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Phylogenetic relationship among six economically important species of *Asparagus* utilizing RAPD, ISSR and isozyme polymorphism

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Abstract

Background: In the present study, an attempt has been taken to evaluate phylogenetic relationship among six different species of *Asparagus* by utilizing 20 RAPD, 6 ISSR and 2 isozyme markers. The species of *Asparagus* of *Asparagaceae* (formerly Liliaceae) are important as ornamental, vegetable and medicinal plants.
Result and conclusion: Generated RAPD and ISSR fragments showed high variance among species and population level. The UPGMA cluster analysis and Squared Euclidean matrix prepared from RAPD, ISSR and isozyme data revealed a distinct relationship between these different species of *Asparagus.*

Keywords: *Asparagus*, Distance matrix, Internal simple sequence repeat (ISSR), Phylogeny, Random amplified polymorphic DNA (RAPD), Squared euclidean distance (SQD) values, Unweighted pair wise methods with arithmetic averages (UPGMA).

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INTRODUCTION

The genus *Asparagus* belonging to the family *Asparagaceae* has about 150 species distributed throughout the world (Wealth of India, 1974). The intrageneric classification divided *Asparagus* into three subgenera: *Asparagus, Proto asparagus and Myrsiphyllum* (Clifford and Conran, 1987). The species of *Asparagus* has high heterozygosity (Wealth of India, 1974). *Asparagus officinalis* is an important vegetable crop, used worldwide whereas *A. plumosus, A. pyramidalis*, *A. cooperi* and *A. densiflorus* have both horticultural and ornamental values. The only species, *A. racemosus* has medicinal importance (Sabnis et al, 1968). The species of *Asparagus* contain important active principles including saponins, sapogenins of pharmaceutical importance. Genetic diversity evaluation among different species and population is necessary for crop improvement and conservation of gene pool. The analysis of molecular phylogeny may be utilized for the species of wide ecological adaptation. Molecular 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). These have quantified the extent of genetic diversity accurately within and between different species. The major disadvantages of these markers lie in the limited number of informative markers and its proneness to environmental and developmental variation (Rani et al, 1995). DNA markers provide a powerful tool for genetic evaluation and marker assisted breeding of crops and specially, for cultivar identification. Among the different types of molecular markers the widely used are RAPD, RFLP and AFLP markers.

Among them, RAPD markers are most widely used because of their simplicity, speed and low cost. Caporali et al (1996) and Jiang et al (1997) have published independent molecular marker based linkage maps on *A. officinalis* based on RFLP and RAPD. Among a wide range of DNA markers methods available, single sequence repeats (SSR) or microsatellites tend to be the most variable for discriminating among genotypes (Pejic et al, 1998, Russel 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). A variant of microsatellites called ISSR (Inter Simple Sequence Repeat) involves the amplification of DNA region located between two microsatellites loci (Zietkiewicz et al, 1994).

The objective of our present investigation is, to create a phylogenetic relationship on six economically important species of *Asparagus* as well as to compare the resolving power and compatibility of three molecular markers including isozyme, RAPD and ISSR markers.

MATERIALS AND METHODS

Materials

Six different species of *Asparagus* including two populations each of *A. densiflorus* Kunth and *A. racemosus* Wild was obtained from different ecological regions of India. *A. officinalis* L, population I of *A. densiflorus* Kunth cv. *sprengeri* and population II of *A. racemosus* Wild were obtained from Salem, Tamil Nadu. Population I of *A. racemosus* Wild, *A. plumosus* Baker and *A. cooperi* Baker were collected from local nursery in Kolkata whereas *A. pyramidalis* and population II of *A. densiflorus* were obtained from Kalimpong, West Bengal. The plant materials (shoots) from all the species and populations were collected at the same day during the months of July and August, to avoid any error.

Methods

Isozyme analysis

1gm frozen tissue sample from each species was ground to fine powder in a pre-chilled mortar and pestle with 1 ml protein extraction buffer (PEB). The homogenates were centrifuged at 15000 rpm for 15 min at 4ºC and supernatants were stored in -20ºC for further analysis. The protein samples were quantified by Lowry's method (Lowry et al, 1951) and about 30 µg of protein ran in each lane of a native discontinuous polyacrylamide gel.

For esterase analysis the gel was incubated in 0.1 M sodium phosphate buffer (pH 6.0) for 30 min in dark following staining in a 50 ml solution of 25 mg α-naphthyl acetate, 25 mg ß-naphthyl acetate and 50 mg Fast Garnet GBC salt. For peroxidase, the gel was incubated in 0.1 M sodium phosphate buffer (pH 6.0) for 30 min in dark following staining in a 50 ml solution of 50 mg $CaCl_2$, 0.25 ml of 3% H_2O_2, 25 mg 3-amino-9-ethylcarbazole and 2 ml of N-N-Dimethylformamide. In both the cases, the incubated gel was photographed under illuminator.

RAPD and ISSR analysis

Genomic DNA was extracted from frozen

leaf tissue following Doyle's and Doyle's method (Doyle and Doyle, 1987). RAPD and ISSR amplification reactions were performed following modified protocol of Williams et al. (1990) and Goldman (2008) respectively. For each primer, the experiment was carried out thrice to avoid ambiguity, if any.

The amplification reaction products were size separated by 1.2% agarose gel electrophoresis in 0.5 X TBE and finally gel was visualized under UV light after ethidium bromide staining.

Statistical analysis

In both isozyme and DNA marker based RAPD-ISSR studies, the presence or absence of the band was taken into consideration and the difference in the intensity of the band was ignored. For all the species, bands on gels were scored as '1' when present and '0' when absent. From this data, a squared euclidean distance matrix was formed and the matrix was subjected to UPGMA (Unweighted Pair wise Methods with Arithmetic averages) cluster analysis using joining tree clustering algorithms through *Statistica* version 6.0 software to generate a dendogram. The distance matrices were compared by sample correlation coefficient.

RESULTS AND DISCUSSION

Zymograms obtained from two enzyme systems showed good resolution. Staining and banding patterns were reproducible for each enzyme system among all the species. A total of 37 highly reproducible bands were obtained (16 from peroxidase and 21 from esterase) (**Table 1 & Figs 1a-b**). Genetic dissimilarities among all the species ranged from 1.41 SQD (Squared Euclidean distances) values to 3.00 SQD values. UPGMA dendogram, showed four different sub clusters (**Fig 2**). The first sub cluster consisted of two populations of *A. racemosus* as well as two populations of *A. densiflorus*. The second sub cluster included *A. pyramidalis*. *Asparagus plumosus* and *A. cooperi* made a different sub cluster and the fourth sub cluster was made by *A. officinalis*. The low level of polymorphism among each population suggested that domesticated *Asparagus* germplasm was limited (Khandka et al, 1996). The results of two isozyme systems in the present study indicated narrow genetic base of *Asparagus* (Geoffraio et al, 1992). The present study also suggests that the low level of polymorphisms among population level may be attributed to their vegetative nature of propagation.

RAPD analysis was performed with twenty random primers out of which ten primers produced reproducible and unambiguous data for phylogenetic analysis. These ten primers produced a total of 281 RAPD fragments (**Table 2**) with an average of 28.1 bands per primer. Among the ten primers, OPA- 09 produced most reproducible banding pattern and OPP- 09 produced the least

Fig. 1a: Zymogram on esterase profile in different species and populations of *Asparagus*.

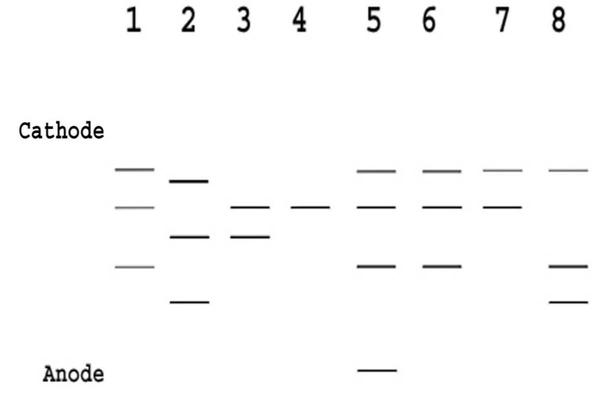

Fig 1a

Lane 1- *A. officinalis,* Lane 2- *A. plumosus*, Lane 3- *A. cooperi*, Lane 4- *A. pyramidalis,* lane 5- *A. racemosus* (Population I), Lane 6- *A. racemosus* (Population II), Lane 7- *A. densiflorus* (Population II), Lane 8- *A. densiflorus* (Population I).

Fig. 1b: Peroxidase profile in different species and populations of *Asparagus.*

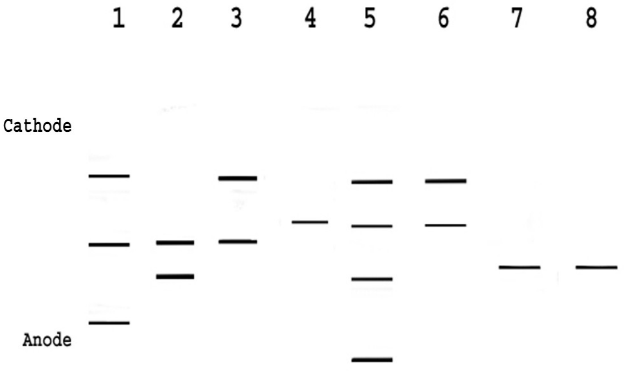

Fig 1b

Lane 1- *A. officinalis,* Lane 2- *A. plumosus*, Lane 3- *A. cooperi,* Lane 4- *A. pyramidalis,* lane 5- *A. racemosus* (Population I), Lane 6- *A. racemosus* (Population II), Lane 7- *A. densiflorus* (Population I), Lane 8- *A. densiflorus* Population II.

Fig. 2: Dendogram obtained from isozyme data showing relationship between different species of *Asparagus.*

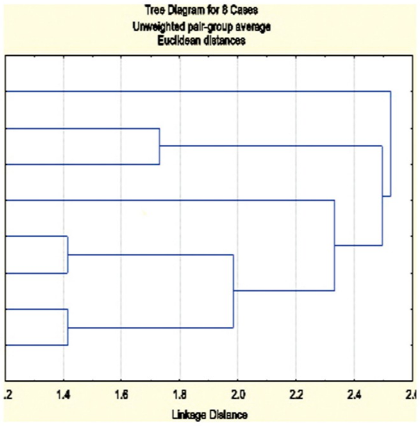

Fig 2

reproducible banding pattern. Amplified product sizes ranged from 76 bp to 10,462 bp (Table 2). **Figs 3a-b** showed the RAPD profile obtained with the primer OPA- 09. Genetic dissimilarities among all the species ranged from 12 to 93 SQD values. UPGMA dendogram, showed four main groups (**Fig 4**). The first sub cluster consisted of two populations of *A. racemosus* as well as *A. pyramidalis* and *A. plumosus*. The second sub cluster consisted of two populations of *A. densiflorus*. *Asparagus cooperi* and *A. officinalis* made different sub clusters and the fourth sub cluster was made by *A. officinalis.*

Out of the six ISSR primers, four produced reproducible data without any ambiguity for phylogenetic analysis. These four primers produced a total of 110 ISSR fragments with an average of 27.5 fragments per primer (**Fig 5**). The size of amplified product ranged from 165 bp to 990 bp (Table 2). Highest number of bands was produced by primer 2 and lowest by number 4 primer. Genetic dissimilarities among all the species ranged from 15 to 43 SQD values. **Fig 6** shows the ISSR profile obtained with the primer 1. From the UPGMA dendogram, it was found that the first sub cluster was made by two populations of *A. racemosus* and two populations of *A. densiflorus* (Fig 6). The second sub cluster contained *A. pyramidalis* and *A. plumosus*. *Asparagus cooperi* and *A. officinalis* produced two separate sub clusters (Fig 6).

The 26 (RAPD and ISSR) primers produced a total of 391 bands, which were polymorphic. The dendrogram generated by the RAPD and ISSR primers was largely in agreement with previous taxonomic studies on *Asparagus*. The difference between *A. officinalis* and other species of *Asparagus* generally corroborated the taxonomic classifications put forwarded by Clifford and Conran (1987) on the basis of morphological traits. In all the UPGMA based dendograms, we obtained the species under the same sub genus clustered together as in they are in Clifford and Conran's classifications. However relationship among the different species differed to some extent with the marker type. The correlation between RAPD and

Fig. 3a: RAPD profiles in different species of *Asparagus.*
Fig. 3b: RAPD profiles in different populations (Pop) of *Asparagus.*

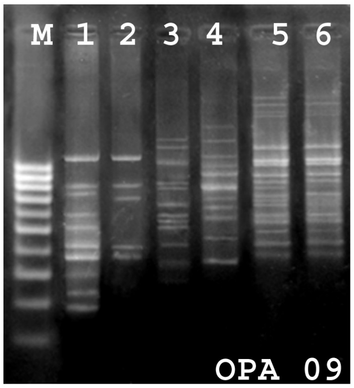

Fig 3a

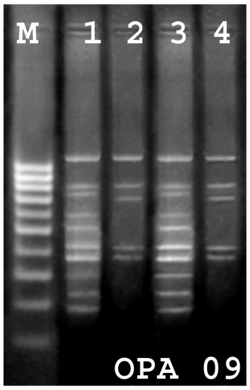

Fig 3b

Fig. 3a: Lane 1 - *A. densiflorus,* Lane 2 - *A. racemosus,* Lane 3 - *A. pyramidalis,* Lane 4 - *A. cooperi,* Lane 5 - *A. officinalis,* Lane 6 - *A. plumosus.* 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: OPA-09.
Fig. 3b: Lane 1 - *A. densiflorus* PopI, Lane 2 - *A. racemosus* PopI Lane 3 - *A. densiflorus* PopII, Lane 4 - *A. racemosus* PopII. 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: OPA-09.

Fig. 4: Dendogram obtained from RAPD data showing relationship between different species of *Asparagus*.

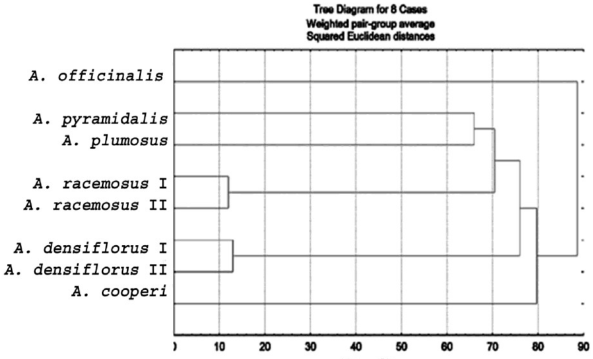

Fig 4

ISSR dissimilarity matrices generally was moderate in magnitude (r = 0.76) and there were some differences between RAPD and ISSR based grouping of the 6 species.

The target difference in the genome of these two markers based techniques might be responsible (Sikder et al, 2010). RAPD marker targets mutation locations in coding as well as non-coding regions of the entire genome. On the other hand, ISSR analysis is based on the variable number of simple sequence repeats present in different species. Again, clustering of different species within different sub clusters was not exactly similar when RAPD and ISSR derived dendograms were compared. These differences might 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 presence of similar RAPD fragments may not be always homologous, although these may be of same sizes in base pairs. It is obvious that during evaluating genetic relationships this may lead to erroneous results (Fernandez et al, 2002). Again, marker based differences in the genetic relationship between species of *Asparagus*, emphasized the need of using a combination of different marker systems.

Fig. 5: ISSR profiles in different species and populations of *Asparagus*.

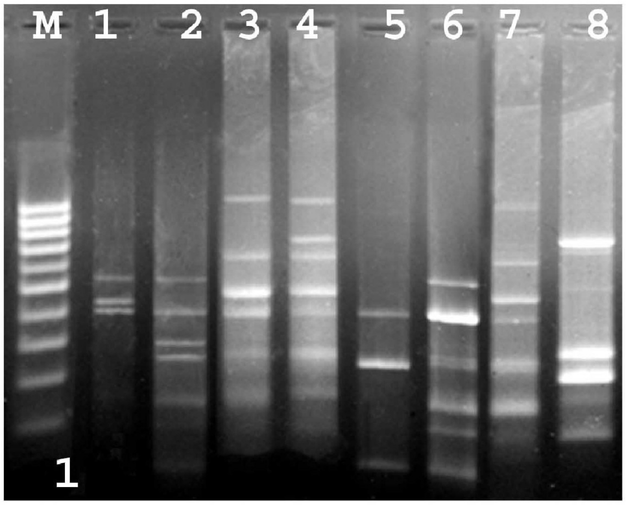

Fig 5

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.

In the present investigation, variation has been observed using RAPD and ISSR marker systems rather than isozyme marker. The clustering of UPGMA based on RAPD and ISSR distance were consistent with the morphogenetic classification of *Asparagus* in all aspects. In addition, these clustering supported the relationship of *A. pyramidalis* and *A. plumosus* according to Clifford and Conran, 1987.

The UPGMA clustering based on ISSR markers and isozyme markers placed *A. racemosus* and *A. densiflorus* in same sub cluster, in agreement with the classification structure of Fukunda and his colleagues (Fukunda et al, 2005). But isozyme based dendogram placed *A. pyramidalis* and *A. plumosus* in different sub cluster, whereas *A. pyramidalis* and *A. plumosus* were placed in same sub cluster in ISSR and RAPD based dendograms. Discordance in genetic distance measured with two molecular markers has been reported (Lebot et al, 2003). It might be due to the fact that isozyme represents the allelic expression of the same locus and shows a higher proportion of non-neutral markers; while the fragments produced by the RAPD primer are independent genetic marker (Ochiai et al, 2001).

CONCLUSION

RAPD and ISSR markers are extremely useful for genetic diversity evaluation in different species of *Asparagus*. However, a different relationship might be obtained when more isozyme markers and more numbers of species are taken for analysis. The present study has, however, indicated

Fig. 6: Dendogram obtained from ISSR data showing relationship between different species and populations of *Asparagus*.

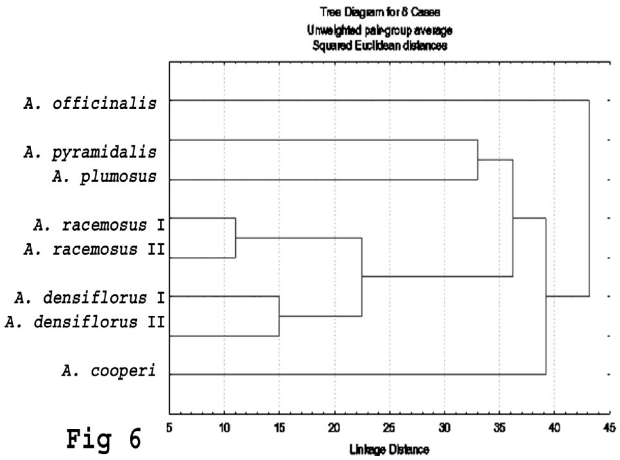

Fig 6

Table 1: Number of polymorphic bands for each isozyme markers in different species & populations of *Asparagus*

Species	**Esterase**					**Peroxidase**				
	No. of observed bands	**R_m values**				**No. of observed bands**	**R_m values**			
A. officinalis L	3	0.0001	0.1894	0.3570		3	0.006	0.197	0.447	
A. plumosus Baker	3	0.0840	0.3370	0.5890		2	0.197	0.260		
A. cooperi Baker	2	0.1894	0.3370			2	0.006	0.197		
A. pyramidalis	1	0.1894				1	0.151			
Population I of *A. densiflorus* Kunth cv *sprengeri*	3	0.0001	0.3570	0.5890		1	0.260			
Population II *A. densiflorus* Kunth cv *sprengeri*	2	0.0001	0.1894			1	0.260			
Population I *A. racemosus* Wild	4	0.0001	0.1894	0.3570	0.6670	4	0.006	0.151	0.260	0.520
Population II *A. racemosus* Wild	3	0.0001	0.1894	0.3570		2	0.006	0.151		

that both RAPD and ISSR were more convincing in the assessment of genetic relationship between species and genotypes of *Asparagus*.

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Table 2: RAPD and ISSR fragments generated in species of *Asparagus*

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		GGACTGGAGT	612-1100	15
OPP 09		GTGGTCCGCA	415-1365	15
OPP 11		AACGCGTCGG	360-6600	27
OPA 01		CAGGCCCTTC		0
OPA 02		TGCCGAGCTG		0
OPA 04		AATCGGGCTG		0
OPA 05		AGGGGTCTTG		0
OPA 06		GGTCCCTGAC		0
OPA 08		GTGACGTAGG		0
OPA 16		AGCCAGCGAA		0
OPA 19		CAAACGTCGG		0
OPA 20		GTTGCGATCC		0
A 31		AGTCAGTCAC		0
	1	CACACACACACACACACAAT	165-960	31
	2	GCTCACACACACACACACAC	291-990	53
	3	CACACACACACACACACC	242-740	32
	4	GAGTCTCTCTCTCTCTCTC	233-615	22
	CR-2	(CA)$_8$AG		0
	CR-8	(GTG)$_5$GC		0

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