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ASSESSMENT OF MICROSATELLITE MARKERS (SSRs) FOR GENETIC DIVERSITY IN *ASPARAGUS OFFICINALIS* L. AND ALLIED SPECIES

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Abstract

The present study was hypothesized to evaluate a set of SSRs for the assessment of genetic variations in *Asparagus officinalis* L. and their allied species. Nine genic SSR markers were especially developed for *Asparagus* genome and employed for DNA profiling studies of *Asparagus* species. These SSRs markers have revealed the allelic polymorphism ranging from 1.0 to 2.0. Allele frequency was found highest for psbD-trnL (1.0), petB (1.0) and AG7 (1.0), while it was lowest for ZHD1 (0.1). Polymorphism Information content (PIC) was highest for TC7 (0.9) while it was lowest (0.0) for psbD-trnL, petB and AG7 respectively. The genetic similarity coefficients were found to range from 0.42 to 1.0. The UPGMA clustering algorithm based on SSRs data have clustered *Asparagus* species into 4 groups (I, II, III & IV) indicating *Asparagus officinalis* (L.) cultivars and allied species in the first clade, while *Asparagus officinalis* (L.) 'Gersengum', *Asparagus densiflorus* (Kunth) Jessop, and *Asparagus racemosus* willd. were clustered in separate clades respectively. The present study has endorsed the origin status of *Asparagus officinalis* and their allied species. *A. officinalis* cultivars and other allied *Asparagus* species are clustered in separate clades, and it was revealed that they have monophyletic origin. It was established that SSRs markers could be informative markers for the differentiation of *Asparagus officinalis* cultivars, and their allied *Asparagus* species.

Introduction

Asparagus L. (Asparagaceae) contains more than 210 species, dispersed throughout the world from temperate to tropical regions (Kanno and Yokoyama 2011). *Asparagus* is grown particularly for their nutritional, ornamental and medicinal purposes. *Asparagus officinalis* L. (Garden *Asparagus*) is an important vegetable crop grown in a wide diversity of environments, while other species have long been used for medicinal purposes and are included in traditional pharmacopoeia (*Asparagus racemosus* willd., *A. verticillatus* L., *A. adscendens* Roxb.), and many others are considered as ornamental (*A. plumosus* Baker, *A. densiflorus* (Kunth) Jossop, *A. virgatus* Baker) (Kumar *et al*. 2015). Recent phylogenetic studies on the genus *Asparagus* (Kubota *et al*. 2012, Norup *et al*. 2015) have confirmed their monophyletic origin with sexual dimorphism and polyploidy as the main force of evolution (Castro *et al*. 2013). *Asparagus officinalis* L. has been used as a vegetable globally for a long time, making it as an economically valuable plant. It has also been reported that continuous use of *Asparagus officinalis* can stimulate insulin levels in the blood, which is helpful for improving type 2 diabetes and protecting liver cells (Hafizur *et al*. 2012). Furthermore, they also possess anti-inflammatory, antimicrobial, and anti-cancerous

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components (Fuentes-Alventosa *et al.* 2009). These are distributed in two forms including white *Asparagus*, in which the spears are white, and their young stems are harvested from raised beds, and green *Asparagus*, in which the spears are green, and harvested at ground level. These *Asparagus* becomes green when it receives light, while it becomes white when their exposure to light is blocked. This variation is associated with differences in rutin and protodioscin contents of *Asparagus* (Maeda *et al*. 2005). Green *Asparagus* has a higher content of rutin, while white *Asparagus* has a higher content of protodioscin (Lee *et al*. 2010). Recently, there is great attention on the breeding programs of *Asparagus* species to increase the concentrations of valuable phytochemicals. However, the feasibility of genetic improvement must need to be first established, including an investigation into the presence of heritable variability in available populations. Genetic diversity plays a vital role in identifying the scenario for genetic improvement of genotypes, germplasm management and selection of the elite genotypes (Engles *et al.* 2002). Various types of data sets have been used for such purposes, for which DNA markers are more reliable (Ray *et al.* 2010).

DNA based molecular markers are independent of environmental influences (Kalpana *et al.* 2004). Recently, microsatellites or simple sequence repeats (SSRs) are widely used for assessing genetic variations, gene flow characterization and linkage disequilibrium (Koppolu *et al.* 2010). Limited studies are available for the assessment of genetic polymorphism, origin, and evolution of *Asparagus* species available in Pakistan. Thus, the present study was hypothesized to evaluate a set of SSRs markers for the assessment of genetic variations of *Asparagus officinalis* and their allied *Asparagus* species

Materials and Methods

Six *Asparagus officinalis* (L.) cultivars and eight *Asparagus* allied species, which were cultivated in *Asparagus* cultivation and breeding programs of Pakistan, were collected from Agriculture Research Institute, Mingora and other temperate or tropical regions of Pakistan (Table S1).

Table S1. List of *Asparagus* species, locality, and their use

No.	Names	Locality	Use
1	*Asparagus officinalis* (L.)	Punjab, Islamabad	Vegetative
2	*Asparagus officinalis* (L.) ‘Apollo'	KPK*, Swat	"
3	*Asparagus officinalis* (L.) ‘Abril'	KPK*, Swat	"
4	*Asparagus officinalis* (L.) ‘Huchel'	KPK*, Swat	"
5	*Asparagus officinalis* (L.) ‘Gersengum'	KPK*, Swat	"
6	*Asparagus officinalis* (L.) ‘Para'	KPK*, Swat	"
7	*Asparagus officinalis* (L.) ‘Taranga'	KPK*, Swat	"
8	*Asparagus adscendens* Roxb.	KPK*, Kohat	Medicinal
9	*Asparagus capitatus* Baker	KPK*,Swat	"
10	*Asparagus gracilis* Salisb	KPK*, Swat	"
11	*Asparagus racemosus* Willd.	KPK*, Swat	"
12	*Asparagus densiflorus* (Kunth) Jessop	Punjab, Lahore	Ornamental
13	*Asparagus setaceus* (Kunth) Jessop	Punjab, Lahore	"
14	*Asparagus plumosus* Baker	KPK*, Swat	"

*KPK: Khyber PakhtunKhwa.

Healthy fresh green leaves were collected, placed in a labeled sterile plastic cover, and stored in silica bags for further. DNA extraction method was employed to extract the genomic DNA from fresh green leaves of *Asparagus* as described by Doyle and Doyle (1987) with slight modifications. Around 20 mg of the leaf material was well homogenized in 400 µL of extraction buffer containing 100 mM tris HCL, 25mM EDTA, 1.5 M NaCl, 2.5% CTAB, 1% PVP, 20 ml β-mercapto-ethanol with the help of sterilized pestle and mortar. The mixture was incubated at 65ºC for 30 min in a water bath with intermittent shaking and vortexing. The mixture was then placed at room temperature for 10 minutes. Equal volume of Chloroform: isoamylalcohol (24:1, v/v) was added to the tube and gently mixed. The mixture was centrifuged at 12,000 rpm for 10 minutes at 24 ºC. The aqueous phase was pipetted out in another centrifuge tube. Then equal volume of ice-cold isopropanol was added with addition of 5 M NaCl. The DNA pellet was washed with wash buffer (Tris HCl 5mM, Nacl 25mM and Etnanol 25%) and then with 70 % ethanol, air dried and finally resuspended in TE buffer (pH 8). DNA quality was visually evaluated on 0.8% of agarose gel electrophoresis and stored at -20 ^{o}C for PCR amplification.

SSR markers especially developed for *Asparagus* genome (Idrees *et al.* 2019, Table S2) were amplified for different *Asparagus* species in a 25 µL volume reaction mixture, containing 2.5 µL of 10 X EasyTaq buffer with $MgCl_2$, 10 mM of 0.5 µL of dNTP Mix, 1 µL of each forward and reverse primer (10 mmol/L), 1 U/µL of EasyTaq DNA polymerase and 2 µl of 50 ng of template DNA. PCR amplification reactions were performed in a 96 well thermal cycler (Applied Biosystem Inc. USA) using touchdown PCR protocol consisting of an initial step of denaturation at 95^{0}C for 5 minutes. This was continued for 35 cycles of denaturation at 94 ^{o}C for 1 minute, primer annealing at 50 to 60 ^{o}C for 1.5 minutes, extension at 72 ^{o}C for 7 minutes and final extension at 72 ^{o}C for 7 minutes, with a hold temperature of 4^{0}C. PCR products were electrophoretically resolved on 2 % agarose gels in 1X TBE buffer at 125 V for 90 min. Gels were stained with ethidium bromide and photographed under UV transilluminator. The sizes of the amplified products were determined by comparing with a 1 Kb DNA ladder.

Table S2. Lists of 9 SSR markers and its forward and reverse sequences.

No.	Primers	Sequences (Forward)	Sequences (Reverse)
1	psbD-trnL	CGTCCAATGCCCTTTACAAT	AATTTAGGGGCAGGGAAAAA
2	PetB	AAGAGGCCTGTAACGAGCAA	CACAAATACTGATTTCACCGGATA
3	ZHD1	GGAAGAGGGTGCGTGTTTTA	AAACGAACCAAAGTGCCATC
4	AG7	TTTTGCTCCGATCATTTTCA	CCTCTTCGTCTTCATCAGCC
5	ITS1&2	CCGTGAACCATCGAGTCTTT	CAGCGTCTTTTGTCTGTCCA
6	TC7	CGCCCCGAATCAACTAATAA	TACTGCGGAGGTATGTGGGT
7	rps16-trnK	TTCCTTGAAAAAGGTGCTCAA	GGTGGATTCCCACAACAAGA
8	AoAS1	CTCATGCCCACTCCGATATT	TCAGCCTCCACGAACTCTCT
9	trnQ-rps16	ATGATTCACCATCCCGAAAA	TGAATAGTCATTGGATCAACGGTA

Analysis of electrophoresis patterns were scored for the presence and absence of bands. Numerical Taxonomy System (NT-SYS), version 2.11 from applied biostatistics Inc. (2002), was used to analyze the result obtained after bands scoring. Nei and Li genetic similarity coefficient was used to estimate the genetic similarity and genetic distances (Nei and Li 1979). Dendrogram for SSR fragments were generated using an Un-Weighted Pair Group Method Arithmetic Averages (UPGMA).

Results and Discussion

DNA profiling of *Asparagus* species using nine genic SSR markers were performed which showed consistent reproducibility in their amplification (Table 1). These SSRs markers were found to reveal the allele polymorphism ranging from 1.0 to 2.0 for different *Asparagus* species. Majority of SSRs markers including psbD-trnL, petB, ZHD1, AG7, rps16-trnK and trnQ-rps16 have showed single allele for all *Asparagus* species, except TC7 and AoAS1, which have not showed any allele across all *Asparagus* species. Similarly, ITS1 and ITS2 have shown 2 alleles for all *Asparagus* species, except *Asparagus densiflorus* (Kunth) Jessop, in which only one allele was appeared (Table 1). Ginwal *et al.* (2011) has evaluated the cross-species amplification of microsatellite loci for different accessions of *Asparagus racemosus.* They have screened 18 cpSSR markers for amplification, of which 5 cpSSR markers around 27.77 % (AC03; AC-05; AC-09; AC-13 and AC-17) have revealed good cross-species amplification across 20 different individuals of *A. racemosus.* In the present study, Polymorphic Information Content, allele size range, and allele frequency for the SSRs alleles of *Asparagus officinalis*, cultivars and allied *Asparagus* species were also noticed. Size range (bp) for SSR alleles was found to range from 183 bp (petB) to 1000 bp (ITS1 and ITS2). Allele frequency was ranged from 0.1 (ZHD1-210) to 1.0 (psbD-trnL, petB and AG7). Polymorphism Information content (PIC) was highest for TC7 (0.9), while it was lowest (0.0) for psbD-trnL, petB and AG7 respectively. Most of the SSRs markers are monomorphic except ZHD1, TC7 and ITS1 and ITS2 (Table 2). Lal *et al.* (2010) have assessed the genetic diversity among five economically important *Asparagus* species using RAPDs markers. They noticed that the RAPD primers have yielded the amplification of 273 bands, among which 258 were polymorphic (94.50%) while only 15 were monomorphic (5.50%). The have noticed the high level of genetic diversity and low level of genetic similarity among five economically important *Asparagus* species. They revealed that high level of polymorphism could reflect the out-crossing nature of *Asparagus* species. These investigations provide an important baseline data for conservation and inter breeding programs. Similarly, Sica *et al.* (2005), have evaluated the ISSRs for the estimation of study genetic diversity in *A. acutifolius* italian populations. They have utilized twenty-three primers, which produced a total of 228 polymorphic fragments. Their diversity indices including FST (0.4561) and Theta B (0.4776) values have indicated a wide genetic variation among the *A. acutifolius* genotypes. UPGMA tree have grouped all these genotypes according to their geographical origin, revealing the level of genetic structure and distinct nature.

In the present study, the genetic similarity coefficients were also estimated that ranged from 0.42 to 1.0, revealing a high level of genetic variations for *Asparagus officinalis*, cultivars, and allied *Asparagus* species. This finding is in close agreement with the findings of Caruso *et al.* (2007). They have reported the methodology for generating Simple sequence repeat (SSR) markers from expressed sequence tag (EST) sequences. They have also applied these EST–SSRs for the level of estimation of genetic diversity among thirty-five *Asparagus* cultivars. Eight EST–SSR loci were found to be efficient for differentiating all the analyzed cultivars. Moreover, UPGMA (unweighted pair group method with arithmetic mean) and neighbor-joining trees, as well as principal components analysis have separated all these cultivars into clusters corresponding to their geographical origins. Similarly, Singh *et al.* (2013) have performed the molecular diversity analysis of 28 genotypes of 4 species of the genus Asparagus, namely: *Asparagus racemosus, Asparagus falcatus, Asparagus officinalis and Asparagus plumosus* using random amplified polymorphic DNA (RAPD). Around 25 RAPD primers have yielded a total of 296 RAPD fragments, of which 287 bands (96.95%) were found to be polymorphic in the four species. Average polymorphic information content (PIC) value was 0.23. Fourteen species-specific bands were found for A. racemosus species. These RAPDs markers have yielded high

Table 1. Allele profiling of SSRs markers for *Asparagus officinalis*, cultivars and allied *Asparagus* species.

	AO	*AO* 'Abril'	*AO* 'Apollo'	*AO* 'Gersengum'	*AO* 'Huchel'	*AO* 'Para selection'	*AO* 'Taranga'	*AA*	*AC*	*AD*	*AP*	*AR*	*AS*	*AG*
psbD-trnL	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
petB	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
ZHD1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AG7	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
TC7.	1.00	0.00	0.00	1.00	0.00	1.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
ITS1&2	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	1.00	2.00	2.00	2.00	2.00
AoAS1	0.00	0.00	1.00	0.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00	1.00
rps16-trnK	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00
trnQ-rps16	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Diversity Indices (DI)	2.20	2.08	2.20	2.08	2.08	2.30	2.20	2.30	2.30	2.08	2.30	2.20	2.30	2.30

AO= *Asparagus officinalis* (L.), *AA* = *Asparagus adscenden* Roxb., *AC* = *Asparagus capitatus* Baker, *AD* = *Asparagus densiflorus* (Kunth) Jessop
AP = *Asparagus plumosus* Baker, *AR* = *Asparagus racemosus* willd. *AS* = *Asparagus setaceus* (Kunth) Jessop, *AG* = *Asparagus gracilus* Baker.

level of genetic variations for these *Asparagus* species. Genetic diversity among *Asparagus* species was also studied by other investigators including Lal *et al.* (2011), who had identified the similarity index ranging from 0.22 to 0.48 with a mean similarity of 0.36 indicating reasonable variability as obtained by RAPD markers. They have detected the high level of genetic diversity and low level of genetic similarity among five economically important *Asparagus* species. The polymorphism observed in present study, using SSR markers for *Asparagus* species and its cultivars were effective to determine genetic variation at species level.

Table 2. Polymorphic characteristics, allele size range and allele frequency for alleles of SSRs markers revealed for *Asparagus officinalis*, cultivars, and allied *Asparagus* species.

	Size range (bp)	Allele Frequency	PIC *	Polymorphism	Monomorphism	Diversity Indices (DI)
psbD-trnL.	227	1.0	0.0	0.0	100.0	2.639
petB	183	1.0	0.0	0.0	100.0	2.639
ZHD1	200	0.9	0.1	92.9	0.0	2.565
	210	0.1	1.0	7.1	0.0	0
AG7	1000	1.0	0.0	0.0	100.0	2.639
TC7	220	0.4	0.9	35.7	0.0	1.609
	230	0.4	0.9	35.7	0.0	1.609
ITS1&2	1000	0.9	0.1	92.9	0.0	2.565
	500	1.0	0.0	0.0	100.0	2.639
AoAS1	220	0.6	0.6	0.0	100.0	2.197
rps16-trnK	200	0.9	0.1	0.0	100.0	2.565
trnQ-rps16	197	0.9	0.1	0.0	100.0	2.565

*Polymorphism Information content (PIC)

Cluster analysis using Nei and Li's coefficients has clustered *Asparagus officinalis* (L.), cultivars and allied *Asparagus* species into 4 groups (I, II, III & IV) as shown in Fig. 1. Group 1 was further clustered into 2 subgroups including subgroup 'a', which was comprised of *Asparagus officinalis* (L.) that formed a clade with *Asparagus officinalis* (L.) 'Abril' and *Asparagus officinalis* (L.) 'Huchel'. Subgroup 'b' was further clustered into two clusters including cluster 'i' that was comprised of *Asparagus officinalis* (L.) 'Apollo', *Asparagus officinalis* (L.) 'Taranga', *Asparagus officinalis* (L.) 'Para selection', *Asparagus adscenden* Roxb., and *Asparagus capitatus* Baker. Similarly, Subgroup 'b' was clustered into another cluster "ii" that was comprised of *Asparagus plumosus* Baker, *Asparagus setaceus* (Kunth) Jessop, and *Asparagus gracilus* Salisb. Similarly, *Asparagus officinalis* (L.) 'Gersengum' was clustered into group II, *Asparagus densiflorus* (Kunth) Jessop was clustered to group III while *Asparagus racemosus* willd. was clustered into group IV. It was revealed that *Asparagus* species of group II, III and IV were variable from *Asparagus officinalis* (L.) species of group I, suggesting that these were the most primitive species of genus *Asparagus.*

The results of the present study are in agreement with the results of Altıntaş *et al.* (2019), which have revealed similar pattern of genetic diversity for *Asparagus* species. They have evaluated *Asparagus* species using rDNA ITS, cpDNA trnL Intron sequences. Phylogenetic analysis based on ITS data have revealed *Asparagus* species into two clades: the first clade consisted of *Asparagus persicus* and *Asparagus officinalis,* and clade II consisted of *Asparagus palaestinus.* Similarly, Castro *et al.* (2012) have also assessed the genetic diversity and phylogenetic relationships of *Asparagus* species and allied *Asparagus officinalis.* Phylogenetic

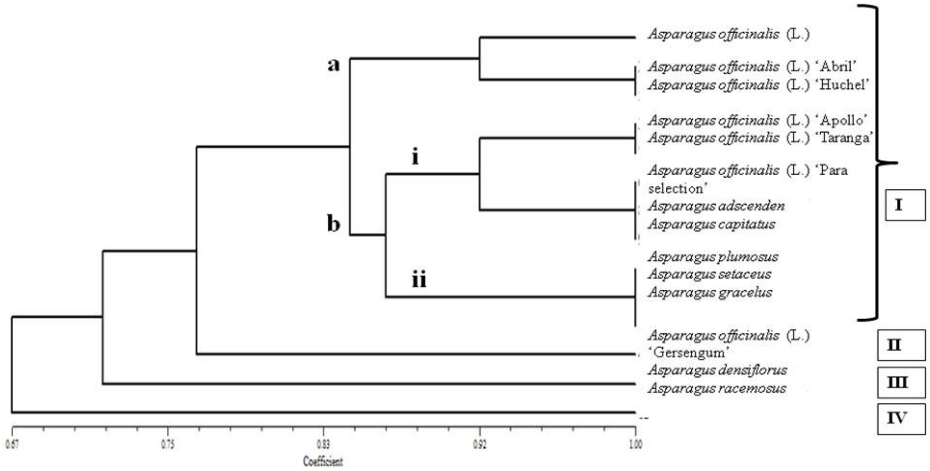

Fig. 1. Cluster analysis of *Asparagus officinalis*, cultivars and allied *Asparagus* species using Nei and Li's coefficients.

analysis using ITS data have clustered *Asparagus* species into two major clades including clade I, which was consisted of *A. acutifolius* while clade II (referred to in this study as the 'officinalis group') was comprised of sequences derived from species closely related to *A. officinalis.* It was further revealed that group of species classified in the 'officinalis group' are the primary gene pool, indicating that these species can be used to increase the genetic diversity of the cultivated *A. officinalis* genotypes. The present study has endorsed the origin status of *Asparagus officinalis* and their allied species. *A. officinalis* cultivars and other allied *Asparagus* species are clustered in separate clades, and it was revealed that they have monophyletic origin. It was established that these SSRs markers could be informative markers for the differentiation of *Asparagus officinalis* cultivars, and allied *Asparagus* species, and these markers can be used for association mapping and for phylogenetic studies.

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