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GENETIC RELATIONSHIPS OF *ASPARAGUS* SPECIES (ASPARAGACEAE) BASED ON BIOCHEMICAL AND MOLECULAR MARKERS

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Abstract

The genetic relationships of *Asparagus* species (Asparagaceae) from Pakistan were evaluated using sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) and PCR-based random decamer markers. Protein profiling of seed proteins of *Asparagus* species yielded 19 bands with 63.2% polymorphism. The indices of similarity ranged from 0.61 to 0.97, with an average of 0.55. *Asparagus* species were divided into six clusters based on cluster analysis, Cluster I consists of *A. officinalis* and *A. officinalis* cv. Taranga, cv. Gersengum, and cv. Para selection. Cluster II consists of *A. officinalis* cv. Appolo, cv. Abril and cv. Huchel. Cluster III consists of *A. capitatus* and *A. gracilis*. Cluster IV, V and VI contain *A. adscendens, A. racemosus* and *A. densiflorus*, which have formed distinct clades. RAPD markers using eight primers produced a total of 50 fragments, of which 36 (72%) were polymorphic and 14 (28%) were monomorphic bands. The *Asparagus* species were divided into four major clusters based on neighbor joining method of cluster analysis. Cluster I consists of *Asparagus officinalis*, cluster II comprise of *Asparagus officinalis* cultivars (Abril, Apollo, Gersengum, Huchel, Para and Taranga). Cluster III consists of six *Asparagus* wild species (*A. adscendens, A. densiflorus, A. racemosus, A. setaceus, A. plumosus* and *A. capitatus*), whereas Cluster IV contain only *Asparagus gracilis* (synonym of *A. asiaticus*), which was formed a separate clade from the rest of the species. It was noticed that PCR-based RAPD markers are more effective at resolving species relationships than allelic patterns of seed storage proteins.

Key words: *Asparagus*, Asparagaceae, Genetics, Protein profiling, Random amplified polymorphic DNA, Molecular markers.

Introduction

The genus *Asparagus* L. (Linnaeus, 1753) belongs to the family Asparagaceae of Asparagale (APG III, 2009), and comprises ca. 214 species (Ali & Khan, 2009; Baker, 1875; Chen & Tamanian, 2000; Govaerts, 1995). The genus is widely distributed in the old-world continents (Dahlgren *et al*., 1985), and has been suggested to originate from Africa, especially South Africa and adjacent regions (Kanno & Yokoyama, 2011; Sebsebe, 2008; Hamdi & Assadi, 2009), subsequently spreading throughout the old world (Asia and Europe) through intensive speciation and dispersal (Kubota *et al*., 2012). In flora of Pakistan, fourteen species are recorded, and are mainly distributed in Sindh (Thatta, Ghulamullah, and Gharo), Khyber Pakhtunkhwa (Swat, Malakand, and Kohat), Punjab (Rawalpindi, and Islamabad), Baluchistan, and Kashmir (Ali & Khan, 2009). The most recent intrageneric classification split *Asparagus* species into three subgenera: *Asparagus*, *Myrsiphyllum* and *Protoasparagus* (Clifford & Conran, 1987). Generally, subg. *Asparagus* species are perennial herbs, vines and shrubs or sub-shrubs with leaves scale like, scariose, spurred at the base, spurs sometimes extending into spines, subtending 1-many cladodes. Inflorescence axillary or terminal solitary or cluster of flowers, sometimes a individual with staminate flowers exhibit much variation in reduction of pistil, from fully functional to rudimentary. Pedicel articulated. Perianth campanulate or subglobose, tepals 6 in two series, free or connate at base. Stamens 6, filaments free and epiphyllous. Ovary superior, trilocular, axile placentation, ovules 2-many per locule, style with 3 short stigmatic branches or with capitate or lobate stigma. Fruit a berry (Ali & Khan, 2009). *Asparagus* is known as the "King of Vegetables" and one of the top 10 most popular vegetables, because of unique texture and taste as well as high nutrition (Baxter *et al*., 2003). Most of the *Asparagus* species are economically important for use as an ornamental, edible as well as medicinal purposes (Sharma *et al*., 2000; Negi *et al*., 2010; Al-Snafi, 2015; Takahashi *et al*., 2019). Recent phylogenetic studies on the genus *Asparagus* have confirmed their monophyletic origin with sexual dimorphism and polyploidy as the main force of evolution (Kubota *et al*., 2012; Norup *et al*., 2015; Castro *et al*., 2013; Idrees *et al*., 2021).

Genetic relationships and diversity play a vital role in plant breeding programmes to provide raw materials for developing new and high yielding varieties, abiotic stresses tolerance and used to enhance diseases resistance (Allard, 1999). It also plays a vital role in identifying the scenario for genetic improvement of genotypes, germplasm management and selection of the elite genotypes using different DNA profiling techniques (Engles *et al*., 2002). Various types of data sets have been used for such purposes, of these, the most important are

morphological (Zhang *et al*., 2017), storage protein (Malviya *et al*., 2018), and DNA markers (Nkatha *et al*., 2018). Traditionally, crop species characterization and evaluation based on vegetative and floral characters. They are simple, rapid and inexpensive to measure and score. Phenotypic estimates are used to present the degree of genetic relationship and variation between lines. It is assumed that similarity in phenotype characteristics reflects genetic similarity of genotypes. Morphological markers are important for detection and characterization of species and cultivars. Despite simple monitoring, expression of morphological markers is influenced by epistatic-pleiotropic interaction and dominant-recessive relationship. The study of genetic variation by morphological characterization has been criticized because of time consuming and labors (Cooke, 1987). Biochemical markers that are discriminated based on difference at the expressed protein level and can also be separated by electrophoresis to detect polymorphisms. Now large-scale analysis such markers are limited used because of low level of species and subspecies genetic variation (Rafinski & Babik, 2000). Recently various types of molecular markers proven to be valuable tool for detection and analyzed evolutionary relationships and genetic diversity among and within plant species and varieties. Randomly amplified polymorphic DNA (RAPD) is the PCR amplification of genomic DNA, with random nucleotide sequence of single primers (Williams *et al*., 1990). RAPD approach has been used to analyze genetic diversity in various medicinal plant species (Li *et al*., 2002), due its speed and simplicity. RAPD are being widely used to study the phylogenetic position (Zhao *et al*., 2017; Idrees *et al*., 2021), genetic linkage mapping (Venkateswarlu *et al*., 2006). In recent years, RAPD markers were used for resolving genetic relationships and genetic diversity in many medicinal plants, such as *Catharanthus roseus*, *Cuscuta relexa* and *Asparagus* (Khan *et al*., 2010; Shaw *et al*., 2009).

The utilization of biochemical and genetic markers for the estimation of genetic relationships in *Asparagus* are more important because very little information is available about its genomic and phylogenetic studies. Morphological characterization is still problematic and time-consuming procedure for the estimation of genetic differentiation among *Asparagus* species and its cultivars. The evaluation of the genetic polymorphism, origin, and evolution of the *Asparagus* species that are grown in Pakistan has received relatively little research. Thus, in the current study was hypothesized to utilize the genetic differentiation patterns of eight *Asparagus* species and six *A. officinalis* cultivars using SDS-PAGE and RAPD markers.

Material and Methods

Taxon sampling: A total of eight *Asparagus* species and six *Asparagus officinalis* cultivars were collected from Pakistan regions (Swat, Islamabad, Kohat and Lahore) as indicated in the flora of Pakistan (Ali & Khan, 2009) (Table 1). The seeds were grown in the greenhouse for DNA isolation. The leaves samples were immediately stored in silica bag.

Protein extraction: Seeds were crushed to a fine powder with mortar and pestle. Around 10 mg powder was homogenized with 700 µl of protein buffer solution (0.125 M Tris) pH 6.8; 8 M Urea, 4 % SDS, 20 % Glycerin, 5 % 2-β-mercaptoethanol), and placed in shaker at 40 rpm/min for 12 h. The sample was denatured in water bath at 100ºC for 3 minute and centrifuged for 10 minutes at 10,000 g. The aqueous layer was taken on another sterile tube and kept at -20ºC for further protein analysis.

Seed protein was electrophoresis using SDS-PAGE by the procedure described by Hong *et al*., (2001) having 5% stacking gel and 12% separating gel concentrations. A total volume of 12 µl protein samples was loaded into each well. The electrophoresis was carried out at 80 V, when the bromophenol blue dye reached the end of the gels. After electrophoresis, gels were stained in a staining solution comprising of 4% Acetic acid, 18% methanol and 0.05% coomassie brilliant blue (R-250). The gel was placed in shaker at 40 rpm/min for 3 h and destained in destaining solution comprising of 4% acetic acid and 18% methanol for 8 h until the background become clear and protein bands were clearly visible. The image was photographed by scanner.

Table 1. A list of species, origin, altitude and longitude used in the present study.

Species and cultivars	Type	Origin	Latitude	Longitude	Altitude
A. officinalis L.	Cultivated	NARC, Islamabad	33°43'N	73°04'E	490 m
A. officinalis cv. Abril	Current cultivar	ARI, Mingora	34°78'N	72°36'E	984 m
A. officinalis cv. Apollo	Current cultivar	ARI, Mingora	34°78'N	72°36'E	984 m
A. officinalis cv. Gersengum	Current cultivar	ARI, Mingora	34°78'N	72°36'E	984 m
A. officinalis cv. Huchel	Current cultivar	ARI, Mingora	34°78'N	72°36'E	984 m
A. officinalis cv. Para	Current cultivar	ARI, Mingora	34°78'N	72°36'E	984 m
A. officinalis cv. Taranga	Current cultivar	ARI, Mingora	34°78'N	72°36'E	984 m
A. adscendens Roxb.	Wild species	Jerma (Kohat)	33°58'N	71°43'E	489 m
A. capitatus Baker	Wild species	Ghalegay (Swat)	34°69'N	72°26'E	950 m
A. densiflorus (Kunth) Jessop	Ornamental	More green Nursery (Lahore)	31°34'N	74°22'E	217 m
A. plumosus Baker	Ornamental	Mingora (Swat)	34°69'N	72°26'E	950 m
A. racemosus Willd.	Wild species	Charbhage (Swat)	34°83'N	72°44' E	1032 m
A. setaceus (Kunth) Jessop	Ornamental	Bhage Jinnah (Lahore)	31°54′N	74°33′ E	209 m
A. gracilis Salisb. (synonym of *A. asiaticus* L.)	Wild species	Shamozu (Swat)	34°51'N	72°26' E	974 m

DNA extraction and PCR amplifications: The procedure developed by Doyle & Doyle (1987), with slight modifications (addition of high concentration of polyvinyl pyrrolidine (PVP), were used to extract the DNA from *Asparagus* species and its cultivars. The DNA pellet was diluted in 50 µL TE buffer (10 mM Tris-HCL; pH 8.0 and 0.1 mM EDTA; pH 8.0) and stored at 4ºC. DNA quality and quantity was assessed by spectrophotometrically at 260/280 nm and also check on 0.8% agarose gels. A total of eight RAPD primers including OPC-09, OPC-10, OPC-13, OPC-20, OPD-07, OPE07, RPI-2 and RPI-3 (Operon Technologies, Alameda, CA, USA) were employed for PCR amplification (Table 2). Polymerase Chain Reaction (PCR) was performed in 25 µl reaction mixture containing 0.5 µl of assay buffer, 0.2 mM dNTPs, 1U Taq DNA polymerase, 0.2 pM primers, 1.5 mM $MgCl_2$, and 50 ng template DNA. DNA amplification was performed with arbitrary primers in an ABI thermal cycler (Applied Biosystem Inc, USA). PCR reactions were performed for 35 cycles and each cycle standardized for this study were comprised of denaturation at 94ºC for 45 seconds, annealing temperature at 37ºC for 1 minute and extension at 72ºC for 1 minute with initial denaturation at 95ºC for 5 minutes and final extension at 72ºC for 10 minutes followed by hold temperature at 4ºC. PCR products were electrophoreses at 25 V (4.2 V/cm) on 1.5% (w/v) agarose gel (1X TAE buffer) for 1.5 h. The gels were stained with ethidium bromide (10mg/ml solution) for 45 minutes, visualization and photographed using UV transilluminator (UVITEC, Japan).

Table 2. RAPD primers amplified for different *Asparagus* species and its cultivars.

Primers name	Sequence (5'–3')	GC %	Tm (°C)
OPC-09	CTCACCGTCC	70	28 °C – 37 °C
OPC-10	TGTCTGGGTG	60	28 °C – 37 °C
OPC-13	AAGCCTCGTC	60	28 °C – 37 °C
OPC-20	ACTTCGCCAC	60	28 °C – 37 °C
OPD-07	TTGGCACGGG	70	28 °C – 37 °C
OPE-07	AGATGCAGCC	60	28 °C – 37 °C
RPI-2	AACGCGTCGG	70	28 °C – 37 °C
RPI-3	ACGCGACCTG	70	28 °C – 37 °C

Data analysis: Fragments for both seed protein and RAPD bands were scored manually for presence (1) or absence (0) of bands, and a binary data matrix was generated and similarity index was calculated for all possible pairs of bands. The % polymorphic loci and heterozygosity at each locus were calculated. Nei and Li genetic similarity coefficient was used to estimate the genetic similarity and genetic distances. Genetic distances were calculated between pairs of populations (D) and UPGMA cluster analysis of distance values was develop using Numerical Taxonomy System for PC (NT-SYS version 2.11) from applied biostatis Inc. (2002) (Nei & Li, 1979).

Results

Seed protein profiling: Seed protein was studied for the first time to investigate the genetic variations among *Asparagus* species using SDS-PAGE analysis. The SDS banding pattern of seed protein revealed a total of 19 bands, of which twelve bands were polymorphic and seven were monomorphic bands. The molecular weight ranges from 10 KDa-72 KDa (Figs. 1 & 2). The protein bands of 46KDa, 20 KDa, 19 KDa and 10 KDa were the most prevalent (92 %) among *Asparagus* species and cultivars, whereas 45 KDa protein was the least present (17 %) (Fig. 3). The genetic similarity coefficient matrix showed a similarity index ranged from 0.61 to 0.97 with an average of 0.55 (Table 3). The highest genetic similarity was observed between cv. Abril and cv. Apollo, with a value of 0.97, whereas the lowest genetic similarity was reported (with a value of 0.61) between *A. capitatus* and *A. densiflorus*. The UPGMA clustering algorithm based on protein data grouped *Asparagus* species and cultivars into 6 clusters (Fig. 4). Cluster I consisted of *A. officinalis* and cv. Gersengum, cv. Taranga and cv. Para selection. Cluster II consisted of *A. officinalis* cv. Appolo, cv. Abril and cv. Huchel. Cluster III consists of *A. capitatus* and *A. gracilis* (synonym of *A. asiaticus*). Cluster IV (*A. adscendens*), Cluster V (*A. racemosus*), Cluster VI (*A. densiflorus*) formed a distinct clade, and which showed these species are distinct from the rest of the species and cultivars.

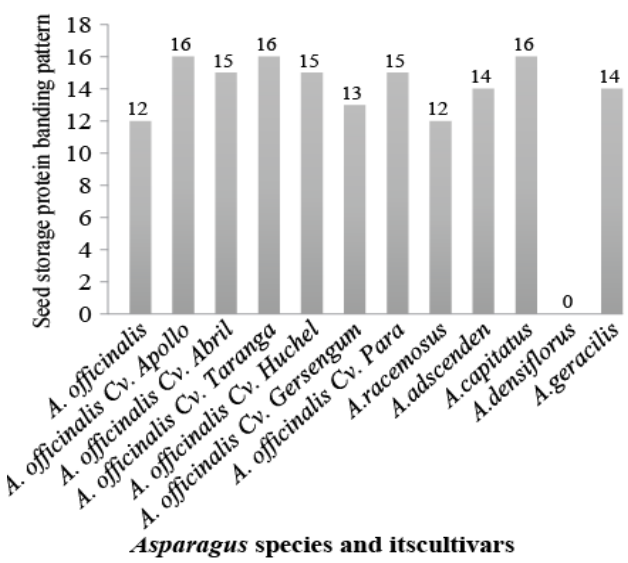


Fig. 1. Protein banding profile of seed storage protein of *Asparagus* species and its cultivars.

RAPD analysis: A total of eight RAPD primers produced 50 fragments, of which 36 (72%) were polymorphic, and the remaining 14 (28%) were monomorphic (Fig. S1). The average number of selected RAPD markers per primer was 6.25 (Table 3). However, 36 polymorphic fragments turned out to be reproducible and regarded as reliable RAPD markers for further analyses. The RAPD markers evaluated in the current study were used to calculate both the similarity value and the marker differences that were used to reconstruct the genetic relationships as shown in Table 4. The similarity matrix obtained from RAPD analysis showed similarity coefficient ranged from 0.66 to 0.93. The highest similarity (0.93) was observed between cultivars Abril and Apollo, followed by 0.92 similarity index between cultivars, cv. Taranga with cv. Abril, and cv. Taranga with cv. Apollo, whereas the lowest similarity (0.66) was observed between cv. Apollo with *Asparagus gracilis*. The UPGMA clustering algorithm based on

RAPD data divided *Asparagus* species into 4 clusters. Cluster I consist of *Asparagus officinalis*, whereas cluster II was comprised of *A. officinalis* cultivars (cv. Abril, cv. Apollo, cv. Gersengum, cv. Huchel, cv. Para and cv. Taranga). Cluster III consists of six *Asparagus* wild species (*A. adscendens, A. racemosus, A. densiflorus, A. setaceus, A. plumosus* and *A. capitatus.* Cluster IV consists of *Asparagus gracilis* (synonym of *A. asiaticus*) which make a distinct clade with the rest of the genus (Fig. 5).

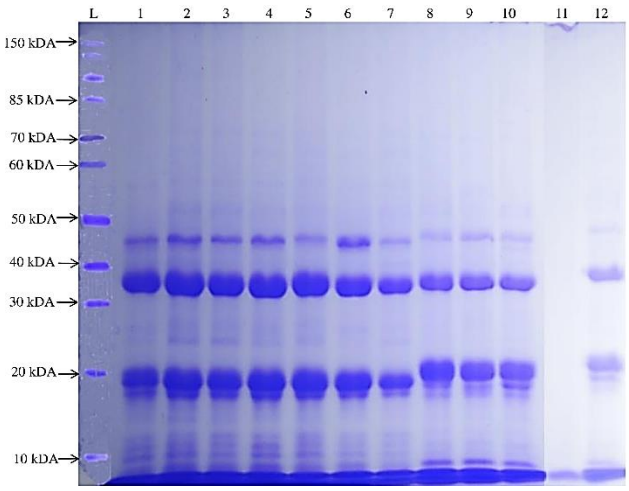


Fig. 2. SDS-PAGE analysis of total seed storage protein of *Asparagus* species and its cultivars.
Lane 1-12 represents (1) *A. officinalis* (2) *A. officinalis* cv. Apollo (3) *A. officinalis* cv. Abril (4) *A. officinalis* cv. Taranga (5) *A. officinalis* cv. Huchel (6) *A. officinalis* cv. Gersengum (7) *A. officinalis* cv. Para (8) *A. racemosus* (9) *A. adscendens* (10) *A. capitatus* (11) *A. densiflorus* (12) *A. gracilis.*

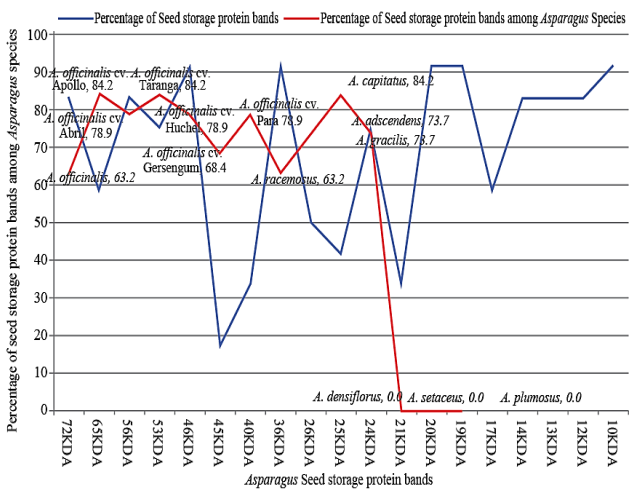


Fig. 3. Protein profiling of seed proteins of *Asparagus* species and cultivars.

Discussion

Seed protein was a method of choice in earlier eras for the investigation of genetic differentiation patterns and to classify plant species, because these proteins are highly preserved (Javid *et al*., 2004). Recently, genetic maps are gaining attention and these can be used in breeding programs to identify loci that are involved in agronomic characters and disease resistance plant. No attempts have been made to evaluate the seed storage proteins for the differentiation of *Asparagus*. In the present study, protein profiling using seed proteins was evaluated for the determination of genetic variations among *Asparagus* species and cultivars. The present result revealed a total of 19 bands with molecular weight ranging from 10 KDa-72 KDa. The highest numbers of bands were observed in *A. capitatus* and *A. officinalis* cultivars (Apollo), whereas the lowest numbers of bands were detected in *A. densiflorus*. The result analyzed in the present study was in agreement with previous studies (Nair *et al*., 2006; Vladova *et al*., 2004), SDS-PAGE for the evaluation of genetic variation and relationship among different species in *Gymnema Sylvester* and *Cucurbitaceae*. Similarly, Berezovskaya *et al*., (2005), studies 95 durum wheat genotypes using gliadin and total reduced protein electrophoresis and showed that some significant correlation between electerophoretic subunits and agronomic traits was existed, whereas significant correlation between gliadin polymorphism and some agronomic traits such as growth type was also noticed. The study of seed protein subunits could be considered as key component for varietal characterization and purity (Branlard *et al*., 2001). The current study revealed that protein profiling of *Asparagus* species and cultivars similarity indices ranging from 0.61 to 0.97. The highest similarity indices were observed between *A. officinalis* cultivars (Abril and Apollo), whereas *A. capitatus* and *A. densiflorus* showed minimum similarity coefficient. This indicated that the protein banding patterns of *A. officinalis* cultivars are more closely related than those of Wild *Asparagus* species. Our result is consistent with Irshad *et al*. (2014) that showed a genetic similarity index ranging from 0.75 to 0.96 and the highest similarity indices were observed between *A. officinalis* cultivars (Abril, Gersengum and Apollo), whereas *A. capitatus* subsp. *gracilis* and cv. Abril showed minimum similarity coefficient. Cluster analysis using neighbor joining method was performed, which grouped *Asparagus* species and cultivars into 6 clusters. Cluster I consist of *A. officinalis* and *A. officinalis* cv. Gersengum, cv. Taranga and cv. Para selection, whereas Cluster II was consists of *A. officinalis* cultivars including cv. Appolo, cv. Abril and cv. Huchel. Cluster III includes *A. capitatus* and *A. gracilis.* Cluster IV *(A. adscendens*), Cluster V *(A. racemosus*), Cluster VI (*A. densiflorus*) formed a distinct clade, which showed the Wild *Asparagus* species have distinct protein banding pattern from *A. officinalis* and its cultivars. Our result is consistent with earlier study of Idrees *et al*., (2021), that showed *A. officinalis* and its cultivars had distinct electrophoretic patterns from wild *Asparagus* species. Masoumi *et al*., (2012) noticed that cluster analysis using seed storage proteins was good enough to cluster the ecotypes into groups. They further concluded that the cophenetic correlation values were too high which was suitable for the clustering of medicinal plant species using seed protein (Williams *et al*., 1990).

The RAPD techniques are quite sensitive because different DNA profiles were generated by each primer for each of the cultivar and species. Using RAPD techniques, unique DNA profiles were obtained in *Asparagus* species

and cultivars. In the present study, a total of 8 different *Asparagus* species and six *A. officinalis* cultivars were used using eight RAPD primers. A total of 50 amplification fragments were scored, among these, 36 (72%) were polymorphic, and 14 (28%) were monomorphic bands, which is closely consistent with our previous studies (Irshad *et al*., 2014). Lal *et al*., (2011), determined five *Asparagus* species using 20 RAPD primers, of which 6 primers were reproducible and polymorphic, and produced a total of 258 polymorphic bands with 43 average bands per primer. The current study showed that the similarity matrix produced from RAPD analysis ranging from 0.66 to 0.93 revealing most of the *Asparagus* species are genetically similar and suggesting the presence of same gene pool. The highest similarities were observed among *Asparagus* cultivars, whereas the most distant *Asparagus* species was *A. gracilis* (synonym of *A. asiaticus*). Neighbor joining method divided *Asparagus* species and its cultivars into 4 main clusters. Cluster I was comprised of only *Asparagus officinalis* species, cluster II was comprised of all *Asparagus officinalis* cultivars. Cluster III was consisting of most of wild *Asparagus* species evaluated in the present study, whereas cluster IV consist of *Asparagus gracilis*, suggesting the presence of genetic changes in their genome which occurred due to the mutational events. Such a high level of polymorphism as compared to previous studies indicated the broadness of genetic base and wild nature of the *Asparagus* species and cultivars. Similarly, Khandka *et al*., (1996), suggested that level of genetic polymorphism between *Asparagus* cultivars obtained in a pairwise marker difference test ranged from 0.08 to 0.17 and relate to domestication among *Asparagus* germplasms. In the present study, the use of both wild species and distantly related cultivars would enrich the breeding material and genetic relationships of *Asparagus*.

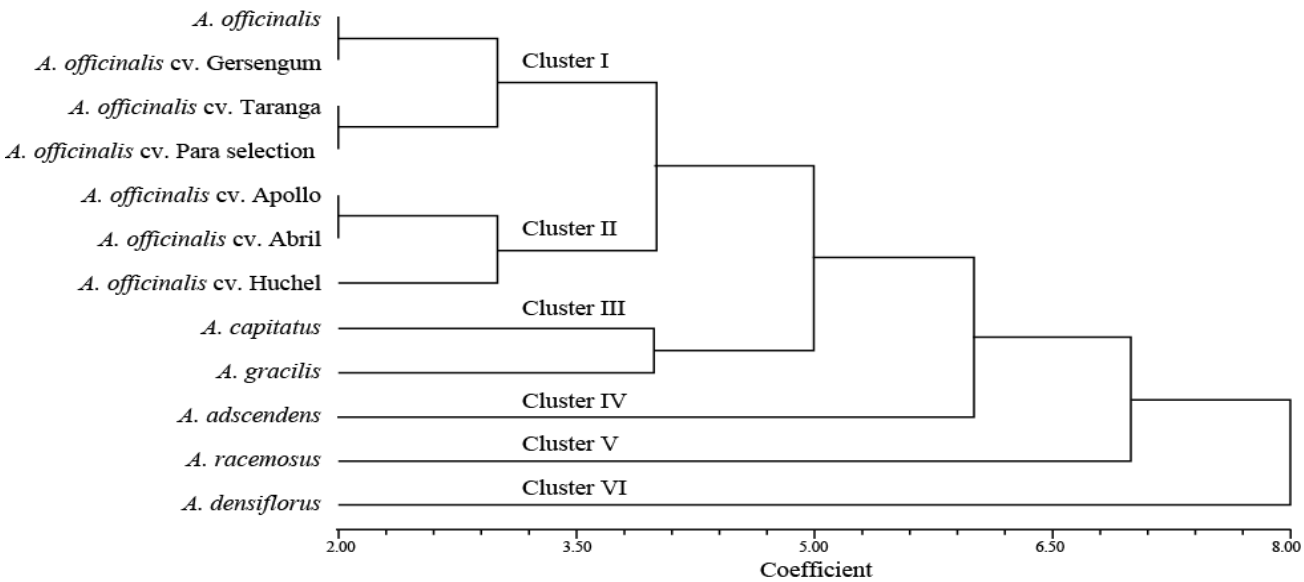


Fig. 4. Neighbor joining method for seed storage protein of *Asparagus* species and its cultivars.

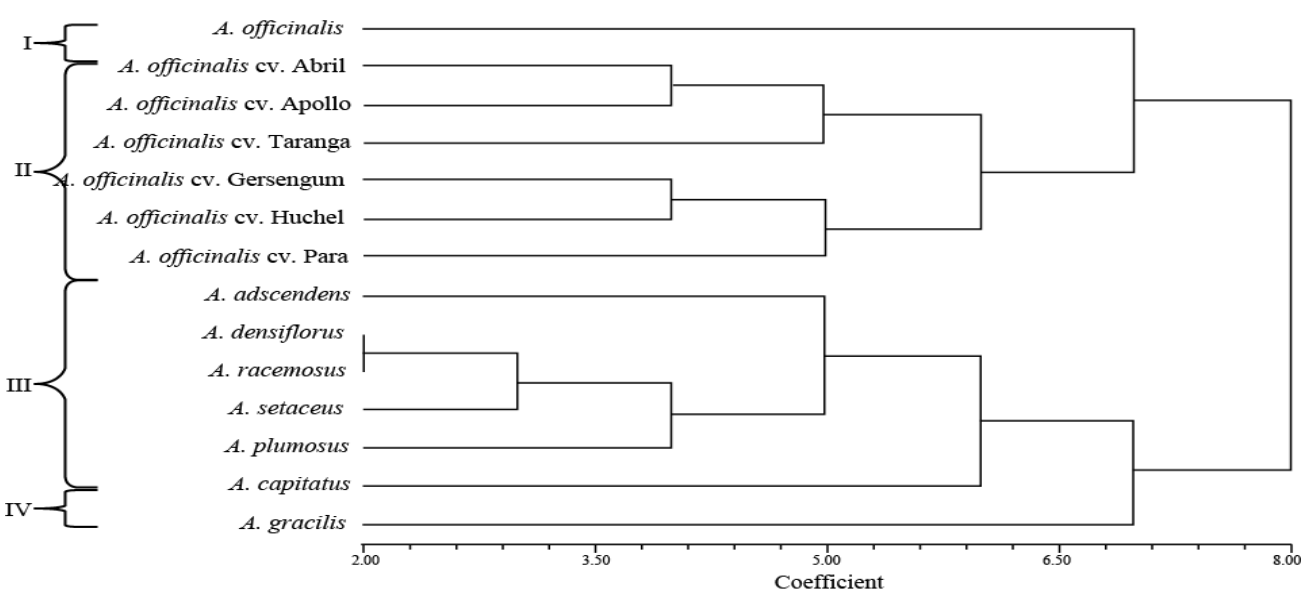
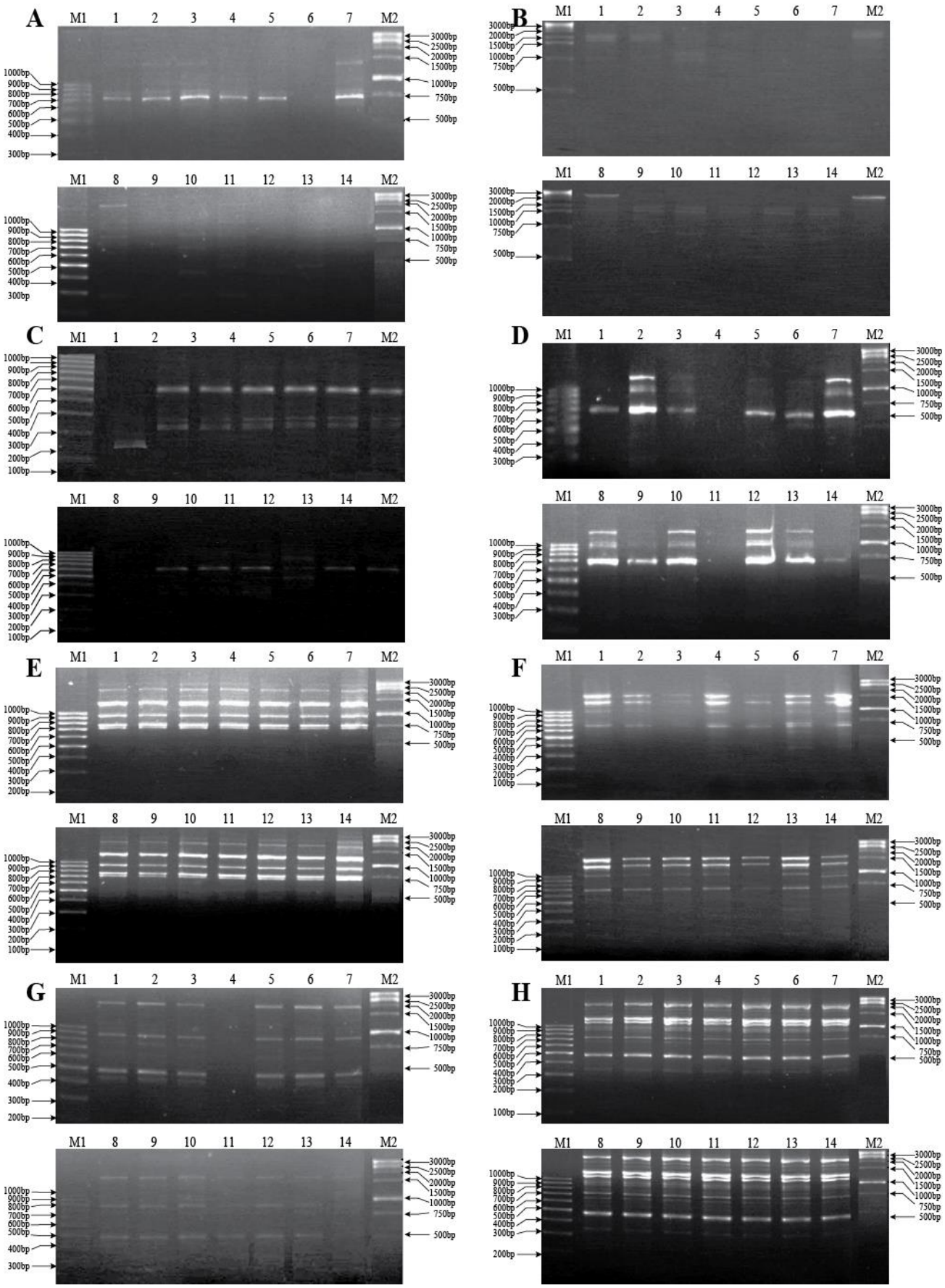


Fig. 5. Neighbor joining method for the clustering of RAPD profiles amplified with RAPD markers evaluated for *Asparagus* species and cultivars.



S1. RAPD pattern generated using primer **A-** OPC-09, **B-** OPC-10, **C-** OPC-13, **D-** OPC-20, **E-** OPD-07, **F-** OPE-07, **G-** RPI-2, **H-** RPI-3. M1 = 100bp marker, M2 = 1kb marker, 1. *A. officinalis*, 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. adsendens*, 9. *A. capitatus*, 10. *A. densiflorus*, 11. *A. plumosus*, 12. *A. racemosus*, 13. *A.setaceus*, 14. *A. gracilis* (synonym of *A. asiaticus*).

Table 3. Polymorphism of seed storage proteins and RAPD markers for *Asparagus* species and cultivars.

	Seed storage protein bands	RAPD Markers							
		OPC-09	OPC-10	OPC-13	OPC-20	OPD-07	OPE-07	RPI- 2	RPI- 3
Total bands	19.0	7.0	5.0	6.0	3.0	7.0	8.0	3.0	11.0
Polymorphic bands	12.0	7.0	5.0	6.0	3.0	3.0	6.0	3.0	3.0
Monomorphic bands	7.0	0.0	0.0	0.0	0.0	4.0	2.0	0.0	8.0
PIC	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Polymorphism	63.2	100.0	100.0	100.0	100.0	42.9	75.0	100.0	27.3

Conclusions

The result of the present study suggested that seed protein and RAPD markers were successfully used to distinguish the *Asparagus* species and its cultivars. It is concluded that seed proteins are also useful for species differentiation, whereas RAPD markers are better to discriminate and identify the broadness of the genetic base in *Asparagus* species and its cultivars. It is further recommended that RAPD markers could be used for the estimation of evolutionary, genetics population and diversity of *Asparagus.*

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