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Genetic diversity among *Asparagus* species and cultivars of *Asparagus officinalis* L. using random amplified polymorphic DNA (RAPD) markers

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The species of *Asparagus* are very important as they are used for ornamental, vegetable and medicinal purposes since ancient time. In the present study, random amplified polymorphic DNA (RAPD) markers were used to evaluate genetic diversity among nine species of *Asparagus* and six cultivars of *Asparagus officinalis* L. RAPD analysis using seven random oligonucleotide primers yielded a total amplification of 245 bands, among which 220 (89.80%) were polymorphic with an average of 31.4 bands per primers. Highest number of 39 (97.50%) polymorphic bands were obtained with primer OPC-07, while minimum polymorphic bands were 18 (69.23%) with primers OPA-01. Genetic similarity coefficient ranged from 0.75 to 0.96 with an average of 0.85. Phenogram clustered all *Asparagus* species and *A. officinalis* L. cultivars into two clear clusters. One cluster comprised of all cultivars of *A. officinalis* L. while the second cluster comprised of all the *Asparagus* species. The present study reveals that RAPD markers were more convincing for analyzing genetic diversity among *Asparagus* species and cultivars of *A. officinalis* L.

Key words: *Asparagus officinalis* L., genetic diversity, random amplified polymorphic DNA (RAPD), Phenogram.

INTRODUCTION

Asparagus is an herbaceous, perennial plant belonging to the *Asparagaceae* family, comprising of about 150 species and is widely distributed in tropical and sub-tropical region up to an altitude of about 1500 m (Velvan et al., 2007). In Pakistan, 14 different species of *Asparagus* has been found (Ali and Khan, 2009). *Asparagus officinalis* L. is an important vegetable crop, while *Asparagus adscendens* Roxb, *Asparagus capitatus* Baker and *Asparagus racemosus* Willd. are extensively used for various medicinal purposes (Goyal et al., 2003; Sharma and Bhatnagar, 2011), whereas *Asparagus densiflorus* (Kunth) Jessop, *Asparagus setaceus* (Kunth)

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Jessop, *Asparagus plumosus* Baker and *Asparagus monophyllus* Baker have economic importance both for horticultural and ornamental purposes.

Asparagus act as a highly valuable plant species having both therapeutic and nutraceutical importance as well as used for food consumption (Shasnay et al., 2003). *Asparagus* contains saponins that possess antitumor activity while it also contains fructans that help to reduce the risk of disorder such as constipation, diarrhea as well as disease like osteoporosis, obesity, cardiovascular disease, rheumatism and diabetes (Shao et al., 1997). Fruit is eaten to treat pimples. Seeds are also used for blood purification. Its pharmacological activities include anticancer, antioxidant, antifungal, antibacterial, anti-dysenteric, anti-inflammatory, anti-abortifacient, anti-oxytoxic, antiulcer, hypertensive and anticoagulant effects (Sharma et al., 2000).

The availability of a variety of DNA markers including restriction fragment length polymorphism (RFLP) (Carreel et al., 2002), amplified fragment length polymorphism (AFLP) (Loth et al., 2000), simple sequence repeat (SSR) (Silvana et al., 2003), and inter simple sequence repeat (ISSR) (Rout et al., 2009), has enabled researchers to examine genetic variation among various plant species across natural populations (Archak et al., 2003). Among these, PCR-based techniques of random amplified polymorphic DNA analysis (RAPD) have been successfully used, due to technical simplicity and speed, RAPD methodology have been used for genetic diversity analysis, genotyping and genome mapping in various medicinal plant species such as *Berberis lycium* Royle (Tripathi and Sandhya, 2013), and Rose (Jan and Byrne, 1999). The objective of the present study was used to analyze the genetic diversity among 14 different *Asparagus* species and *A. officinalis* L. cultivars using RAPD markers.

MATERIALS AND METHODS

Plant materials

A total of eight *Asparagus* species and six cultivars of *A. officinalis* L. were collected from different region of Pakistan including Islamabad, Lahore, Kohat and Swat (Table 1). The leaves were collected and stored at -20°C in a freezer until their DNA was extracted.

DNA isolation

Genomic DNA was isolated from fresh and young leaves of *Asparagus* by standard cetyl trimethyl ammonium bromide (CTAB) method with few modifications (Doyle and Doyle, 1987). Modifications were designed to counter the high level of secondary compounds and polysaccharides present in *Asparagus* leaves. These compounds degrading DNA, inhibit subsequent enzyme digests and PCR reactions (Pirttila et al., 2001). The modifications included the use of high concentration of PVP (polyvinyl-pyrrolidine), repetition of purification step with chloroform : Isoamyl alcohol, DNA pellet wash with wash buffer and ethanol (70%). *Asparagus* young leaves samples were crushed in liquid nitrogen, about 100 mg were weight and transfer in a 1.5 mL tube. The powder was then mixed with 800 µL extraction buffer (100 mM Tris HCl pH (8.5), 50 mM EDTA, 500 mM NaCl and 1% PVP) and 20 µL β-2-mercaptoethanol. The homogenate was incubated in water bath at 65°C for 1 h with periodic gentle vortexing and the DNA was extracted twice with chloroform-isoamyl alcohol (24:1). The DNA was precipitated by adding equal volume (0.6V) of chilled isopropanol and 30 µl of 5 M NaCl was added, the tube was mixed gently to form fibrous DNA, DNA pellet was first washed with 800 µl of wash buffer (5 mM Tris HCl; pH (8), 25 mM NaCl, 75 % ethanol), and again washed with 300 µl of 70% ethanol. The DNA pellet was dissolved in 30 µL TE buffer (10 mM Tris-HCl, pH 8, 2 mM EDTA) and stored at -20°C. DNA concentration was determined by running the DNA samples on 0.8% agarose gel electrophoresis and comparison of band intensities with lambda DNA standards was done.

RAPD primers

A total of 15 RAPD primers (Operon Technologies, Alameda, CA, USA) were used. Seven random primers including OPA-01, OPA-03, OPA-09, OPA-10, OPB-07, OPC-05 and OPC-07 were used (Table 2).

RAPD PCR amplification

DNA amplification was performed for arbitrary polymerase chain reaction (PCR) in an ABI (Applied Biosystem Inc, USA) thermal cycler. PCR was performed in a reaction mixture with a total volume of 25 µl containing 10X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1U Taq DNA polymerase, 0.2 picomole primers, 40 ng of template DNA and PCR water. After initial denaturation of the DNA at 94°C for 5 min, the thermal cycling was performed with denaturation at 94°C for 45 s, annealing at 37°C for 1 minand extension at 72°C for 1.5 min and final extension at 72°C for 10 min, while hold a temperature at 4°C.

Agarose gel electrophoresis

Amplified RAPD products were size separated by on 1.5% agarose gels electrophoresis at 125 V in 1X TBE buffer for 1 h, stained with ethidium bromide and photographed by gel documentation system (Alpha Innotech, Alpha Imager EP, and U.S.A). All PCR experiments were done at least twice and best gels of the replicates were used for band scoring.

RAPD data analysis and scoring

Electrophoretic patterns of each RAPD primers were scored manually as ‘1’ or ‘0’ for presence and absence of the bands. The results were analyzed 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 individuals. Using Nei and Li genetic similarity coefficient (Nei and Li, 1979), a similarity matrix involving eight *Asparagus* species and six cultivars was generated with NTSYS-pc (Numerical taxonomy system, applied biostatistics, Inc., New York, USA, software version 2.02e (Sonnante et al., 2002). A phenogram was constructed using the Neighbor Joining Method.

RESULTS AND DISCUSSION

The identification is more difficult through vegetative

Table 1. Collection sites and environmental parameters for *Asparagus* species and *A. officinalis* cultivars.

Species and cultivars	Type	Collection sites	Herbarium specimen
A. racemosus Willd.	Species	Charbhage, Swat	
A. capitatus subsp. *capitatus*	Species	Ghalegay, Swat	
A. *capitatus* subsp. *gracilis*	Species	Shamozu, Swat	
A. adscendens	Species	Jerma, Kohat	
A. setaceus	Ornamental	Bhage Jinnah,Lahore	
A. densiflorus	Ornamental	More green, Lahore	
A. plumosus	Ornamental	Mingora, Swat	
A. officinalis L.	Vegetative	NARC, Islambad	
A. officinalis Cv. Abril	Cultivar	ARI, Mingora	
A. officinalis Cv. Apollo	Cultivar	ARI, Mingora	
A. officinalis Cv. Gersengum	Cultivar	ARI, Mingora	
A. officinalis Cv. Huchel	Cultivar	ARI, Mingora	
A. officinalis Cv. Taranga	Cultivar	ARI, Mingora	
A. officinalis Cv. Para selection	Cultivar	ARI, Mingora	

Table 2. Lists of RAPD primers with their sequences and GC (%).

Primer	Sequence (5'-3')	GC%
OPA-01	CAGGCCCTTC	60
OPA-03	AGTCAGCCAC	60
OPA-09	GGGTAACGCC	70
OPA-10	GTGATCGCAG	60
OPB-07	GGTGACGCAG	70
OPC-05	GATGACCGCC	70
OPC-07	GTCCCGACGA	70

characters, although true phenotypic expression showed variation. Beside this, morphological and biochemical character cannot determine genetic differentiation and plasticity in population adaptation and variations (Gepts, 1993). So they lack the resolving power for individual genotype identification. Sometimes in early stage in *Asparagus* species, identification is more difficult from the other member of *Asparagus* family. Beside this, for *Asparagus* species due to the erratic flowering and lack of morphological differences, the recognition of genetic relationship is extremely difficult. Reliable identification of

Table 3. Polymorphism of RAPD Primers for *Asparagus* species and cultivars of *A. officinalis* L.

Primer	Number of bands	Monomorphic bands	Polymorphic bands	Monomorphic (%)	Polymorphic (%)
OPA 01	26	8	18	30.77	69.23
OPA 03	77	7	71	9.09	92
OPA 09	28	6	22	21.42	78.57
OPA 10	34	2	32	5.88	94.11
OPB 07	25	1	24	4	96
OPC 05	15	1	14	6.66	93.33
OPC 07	40	1	39	2.5	97.50
Total	245	26	220	80.32	89.80
Average	35	3.71	31.4	11.40%	88.71

taxa is not only necessary for breeders but also necessary for propagation and consumers. Nowadays, traditional method of species identification by morphological parameters is gradually being replaced by DNA profiling which is more reliable because of various limitations of morphological data. In recent year, DNA based RAPD markers have been widely used due to its rapid and simplicity, for the identification of variety, management of genetic resources, genetic diversity and phylogenetic relationship (Hu and Quiros, 1991; He et al., 1992).

In the present study, RAPD markers have been used for the genetic diversity of eight *Asparagus* species and six cultivars of *A. officinalis* L. from different regions of Pakistan (Table 1). Fifteen RAPD markers were selected for this purpose, to identify DNA polymorphisms and relationships among *Asparagus* species and its cultivars. In the present study, only seven random RAPD primers (OPA-01, OPA-03, OPA-09, OPA-10, OPB-07, OPC-05 and OPC-07) were reproducible and satisfactory, while the rest of the primers gave smear and unreadable band pattern.

A total of 245 bands were produced, among which 26 bands were monomorphic (11.40%), whereas 220 bands were polymorphic (88.71%). Lal et al. (2011) in their studies on five different species of *Asparagus*, utilizing 6 RAPD primers yielded 258 polymorphic DNA fragment. Determination of high level of genetic diversity of *Asparagus* species and cultivars of *A. officinalis* L. is very important to conserve for easy management of genetic resources and high level of variation for the breeding programs.

In the present study, the average numbers of bands per primers were 31.4, which were higher than that reported by Ray et al. (2010) for *Asparagus* species (28.1). These differences might be due to different primer sequences as well as different geographical origin. All RAPD primers showed a wide range of amplicons ranging from 300 to 3000 bp. The highest number of bands was obtained with primers OPC-07 which revealed 39 polymorphic bands and 1 monomorphic band with 97.50% polymorphism, while the lowest number of polymorphic bands was obtained by the primer OPA-01 which were 18 polymorphic bands and 8 monomorphic bands with 69.23% polymorphism (Table 3). Among *Asparagus* species and cultivars of *A. officinalis*, *A. officinalis* Cv. Huchel showed maximum number of bands (45), whereas *A. racemosus* showed lowest number of bands (37) (Table 4).

Estimation of genetic similarity using genetic fingerprinting data are useful tool in plant breeding which allows plant breeders to create better decisions regarding the selection of germplasm to be used in crossing schemes (Milbourne et al., 1997; Russell et al., 1997). The genetic similarity index obtained from RAPD analysis showed a genetic similarity coefficient ranging from 0.75 to 0.96 with mean genetic similarity of 0.85. The highest genetic similarity was observed between (*A. officinalis* and *A. officinalis* Cv. Apollo) and *(A. officinalis* Cv. Gersengum and Abril) with a value of 0.96, while the lowest genetic similarity value was 0.75 between (*A. officinalis* Cv. Abril and *A. capitatus* subsp. *gracilis)* and (*A. setaceus* and *A. officinalis* Cv. Apollo) (Table 5).

The genetic similarity value were used for cluster analysis using neighbor joining algorithm, grouped *Asparagus* species and cultivars of *A. officinalis* L. into two main clusters (cluster I and cluster II). The cluster I was comprised of *A. officinalis* L., *A. officinalis* Cultivars Abril, Apollo, Gersengum, Huchel, Para selection, Taranga and *A. adscendens*, whereas Cluster II comprised of *A. capitatus* subsp. *capitatus, A. capitatus* subsp. *gracilis, A. densiflorus, A. racemosus, A. plumosus* and *A. setaceus*.

The data matrix of genetic similarities and phenogram is illustrated in Table 5 and Figure 2. The clarity of the differentiation for wild species by RAPD in the present work agreement with those of Lal et al. (2011), where they clustered the *Asparagus* species on the basis of their geographical isolation.

RAPD analysis to obtain information on genetic variations among *Asparagus* species was applied for the first time in Persia and this was the beginning of further studies by more powerful markers. To preserve this valuable plant, more *Asparagus* samples should be

Table 4. RAPD primers and total number of bands among *Asparagus* species and cultivars of *A. officinalis* L.

	A. officinalis L.	*A. officinalis* Cv. Abril	*A. officinalis Cv. Apollo*	*A. officinalis* Cv. Gersengam	*A. officinalis* Cv. Huchel	*A. officinalis* Cv. Para	*A.officinalis* Cv. Taranga	*A. adscendens*	*A.capitatus* subsp. *capitatus*	*A. densiflorus*	*A. plumosus*	*A. racemosis*	*A. setaceus*	*A.capitatus* subsp. *gracilis*
OPA 01	9	8	9	9	8	9	9	10	10	10	9	10	10	10
OPA 03	12	12	8	12	12	12	11	11	9	11	11	11	10	8
OPA 09	7	7	5	7	8	8	8	8	6	8	7	7	8	9
OPA 10	4	5	2	4	5	6	3	2	5	5	4		4	4
OPB 07	3	1	3	4	4	3	1	3	3	3	3	3	3	2
OPC 05	3	3	3	3	3	3	3	1	1	1	1	1	1	1
OPC 07	5	5	5	5	5	3	5	3	3	2	2	2	2	4
Total	43	41	35	44	45	44	40	38	37	40	37	34	38	38

Table 5. Genetic similarities index based on Nei and Li coefficient using RAPD primers for *Asparagus* species and cultivars of *A. officinalis* L.

***Asparagus* species**	***A.officinalis* L.**	***A.officinalis* Cv. Abril**	***A.officinalis* Cv. Apollo**	***A.officinalis* Cv. Gersengum**	***A.officinalis* Cv. Huchel**	***A.officinalis* Cv. Para**	***A.officinalis* Cv. Taranga**	***A.adscendens***	***A.capitatus* subsp. *capitatus***	***A.densiflorus***	***A.plumosus***	***A.racemsus***	***A.setaceus***	***A.capitatus* subsp. *gracilis***
A. officinalis L.	1													
A. officinalis Cv. Abril	0.95	1												
A. officinalis Cv. Apollo	0.96	0.96	1											
A. officinalis Cv. Gersengum	0.96	0.94	0.93	1										
A. officinalis Cv. Huchel	0.95	0.93	0.92	0.96	1									
A. officinalis Cv. Para	0.92	0.89	0.90	0.88	0.92	1								
A. officinalis Cv. Taranga	0.94	0.93	0.92	0.90	0.91	0.90	1							
A. adscendens	0.84	0.83	0.80	0.85	0.86	0.85	0.87	1						
A. capitatus subsp.*capitatus*	0.80	0.79	0.76	0.81	0.82	0.79	0.80	0.88	1					
A. densiflorus	0.79	0.84	0.81	0.81	0.84	0.83	0.85	0.89	0.85	1				
A. plumosus	0.82	0.82	0.79	0.86	0.87	0.84	0.85	0.88	0.86	0.90	1			
A. racemsus	0.84	0.83	0.80	0.85	0.86	0.82	0.84	0.92	0.90	0.94	0.93	1		
A. setaceus	0.79	0.78	0.75	0.80	0.84	0.80	0.82	0.89	0.90	0.92	0.93	0.94	1	
A. capitatus subsp. *gracilis*	0.76	0.78	0.78	0.78	0.81	0.75	0.79	0.81	0.82	0.84	0.80	0.84	0.84	1

gathered, cultivated and domesticated in collections. Prohens et al. (2008) showed Asia, Africa and Europe as the main centers for the genetic diversity of edible *Asparagus*. The results of the current study showed that Pakistan could be considered as another center for genetic diversity of *Asparagus*.

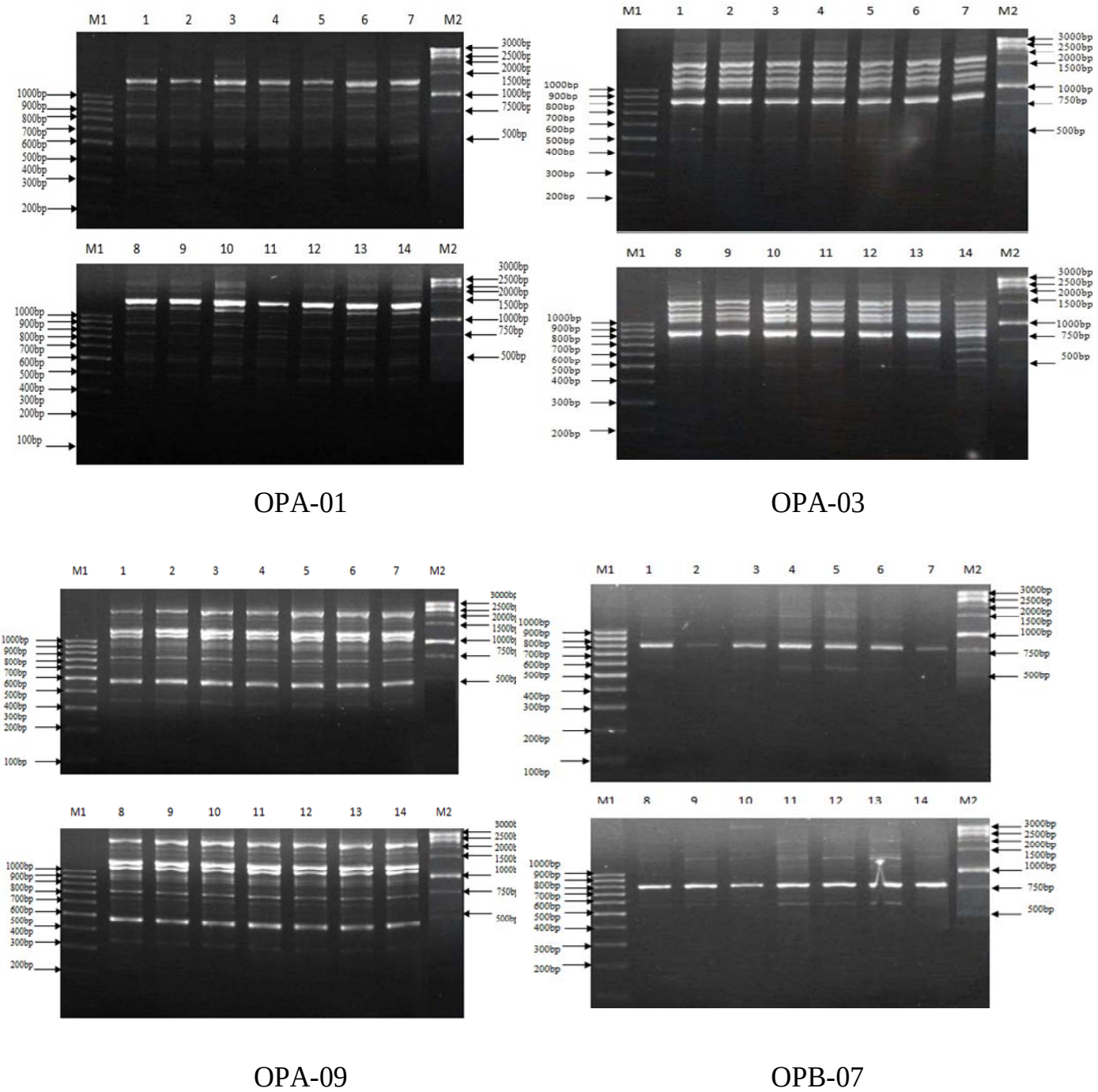

Figure 1. RAPD pattern generated using primer (A) OPA-09 (B) OPA-03 (C) OPA-01 (D) OPB-07: Lane 1-14 represents M1 = 100 bp marker, Lane M2 = 1 kb marker, (1) *A. officinalis* L. (2) *A. officinalis* Cv. Abril, (3) *A. officinalis* Cv. Apollo, (4) *A. officinalis* Cv. Gersengum, (5) *A. officinalis* Cv. Huchel, (6) *A. officinalis* Cv. Para, (7) *A. officinalis* Cv. Taranga, (8) *A. adscendens,* (9) *A. capitatus* subsp. *capitatus*, (10) *A. densiflorus,* (11) *A. plumosus*, (12) *A. racemosus*, (13) *A. setecus*, (14) *A.capitatus* subsp. *gracilis*.

Conclusion

RAPD markers are very useful for analyzing genetic diversity as well as pattern of genetic relationship among *Asparagus* species and cultivars of *A. officinalis* L. Further, more numbers of primers and large number of samples with wide range of collection sites should be used to obtain a clear picture of genetic diversity. This study will be particularly useful for the conservation, breeding and germplasm management of *Asparagus.*

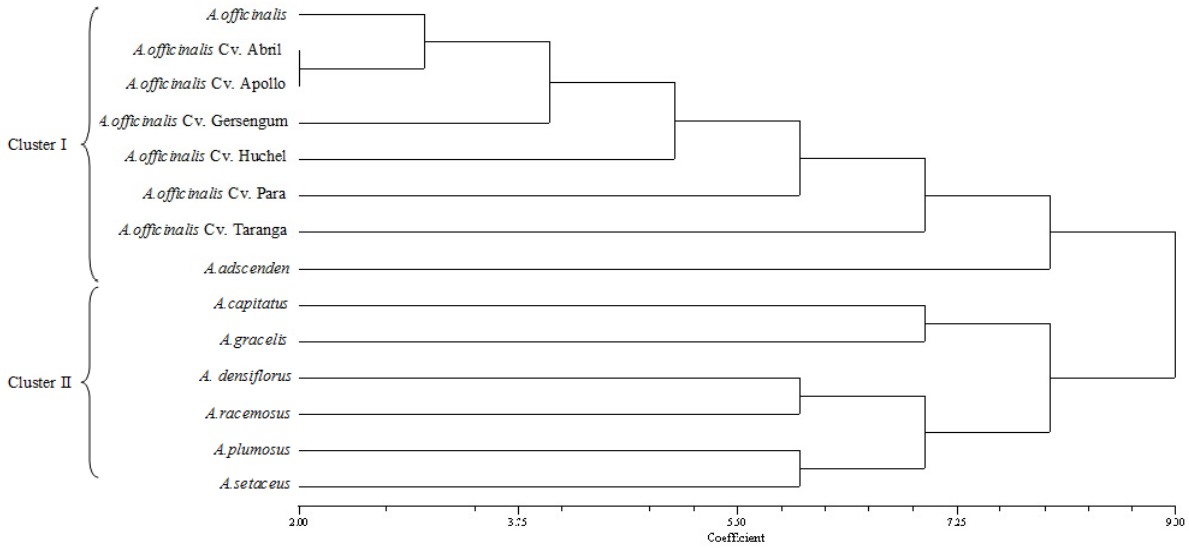

Figure 2. Neighbor Joining method of cluster analysis of *Asparagus* species and cultivars of *A. officinalis* L. using RAPD primer.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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