoreplication of chromosomes and is probably caused by environmental factors (Jackson 1976).

The individual chromosome morphology studied in these species and populations of *Asparagus* is quite distinct with asymmetric karyotypes. The differences in karyotypic details (Table 1, Figs. 1a–x) are principally the number and combination of different chromosome types, including those with secondary constrictions. Among all the species studied, the value of the inter-chromosomal asymmetry index (A_1) varied from 0.902 in population I of *A. racemosus* to 0.977 in population I of *A. densiflorus* (Fig. 1(2b)), while the intra-chromosomal asymmetry index (A_2) varied from 0.197 in *A. plumosus* to 0.486 in population I of *A. racemosus*. The variation is much less in the two populations of the hexaploid species. In *A. pyramidalis*, a specific type of secondary constricted chromosome was observed, which was absent in all other species and populations. The number of chromosomes with secondary constrictions varied from 2–8 in all the species and populations of *Asparagus* studied in the present investigation. The increase or decrease in the number of chromosomes with secondary constrictions might have been due to the duplication of such chromosomes or translocation between the chromosome with or without secondary constrictions at an early stage of evolution. The two types of centromeric chromosomes (median, submedian) that have been observed in variable numbers differ progressively with increase in ploidy level.

Among all the species studied, the longest chromosome was found in *A. officinalis* ($5.83 \mu\text{m}$) and the shortest one in *A. racemosus* Population I ($0.90 \mu\text{m}$). The total chromosome length varied from 0.90 to $5.83 \mu\text{m}$ in all the species of *Asparagus*. The highest total chromosome length (TCL) and volume (TCV) were found in population II of *A. densiflorus* (137.02 , $102.00 \mu\text{m}^3$), while the lowest TCL was found in *A. pyramidalis* ($33.18 \mu\text{m}$), and the lowest TCV was recorded in

Table 1. Karyological data on different species and populations of *Asparagus*.

Name of the species	2n	TCL (μm)	ACL (μm)	RCL (μm)	Karyotype asymmetry index		TCV (μm^3)
					A ₁	A ₂	
<i>A. officinalis</i>	20	61.76	3.08	1.60–5.83	0.944	0.341	49.45
<i>A. pyramidalis</i>	20	33.18	1.65	1.00–2.38	0.933	0.247	18.10
<i>A. plumosus</i>	20	34.00	1.70	1.00–2.75	0.934	0.197	20.43
<i>A. racemosus</i> Population I	20	40.25	2.01	0.90–3.35	0.924	0.486	18.00
<i>A. racemosus</i> Population II	20	37.70	1.88	0.90–3.30	0.928	0.398	19.09
<i>A. cooperi</i>	40	65.00	1.62	0.64–3.67	0.960	0.390	62.00
<i>A. densiflorus</i> Population I	60	123.38	2.05	1.20–4.34	0.967	0.368	96.85
<i>A. densiflorus</i> Population II	60	130.02	2.20	1.46–4.32	0.965	0.282	102.00
		*7.58					*9.81

Name of the species	Type of primary chromosomes		Type of secondary chromosomes				Karyotype formula
	M	Sm	M, St	Sm, Sm	Sm, St	St, St	
<i>A. officinalis</i>	10 (D)	6 (C)	2 (A)	2 (B)	0		A ₂ B ₂ C ₆ D ₁₀
<i>A. pyramidalis</i>	14 (C)	2 (D)	2 (A)	0	0	2 (B)	A ₂ B ₂ C ₁₄ D ₂
<i>A. plumosus</i>	12 (C)	4 (B)	4 (A)	0	0		A ₄ B ₄ C ₁₂
<i>A. racemosus</i> Population I	16 (C)	0	2 (A)	2 (B)	0		A ₂ B ₂ C ₁₆
<i>A. racemosus</i> Population II	14 (D)	2 (C)	2 (A)	2 (B)	0		A ₂ B ₂ C ₂ D ₁₄
<i>A. cooperi</i>	26 (C)	8 (D)	4 (B)	0	2 (A)		A ₂ B ₄ C ₂₆ D ₈
<i>A. densiflorus</i> Population I	38 (B)	16 (C)	6 (A)	0	0		A ₆ B ₃₈ C ₁₆
<i>A. densiflorus</i> Population II	38 (D)	14 (E)	2 (B)	2 (C)	4 (A)		A ₄ B ₂ C ₂ D ₃₈ E ₁₄

* Indicates means within each column at species level. $p \leq 0.05$

The following abbreviations are used—number (No), average (avg), chromosome (ch), length (lth), Median (M), sub median (Sm), sub terminal (St), terminal (T).

Chromosomes with secondary constrictions—one constriction median and other sub-median (M, Sm), one median and other sub-terminal (M, St), both constrictions sub-median (Sm, Sm) and one constriction sub-median and one sub-terminal (Sm, St).

A. racemosus Population I ($18.00\mu\text{m}^3$) (Table 1). In several angiospermic species, a reduction in chromosome length has often been recorded along with polyploidy, where differential condensation, differential polynemy, and other factors might have been responsible for such reduction. However, a higher degree of correlations ($r=0.96$) has been found between the total chromosome length and total chromosome volume (Fig. 1(2a)). There are reports where diploids and polyploids have the same chromosome size (Jha and Sen 1983). Such observations have revealed that every species and population differ in minute details of karyotype, indicating the role of the structural changes of chromosomes in the species evolution in *Asparagus*.

4C Nuclear DNA content analysis

4C nuclear DNA contents were quantified through *in-situ* cytophotometry in different species of *Asparagus* (Table 2). The highest value was recorded in population I of *A. densiflorus* (34.02 pg) and the lowest one in *A. pyramidalis* (20.66 pg). One population of *A. densiflorus* showed a lower 4C value (23.24 pg) than the other population. The 4C DNA values did not differ much between *A. pyramidalis*, *A. racemosus* Population I, *A. racemosus* Population II, and *A. plumosus* (Table 2), while tetraploid *A. cooperi* presented a higher nuclear DNA value (30.04 pg). The correlation of coefficient was found to be 0.97 between the total chromosome length and 4C nuclear DNA content (Fig. 2(1a)). The amount of 4C nuclear DNA correlated with the total chromosome length in diploids and tetraploids. On the one hand, population I of *A. densiflorus* revealed the highest amount of

Fig. 1(1a-x). Somatic metaphase plates (photograph and drawing) of different species and populations of *Asparagus*. a-c: Somatic metaphase plates and karyogram of ($2n=2x=20$) of *A. officinalis*, d-f: Somatic metaphase plates and karyogram of ($2n=2x=20$) of *A. pyramidalis*, g-i: Somatic metaphase plates and karyogram of ($2n=2x=20$) of *A. plumosus*, j-l: Somatic metaphase plates and karyogram of ($2n=2x=20$) of *A. racemosus* Population I, m-o: Somatic metaphase plates and karyogram of ($2n=2x=20$) of *A. racemosus* Population II, p-r: Somatic metaphase plates and karyogram of ($2n=2x=40$) of *A. cooperi*, s-u: Somatic metaphase plates and karyogram of ($2n=2x=60$) of *A. densiflorus* Population I, v-x: Somatic metaphase plates and karyogram of ($2n=2x=60$) of *A. densiflorus* Population II.

4C nuclear DNA and the highest value of total chromosome length. On the other hand, the other population of this species revealed quite a low value of 4C nuclear DNA, even though the total chromosome length was quite high, indicating a lack of correlation between these two parameters. In the absence of any correlation, the difference in length may be attributed to the differential condensation and spiralization of the chromosome arm (Sharma and Mukhopadhyay 1984, Lahiri *et al.* 2011). The extent to which such processes are associated with the expression of specific genetic

Fig 1.2a

Fig 1.2b

Fig. 1(2a, b). Scatter diagrams presenting total chromosome length and total chromosome volume relationship and inter and intra chromosomal karyotype asymmetry in different species and populations of *Asparagus*, respectively.

Fig 2.1a

Fig 2.1b

Fig. 2 (1a, b) Scatter diagram on total chromosome length and 4C nuclear DNA contents and on total chromosome volume and 4C nuclear DNA contents. Ao=*A. officinalis*, Apy=*A. pyramidalis*, Apl=*A. plumosus*, ArI=*A. racemosus* Population I, ArII=*A. racemosus* Population II, Ac=*A. cooperi*, AdI=*A. densiflorus* Population I, AdII=*A. densiflorus* Population II.

Table 2. DNA values obtained by Feulgen *in situ* cytophotometry of species and populations of *Asparagus*.

Species name	Ploidy level (2n)	Total chromosome length (μm)	Total chromosome volume (μm ³)	4C DNA content (pg)*
<i>A. officinalis</i>	20	61.76	49.45	31.60±1.02
<i>A. plumosus</i>	20	33.18	20.43	23.88±0.67
<i>A. pyramidalis</i>	20	34.00	18.10	20.66±0.71
<i>A. racemosus</i> Population I	20	40.07	18.00	24.82±0.54
<i>A. racemosus</i> Population II	20	37.70	19.09	20.82±1.11
<i>A. cooperi</i>	40	65.00	62.00	30.04±1.13
<i>A. densiflorus</i> Population I	60	123.38	96.85	34.02±0.81
<i>A. densiflorus</i> Population II	60	137.02	102.00	23.24±0.78

**412, **5.02

*Data represents Mean±S.E. from 20 replicates. **F value ($p \leq 0.05$).

Table 3. Qualitative total protein assay and number of polymorphic bands for each isozyme marker in different species and populations of *Asparagus*.

Name of the species	Number of polymorphic bands	Range of protein-size in molecular weight (kilodalton or kD)	Bands obtained from isozyme analysis	
			Esterase	Peroxidase
<i>A. officinalis</i>	15	5.39–142.26	3	3
<i>A. pyramidalis</i>	17	2.48–134.13	1	1
<i>A. plumosus</i>	23	4.37–142.26	3	2
<i>A. racemosus</i> (I)	20	1.24–150.69	4	4
<i>A. racemosus</i> (II)	13	1.39–168.24	3	2
<i>A. cooperi</i>	20	2.19–168.24	2	2
<i>A. densiflorus</i> (I)	14	2.19–088.38	3	1
<i>A. densiflorus</i> (II)	16	2.48–092.63	2	1

Fig 3.1 **Fig 3.2**

Fig. 3. (1) Protein profile in different species and populations of *Asparagus*. Lane 1: *A. officinalis*, Lane 2: *A. plumosus*, Lane 3: *A. pyramidalis*, Lane 4: *A. racemosus* (Population I), Lane 5: *A. racemosus* (Population II), lane 6: *A. cooperi*, Lane 7: *A. densiflorus* (Population I), Lane 8: *A. densiflorus* (Population II). Protein molecular weight marker gave the bands of 205 kD, 97.4 kD, 66 kD, 43 kD, 20.1 kD, 14 kD, 6.5 kD and 3 kD, respectively. (2) Dendrogram obtained from total protein profile data showing relationship between different species and populations of *Asparagus*.

characters is yet to be ascertained. However, the scatter diagram revealed a close association between the diploid species, one diploid and one tetraploid species, and two hexaploid populations when these two parameters were considered.

Additionally, a high degree of correlation coefficient ($r=0.94$) was found between the total chromosome volume and 4C nuclear DNA contents (Fig. 2(1b)). The variation in total chromosome volume in these species might have been under genetic control. Moreover, in a eukaryotic system, the chromosome volume is determined not only by DNA but also by both basic and non-basic proteins. A detailed cytochemical assay utilizing specific enzymes and interferometry for protein estimation may provide positive clues to this direction.

Biochemical and molecular marker-based studies

A total of 214 polypeptide bands were observed in all the species studied (Table 3). The total protein profile revealed differences in number of polymorphic bands ranging from 13 in *A. racemosus* II to 23 in *A. plumosus*. The qualitative as well as the polymorphisms observed among these species suggested differences in genomic constitution at the functional level as the tissues were collected in the same period during their maximum growth in the field. The dendrogram (Fig. 3) obtained from the SDS-PAGE study showed two main clusters. One of the clusters was composed of two sub-clusters. *Asparagus racemosus* (two populations) and *A. pyramidalis* are under the same branch of a common sub-cluster. *Asparagus plumosus* and *A. cooperi* are in another branch of the same common sub-cluster. However, *A. densiflorus* is on a different sub-cluster of the common cluster. *Asparagus officinalis* is in a single distant cluster. The percent disagreement values of dis-

Table 4. Dissimilarity matrix of total protein profile among different species and populations of *Asparagus*.

	<i>A. officinalis</i>	<i>A. plumosus</i>	<i>A. cooperi</i>	<i>A. pyramidalis</i>	<i>A. densiflorus</i> I	<i>A. densiflorus</i> II	<i>A. racemosus</i> I	<i>A. racemosus</i> II
<i>A. officinalis</i>	00							
<i>A. plumosus</i>	24	00						
<i>A. cooperi</i>	24	26	00					
<i>A. pyramidalis</i>	26	26	20	00				
<i>A. densiflorus</i> I	30	32	26	30	00			
<i>A. densiflorus</i> II	37	39	33	37	35	00		
<i>A. racemosus</i> I	25	27	21	25	27	30	00	
<i>A. racemosus</i> II	29	31	25	29	15	34	26	00

Table 5. Dissimilarity matrix of isozyme analysis among different species and populations of *Asparagus*.

	<i>A. officinalis</i>	<i>A. plumosus</i>	<i>A. cooperi</i>	<i>A. pyramidalis</i>	<i>A. densiflorus</i> I	<i>A. densiflorus</i> II	<i>A. racemosus</i> I	<i>A. racemosus</i> II
<i>A. officinalis</i>	00.00							
<i>A. plumosus</i>	67.00	0.00						
<i>A. cooperi</i>	42.00	25.00	0.00					
<i>A. pyramidalis</i>	58.00	42.00	33.00	0.00				
<i>A. densiflorus</i> I	42.00	42.00	67.00	50.00	0.00			
<i>A. densiflorus</i> II	50.00	33.00	58.00	42.00	8.00	0.00		
<i>A. racemosus</i> I	58.00	75.00	67.00	50.00	50.00	42.00	0.00	
<i>A. racemosus</i> II	33.00	67.00	42.00	25.00	42.00	33.00	25.00	0.00

Fig. 4. Dendrogram obtained from isozyme data showing relationship between different species of *Asparagus*.

similarity ranged from 15 to 39. The dissimilarity value is 15 between the two populations of *A. densiflorus*, and 20 between the two populations of *A. racemosus* (Table 4). The present study also indicated that the SDS-PAGE of leaf proteins could easily be used to reveal the phylogenetic relationships among different plant species and populations (Ray *et al.* 2010, Mirali *et al.* 2007).

Zymograms obtained from two enzyme systems showed good resolution, and a total of 37 highly reproducible bands were obtained (16 from peroxidase and 21 from esterase) (Table 3). In both the cases, the highest number of polymorphic bands (four in each enzyme systems) was found in *A. racemosus* (Population I). Genetic dissimilarities among all the species ranged from 0.08PDV (Percent Disagreement Values) to 67.0PDV (Table 5). The dendrogram (Fig. 4) obtained from the isozyme study showed two main clusters. *Asparagus racemosus*, *A. densiflorus*, and *A. pyramidalis* are under the same branch of a common cluster. *Asparagus officinalis* is on another distinct branch of the same common cluster. *Asparagus plumosus* and *A. cooperi* are in same branch of a different common cluster. Variation at the population level, however, is very low.

RAPD analysis was performed with 24 random primers out of which 10 primers produced informative and unambiguous data for phylogenetic analysis. These 10 primers produced a total of

Table 6. RAPD and ISSR fragments generated in all the species of *Asparagus* surveyed.

Primer		Sequence (5'-3')	Range of fragment size (bp)	Number of observed fragments
RAPD	ISSR			
B 01		TCGAAGTCCT	310–976	10
B 02		AGATGCAGCC	413–2523	40
B 08		TCACCACGGT	403–3560	46
B 10		CAGGCACTAG	076–700	26
OPA 07		GAAACGGGTG	414–8110	21
OPA 09		GGGTAACGCC	489–10462	56
OPA 15		TTCCGAACCC	435–1303	25
OPB 04		GGA CTGGAGT	612–1100	15
OPP 09		GTGGTCCGCA	415–1365	15
OPP 11		AACGCGTCGG	360–6600	27
OPA 01		CAGGCCCTTC		
OPA 02		TGCCGAGCTG		
OPA 04		AATCGGGCTG		
OPA 05		AGGGGTCTTG		
OPA 06		GGTCCCTGAC		
OPA 08		GTGACGTAGG		
OPA 16		AGCCAGCGAA		
OPA 19		CAAACGTCCG		
OPA 20		GTTGCGATCC		
A 31		AGTCAGTCAC		
OPD 01		GTCAGGGCAA		
OPD 02		GGTCGGAGAA		
OPD 04		GGTGAGGTCA		
P 28		TGGGGGACTC		
	1	CACACACACACACAAT	165–960	31
	2	GCTCACACACACACAC	291–990	53
	3	CACACACACACACACC	242–740	32
	4	GAGTCTCTCTCTCTCTC	233–615	22
	CR-2	(CA) ₈ AG		
	CR-8	(GTG) ₅ GC		
	UBC	(CA) ₆ GG		
	816	TCGAAGTCCT		

281 RAPD fragments (Table 6) with an average of 28.1 bands per primer, and 76 to 10,462 bp amplified fragments. The primer OPA 09 produced the most reproducible banding pattern, and B 01 produced the lowest reproducible banding pattern. Figure 5a, b shows the RAPD profile obtained with the primer B 10. Genetic dissimilarities among all the species ranged from 0.05 PDV to 0.40 PDV (Table 7). The UPGMA dendrogram showed four main groups (Fig. 6). The first sub-cluster consisted of the two populations of *A. racemosus* as well as *A. pyramidalis* and *A. plumosus*. The second sub-cluster consisted of the two populations of *A. densiflorus*. *Asparagus cooperi* made a different sub-cluster, and the fourth sub-cluster was made by *A. officinalis*.

Out of the seven ISSR primers, four produced reproducible data without any ambiguity for phylogenetic analysis. These four primers produced a total of 138 ISSR fragments with an average of 34.5 fragments per primer. The size of the amplified product ranged from 165 to 990 bp (Table 6). The highest number of bands was observed in primer 2, and the lowest in primer 4. Genetic dissimilarities among all the species ranged from 0.09 PDV to 0.40 PDV (Table 8). Figure 7 shows the ISSR profile obtained with primer 1. The UPGMA dendrogram revealed four main groups (Fig. 8). The first sub-cluster contained the two populations of *A. racemosus* as well as the two populations of *A. densiflorus*. The second sub-cluster contained *A. pyramidalis* and *A. plumosus*. *Asparagus cooperi* formed a different sub-cluster, and the fourth sub-cluster contained only *A. officinalis*.

Fig. 5. (a) RAPD profiles in different species of *Asparagus*. Lane 1: *A. officinalis*, Lane 2: *A. pyramidalis*, Lane 3: *A. plumosus*, Lane 4: *A. cooperi*, Lane 5: *A. racemosus*, Lane 6: *A. densiflorus*. Lane M: DNA ladder with 3000bp, 2000bp, 1000bp, 900bp, 800bp, 700bp, 600bp, 500bp, 400bp, 300bp marker bands. Primer: B-10. (b) RAPD profiles in different populations (Pop) of *Asparagus*. Lane 1: *A. racemosus* Pop I, Lane 2: *A. racemosus* Pop II, Lane 3: *A. densiflorus* Pop I, Lane 4: *A. densiflorus* Pop II. Lane M—DNA ladder with 3000bp, 2000bp, 1000bp, 900bp, 800bp, 700bp, 600bp, 500bp, 400bp, 300bp marker bands. Primer: B 10.

Fig. 6. Dendrogram obtained from RAPD data showing relationship between different species of *Asparagus*.

Table 7. Dissimilarity matrix of RAPD analysis among different species and populations of *Asparagus*.

	<i>A. officinalis</i>	<i>A. pyramidalis</i>	<i>A. plumosus</i>	<i>A. cooperi</i>	<i>A. densiflorus</i> I	<i>A. densiflorus</i> II	<i>A. racemosus</i> I	<i>A. racemosus</i> II
<i>A. officinalis</i>	0.00							
<i>A. pyramidalis</i>	0.37	0.00						
<i>A. plumosus</i>	0.39	0.28	0.00					
<i>A. cooperi</i>	0.37	0.34	0.36	0.00				
<i>A. densiflorus</i> I	0.39	0.32	0.35	0.34	0.00			
<i>A. densiflorus</i> II	0.40	0.32	0.36	0.34	0.06	0.00		
<i>A. racemosus</i> I	0.36	0.30	0.32	0.34	0.31	0.31	0.00	
<i>A. racemosus</i> II	0.34	0.29	0.29	0.33	0.30	0.31	0.05	0.00

Table 8. Dissimilarity matrix of ISSR analysis among different species and populations of *Asparagus*.

	<i>A. officinalis</i>	<i>A. pyramidalis</i>	<i>A. plumosus</i>	<i>A. cooperi</i>	<i>A. racemosus</i> I	<i>A. racemosus</i> II	<i>A. densiflorus</i> I	<i>A. densiflorus</i> II
<i>A. officinalis</i>	0.00							
<i>A. pyramidalis</i>	0.40	0.00						
<i>A. plumosus</i>	0.39	0.28	0.00					
<i>A. cooperi</i>	0.39	0.39	0.36	0.00				
<i>A. racemosus</i> I	0.34	0.30	0.28	0.29	0.00			
<i>A. racemosus</i> II	0.33	0.29	0.27	0.28	0.09	0.00		
<i>A. densiflorus</i> I	0.40	0.34	0.34	0.35	0.20	0.19	0.00	
<i>A. densiflorus</i> II	0.37	0.35	0.33	0.34	0.21	0.18	0.13	0.00

Fig. 7. ISSR profiles in different species and populations of *Asparagus*. Lane 1: *A. densiflorus* Pop I, Lane 2: *A. racemosus* Pop I, Lane 3: *A. cooperi*, Lane 4: *A. pyramidalis*, Lane 5: *A. densiflorus* Pop II, Lane 6: *A. racemosus* Pop II, Lane 7: *A. plumosus*, Lane 8: *A. officinalis*. Lane M: DNA ladder with 3000 bp, 2000 bp, 1000 bp, 900 bp, 800 bp, 700 bp, 600 bp, 500 bp, 400 bp, 300 bp marker bands. Primer: ISSR 1.

Fig. 8. Dendrogram obtained from ISSR data showing relationship between different species and populations of *Asparagus*.

On the one hand, RAPD and ISSR analyses have been done in both wild and cultivated species. On the other hand, isozyme and SDS-PAGE studies have been carried out on different plant species such as *Vicia* or plants of the Liliaceae family. The low level of polymorphism among each population suggested that domesticated *Asparagus* germplasm was limited (Khandka *et al.* 1996). The dendrogram generated by RAPD, ISSR, and SDS-PAGE studies is largely in agreement with previous taxonomic studies of *Asparagus*. The difference between *A. officinalis* and other species of *Asparagus* generally corroborate the taxonomic classification established by Clifford and Conran (1987) on the basis of morphological traits. However, relationships among the different species differ to some extent with the isozyme marker type. The isozyme-based data placed *A. officinalis* in a common cluster with *A. racemosus*, *A. densiflorus*, and *A. pyramidalis*. The correlations between isozyme and RAPD-based distance matrices differed greatly from each other, as did those between isozyme and SDS-PAGE based distance matrices. The correlations between ISSR and RAPD dissimilarity matrices were higher in magnitude ($r=0.86$), and there was little difference between the RAPD and ISSR profile-based grouping of the six species. These differences can be attributed to marker sampling error or the level of polymorphism detected, reinforcing the importance of the number of loci and their coverage of the overall genome to obtain reliable estimates of genetic relationships among taxa (Loarce *et al.* 1996). The efficiency of DNA markers in *Asparagus* species is determined by the possibility to apply each of them individually or in combination for species identification and differentiation. The level of polymorphisms was much higher in RAPD- or ISSR-based marker types. However, the level of polymorphism was lower in isozyme-based data. Again, the obtained clustering pattern could be changed if more isozyme markers were taken into consideration. The range of values of the dissimilarity index is almost similar in both RAPD (0.05–0.40) and ISSR (0.09–0.40) studies. These findings again reinforce the need for using a combination of mark-

ers rather than a specified one. The presence of similar RAPD fragments may not always be homologous, although they may be of the same size in base pairs.

Conclusion

The importance of molecular phylogenetic studies to understand changes in plant genome organization and chromosome evolution has been emphasized. Two species of *A. densiflorus* revealed a primitive nature as evident from the higher number of median chromosomes. *Asparagus densiflorus* originated from southern Africa and may be considered as the primitive species under the genus *Asparagus*. Clear evolutionary relationships cannot be drawn from these six species analysis. However, a compiled picture can be drawn using a combination of cytological and molecular marker-based analyses, which may assist the breeding pattern or germplasm conservation of the gene pool of specified crops. Markers such as DNA-based RAPD and ISSR markers gave very consistent results and can be reliably used for phylogenetic analysis. The high genetic variability detected using RAPD and ISSR, and the correlation between cytogenetic and molecular markers give additional support to the idea that a major evolutionary pathway operated toward genome organization and karyotype differentiation, leading to the separation of different species of *Asparagus*.

Acknowledgements

Financial assistance from the UGC in the form of University Fellowship [Ref no UGC/339/Fellow (UNIV)] to the second author is gratefully acknowledged.

References

- Aitkin, S. A., Tinker, N. A., Mather, D. E., and Fortin, M. G. 1994. A method for detecting DNA polymorphisms in large populations. *Genome* **37**: 507–508.
- Bennett, M. D., and Leitch, I. J. 1997. Nuclear DNA amount in angiosperms 583 new estimates. *Ann. Bot.* **80**: 169–196.
- Bennett, M. D., and Smith, J. B. 1976. Nuclear DNA amount in angiosperms. *Phil. Transact. Roy. Soc. London B* **274**: 227–274.
- Caporali, E., Carboni, A., Spada, A., and Marziani, G. 1996. Construction of Linkage map in *Asparagus officinalis* through RFLP and RAPD analysis. *Proc. VIII Int. Symp. on Asparagus*. *Acta Hort.* **415**: 435–440.
- Clifford, H. T., and Conran, J. G. 1987. 2 *Asparagus* 3 *Protoasparagus* 4 *Myrsiphyllum* in Flora of Australia. In: George, A. S. (ed.). Australian Government Publishing Service, Canberra.
- Doyle, J. J., and Doyle, J. L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19**: 11–15.
- Everett, N. P., and Wach, M. J. 1985. Biochemical markers of embryogenesis in tissue cultures of maize inbred line B73. *Plant Sci.* **41**: 133–140.
- Goldman, J. J. 2008. The use of ISSR markers to identify Texas bluegrass interspecific hybrids. *Plant Breed.* **127**: 644–646.
- Jackson, R. C. 1976. Evolution and systematic significance of polyploidy. *Annu. Rev. Ecol. Syst.* **7**: 209–234.
- Jena, S. N., Vasundharan, S. K., Sahoo, M. K., and Das, A. 2007. Assessment of genetic diversity in nine Indian species of *Asparagus*: in sights from karyotype, *in situ* DNA content and RAPD markers. *Bot. Steci.* **11**: 95–106.
- Jha, S., and Sen, S. 1983. Chromosome study of polyploid Indian squill, *Urginea indica* Kunth. *Cytologia* **48**: 407–418.
- Jiang, C., Lewis, M. E., and Sink, K. C. 1997. Combined RFLP and RAPD molecular linkage map of *Asparagus*. *Genome* **40**: 69–76.
- Kar, D., and Sen, S. 1985. Chromosome Characteristics of *Asparagus*- Sapogenin Yielding Plant. *Cytologia* **50**: 147–155.
- Khandka, D. K., Nejidat, A., and Golan-Goldhirsh, A. 1996. Polymorphism and DNA markers for asparagus cultivars identified by random amplified polymorphic DNA. *Euphytica* **87**: 39–44.
- Khasa, P. D., and Dancik, B. P. 1996. Rapid identification of white-Engelman spruce species by RAPD markers. *Theor. Appl. Genet.* **92**: 46–52.
- Lahiri, K., Mukhopadhyay, M. J., and Mukhopadhyay, S. 2011. Karyotype analysis and *in situ* 4C nuclear DNA quantification in two varieties of *Mucuna pruriens* L. *J. Trop. Med. Plant* **11**: 219–225.
- Loarce, Y., Gallego, R., and Ferrer, E. 1996. A comparative analysis of genetic relationships between rye cultivars using RFLP

- and RAPD markers. *Euphytica* **88**: 107–115.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
- Manjunath, B. L. (ed.) 1948. *Wealth of India: Raw Materials*, Vol. 1. CSIR, New Delhi.
- Mirali, N., El-Khouri, S., and Rizq, F. 2007. Genetic diversity and relationships in some *Vicia* species as determined by SDS-PAGE of seed proteins. *Biol. Plant.* **51**: 660–666.
- Mukhopadhyay, S., and Sharma, A. K. 1987. Karyomorphological analysis of different species and varieties of *Calathea*, *Maranta* and *Stromanthe* of Marantaceae. *Cytologia* **52**: 821–831.
- Nag, C. 2006. *In vitro* studies on *Asparagus* with special emphasis on chromosomal and biochemical aspects. Ph.D. Thesis, University of Calcutta, Kolkata.
- Ray, S., Mukhopadhyay, M. J., and Mukhopadhyay, S. 2010. Phylogenetic relationship among six different species of *Asparagus* utilizing RAPD, isozyme and ISSR markers. *Biores. Bull.* **3**: 153–160.
- Romero Zarco, C. 1986. A new method for estimating karyotype asymmetry. *Taxon* **35**: 526–530.
- Sharma, A. K., and Mukhopadhyay, S. 1984. Feulgen micro-spectrophotometric estimation of nuclear DNA of species and varieties of three different genera of Marantaceae. *Proc. Ind. Acad. Sci.* **93**: 337–347.
- Sharma, A. K., and Sharma, A. 1980. *Chromosome Technique: Theory and Practice*, 3rd ed. Butterworths and Co. Pvt. Ltd., London.
- Sheidai, M., and Jalilian, N. 2008. Karyotypic studies in some species and populations of the genus *Lotus* L. in Iran. *Acta Bot. Croat.* **67**: 45–52.
- Souframanien, J., and Gopalakrishna, T. 2004. A comparative analysis of genetic diversity of blackgram genotypes using RAPD and ISSR markers. *Theor. Appl. Genet.* **109**: 1687–1693.
- Van't Hof, J. 1965. Relationships between mitotic cycle duration, S period duration and the average rate of DNA synthesis in the root meristem cells of several plants. *Exp. Cell Res.* **39**: 48–58.
- Weeden, N. F. 1983. Evolution of plant isozymes. In: Tanksley, S. D. and Orton, T. J. (eds.). *Isozymes in Plant Genetics and Breeding*. Elsevier Science Publishers B. V., Amsterdam. pp. 177–208.
- Wendel, L. F. 1989. Visualization and interpretation of plant isozymes. In: Soltis, E. D. and Soltis, P. S. (eds.). *Isozymes in Plant Biology*. Discorides Press, Portland. pp. 5–45.
- Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rofalski, J. A., and Tinjey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6531–6535.
- Zietkiewicz, E., Rafalski, A., and Labuda, D. 1994. Genome fingerprinting by simple sequence repeat anchored polymerase chain reaction amplification. *Genomics* **20**: 176–183.
-