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Molecular diversity analysis of *Asparagus racemosus* and its adulterants using random amplified polymorphic DNA (RAPD)

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Leaves of 28 genotypes of 4 species of the genus *Asparagus*, namely: *Asparagus racemosus*, *Asparagus falcatus*, *Asparagus officinalis* and *Asparagus plumosus* were analyzed for genetic diversity by random amplified polymorphic DNA (RAPD). Out of one hundred 10-mer RAPD primers screened, 25 were selected for comparative analysis of different accessions of *A. racemosus* and its closely related species. High genetic variations were found between the different *Asparagus* species studied. Among a total of 296 RAPD fragments amplified, 287 bands (96.95%) were found polymorphic in the four species. Average polymorphic information content (PIC) value for the amplification product was 0.23. Fourteen species-specific bands were found in *A. racemosus* species. These species-specific RAPD markers could potentially be used for identifying authentic *A. racemosus* spp. and could be sequenced for conversion to a sequence-characterized amplified region (SCAR) marker so as to serve as species specific marker. Jaccard's genetic similarity co-efficient varied from 0.19 to 0.85. The unweighted pair group method with arithmetic average (UPGMA) dendrogram was constructed on the basis of Jaccard's genetic similarity co-efficient values that separate the three other species of *Asparagus* from *A. racemosus* accessions. Results of this study will facilitate authentic *A. racemosus* identification, thereby aiding drug standardization, its collection, management and conservation.

Key words: Medicinal herb, genetic diversity, molecular marker, authentication, conservation.

INTRODUCTION

Herbal medicine is the mainstay of about 75 to 80% of the world population, mainly in the developing countries for primary health care, because of better cultural acceptability, better compatibility with the human body and lesser side effects (Kamboj, 2000). *Asparagus racemosus* Willd. (Asparagaceae; Liliaceae), has been known in India for its therapeutic properties since thousands of years. It is commonly called 'satavari', 'satawar' or 'satmuli' in hindi and 'shatavari' in sanskrit.

The plant grows throughout the tropical and subtropical parts of India up to an altitude of 1500 m. Tuberous roots of this herb find use in about 64 'ayurvedic' formulations that are prescribed, apart as galactogogue (Gupta and Shaw, 2011), for the treatment of a wide range of

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ailments including dysentery, tumors, inflammations, nervous disorders, bronchitis, hyperacidity, neuropathy (Goyal et al., 2003), conjunctivitis (Sharma and Singh, 2002), spasm, chronic fever, rheumatism (Capasso et al., 2003), gonorrhoea, leucorrhoea, menorrhagia and threatened abortion (Sharma and Bhatnagar, 2011).

Further, the roots of this herb have several activities, such as antioxidation (Kamat et al., 2000; Jain et al., 2008), antimicrobial (Mandal et al., 2000), antidepressant (Singh et al., 2009), antiaging (Velavan and Hazeena Begum, 2007), hepatoprotection (Kumar et al., 2011), prevention of stone formation (Christina et al., 2005), immunomodulation (Gautam et al., 2004; Kumari et al., 2012) and memory enhancement (Dhwaj and Singh, 2011).

The major active constituents of *A. racemosus* roots are steroidal saponins. Other primary chemical constituents being essential oils, asparagine, arginine, tyrosine, flavonoids (kaempferol, quercetin and rutin), resin and tannin. Its powdered root contains 2.95% protein, 5.44% saponins, 52.89% carbohydrate, 17.93% crude fiber, 4.18% inorganic matter and 5% oil (Negi et al., 2010). Since it is the active principle that imparts medicinal value to a plant, consistency in quality and quantity needs to be maintained, to ensure uniform drug efficacy by cultivation of superior genotypes. It has been well documented that geographical conditions affect the active constituents of the medicinal plant and hence their activity profiles (Oleszek, 2002). Many researchers have studied geographical variation at the genetic level.

DNA fingerprinting techniques have been widely used in genetic diversity analysis and, to differentiate species or populations in plant conservation management (Morell et al., 1995; Rodriguez et al., 1999). In order to establish a marker for identification of a particular species, genotypes etc., DNA analysis of closely related species and/or genotypes for common botanical contaminants and adulterants is necessary. Welsh and McClelland (1990) and Williams et al. (1990) first described the random amplified polymorphic DNA (RAPD) fingerprinting method, which is a polymerase chain reaction (PCR) based method. RAPD analysis is simple, less expensive and rapid. It has the ability to detect extensive polymorphisms which require minute amounts of genomic DNA, even without prior knowledge of DNA sequences (Clark and Lanigan, 1993). Compared to other genetic markers, RAPD can provide vital information for the development of genetic sampling, conservation and improvement strategies (Chalmers, 1994). RAPD-based molecular markers have been found to be useful in differentiating different accessions of *Asparagus officinalis* (Khandka et al., 1996; Hollingsworth et al., 1998), *Azadirachta indica* (Farooqui et al., 1998), *Phyllanthus amarus* (Jain et al., 2003), *Panax quinquefolius* (Lim et al., 2007), *Tribulus terrestris* (Sarwat et al., 2008), *Trichodesma indicum* (Verma et al., 2009), *Cuscuta reflexa* (Khan et al., 2010), etc.

The ever-increasing demand for herbal drugs is causing loss of precious biodiversity in addition to creating a shortage of raw material. Since, there have been few studies on genetic diversity of *A. racemosus*, a medicinal herb (Shasany et al., 2003; Vijay et al., 2009), for the efficient conservation and management of this important genetic resource, it is imperative to analyze the genetic composition of genotypes of different phytogeographical regions. Further, quality herbal drug preparation from *A. racemosus* roots is affected by the presence of adulterants such as, roots of *A. officinalis* and *A. falcatus*. Therefore, identification of these adulterants is also necessary for aiding, A. racemosus herbal drug standardization. Thus, in this study, RAPD markers were used for detection of genetic polymorphism in *A. racemosus* and three other species of *Asparagus,* collected from various geographical regions of India and pot cultured at Banaras Hindu University.

MATERIALS AND METHODS

Plant

A total of 28 accessions belonging to four species of *Asparagus* were used in the present study. Out of 25 accessions of *A. racemosus*, 24 accessions, namely, NDAS-5 to NDAS-28 were selected from the collection of Northern India, maintained at Narendra Dev University of Agriculture and Technology, Faizabad, Uttar Pradesh, India, while one accession, was collected from the Vindhyan region of Uttar Pradesh. The rest three accessions belonging to the species *Asparagus falcatus*, *Asparagus plumosus* and *A. officinalis,* were collected from the Pune region, Maharashtra. Accession of *Abbottina springeri* and *Asperagus densiflora myersii*, collected from the Pune region, Maharashtra and an accession collected from Trichur, Kerala, could not survive the harsh summer climate of Varanasi. Plants of each accession were pot cultured in a greenhouse at the Institute of Agricultural Sciences, BHU.

DNA extraction

Young leaves from individual plants of each genotype were collected and stored at -80°C until use. Leaf samples were grinded in liquid nitrogen to a fine powder, and genomic DNA was extracted using modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). The DNA was precipitated by chilled isopropanol, and DNA pellet obtained after centrifugation was washed with 70% alcohol, dried at room temperature and dissolved in TE buffer (pH 8.0). The integrity of the extracted DNA was confirmed to be consistent, both spectrophotometrically as well as by running the extracted DNA in 0.8% agarose gel; stained with ethidium bromide (1.5 µg/ml). Quantity of the isolated DNA was determined by spectrophotometer (Gene Space-III, Hitachi, Japan). The extinction ratio (260/280 nm), found between 1.4 and 1.8, indicated that the DNA was pure enough for RAPD analysis.

RAPD analysis

One hundred RAPD primers procured from Operon technologies (U.S.A.), were initially screened for their repeatable amplifications with 5 accessions. Out of these, 25 RAPD primers were selected for

further analysis, based on their ability to detect distinct polymorphic amplified products across the accessions. To ensure reproducibility, primers generating weak products were discarded. The PCR was performed in a DNA engine Dyad thermal cycler (Bio-Rad Laboratories, USA) following the protocol of Williams et al. (1990) with minor modifications. Amplifications were carried out in 25 µl of reaction mixture containing 15 ng genomic DNA, 1.5 mM PCR buffer (MBI Fermentas, USA), 400 µM dNTPs (MBI Fermentas), 1.5 U Taq DNA polymerase (MBI Fermentas) and 0.4 pM primer. Thermal cycler program for RAPD involved an initial denaturation at 94°C for 4 min, followed by 40 cycles each of 1 min denaturation at 94°C, 1 min primer annealing at 32°C, 1 min primer extension at 72°C and one final extension cycle of 10 min at 72°C. The amplified PCR products and 1 Kb DNA marker ladder (MBI Fermentas), were size fractioned through electrophoresis in 1.5% agarose gel prepared in 1 × TAE buffer [40.0 mMTris-base, 16.65 M acetic acid, 0.5 M ethylenediaminetetraacetic acid (EDTA) (PH 8.0)]. After electrophoresis, gels were stained with ethidium bromide (1.5 µg/ml) and visualized in a gel documentation system (Alfa Imager 2200, Alfa Innotech Corporation, California). Amplifications were repeated 2 times to ensure that the amplifications obtained with the primers were reproducible and consistent.

Diversity analysis

RAPD bands were scored manually for the presence (1) or absence (0) across all the accessions. Only the clearest and strongest bands were recorded and used for the analysis. Bands with negligible intensity were not considered. Polymorphic information content (PIC) was calculated according to Ghislain et al. (1999). Pair-wise similarity matrices were generated using Jaccard's coefficient (Jaccard, 1908), by using the SIMQUAL format of NTSYS-pc (Rohlf, 2002). A dendrogram was constructed by using the unweighted pair group method with arithmetic average (UPGMA), with the SAHN module of NTSYS-pc to show a phenetic representation of genetic relationships, as revealed by the similarity coefficient (Sneath and Sokal, 1973). The binary data was also subjected to principal component analysis (PCA), using the EIGEN and PROJ modules of NTSYS Pc.

RESULTS

RAPD analysis

296 randomly amplified DNA fragments were detected with 25 RAPD primers, from leaves of four *Asparagus* species, including, 24 accessions of *A. racemosus*. Out of these 296 RAPD fragments amplified, 287 (96.95%) were polymorphic (Table 1). All the 25 RAPD primers were observed to be polymorphic as well. The number of fragments amplified per primer, ranged from 7 (OPW-04, OPAA-07) to 21 (OPAC-05) with an average of 11.88, while fragment size ranged from ~200 to ~2400 bp (Table 1).

Percent polymorphism ranged from 80 to 100. Most of the primers showed 100% polymorphic bands, whereas bands amplified by the primer, OPAC-10, were least polymorphic (80%). The PIC ranged from 0.12 (OPAA-02) to 0.33 (OPW-7) with a mean of 0.24. Variations in the RAPD pattern of 28 accessions with primer OPY-05 are as shown in Figure 1.

Species-specific markers

Several RAPD fragments were amplified in *A. racemosus* accessions, they were observed to be absent in three other species. These can be used as species-specific markers. Fourteen RAPD fragments amplified by eleven primers were identified as species-specific markers for *A. racemosus*. The primer OPY-05 and OPW-04, amplified three and two unique bands in *A. racemosus* accessions, respectively. Details of species-specific bands detected in *A. racemosus* species are shown in Table 2.

Genetic diversity among *Asparagus* accessions

UPGMA based dendrogram was constructed on the basis of Jaccard's genetic similarity co-efficient values (Figure 2) whereby, the overall genetic relationship among the genotypes was surveyed. Jaccard's genetic similarity co-efficient ranged from 0.19 to 0.85 (Figure 2). The highest genetic similarity co-efficient was observed between NDAS-26 and NDAS-28 (0.85) *A. racemosus* genotypes, whereas, lowest genetic similarity co-efficient was observed between *A. officinalis* and *A. racemosus* (Vindhyan region) genotype (0.19), followed by *A. falcatus* and *A. racemosus* (Vindhyan region) (0.21). Within *A. racemosus,* lowest genetic similarity co-efficient was observed between the genotypes NDAS-24 and that from the Vindhyan region (0.56). Cluster analysis clearly separated the three different species of *Asparagus* from the *A. racemosus* accessions. *A. racemosus* accessions clustered together and formed one major group consisting of 25 genotypes namely, NDAS-5, NDAS-6, NDAS-7, NDAS-8, NDAS-9, NDAS-10,NDAS-11, NDAS-12, NDAS-13, NDAS-14, NDAS-15, NDAS-16, NDAS-17, NDAS-18, NDAS-19, NDAS-20, NDAS-21, NDAS-22, NDAS-23, NDAS-24, NDAS-25, NDAS-26, NDAS-27, NDAS-28 and the Vindhyan region genotype. Principal component analysis carried out using the RAPD data is shown in Figure 3. The eigen value obtained from the first three principal components, cumulatively accounted for 74.53% of the total variation in which, 67.52% was accounted for by component 1 and 4.14% by the component 2. The PCA method yielded an obvious separation among genotypes with distinct species.

DISCUSSION

Information on genetic diversity is vital for strategic collection, conservation, maintenance and the effective genetic improvement of any plant species. *A. racemosus,* a species of the genus *Asparagus,* is of immense multipurpose medicinal importance yet, it remains genetically uncharacterized. Most of the *A. racemosus* accessions belonging to different geographical regions of India, are found to be similar in morphological characters and seem to be non-discriminable on this basis. In

Table 1. Genetic variations among four species of *Asparagus* and within twenty four lines of Asparagus racemosus collected from different locations in India.

S/N	Primer code	Sequence	Total no. of bands	Amplified range of size (bp)	Polymorphic bands		PIC
					Number	%	
1	OPV6	AAGGCTCACC	8	250-1700	8	100	0.29
2	OPV7	AGAGCCGTCA	18	350-1600	17	94.44	0.23
3	OPV12	AAGCCTGCGA	8	350-1600	8	100	0.24
4	OPV16	GGGCCAATGT	19	300-1800	19	100	0.25
5	OPV19	TGAGGGTCCC	12	350-1100	12	100	0.17
6	OPV20	AGCCGTGAAA	8	400-2200	8	100	0.19
7	OPW4	CAGAAGCGGA	7	300-2200	7	100	0.27
8	OPW7	CTGGACGTCA	15	250-1600	15	100	0.33
9	OPW16	CAGCCTACCA	8	200-1500	7	87.5	0.29
10	OPW19	CAAAGCGCTC	9	200-1300	9	100	0.25
11	OPY5	GGCTGCGACA	14	350-1300	14	100	0.18
12	OPY7	AGAGCCGTCA	13	350-1000	13	100	0.23
13	OPY16	GGGCCAATGT	9	300-1800	8	88.89	0.22
14	OPY18	GTGGAGTCAG	10	250-1500	9	90	0.23
15	OPY19	TGAGGGTCCC	11	350-1100	10	90.9	0.2
16	OPY20	AGCCGTGGAA	14	250-1500	14	100	0.24
17	OPN02	ACCAGGGGCA	16	300-1700	15	93.75	0.2
18	OPN03	GGTACTCCCC	10	350-1550	9	90	0.25
19	OPAA02	GAGACCAGAC	13	400-1500	13	100	0.12
20	OPAA07	CTACGCTCAC	7	300-1750	7	100	0.27
21	OPAA11	ACCCGACCTG	11	400-2000	11	100	0.31
22	OPAA12	GGACCTCTTG	11	450-2000	11	100	0.23
23	OPAB03	TGGCGCACAC	14	500-2400	14	100	0.2
24	OPAC05	GTTAGTGCGG	21	400-1600	21	100	0.21
25	OPAC10	AGCAGCGAGG	10	400-1300	8	80	0.17
-	Total	-	296	-	287	96.95	-
-	Mean	-	11.88	-	11.48	-	0.24

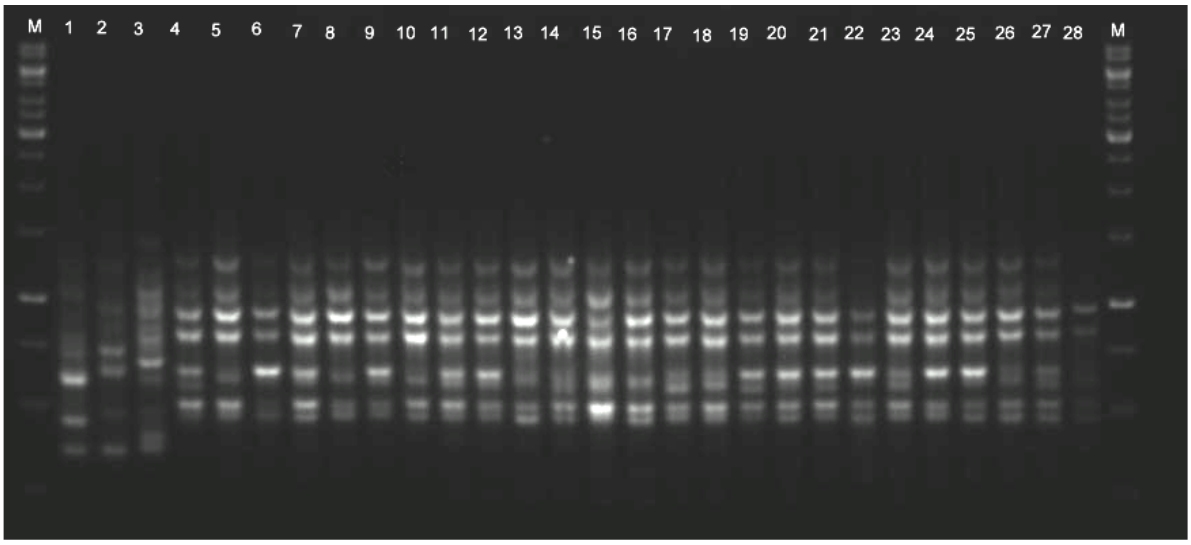

Figure 1. RAPD pattern of *Asparagus* species with primer OPY-05. M: molecular weight marker, 1 Kb DNA marker ladder (MBI Fermentas).

Table 2. *Asparagus racemosus* species-specific RAPD markers with size in bp.

S/N	Primer	Fragment number	Fragment size (bp)
1	OPY-05	3	~ 1000
2	OPY-05	4	~ 875
3	OPY-05	6	~ 500
4	OPY-19	11	~ 250
5	OPW-04	7	~ 450
6	OPW-04	8	~ 400
7	OPW-16	8	~ 400
8	OPV-06	12	~ 750
9	OPAC-10	10	~ 550
10	OPN-03	4	~ 800
11	OPAA-11	6	~ 900
12	OPAC-5	21	~ 450
13	OPAA-12	5	~ 1200
14	OPAB-3	14	~ 650

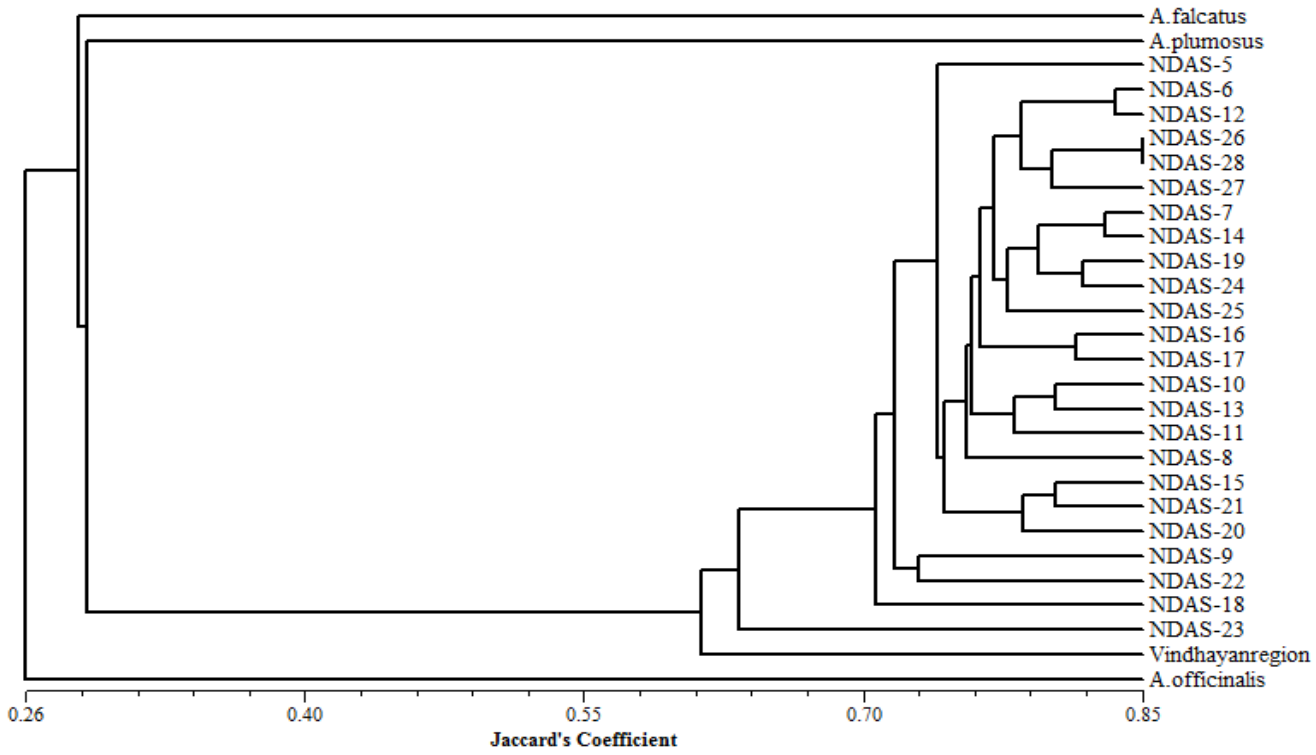

Figure 2. Dendrogram (UPGMA) pattern of RAPD analysis.

contrast, DNA markers are found in abundance and are not influenced by the environment or developmental stage of a plant, making them an ideal tool for genetic relationship studies (Reddy et al., 2002).

Thus, diversity analysis was conducted on 28 *Asparagus* accessions with varied saponin content using 25 RAPD markers. The Jaccard's genetic similarity co-efficient value ranged from 0.19 to 0.85, indicating the existence of a high level of diversity among the genotypes. The twenty five primers used, proved to be highly informative, as they detected a high level of polymorphic fragments (96.95%), thereby discriminating all 28 accessions. These results, in addition to confirming the ability of RAPDs in discriminating the genotypes also, suggest their application for cultivar identification. In this study, fourteen *A. racemosus* species specific diagnostic

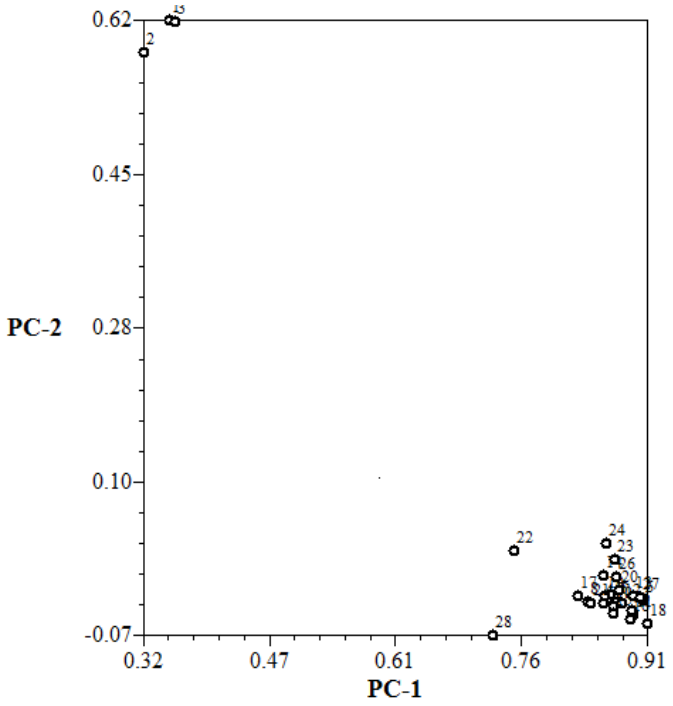

Figure 3. Principal component analysis.

markers, suitable for discriminating authentic *A. racemosus* material from its closely related co-species/adulterants, were identified. These species-specific RAPD markers could potentially be used for identifying *A. racemosus* spp., from any mixed population comprising other species of the genus *Asparagus*. Similar approach has been successfully used for molecular diagnosis of species by several workers (Ram et al., 2008; Sen et al., 2010). Moreover, some promising RAPD markers could be sequenced for conversion to SCAR markers, so as to serve as species specific markers. Several workers have demonstrated that the RAPD–PCR method is a powerful tool for discriminating a large number of medicinal species from their close relatives or adulterants, including *A. officinalis* (Khandka et al., 1996; Hollingsworth et al., 1998), *Panax* species (Shaw and But, 1995) and *Coptis* species (Cheng et al., 1997). PIC analysis can be used to evaluate markers so that, the most appropriate marker can be selected for genetic mapping and phylogenetic analysis (Anderson et al., 1993; Powell et al., 1996).

In the present study, PIC values for RAPD primers ranged from 0.17 to 0.33. As the PIC provides a measure that is influenced by the number and frequency of alleles, the maximum PIC value for a RAPD marker is 0.5, since two alleles per locus are assumed in the RAPD analysis (Henry, 1997).

Further, three *Asparagus* spp., namely *A. falcatus, A officinalis* and *A. plumosus* were separated from the rest accessions of *A. racemosus*. Though, genetic diversity within *A. racemosus* accessions was low, all accessions were separated from each other. Accessions NDAS-23 and that from the Vindhyan region were most diverse from the rest. Shasany et al. (2003) used 40 RAPD primers and observed significant, but low diversity among *A. racemosus* accessions (51.7%), and also within species (48%). Vijay et al. (2009) assessed genetic diversity within *A. racemosus* genotypes collected from 7 different locations, with 6 primers.

We herein report an efficient, precise and sensitive method for identifying genuine medicinal plant material that will contribute significantly in regulating the quality control of herbal drugs. Furthermore, we suggest that, RAPD method is convenient for a better understanding of distribution of genetic variation at the intraspecific level. This approach would help identify superior genotype(s) for cultivar upgradation as well as, evolving strategies for effective conservation.

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