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ASSESSMENT OF GENETIC DIVERSITY OF A*sparagus racemosus* Willd. FROM DIFFERENT AGRO-ECOLOGICAL ZONES OF KERALA USING RAPD MARKERS

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Twenty accessions of *Asparagus racemosus* from ten agro-ecological zones of Kerala were evaluated for genetic diversity using Random Amplified Polymorphic DNA markers. Twenty random primers were selected based on reproducibility and clarity of bands. A total of 1209 bands were scored out of which, 1181 loci were found to be polymorphic (97.75%). Efficiency parameters of primers *viz.*, Total Number of Loci, Total Number of Polymorphic Loci, Percentage of Polymorphism, Polymorphism Information Content (PIC), Resolving Power, Marker Index and Number of Unique Bands were estimated. Value of PIC varied from 0.059 to 0.209 and OPI19 is proved to be most polymorphic marker. Evaluation of relationship among seven efficiency parameters revealed positive and significant ($P<0.01$) correlation of Total Number of Loci with Total number of Polymorphic Loci, Resolving Power and Unique Bands. Jaccard's similarity coefficient varied from 0.118 to 0.566. It indicates presence of large genetic variations within the accessions. The UPGMA dendrogram data revealed genetic relationships within accessions and there is no geographic isolation in the clustering. The

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present observations suggest that RAPD markers can be used as an effective tool for deriving intra-species genetic diversity among *A. racemosus* accessions.

Keywords: Efficiency parameters, Genetic diversity, Polymorphic Information Content (PIC), RAPD, UPGMA, *Asparagus racemosus*.

INTRODUCTION

Medicinal plants may play a significant role in preserving human health. In the ancient World, herbal medicines were the sole source of health care. Indian system of medicine, *Ayurveda*, considered as the oldest system, is totally based on herbs assuming that they are safe to use, cheap and easily available, and have few side effect (KRISHANA *et al*., 2005). *Asparagus racemosus* Willd. (Shatavari), is one such important medicinal plant which is regarded as a *'rasayana'* that promote general well-being by increasing cellular vitality and resistance in the *Ayurvedic* system of medicine (GOYAL *et al.*, 2003) and belongs to the family Asparagaceae. The genus *Asparagus* includes about 300 species and 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).

There are 22 species of *Asparagus* recorded in India. *A. racemosus*, is a much branched, spinous under-shrub. The plant is found wild in tropical and subtropical regions of India including Andaman and Nicobar Islands. It is distributed from mean sea level up to 1500 m in the Himalayas from Kashmir Eastwards. It has an adventitious root system with tuberous roots that measure about one meter in length, tapering at both ends, with roughly a hundred on each plant and the root is taken up in gravelly, rocky soils. The leaves and the tuberous roots of *A. racemosus* are medically important in several diseases. The amazing herb is also known as the 'Queen of Herbs' in *Ayurvedic* system of medicine with widespread applications as diuretic, cooling agent and an excellent safe herbal medicine for antenatal care.

A. racemosus is mainly known for its phytoestrogenic properties (ASHAJYOTHI *et al*., 2009) due to the presence of isoflavones in the roots (SAXENA and CHOURASIA, 2001). Shathavari is used in combating menopausal symptoms and increasing lactation (MAYO *et al*., 1998; MITRA *et al.*, 1999). The increasing demand of *A. racemosus* for medicinal uses, unsustainable and destructive harvesting had led to the shrinkage of natural populations of *A. racemosus*. Poor cultivation coupled with over exploitation of tubers for pharmaceutical use widening the demand and thus putting pressure on the availability of this plant resource. It is now considered endangered in its natural habitat and has also been recognized as 'vulnerable' (WARRIER *et al*., 2001) in south Western Ghats, of India. The National Medicinal Plants Board, Government of India has included *A. racemosus* as one of the 32 highly prioritized medicinal plants of India (NATIONAL MEDICINAL PLANTS BOARD, 2002). Nowadays the interest in plant-derived estrogens, known as phytoestrogen has increased enormously making *A. racemosus* more important. It demands assessment of the plant genetic diversity, improvement, conservation and cultivation.

Genetic variation is essential for long term survival of species and is a vital feature for conservation. Assessment of genetic variation in a species provides information about the level of genetic divergence and is a prerequisite for initiating an efficient breeding program. It serves a platform for specific breeding program (THOMPSON *et al*., 1998) and it provides the basis for molding desirable genotypes. For efficient conservation and management, the genetic composition of the species in different geographic locations needs to be assessed.

Other than the morphological markers, the molecular markers provide additional tools for germplasm characterization and assessment of genetic relatedness and diversity in collections. They have been found to be more reliable than the phenotypic observations for evaluating the variations and in the assessment of the genetic stability (LEROY *et al*., 2000).

In the recent years, several molecular techniques have been used for germplasm characterization. Polymerase chain reaction (PCR) based techniques has now led to the development of simple, quick and efficient tools like RAPD (Random Amplified Polymorphic DNA) which in contrast to allozyme/protein markers is independent of the environmental factors and the developmental stages of the plant.

The RAPD technique has several advantages such as simplicity, rapidity, less expensive, reliability, no prior requirement of genetic information and availability of large number of primers. In RAPD technology random short synthetic oligonucleotide primers (10–12 base pairs) are used to amplify the genomic DNA through polymerase chain reaction under low annealing temperature. RAPDs have been widely used for authentication of plant species of medicinal importance (KHAN *et al*., 2009; GAHLAUT *et a*l., 2013; PATEL *et al*., 2015). The RAPD technique also has been used extensively for diversity analysis in many crop species like *Fagopyram tartaricum* (SHARMA and JANA, 2002), *Brassica carinata* (TEKLEWOLD and BECKER, 2006), *Eleusine coracana* (PANWAR *et al*., 2013), *Crocus sativus* (IZADPANAH *et al*., 2014), *Annona muricata* (BRISIBE *et al*., 2016) etc. It also finds its applications to measure the genetic diversity among accessions of different species and among accessions within species. This technical simplicity and the advantages associated with RAPDs have made them among favorite markers in the determination of the phylogenetic relationships.

There have been extensive studies carried out on genetic diversity in different *Asparagus* species like *A. racemosus*, *A. srpingeri*, *A. officinalis*, *A. plumosus* and *A. densiflors myersii* (LAL *et al*., 2011) especially *A. officinalis* using RAPD markers (RAIMONDI *et al*., 2001; LI *et al*., 2012; IRSHAD *et al*., 2014). However, there are scares of literature on genetic diversity studies in *A. racemosus* (VIJAY *et al*., 2009; SINGH *et al*., 2013b; KUMAR *et al*., 2016). Hence, the present study was attempted to analyze the genetic diversity among *A. racemosus* accessions using RAPD markers to provide helpful genetic data for measuring the pattern of genetic diversity.

The objectives of our study were to (i) determine the efficiency of applying RAPD as a diversity analysis tool in *A. racemosus*, (ii) cluster *A. racemosus* accessions collected from different agro-ecological zones on the basis of their genetic diversity, and (iii) analyze the relatedness of *A. racemosus* accessions.

MATERIALS AND METHODS

Plant materials

A comprehensive collection of *A. racemosus* germplasm was made through continuous field exploration from different localities of Kerala and 20 accessions representing ten agro-ecological zones of Kerala were selected (ENVIS, 2017) (Table 1 and Fig. 1). These accessions were maintained in the botanic garden of the Department of Botany, University of Kerala (8º33'03.86" N; 76º52'38.64" E; 18 m alt) under identical cultivation conditions. Fresh young cladodes were collected from three randomly chosen plants of each accession when the plants were approximately 18 months old and pooled to form a bulk sample of cladodes of each accession.

Table 1. Accession of A. racemosus and geographic details of their collection sites in Kerala state, India

Accession Code	Place of Collection	Revenue District	Agro-ecological zone	Zone No.	Latitude	Longitude	Altitude (m)	altitude type[a]	Rainfall Pattern[b]	Soil Type
Ar 1	Nellimoodu	Trivandrum	Red loam	1	8°22'51.81" N	77°02'31.39"E	55	I	I	Laterite without
Ar 2	Kattakada	Trivandrum	Malayoram	3	8°30'11.71"N	77°05'06.81"E	79	I	I	Laterite
Ar 3	Punalur	Kollam	Malayoram	3	9°01'12.34"N	76°55'51.14"E	47	I	I	Laterite without
Ar 4	Arattupuzha	Alappuzha	Costal sandy	6	9°12'53.38"N	76°25'52.65"E	08	I	I	Sandy loam
Ar 5	Kareelakulan	Alappuzha	Onattukara	4	9°11'36.38"N	76°29'06.82"E	09	I	I	Sandy
Ar 6	Chingoli	Alappuzha	Onattukara	4	9°15'01.24"N	76°27'07.09"E	10	I	I	Sandy
Ar 7	Kozhencherr y	Pathanamthitta	Southern midlands	2	9°19'58.97"N	76°42'20.88"E	17	I	I	Laterite without
Ar 8	Vandiperiyar	Idukki	Highranges	7	9°34'19.60"N	77°14'45.77"E	809	II	I$II	Red
Ar 9	Olanad	Ernakulam	Central midlands	8	10°05'30.55"N	76°16'31.54"E	13	I	I$II	Laterite
Ar 10	Vellanikkara	Thrissur	Central midlands	8	10°32'42.77"N	76°16'26.33"E	35	I	I$II	Laterite
Ar 11	Silent Valley	Palakkad	Highranges	7	11°04'06.31"N	76°31'09.80"E	657	II	I$II	Red
Ar 12	Mundur	Palakkad	Palakkad plains	10	10°57'27.44"N	76°30'19.67"E	84	I	II	Red loam
Ar 13	Mannarkkad	Palakkad	Malayoram	3	10°59'35.65"N	76°27'39.59"E	85	I	I	Laterite without
Ar 14	Wandoor	Malappuram	Malayoram	3	11°11'43.83"N	76°14'09.74"E	80	I	I	Laterite
Ar 15	Pandikkad	Malappuram	Malappuram	11	11°05'33.20"N	76°13'23.84"E	40	I	II	Laterite
Ar 16	Janakikkadu	Kozhikode	Northern	12	11°37'47.93"N	75°47'09.09"E	49	I	II	Laterite
Ar 17	Muthanga	Wayanad	Highranges	7	11°38'43.53"N	76°22'32.64"E	862	II	I$II	Red
Ar 18	Thaliparamba	Kannur	Northern	12	12°02'17.77"N	75°22'02.83"E	57	I	II	Laterite
Ar 19	Kanhangad	Kasargode	Malappuram	11	12°19'56.90"N	75°05'46.16"E	49	I	II	Laterite
Ar 20	Ranipuaram	Kasargode	Malappuram type	11	12°25'09.41"N	75°21'03.45"E	1019	II	II	Laterite

Altitude: Type I- Altitude Up to 500 m above Mean Sea Level, Type II- More than 500 m above Mean Sea Level.[b]Rainfall: Pattern I- Both the southwest and northeast monsoons are active and moderately distributed. Southwest monsoon with June maximum, Pattern II- Poorly distributed rainfall; southwest monsoon with July maximum and concentrated in 3-4 months. Northeast during October is mostly sparse

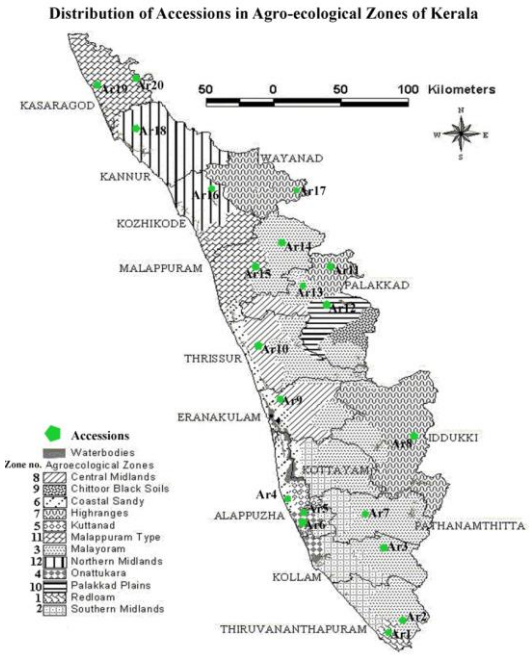

Fig. 1 Distribution of accessions in agro-ecological zones of Kerala (Source: KAU 2011)

DNA Extraction and Quantification

Total genomic DNA was isolated from young cladodes using method of LODHI *et al.* (1994). The DNA quality was checked by running the extracted DNA on 0.8% agarose gel. The genomic DNA was then diluted with TE buffer followed by purity analysis and quantification was done using a biophotometer (Biophotometer plus, Eppendorf, Germany).

RAPD Analysis and Agarose Gel Electrophoresis

A total of 25 random 10-mer primers (Eurofins/Operon, Bangalore, India) belonging to OPI (20 numbers), OPX (3 numbers) and OPAE (2 numbers) series were screened for reproducibility. Out of them, 20 RAPD primers (OPI) were selected (Table 2) based on their reproducibility and clarity of bands. The PCR amplifications was carried out in thermal cycler (Eppendorf, Germany) which consisted of reaction volume of 25µl each consisting of 12.5µl Taq PCR smart mix (Origin X Taq PCR Smart Mix2X with 2.5 µl, 10X PCR buffer, 0.25mM of each dNTPs, 1.5mM $MgCl_2$, and 1.0 Unit of Taq DNA polymerase enzyme) 0.2µM primer, approximately 40ng of template DNA, and the final volume was adjusted to 25µl with sterile double distilled water. The thermo cycler for the RAPD amplification was programmed as: initial denaturation (95°C) for 5 minutes followed by 40 cycles of denaturation (94°C) for 30 seconds, annealing (32-37°C) for 1minute, extension (72°C) for 1 minute and final extension (72°C) for 10 minutes followed by storage step (4°C) till use.

Table 2 . Sequence information and efficiency parameter of 20 RAPD primers

Sl. No.	Primer Code	Primer Sequence	Ta[c] (ºc)	TNL[d]	Polymorphism		PIC[f]	RP[g]	MI[h]	No. of Unique Bands	Amplicon Band Size
					TNP[e]	%					
1	OPI 01	5'ACCTGGACAC3'	37	99	98	98.98	0.059	31.0	5.74	34	296-3935
2	OPI 02	5'GGAGGAGAGG3	34	61	60	98.36	0.135	29.4	7.99	13	158-2474
3	OPI 03	5'CAGAAGCCCA3'	34	53	52	98.11	0.148	24.4	7.57	14	175-2215
4	OPI 04	5'CCGCCTAGTC3'	34	39	39	100.0	0.203	19.3	7.93	9	165-1579
5	OPI 05	5'TGTTCCACGG3'	32	63	63	100.0	0.078	16.7	4.96	23	174-4311
6	OPI 06	5'AAGGCGGCAG3'	34	56	54	96.42	0.151	28.2	7.90	12	299-2575
7	OPI 07	5'CAGCGACAAG3'	35	37	36	97.29	0.209	18.5	7.34	8	286-1619
8	OPI 08	5'TTTGCCCGGT3'	32	43	43	100.0	0.107	11.0	4.63	17	342-5516
9	OPI 09	5'TGGAGAGCAG3'	32	47	46	97.87	0.167	22.5	7.54	8	250-2032
10	OPI 10	5'ACAACGCGAG3'	37	62	62	100.0	0.108	23.6	6.68	25	201-2871
11	OPI 11	5'ACATGCCGTG3'	37	64	64	100.0	0.100	22.7	6.41	18	248-2595
12	OPI 12	5'AGAGGGCACA3'	37	61	60	98.36	0.144	30.6	8.54	4	121-2032
13	OPI 13	5'CTGGGGCTGA3'	37	61	60	98.36	0.135	28.9	7.97	10	250-2429
14	OPI 14	5'TGACGGCGGT3'	37	67	64	95.52	0.125	32.4	7.67	9	191-3680
15	OPI 15	5'TCATCCGAGG3'	34	51	51	100.0	0.130	19.0	6.65	9	299-2461
16	OPI 16	5'TCTCCGCCCT3'	37	69	67	97.10	0.115	30.9	7.48	15	142-3137
17	OPI 17	5'GGTGGTGATG3'	37	56	51	91.07	0.178	34.5	8.30	8	271-2038
18	OPI 18	5'TGCCCAGCCT3'	37	67	62	92.53	0.119	31.5	6.86	14	145-2965
19	OPI 19	5'AATGCGGGAG3'	37	95	92	96.84	0.075	37.1	6.74	18	172-4066
20	OPI 20	5'AAAGTGCGGG3'	37	58	57	98.27	0.108	20.5	6.05	15	222-2823
		Total		1209	1181	1955.08	2.594	512.7	140.95	283	
		Mean		60.45	59.05	97.75	0.129	25.63	7.04	14.15	

[c]Ta- Annealing Temperature,[d] TNL- Total Number of Loci,[e]TNL-Total Number of Polymorphi loci [f]PIC- Polymorphic InformativeContent, [g]RP- Resolving Power, [h]MI- Marker Index

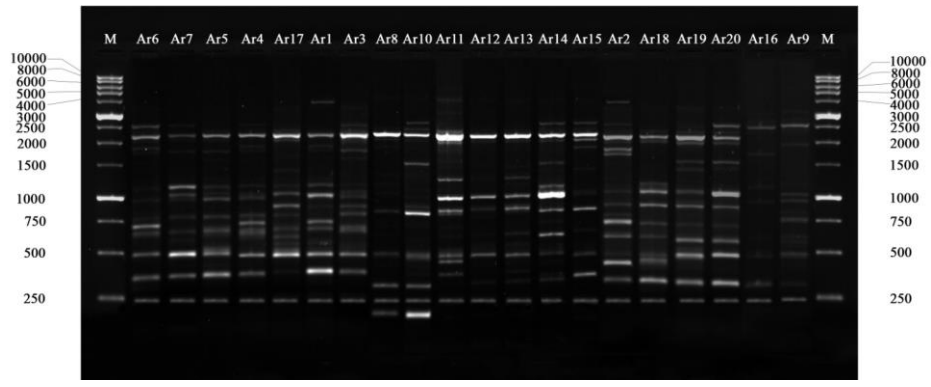

Fig. 2 DNA banding pattern of 20 *A. racemosus* accessions using RAPD primer OPI19. M-1Kb ladder, lanes 2-21 represents 20 *A. racemosus* accessions

The amplified products were resolved by electrophoresis on 1.8% agarose gels in 1x Tris Borate EDTA (TBE) buffer. The gel was stained with ethidium bromide and added 4µl of 6X gel loading dye to each reaction tube. Electrophoresis was carried out at 75v till dye travelled less than 2/3rd the length of gel. DNA (1.0 kb) ladders were used to measure the size of the obtained DNA fragments. The electrophoresis was followed by documentation under ultraviolet light and photographed by a Gel Documentation System (BioRad XR+) (Fig. 2). All PCR reactions were run in duplicates and only clear and reproducible bands were scored for polymorphism study.

Data Analysis

Only the reproducible bands were selected and scored as present (1) or absent (0) to form a binary matrix. The discriminatory power of RAPD markers was evaluated by seven efficiency parameters *viz.*, Total Number of Loci (TNL), Total Number of Polymorphic Loci (TNP), Percentage of Polymorphism (PP), Polymorphism Information Content (PIC), Resolving Power (RP), Marker Index (MI) and Number of Unique Bands (UB). These parameters were assayed as follows;

PP=Total number of polymorphic bands /Total number of bands x 100. PIC=PICi =2fi (1- fi) (ROLDAN-RUIZ *et al*., 2000), where, PICi =the polymorphism information content of marker I, fi = the frequency of the marker bands present, 1- fi = the frequency of absent marker bands. RP is based on the distribution of alleles within the genotypes. RP= ΣIb (PREVOST and WILKINSON, 1999), where, Ib =band informativeness. Ib= 1-[2x (0.5-p)] where, p-represents the proportion of genotypes having that band. MI is a measure of overall efficiency of a molecular marker technique. MI= PIC x Effective Multiplex Ratio (EMR) (POWELL *et al*., 1996), where EMR is the fraction of Polymorphic Loci x The number of Polymorphic Loci.

Data analysis was made by NTSYS-pc 2.01 software (ROHALF, 1997). Data generated from RAPD analysis were analyzed using Jaccard's similarity coefficients (JACARD, 1908). These similarity coefficients were used to calculate the pair wise similarity matrix of the accessions. The similarity matrix was subjected to the cluster analysis by using UPGMA (Unweighted Pair Group Method with Arithmetic average) and dendrogram was generated. The two dimensional and three dimensional Principal Component Analysis (PCA) were also constructed for accurately testing the relationships among 20 *A. racemosus* accessions based on EIGEN program (SNEATH and SOKAL, 1973).

Pearson Correlation Coefficient (PEARSON, 1926) was determined at P<0.01 among Total Number of Loci (TNL), Total Number of Polymorphic Loci (TNP), Percentage of Polymorphism (PP), Polymorphism Information Content (PIC), Resolving Power (RP), Marker Index (MI) and Number of Unique Bands (UB). All these numerical taxonomic analysis were conducted using SPSS package version 22 (IBM Crop, 2013).

RESULTS

A total of 1209 bands were obtained by 20 screened primers with an average of 60.45 bands per primer. Out of which, 1181 loci (an average of 59.05 loci per primer) were found to be polymorphic and 28 loci were monomorphic (Table 2). All the primers were observed to be polymorphic. The size of the amplified fragments varied from 121 bp to 5.50 Kb with an average molecular weight of 1208 bp. Through the present study 97.75% polymorphism was scored with a range of 91.07% (OPI17) to 100% (OPI 04, OPI 05, OPI 08, OPI 10, OPI 11 and OPI15). This

indicates the existence of high level of polymorphism within the accessions of *A. racemosus* under study. Also in our study monomorphic band was absent in the six RAPD primers (OPI 04, OPI 05, OPI 08, OPI 10, OPI 11 and OPI 15). The unique bands makes their presence important by the rarely occurrence in certain accession only. Among the 20 primers tested, OPI1 produced the highest number of unique bands (34 bands) while OPI12 produced the lowest number of unique bands (4 bands). Thus 283 unique bands were scored by all the 20 primers in 20 *A. racemosus* accessions.

Evaluation of efficiency parameters

The estimates of RP were found to be highest for the primer OPI 19 (37.1) (Table 2 and Fig. 2) followed by OPI17 (34.5) and was lowest for the primer OPI 08 (11.0). Here the primer OPI 19 (95 bands) is more efficient whereas OPI 08 (43 bands) was found to be least efficient (Table 2). The primer OPI01 (31.0), OPI12 (30.6), OPI14 (32.4), OPI16 (30.9), OPI17 (34.5) and OPI18 (31.5) also exhibited higher resolving power. The maximum marker index was observed for the primer OPI12 (8.54). OPI 17 also have higher MI (8.30) followed by the primer OPI 02 (7.99), OPI 04 (7.93), OPI 06 (7.90), OPI 13 (7.97) and OPI 14 (7.67). MI also found to be least in OPI08 (4.63). In the present analysis the value of PIC varied from 0.059 (OPI01) to 0.209 (OPI 07).

Association between efficiency parameter

Relationship between seven efficiency parameters *viz.*, TNL, TNP, PP, PIC, RP , MI and UB of twenty primers were evaluated (Table 3) by Pearson's correlation coefficient. It indicated positive and a significant ($P<0.01$) correlation between TNL with TNP ($r = 0.994$), RP ($r = 0.667$), and UB ($r = 0.620$). Similarly, UB has a significant correlation to TNP ($r = 0.666$), while RP showed low magnitude of positive correlation with TNP (0.555). MI showed significant correlation with PIC (0.666) and UB (0.690). However, the PIC was found to be negatively correlated with TNP ($r = -0.814$), TNL (-0.776), PP (-0.245), RP (-0.141) and UB (-0.772).

Table 3. Pearson's correlation coefficients between efficiency parameters of 20 RAPD primers of A. racemosus accessions

Parameters	TNL	TNP	PP	PIC	RP	MI	UB
TNL	1						
TNP	0.994**	1					
PP	-0.123	-0.020	1				
PIC	-0.776**	-0.814**	-0.245	1			
RP	0.667**	0.599**	-0.638**	-0.141	1		
MI	0.184	-0.242	-0.424	0.666**	0.551	1	
UB	0.620**	0.666**	0.323	-0.772**	-0.041	0.690**	1

**Significant at 0.01 level

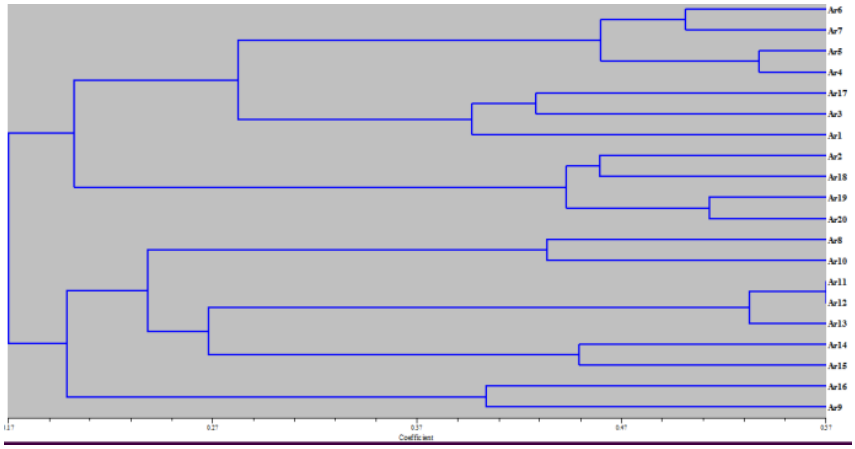

Fig. 3 Dendrogram based on UPGMA among 20 accessions of *A. racemosus* by using RAPD data

Cluster analysis

The value of Jaccard's similarity coefficient (J) obtained from RAPD data across the 20 accessions varied from 0.118 (Ar2 vs. Ar14) to 0.566 (Ar11 vs. Ar12). The average J value was found to be 0.297 (Table 4). The highest genetic similarity coefficient was observed between Ar11 and Ar12 (0.566). It was lowest between the accessions Ar2 and Ar14 which were highly dissimilar accessions based on Jaccard's similarity coefficient. Ar13 also found to be highly similar accession with Ar11 (0.512) and Ar12 (0.546). Jaccard's similarity coefficient was used to construct dendrogram based on UPGMA algorithm, consist of two principal clusters at 17% similarity level. They were further extensively divided into sub clusters. In the first principal cluster, accessions from Chingoli (Ar6; zone 4), Kozhencherry (Ar7; zone 2), Kareelakkulangara (Ar5; zone 4), Arattupuzha (Ar4; zone 6), Muthanga (Ar17; zone 7), Punalur (Ar3; zone 3), Nellimoodu (Ar1; zone 1), Kattakkada (Ar2; zone 3), Thaliparamba (Ar18; zone 12), Kanhangad (Ar19; zone 11) and Ranipuram (Ar20; zone 11) were grouped at overall similarity coefficient of 18%. This first principal cluster is divided into 3 subclusters with Ar6, Ar7, Ar5 and Ar4 constitute the first sub cluster at 46% similarity coefficient while Ar17, Ar3 and Ar1 constitute the second subcluster at 40% similarity coefficient and third subcluster comprised of Ar2, Ar18, Ar19 and Ar20 at 44% similarity coefficient.

Vandiperiyar (Ar8), Vellanikkara (Ar10), Silent Valley (Ar11), Mundur (Ar12), Mannarkkad (Ar13), Wandoor (Ar14), Pandikkad (Ar15), Janakikkadu (Ar16) and Olanad (Ar9) accessions were grouped in the second principal cluster at 19.l5% similarity coefficient and is divided into three subclusters. Among them Ar8 and Ar10 formed the first subcluster at 44% similarity coefficient, while Ar11, Ar12, Ar13 formed the second subcluster of the second principal cluster and the remaining constituted the third subcluster. Interestingly, based on Jaccard's similarity coefficient, most similar accessions Ar11 and Ar12 were placed on a single node and Ar 2 and Ar14 were found to be highly dissimilar accessions from the dendrogram. Accession Ar9 and Ar16 were found to be standing most apart from rest of the accessions studied. These clustering were also confirmed by 2 dimensional and 3 dimensional PCA analysis (Fig. 4 and Fig. 5).

Table 4. Jaccard's similarity coefficient of 20 A. racemosus accessions using RAPD marker

	Ar6	Ar7	Ar5	Ar4	Ar17	Ar1	Ar3	Ar8	Ar10	Ar11	Ar12	Ar13	Ar14	Ar15	Ar2	Ar18	Ar19	Ar20	Ar16	Ar9
Ar6	1.000																			
Ar7	0.498	1.000																		
Ar5	0.445	0.506	1.000																	
Ar4	0.417	0.461	0.534	1.000																
Ar17	0.273	0.290	0.314	0.293	1.000															
Ar1	0.231	0.253	0.264	0.291	0.376	1.000														
Ar3	0.263	0.290	0.303	0.313	0.426	0.414	1.000													
Ar8	0.238	0.216	0.211	0.218	0.184	0.171	0.186	1.000												
Ar10	0.187	0.178	0.185	0.192	0.188	0.184	0.195	0.431	1.000											
Ar11	0.166	0.157	0.139	0.174	0.149	0.153	0.166	0.209	0.284	1.000										
Ar12	0.182	0.162	0.156	0.163	0.153	0.149	0.156	0.220	0.229	**0.566**	1.000									
Ar13	0.178	0.169	0.166	0.178	0.161	0.154	0.168	0.241	0.255	0.512	0.546	1.000								
Ar14	0.210	0.189	0.174	0.179	0.143	0.149	0.158	0.234	0.240	0.265	0.275	0.316	1.000							
Ar15	0.236	0.200	0.193	0.186	0.155	0.164	0.170	0.240	0.223	0.245	0.257	0.244	0.447	1.000						
Ar2	0.227	0.248	0.237	0.232	0.210	0.203	0.197	0.162	0.165	0.155	0.144	0.149	**0.118**	0.159	1.000					
Ar18	0.198	0.203	0.190	0.192	0.158	0.164	0.175	0.134	0.164	0.145	0.133	0.141	0.146	0.158	0.457	1.000				
Ar19	0.222	0.231	0.220	0.212	0.192	0.191	0.207	0.166	0.166	0.154	0.141	0.146	0.156	0.189	0.430	0.457	1.000			
Ar20	0.193	0.216	0.202	0.190	0.177	0.187	0.182	0.154	0.173	0.148	0.142	0.147	0.150	0.167	0.420	0.455	0.510	1.000		
Ar16	0.238	0.232	0.218	0.228	0.177	0.161	0.170	0.208	0.167	0.178	0.200	0.218	0.217	0.256	0.177	0.152	0.160	0.156	1.000	
Ar9	0.200	0.177	0.176	0.178	0.188	0.178	0.178	0.192	0.157	0.188	0.196	0.184	0.204	0.215	0.146	0.150	0.138	0.137	0.402	1.000

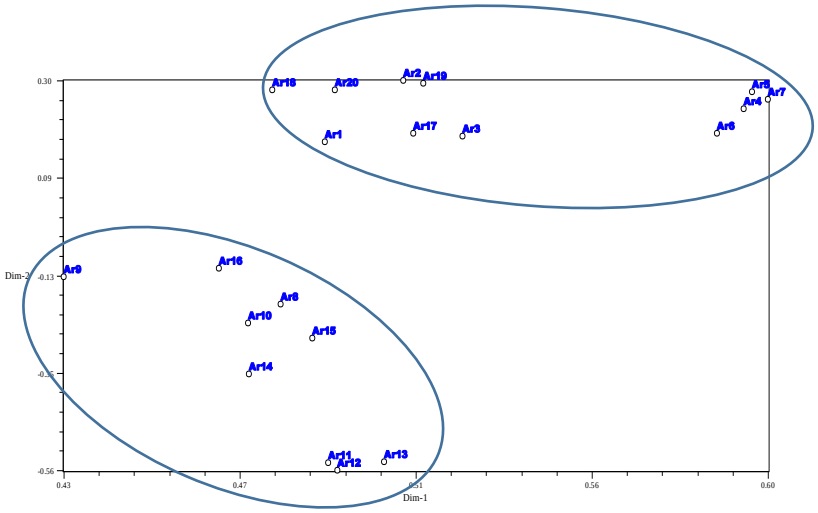

Fig. 4 PCA 2-D clustering based on RAPD

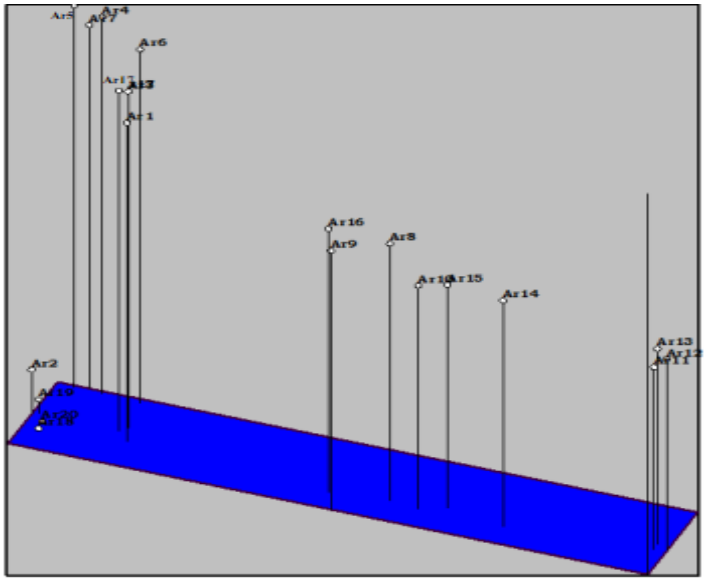

Fig. 5 PCA 3-D clustering based on RAPD

DISCUSSION

Asparagus racemosus is a well-known medicinal plant and has great importance in the field of Ayurveda. In the past the research was mostly focused on phytochemical characterization and pharmacological evaluations. In the present study, 20 *A. racemosus* accessions representing ten agro-ecological zones of Kerala state were chosen for assessing genetic diversity through RAPD markers. Diversity among the plants arises by the differences in the DNA sequences that play a key role in the path of crop improvement. In Kerala, *A. racemosus* is distributed in different agro-ecological zones and in their morphology with respect to certain vegetative characters varied significantly among accessions. Therefore molecular profiling of accessions from ten agro-ecological zones of Kerala was performed as a basic step through RAPD markers. 97.75% of polymorphism reflecting high level of genetic diversity and is higher than in *A. officinalis* as reported by SARABI *et al*. (2010). Similar to a previous study by SINGH *et al*. (2013a) all the primers were observed to be polymorphic. However, only 54.92% of polymorphism was resulted in a study by VIJAY *et al*. (2009) in *A. racemosus*. In a previous study, KUMAR *et al*. (2016) reported 81.42% of polymorphism in 60 *A. racemosus* cultivars. LAL *et al*. (2011) had reported 94.50% of polymorphism between 5 different species of *Asparagus*. In contrast, present study could able to trace out 97.75% polymorphism within *A. racemosus* species itself. In *Ocimum sanctum* higher percentage of polymorphism (96.47%) has been reported using RAPAD markers (PATEL *et al*., 2015).

Resolving power of a primer shows its ability to distinguish between the genotypes. In the present work primer OPI19 which showed highest resolving power (37.1) was found to be most efficient with respect to *A. racemosus*. Comparing to this, genetic diversity analysis in 60 *A. racemosus* cultivars through RAPD revealed RP ranged from 0.8 to 25.17 (KUMAR *et al*., 2016). The highest Marker Index observed for the primer OPI 12 was not evaluated as a genotype diagnosis tool of a primer but provides information about overall utility of a marker. According to HENRY (1997), PIC provides a measure to evaluate a marker that was influenced by the number and frequency of alleles. Since two alleles per locus are assumed in RAPD analysis, maximum PIC value for a RAPD marker is 0.5. The efficiency of RAPD primers for the better resolving of polymorphism in the intra-species level is evident from the broad range of PIC values (0.059-0.209). Previous report (KUMAR *et al*., 2016) suggests the range of PIC from 0.497 to 0.945 in *A. racemosus*, where as in *Aloe barbadensis* PIC value was between 0.13 to 0.44 (PANWAR *et a*l., 2013). From the present study it is clear that both TNP and RP are inversely related to PIC. This is further confirmed by determining correlation among the accessions. Association studies between efficiency parameters revealed positive and significant correlation of TNL with TNP, RP and UB. Close association of MI with PIC and RP also strengthened the selection of OPI 19 as efficient primer among the 20 RAPD primers assessed in the present study.

The presence of large genetic variations within the accessions was clear from the range of Jaccard's similarity coefficient (0.118 to 0.566). Similar to the results the study of KUMAR *et al*. (2016) in *A. racemosus* accessions revealed the range of Jaccard's coefficient from 0.48 to 0.97. Interestingly in our study the most similar accessions Ar11 and Ar12 are from two different agro-ecological zones *viz.*, zone 7 and 10. While Ar2 and Ar14 was highly dissimilar accessions based on Jaccard's similarity coefficient. But they represent same agro-ecological zone 3. This result obviously supports the observations of morphological diversity and confirming the effective role of genetic variations regardless of environmental variations.

The UPGMA generated dendrogram (Fig. 3) based on marker data revealed genetic relationships within accessions. All the 20 accessions evaluated were found to be segregated in to two principal clusters. Each clusters representing accessions of different agro-ecological zones. The most similar accessions Ar11 and Ar12 represent two different agro-ecological zones - 7 & 10 and two different altitude types. Zone 7 represents altitude type II while zone 10 represents altitude type I. Their rainfall pattern was also different. Zone 7 have both type I and type II rainfall pattern, where zone 10 have only type II rainfall pattern. Ar 2 and Ar14 were found to be highly dissimilar accessions though they are from same zone 3. It is clear from the dendrogram that they were placed in the first and second principal cluster respectively. Also they have same altitude type, rainfall pattern and soil type. As revealed by KUMAR *et al.* (2016) the present study also confirmed the clustering by two dimensional and three dimensional principal component analyses. Similar clustering pattern was also obtained by BAKSHI and SHARMA (2011) in *Dalbergia sissoo*. Using dice coefficients VIJAY *et al*. (2009) demonstrated dendrogram similar to our results. From the results it is clear that there is no geographic isolation in the clustering and it is concurring with the results of DESHWAL *et al*. (2005) and PANWAR *et al.* (2013).

There are several reports regarding the efficiency of RAPD markers in varietal characterization of *A. officinalis* (KHANDKA *et al*., 1996; EIMERT *et al*., 2003; MORENO *et al*., 2006; SINGH *et al*., 2013a; IRSHAD *et al*., 2014). However, few reports are available regarding the variation within *A. racemosus* accessions. This is the first report regarding the genetic characterization and diversity analysis within *A. racemosus* accessions from Kerala state, India. In comparison to present study, VIJAY *et al*. (2009) reported low level of genetic diversity among *A. racemosus* accessions representing Madhya Pradesh state, India, possibly due to restricted distribution in a particular area, non-effective gene flow, low fecundity, low pollen flow, local selection procedure and inbreeding systems. In our study, RAPD markers were used as a basic tool to assess genetic diversity within *A. racemosus* accessions and revealed a significant level of genetic polymorphism. Therefore the results of present study are more promising because the sample represents wide areas of different agro-ecological zones and confirms high genetic diversity. Also it is proved that there is sufficient diversity at genetic level though there is some similarity in morphology. Through this investigation, it is substantiated that morphological diversity realized in *A. racemosus* accessions (CHITHRA and SIRIL, 2017) have genetic differences regardless of environmental variations. Further, the present study forwards that RAPD markers can be used as a basic tool for deriving intra- species genetic diversity among *A. racemosus* accessions.

CONCLUSION

The immense demand on *A. racemosus* makes it to consider as endangered in its natural habitat and also been recognized as vulnerable. So the information on genetic diversity is vital for its genetic improvement and conservation. Even though samples are belonging different regions of Kerala, there are convincing similarities in their morphology as revealed by our previous study (CHITHRA and SIRIL, 2017). A scan on literature revealed scarce of work on defining the degree of genetic diversity in *A. racemosus*. In view of this as a stepping stone for screening and conservation of superior germplasm, genetic diversity was analyzed through RAPD markers. Study revealed higher level of polymorphism in genetic makeup and was also supported by various efficiency parameters of markers. The wide range of Jaccard's similarity coefficient indicates higher level of polymorphism within *A. racemosus* accessions. The

outcome of the analysis also highlighted RAPD as an efficient and basic tool for assessing intra-species genetic diversity. Highly reproducing and promising unique bands can be sequenced and convert to 'elite' specific SCAR marker. This may be powerful tool for future. Also it is recommending further study of collected accessions with microsatellites and nuclear ribosomal DNA (nrDNA) sequence information for in depth molecular characterization.

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REFERENCES

ASHAJYOTHI, V., R.S., PIPPALLA, D., SATYAVATI (2009): *Asparagus racemosus*- A Phytoestrogen. Int. J. Pharm. Technol., *1*(1): 36-47.

BAKSHI, M., A., SHARMA (2011): Assessment of genetic diversity in *Dalbergia sissoo* clones through RAPD profiling. J. For. Res., *22*(3): 393-397.

BRISIBE, E.A., N.C., OGBONNA, P.N., CHUKWURAH (2016): Characterization and selection of exploitable genetic diversity in soursop (*Annona muricata* Linn.) accessions based on phenotypic attributes and RAPD markers. Agrofor. Syst., *91*(4): 781-793.

CHITHRA, M.G., E.A., SIRIL (2017): Morphological variability of aerial vegetative characters among 20 Shatavari (*Asparagus racemosus* Willd.) collections from Kerala, India. J. Root Crops, *43*: 21-32.

DESHWAL, R.P.S., R., SINGH, K., MALIK, G.J., RANDHAWA (2005): Assessment of genetic diversity and genetic relationships among 29 populations of *Azadirachta indica* A. Juss. using RAPD markers. Genet. Resour. Crop Evol., *52*:285-292.

EIMERT, K., G., REUTTER, B., STROLKA (2003): Fast and reliable detection of doubled-haploids in *Asparagus officinalis* by stringent RAPD-PCR. J. Agr. Sci. *141*:73-78.

ENVIS Centre Agro-Ecological Zones. http://www.kerenvis.nic.in/Database/AgroEcological_Zones_1507.aspx. Accessed 10 August 2017.

GAHLAUT, A., A., GOTHWAL, V., HOODA, R., DABUR (2013): RAPD patterns of some important medicinal plants and their substitutes used in Ayurveda to identify the genetic variations. Int. J .Pharm. Pharm. Sci., *5*:239-241.

GOYAL, R.K., J., SINGH, H., LAL (2003): *Asparagus racemosus*-anupate. Indian J. Med. Sci., *57*(9): 408-414.

HENRY, R.J. (1997): Practical applications of plant molecular biology. Chapman $ Hall, London, pp.258.

IBM Crop Released (2013): IBM SPSS Statistics for Windows. Version 22.0, IBM Crop, Armonk, New York.

IRSHAD, M., M., IDREES, A., SAEED, MUHAMMAD, R., NAEEM (2014): Genetic diversity among *Asparagus* species and cultivars of *Asparagus officinalis* L. using random amplified polymorphic DNA (RAPD) markers. Int. J. Biodivers. Conserv., *6*(5): 392-399.

IZADPANAH, F., S., KALANTARI, M.E., HASSANI, M.R., NAGHAVI, M., SHOKRPOUR (2014): Variations in Saffron (*Crocus sativas* L.) accessions and Crocus wild species by RAPD analysis. Plant Syst. Evol., *300*: 1941-1944.

JACARD, P. (1908): Nouvellesrecherchessur la distribution florale. Bull.Soc. Vaud. Nat., *44*:223-270.

KERALA AGRICULTURAL UNIVERSITY (2011): Package of practices recommendations: Crops. 14th edn. Kerala Agricultural University, Thrissur, pp360.

KHAN, S., K.J., MIRZA, M., TAYAAB, M.Z., ABDIN (2009): RAPD Profile for Authentication of Medicinal Plant *Glycyrrhizaglabra* Linn. Med. Aromat. Plant Sci. Biotechnol., *3*(1):48-51.

KHANDKA, D.K., A. NEJIDAT, A. GOLAN-GOLDHIRSH (1996): Polymorphism and DNA markers for *Asparagus* cultivars identified by random amplified polymorphic DNA. Euphytica, *87*: 39-44.

KRISHANA, L., D., SWARUP, R.C., PATRA (2005): An overview of prospects of ethano-veterinary medicine in India. Indian J. Anim. Sci., *75* (12):1481-1491.

KUMAR, M., P.K., NAIK, SARALA, V., CHHOKAR (2016). Genetic variations in *Asparagus racemosus*, an endangered medicinal herb endemic to India Using RAPD markers. Br. Biotechnol. J., *10*(2):1-11.

LAL, S., K.N., MISTRY, P.B., VAIDYA, S.M., SHAH, R.A., THAKER (2011): Genetic diversity among five economically important species of *Asparagus* collected from central Gujarat (India) utilizing RAPD markers (Random Amplification of Polymorphic DNA). Int. J .Adva. Biotechnol. Res., *2*(4):414-421.

LEROY, X.J., K., LEON, G., CHARLES, M., BRANCHARD (2000): Cauliflower somatic embryogenesis and analysis of regenerant stability by ISSRs. Plant Cell Rep., *19*:1102-1107.

LI, Y., A., URAGAMI, Y., UNO, M., KANECHI, N., INAGAKI (2012). RAPD-based analysis of differences between male and female genotypes of *Asparagus officinalis.* Hort. Sci., *39*(1): 33-37.

LODHI, M.A., G., YE, N.F., WEEDEN, B.I., REISCH (1994): A simple and efficient method for DNA extraction from Grapevine cultivars and *Vitis* species. Plant Mol. Biol. Rep., *12*(1): 6-13.

MAYO, J.L., M.D., FACOG (1998): Black cohosh and chasteberry: Herbs valued by women for centuries. Clinical Nutrition Insights, *6*(15):1-4.

MITRA, S.K., S., GOPUMADHAVAN, M.V., VENKATARANGANNA, D.N.K., SARMA, S.D., ANTURLIKAR (1999): Uterine tonic activity of U-3107, a herbal preparation, in rats. Int. J. Pharmacol., *31*(3):200-203.

MORENO, R., J.A., ESPEJO, A.. CABRERA, T., MILLAN, J., GIL (2006): Ploidic and molecular analysis of 'Morado de Huetor' asparagus (*Asparagus officinalis* L.) populations; a Spanish tretraploid landrace. Genet. Resour. Crop Evol., *53*:729-736.

NATIONAL MEDICINAL PLANTS BOARD (2002) http://www.nmpb.nic.in

PANWAR, B.S., R., SINGH, V.K., DWIVEDI, A., KUMAR, P., KUMARI (2013): Genetic diversity among Indian *Aloe* accessions based on RAPD analysis. Indian J .Med. Arom. Plants, *3*(3):326-333.

PATEL, H.K., R.S., FOUGAT, S., KUMAR, J.G., MISTRY, M., KUMAR (2015): Detection of genetic variation in *Ocimum* species using RAPD and ISSR markers. 3 Biotech, *5*:697-707.

PEARSON, K. (1926): On the coefficient of racial likeness. Biometrika, *18*:105-117.

POWELL, W., G.C., MACHRAY, J., PROVAN (1996): Polymorphism revealed by simple sequence repeats. Trends. Plant. Sci., *1*: 215-222.

PREVOST, A., M.J., WILKINSON (1999): A new system for comparing PCR primers applied to ISSR fingerprinting of potato cultivars. TAG, *98*:107-112.

RAIMONDI, J.P., R.W., MASUELLI, E.L., CAMADRO (2001): Assessment of somaclonal variations asparagus by RAPD fingerprinting and cytogenetic analysis. Sci. Hortic., *90*:19-29.

ROHALF, F.J. (1997): NTSYS-pc Numerical taxonomy and multivariate analysis system version 2.02i. Applied Biostatistics Inc., Exeter software, New York.

ROLDAN-RUIZ, I., E., CALSYN, T.J., GILLILAND, R., COLL, M.J.T., VAN- EIJK, M.D., LOOSE (2000): Estimating genetic conformity between related ryegrass (*Lolium*) varieties: II AFLP characterization. Mol. Breed., *6*:593-602.

SARABI, B., M.R., HASSANDOKHT, M.E., HASSANI, T.R., MASOUMI, T., RICH (2010): Evaluation of genetic diversity among some Iranian wild asparagus populations using morphological characteristics and RAPD markers. Sci. Hort., *126*:1-7.

SAXENA, V.K., S., CHOURASIA (2001): A new isoflavone from the roots of *Asparagus racemosus*. Fitoterapia, *72*: 307-309.

SHARMA, T.R., S., JANA (2002): Random amplified polymorphic DNA (RAPD) variation in *Fagopyrum tartaricum* Gaertn. accessions from China and Himalayan region. Euphytica, *127*:327-333.

SHASNAY, A.K., P., DAROKAR, D., SAIKIA, S., RAJKUMAR, V., SUNDARESAN, S.P.S., KHANUJA (2003): Genetic diversity and species relationship in *Asparagus* spp. using RAPD analysis. J. Med. Arom. Plant Sci., *25*:698-704.

SINGH, A., V.P., RAI, M., SINGH, A.K., SINGH, B., SINHA (2013a): Molecular diversity analysis of *Asparagus racemosus* and its adulterants using random amplified polymorphic DNA (RAPD). J. Med. Plants Res., *7*(16):1050-1056.

SINGH, A., V.P., RAI, B., SINHA (2013b): Identification of genotype specific RAPD markers for *Asparagus racemosus*. Indian J. Genet., *73*(4):462-465.

SNEATH, P.H.A., R.R., SOKAL (1973): Numerical Taxonomy: the principles and practice of numerical classification. WH Freeman and Company, San Francisco, pp573.

TEKLEWOLD, A., H.C., BECKER (2006): Comparison of phenotypic and molecular distances to predict heterosis and F_1 performance in Ethiopian mustard (*Brassica carinata* A. Braum.). TAG, *112*:752-759.

THOMPSON, J.A., R.L., NELSON, L.O., VODKIN (1998): Identification of diverse soybean germplasm using RAPD markers. Crop Sci., *38*(5):1348-1355.

VIJAY, N., P., SAIRKAR, N., SILAWAT, R.K., GARG, N.N., MEHROTRA (2009): Genetic variability in *Asparagus racemosus* (Willd.) from Madhya Pradesh, India by random amplified polymorphic DNA. Afr. J. Biotechnol., *8*(14):3135-3140.

WARRIER, P.K., V.P.K., NAMBIAR, P.M., GANAPATHY (2001): Some important medicinal plants of the Western Ghats, India: a profile. International Development Research Centre, Artstock, New Delhi, pp398.

PROUČAVANJE GENETIČKOG DIVERZITETA A*sparagus racemosus* Willd. IZ RAZLIČITIH AGRO-EKOLOŠKIH ZONA KERALE UPOTREBOM RAPD MARKERA

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Izvod

Kod dvadeset uzoraka *Asparagus racemosus* iz deset agro-ekoloških zona Kerale ocenjen je genetički diverzitet upotrebom RAPD markera. Dvadeset prajmera je izabrano na osnovu reproduktivnosti i jasnoće traka. Ukupno je zabeleženo 1209 traka od kojih je 1181 lokusa bilo polimorfno (97,75%). Procenjeni su parametri efikasnosti prajmera, ukupnog broja lokusa, ukupnog broja polimorfnih lokusa, procenta polimorfizma, sadržaja informacija o polimorfizmu (PIC), snage rezolucije, indeksa markera i broja jedinstvenih traka. Vrednost PIC varirala je od 0.059 do 0.209 i dokazano je da je OPI19 najpolimorfniji marker. Evaluacija odnosa između sedam parametara efikasnosti pokazala je pozitivnu i signifikantnu (P <0,01) korelaciju ukupnog broja lokusa sa ukupnim brojem polimorfnih lokusa, rezolucijom snage i jedinstvenih traka. Jaccard-ov koeficijent sličnosti bio je u rasponu od 0.118 do 0.566, što ukazuje na veliku genetičku varijabilnost između uzoraka. Podaci iz UPGMA dendrograma otkrili su genetske odnose između uzoraka i da ne postoji geografsko razdvajanje u klasteru. Ova zapažanja ukazuju na to da se RAPD markeri mogu koristiti kao efikasno sredstvo za utvrđivanje genetičke raznolikosti između uzoraka *A. racemosus*.

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