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Full Length Research Paper

Genetic variability in *Asparagus racemosus* (Willd.) from Madhya Pradesh, India by random amplified polymorphic DNA

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Random amplified polymorphic DNA (RAPD) markers were used to assess genetic diversity in *Asparagus racemosus* (Willd.) an important medicinal plant collected from 7 different locations covering Madhya Pradesh. High level of genetic similarity was observed in the collected accessions. 4 random primers generated a total 39 polymorphic bands out of the total 71 bands. A low genetic diversity among the *A. racemosus* samples was revealed by RAPD analysis. Cluster analysis based on dice coefficient showed 2 major groups. This distributive pattern of genetic variation of *A. racemosus* accessions provides important baseline data for conservation and collection strategies for this species.

Key words: Genetic diversity, *Asparagus racemosus*, RAPD, polymorphism.

INTRODUCTION

Medicinal plants play a vital role to preserve our health. The genus, *Asparagus* consists of herbs, shrubs and vines that are widespread all over the world and represents highly valuable plant species having therapeutic and nutraceutical importance in addition to being consumed as food (Shasnay et al., 2003). *Asparagus racemosus* (locally known as Shatavar) is one of the important medicinal plants extensively used by the traditional practioners in India for its medicinal value. The leaves and the tuberous roots of *Asparagus* are medically important in several diseases. *A. racemosus* is distributed throughout tropical and sub-tropical parts of India up to an altitude of 1500 m. (Velvan et al., 2007). The healing qualities of Shatavar are useful in a wide array of ailments. Being a rasayana or rejuvenating herb, its restorative action is beneficial in women's complaints. *A. racemosus* is mainly known for its phytoestrogenic properties. With an increasing realization that hormone replacement therapy with synthetic oestrogens is neither as safe nor as effective as previously envisaged, the interest in plant-derived oestrogens has increased tremendously making *A. racemosus* particularly important (Bopana and Saxena, 2007). Roots of *A. racemosus* were found to possess antioxidant and anti-ADH activity (Kamat et al., 2000; Wiboonpun et al., 2004), anti-tumour and anticancer activity (Senna et al., 1993; Shao et al., 1996; Diwanay et al., 2004), anti-ulcerogenic activity (Datta et al., 2002), anti-inflammatory activity (Mandal et al., 1998) and antimicrobial activity (Mandal et al., 2000).

Genetic variation is essential for long term survival of species and it is a critical feature in conservation. For efficient conservation and management, the genetic composition of the species in different geographic locations needs to be assessed. In recent years, fingerprinting systems based on RAPD analysis have been increasingly utilized for detecting genetic polymorphism in several plant genera. Due to technical simplicity and speed, RAPD methodology has been used for diversity analysis in many medicinal plant species (Li et al., 2002; Padamalata and Prasad, 2006a, b, 2007). PCR based RAPD markers have been widely used in assessing genetic variation with in a species by measuring genetic diversity in many species, including medicinal plants. The most common uses of the technique for scientific and com-mercial purposes include the discrimination among species for post-harvest purposes (Hosokawa and Minami, 2000), identification of crop cultivars to settle lawsuits (Congiu et al., 2000), marker assisted selection

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Table 1. Different variants of *A. racemosus* collected from Jabalpur, Raisen and different locations of Bhopal.

S/N	Location	Sample code
1	Vanita Nursery, Bhopal (a)	Ar-2
2	Park-1, Bhopal (b)	Ar-3
3	Park-3, Bhopal (c)	Ar-4
4	Garden Nursery, Bhopal (d)	Ar-5
5	Indranikunj Nursery, Bhopal (e)	Ar-9
6	Obedullahganj, Raisen	Ar-12
7	Jabalpur	Ar-13

establishment of phylogenetic position of natural populations (Sgorbati et al., 2004; Tsuji and Ohnishi, 2001; Viccini et al., 2004), certification of lack of polymorphism between *in vitro* and conventionally propagated plant stands (Hao et al., 2004; Laia et al., 2000; Salvi et al., 2002) and determination of genetic variation in wild and cultivated populations (Birmeta et al., 2004; Gustafson et al., 1999; Sonnante et al., 2002).

The objective of the present study is to assess genetic diversity of *A. racemosus* to provide genetic data and a theoretical basis for protection of the species. Hence, an attempt was made to investigate variation among the collected accessions of *A. racemosus* by using RAPD markers. RAPD markers are based on the amplification of unknown DNA sequences using single, short and random oligonucleotide primers. Therefore, RAPD polymorphism is the reflection of variation of the whole genomic DNA and would be a better parameter to measure the pattern of genetic diversity of the rare and endangered plants.

MATERIALS AND METHODS

Study species and population sampling

A total of 7 accessions of *A. racemosus* were collected from Madhya Pradesh, India. The germplasm in the form of plants is being maintained at the field experimental site of M P Council of Science and Technology. The sampling localities were Jabalpur, Raisen and Bhopal (Table 1). Accessions are separated geographically by an average minimum distance of 30 km to increase possibility of detecting the variation potential of the collected accessions.

Genomic DNA isolation

Young leaf tissue (300 mg) was used for extracting genomic DNA by following the protocol of Doyle and Doyle (1987) with some modifications. Surface sterilized leaf tissue was crushed in pre-chilled mortar and pestle and immediately transferred to a centrifuge tube containing 5 ml modified CTAB extraction buffer (100 mM Tris HCl (pH 8.0), 25 mM EDTA, 1.5 M NaCl, 2.5% CTAB, 0.2% 2 mercapto-ethanol (v/v), and 1% PVP (w/v), added immediately before use) and mixed well to form a slurry and incubated at 70°C for 30 min. The mixture was cooled at room temperature and an equal volume of the mixture of phenol:chloroform: Isoamyl alcohol (25:24:1) was added prior to centrifugation at 5500 rpm for 20 min. To the aqueous phase equal volumes of ice-cold Isopropanol and NaCl was added and incubated at -20°C for 30 min followed by centrifugation at 5500 rpm for 20 min at 4°C. The supernatant was decanted carefully and pellet was dried at room temperature followed by dissolving in 1 ml Tris- EDTA (TE) buffer and 5 ml RNase solution and incubated at 37°C. An equal volume of the mixture of phenol:chloroform:isoamyl alcohol (25:24:1) was added and centrifuged at 5500 rpm for 20 min at 4°C. To the supernatant 600 µl of chilled absolute ethanol was added and incubated at -20°C for 30 min followed by centrifugation at 10000 rpm for 10 min at 4°C. The pellet was washed with 70% ethanol twice and dried at RT. Finally, the DNA pellet was dissolved in 50 ml deionized (RNase, DNase free) water. The yield of DNA was measured using a UV-Spectrophotometer (ND-1000) at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the samples on 0.8% agarose gel, based on the intensities of band when compared with the DNA marker (used to determine the concentration).

RAPD PCR amplification

DNA amplification was performed in a Corbett Research gradient automatic thermal cycler using RAPD markers. Genomic DNA (50 ng) was amplified via the PCR reaction using 50 µl reaction volumes under the following conditions, 2x red dye PCR mix (25 µl), primer (2 µl) (Bangalore Genei), template DNA (1 µl) and sterile nano pure water (22 µl). After initial denaturation of the DNA at 94°C for 5 min, the thermal cycling was performed with the following temperature regimes- 08 cycles of 45 s at 94°C, 1 min at 55°C and 1.5 min at 72°C; 30 cycles of 45 s at 94°C, 1 min at 58°C and 1 min at 72°C and final extension at 72°C at 10 min and a hold temperature at 4°C. Control reactions without template DNA (negative control) and without enzyme (positive control) were also run in the experiments. All the experiments were repeated thrice to ensure reproducibility. Amplified DNA fragments were separated by electrophoresis at 60 V in 1 X TAE buffer for 3 - 4 h on 1.2% agarose gels stained with ethidium bromide and photographed by gel documentation system (Alpha innotech). Raw gel images were recorded through Alphaview software. All PCR experiments were done at least twice and the best gels of the replicates were used for band scoring.

RAPD data analysis and scoring

The PCR protocol, as adopted in the study, resulted in reproducible pattern of amplicons using specific combination of accession and primer. Only the primers which displayed reproducible, scorable and clear bands were considered for analysis. The image profiles of

Table 2. Primer accession numbers (Banglore Genei, Banglore).

S/N	Primer	Accession number
1	RPI-1	AM765819
2	RPI-2	AM750044
3	RPI-3	AM773310
4	RPI-4	AM773769
5	RPI-5	AM773770
6	RPI-6	AM773771

banding patterns were recorded and molecular weight of each band was determined by Alphaview software. The banding pattern was scored based on the presence or absence of clear, visible and reproducible bands. The results were analyzed based on the principle that a band is considered to be 'polymorphic' if it is present in some individuals and absent in others, and 'monomorphic' if present in all the individuals or accessions. Similarity index of bands which were common between 2 accessions was estimated following Nei and Li (1979). Using dice coefficients, a similarity matrix involving 7 accessions was generated with NTSYS-pc (Numerical taxonomy system, applied biostatistis, Inc., New York, USA, software version 2.02e) and PAST (Paleontological Statistics) Software (Hammer et al., 2001). A dendrogram was constructed using the unweighted pair group method with arithmetical averages (UPGMA). Analysis of UPGMA was carried out following Nei and Li index (Figure 2).

RESULTS AND DISCUSSION

The RAPD technique was used to find out the extent of genetic diversity in *A. racemosus*. 6 primers (RPI 1 - 6) were used for amplification. Out of that, 4 showed clear bands. All accessions were identified by presence of bands. Maximum number of bands were observed with primer RPI-3 followed by RPI-5, RPI-4 and RPI-6 (Table 2). A sum total of 71 bands were amplified with respect to all the 4 primers. About 39 bands (54.92%) were polymorphic (Table 3). Maximum polymorphic bands were produced in case of RPI-3 (10). The number of monomorphic bands and unique bands was 25 and 7, respectively. The maximum number of bands were identified from sample Ar- 9 collected from Indiranikunj nursery, Bhopal followed by the samples Ar- 4 (38) and Ar-2 (34). Least bands were identified from Ar- 5 (32) & Ar-3 (31). The bands obtained ranged in size from 230 - 2652 bp.

In essence, the RAPD method used in this study displayed intra-population variation or molecular polymorphism, which pre-existed in different collections. In spite of their morphological identity, a moderate polymorphism was observed among the accessions under study. The dendrogram based on similarity index (SI) showed distinct separation of the collected accessions, though morphologically they were similar and inseparable. The dendrogram separated the accessions collected from 7 different locations into 2 major groups based on the genetic diversity (GD). The first group includes the accessions collected from Vanita nursery and Park No. 1, Bhopal. The second group is again classified into 3 sub groups, where the first sub group includes the accessions collected from Park No. 3, Bhopal. The second sub group includes the accessions collected from Garden Nursery, Bhopal and Obedullaganj, Raisen, which are genetically similar. The third subgroup includes accessions from Indiranikunj nursery, Bhopal and Jabalpur, which are genetically similar (Figure 2). Ample scope for identification of unique RAPD markers with decreasing genetic distance in this species is discernible. However, some of the unique profiles may be shared by other accessions in a more inclusive group of accessions. The range of genetic distance is 0.412 to 0.690 from which it is evident that GD showed no correlation with geographical distances between the accessions, negating a simple isolation by distance mode (Table 4)

The genetic differentiation of accessions of *A. racemosus* could broadly be explained as a result of abiotic (geographical, e.g., hydrographic connections, or climatic differentiation, e.g., annual rainfall differences) and biotic (pollination between populations and seed dispersal) factors. The % of polymorphism that is, 54.92% shows that the species genetic diversity by itself is low, which might be due to restricted distribution in a particular area, non effective gene flow, low fecundity, low pollen flow, local selection procedure (environment and struggle for existence) and inbreeding systems (Loveless and Hamrick, 1984). Apart from the above mentioned, biotic factors like human interference, habitat destruction, commercial exploitation, etc., also attribute to high genetic similarity. It is, therefore, a good strategy to protect more of their habitats.

It can be inferred that from the accessions grouped into similar groups there is an effective gene flow in those locations. Whereas between the accessions collected from park no. 3 (Ar-4) when compared with other accessions the gene flow is less. It can be predicted that every finite population may not experience genetic drift, but the effect will become more pronounced as population size increases and in due course there might be low genetic variation. The mode of natural propagation is also by root tubers apart from seeds which may also aid in self pollination to some extent. For a species with limited gene flow and over 50% variation among accessions, it is necessary to collect samples from at least 6 accessions in order to conserve 95% of the genetic diversity of the species. For a species with only 20% variation among accessions, the samples taken from 2 accessions are enough to get the same results above (Pei et al., 1995; Yun et al., 1998). The results of RAPD showed that we need to take individuals from more different populations so as to preserve their diversity for the future. The study also confirms the suitability of RAPD as a reliable, simple, easy to handle and elegant tool in molecular diagnosis of different accessions of an important medicinal plant. Concurrently, it is also proved that the entries which were found to be similar in taxonomical classification based on morphological characters

Table 3. Polymorphism pattern demonstrated by RAPD analysis in A. racemosus.

Total no. of bands	Total no. of polymorphic bands	Total no. of monomorphic bands	Total no. of unique bands	Uniqueness	Polymorphism (%)
71	39	25	7	9.85 %	54.92 %

Table 4. Similarity Index (SI) of seven variants of *A. racemosus* through RAPD.

	Vanita Nursery	Park No. 1	Park No. 3	Garden Nursery	Obedull-ahganj	Indira-nikunj Nursery	Jabalpur
Vanita Nursery, Bhopal	1.000						
Park No.1, Bhopal	0.617	1.000					
Park No.3, Bhopal	0.437	0.577	1.000				
Garden Nursery, Bhopal	0.490	0.610	0.607	1.000			
Obedullahganj, Raisen	0.655	0.635	0.440	0.625	1.000		
Indiranikunj Nursery, Bhopal	0.575	0.550	0.425	0.522	0.680	1.000	
Jabalpur	0.640	0.580	0.492	0.412	0.622	0.690	1.000

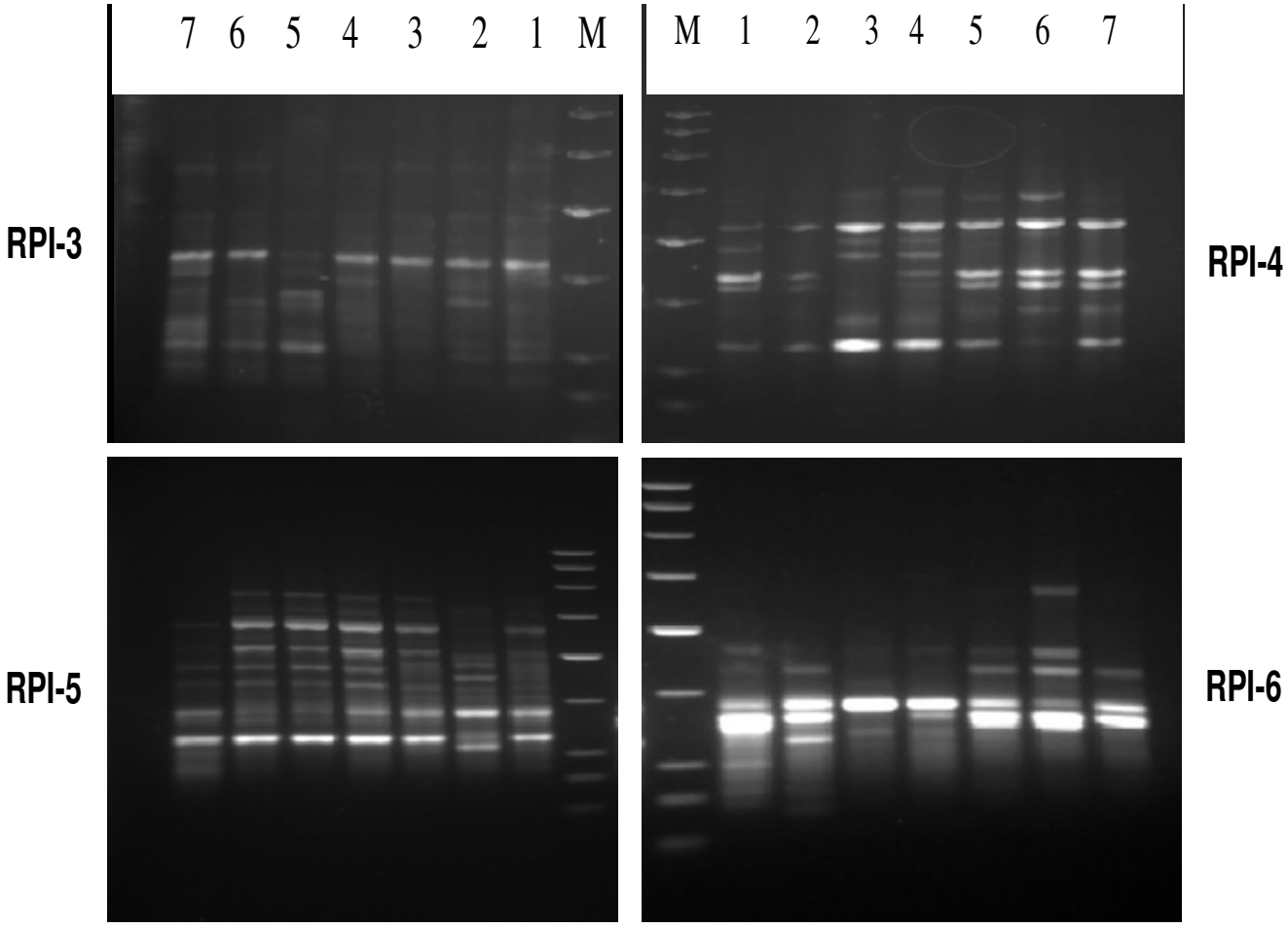

Figure 1. Random amplified polymorphic DNA fragment patterns generated using primers RPI-3, RPI-4, RPI-5 and RPI-6: Lane 1. Vanita Nursery, lane 2. Park 1, lane 3. Park 3, lane 4. Garden, lane 5. Obedullahganj, lane 6. Indranikunj, lane 7. Jabalpur and M is molecular marker (Low range DNA marker).

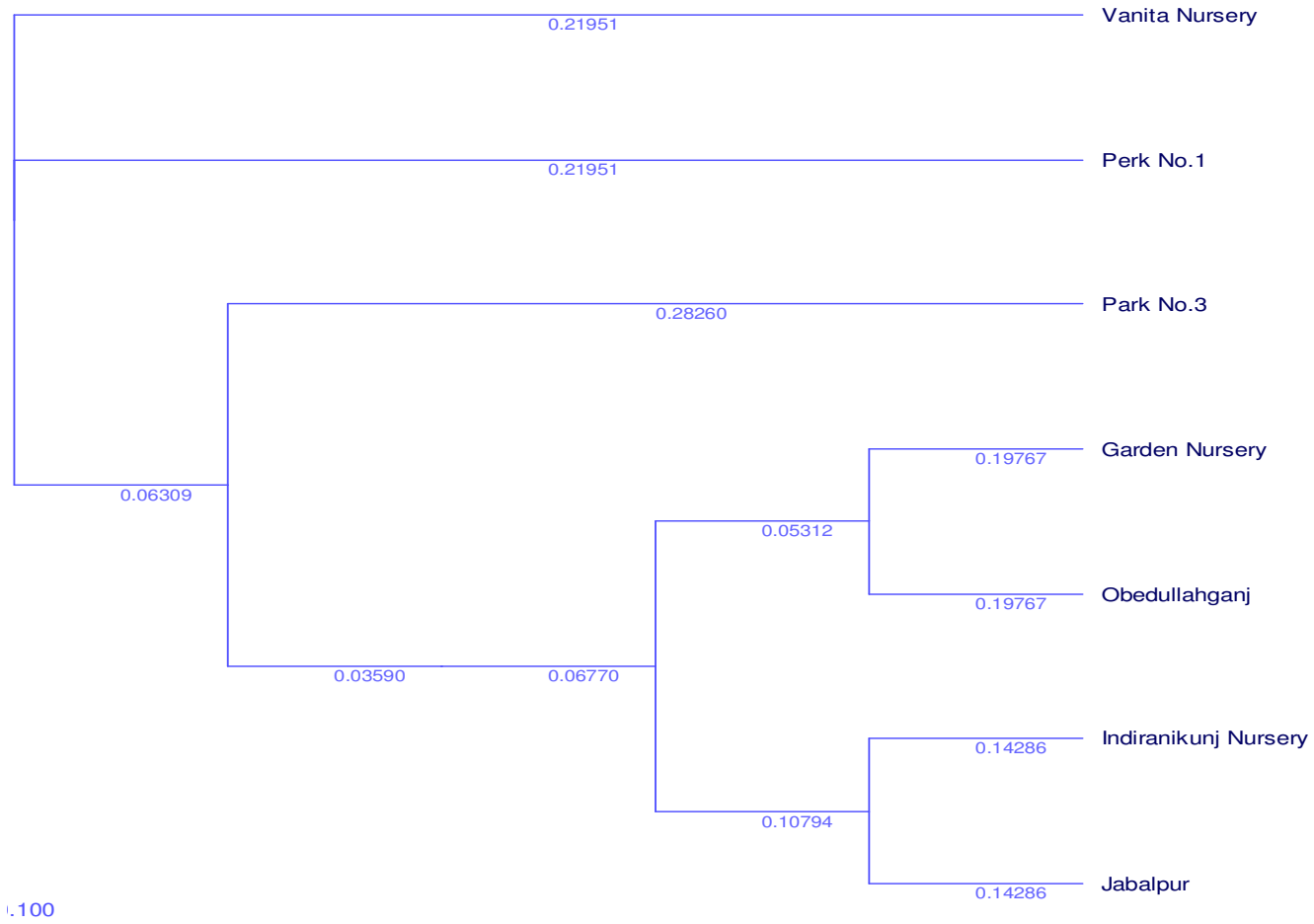

Figure 2. UPGMA cluster analysis of RAPD data for 7 different accessions of *A. racemosus*.

do have divergence at DNA level (Figure 1).

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