

Original Research

Genetic Diversity among Some *Asparagus* Species using rDNA ITS, cpDNA trnL Intron Sequence and Screening for Antioxidant Activity

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Abstract

Three species of genus *Asparagus* native to Lake Van Basin of Turkey were analyzed using internal transcribed spacer (ITS) and cpDNA trnL intron sequence. Furthermore, these species were screened for their antioxidant activity and total phenolic and flavonoid contents. Phylogenetic analysis based on ITS data revealed two clades: the first clade consisted of *Asparagus persicus* and *Asparagus officinalis*, and clade II consisted of *Asparagus palaestinus*. Similarly, phylogenetic analysis using trnL intron sequence revealed two clades. In term of total phenolic content and total flavonoid content, the highest value were measured for acetone extract of *Asparagus persicus* sample No. 2 and *Asparagus persicus* sample No. 6, respectively. The highest antioxidant activity using FRAP assay was found for *Asparagus persicus* sample No. 3. We concluded that these species could be useful for increasing genetic diversity among cultivated species and for breeding.

Keywords: antioxidant, *asparagus*, ITS, molecular systematic

Introduction

The genus *Asparagus* of the Asparagaceae family (formerly in Lilliceae) consist of three subgenera named *Asparagus*, *Protasparagus*, and *Myrsiphyllum* [1-2] and including around 100-300 species [3]. The

species of subgenera *Protasparagus* and *Myrsiphyllum* comprise only hermaphroditic plants and mainly originated from Africa, while the species of *Asparagus* subgenera are dioecious and originated from minor Asia and Europe [4]. Although morphological traits are useful in identifying genotypes, the results are not exactly confidential because morphological traits can be influenced by environmental and growing conditions [5]. The restrictions of phenotype-based genetic markers gave rise to the progress of more general and

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useful direct DNA-based markers known as molecular markers. Given that molecular markers are steady and easy to detect in all tissues without considering growth, differentiation, development, and defense situation, they have many advantages over conventional phenotype-based approaches [6]. An assessment of genetic variability with determination of significant agronomical characteristics could bring new insight into developing new and superior asparagus genotypes.

The ill-defined taxa has paved the way for a different classification of *Asparagus* species, therefore molecular studies have been carried out to provide additional information on the phylogenetic relationship among *Asparagus* species [7]. A molecular approach using rDNA internal transcribed spacer (ITS) has been widely used for revealing phylogenetic variability among the close species of angiosperms. The ITS region has become an important genetic tool for molecular systematic studies of flowering plants [8]. In addition, The *trnL* group I intron and the intergenic spacer between *trnL* and *trnF* are widely used in plant systematics [9]. On the other hand, only a few molecular studies on the genus *Asparagus* was reported to have been carried out based on cpDNA and the rDNA ITS region [10].

Reactive oxygen species (ROS) is a common term used for a group of oxidants that are both free radicals and molecular species capable of producing free radicals [11]. Generally, reactive oxygen species have various detrimental effects on the cell, like damage of DNA, oxidations of poly-desaturated fatty acids in lipids, oxidations of amino acids in proteins, and oxidatively inactivate specific enzymes by oxidation of co-factors [12-13]. Antioxidants are thought to protect cells against specific diseases by prohibiting the harmful effects of the free radical-mediated process [10-14].

The consumption of asparagus has increased due to its being a good source of bioactive compounds [15-17]. The bioactive compounds such as flavonoid, lignin, and steroidal saponin have been found in asparagus [18-19]. According to [20], saponins, flavonoids, and hydroxycinnamates present in asparagus spears are the major bio-compounds responsible for various biological effects such as antioxidant activity and anti-tumor activity. Although there are a number of studies investigating antioxidant activity of *Asparagus officinalis* in literature, the number of studies related to *Asparagus persicus* and *Asparagus palaestinus* has remained limited. So, the objective of our current study is to determine genetic variability among some *Asparagus* species (*Asparagus officinalis*, *Asparagus persicus*, and *Asparagus palaestinus*) by using the rDNA ITS and cpDNA *trnL* intron sequence and screening of their secondary metabolite contents using different solvents.

Materials and Methods

Plant Materials

We analyzed a total of 8 samples from *Asparagus*, including 3 species (*A.persicus*, *A.palaestinus*, and *A. officinalis*). Samples were collected from different regions of Lake Van Basin in Turkey.

Genomic DNA isolation, Amplification of the rDNA ITS and cpDNA *trnL*-F Region and Sequencing

DNA was extracted from young cladodes using the cetyl trimethyl ammonium bromide (CTAB) method [21] with some modification [22]. In order to determine DNA concentrations, each sample was run on 1.0 % (w/v) agarose gel prepared by 1X-TBE (Tris base, boric acid, and 0.5 M EDTA, pH 8.0). The gel was stained with EtBr and visualized under UV illumination (Syngene Co, Cambridge, United kingdom).

For amplification of the region comprising two ITSs (ITS-4, ITS-5) and 5.8S subunit of rDNA, PCR reaction was carried out with a 50 μ L total volume containing of 0.7 μ L units Taq polymerase, 5 μ L Taq buffer, 1 μ L dNTP (10 mM), 4 μ L of template DNA, and 2 μ L of primer (50 pmol/ μ). rDNA ITS regions were amplified using specific primers [23]. Amplification was performed in a BIO-RAD (C1000 Touch) programmable thermal cycler as follows: initial denaturation at 95°C for 10 min, followed by 35 cycles at 94°C for 1 min, annealing at 51°C for 1 min, extension at 72°C for 2 min, final extension at 72°C for 10 min. PCR products were separated by gel electrophoresis on 1.5% agarose gels containing ethidium bromide, and photographed under UV light in a gel doc system. PCR products of ITS region were outsourced (Iontek Co, İstanbul, Turkey).

For amplification of the cpDNA *trnL*-F region, PCR reaction was carried out with a 50 μ L total volume containing 0.7 μ L units Taq polymerase, 5 μ L Taq buffer, 1 μ L dNTP (10 mM), 4 μ L of template DNA, and 2 μ L of each primer (50 pmol/ μ). The cpDNA *trnL*-F intergenic spacer were amplified using specific primers [24]. Amplification was performed in a programmable thermal cycler as follows: initial denaturation at 95°C for 7 min, followed by 35 cycles at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 2 min, final extension at 72°C for 10 min. The PCR products were separated by gel electrophoresis on 1.5% agarose gels containing ethidium bromide and photographed under UV light in a gel doc system. CpDNA *trnL*-F intron PCR amplicon was outsourced (Iontek Co, İstanbul, Turkey).

Sample Preparation, Total Phenolic and Flavonoid Contents, and Ferric-Reducing Antioxidant Power (FRAP) Assay

The cladodes were dried and cut into smaller pieces with the help of a laboratory blender. Then 2 g

of dried material was transferred into the falcon tubes and three different solvents were used. First, 20 ml acidified acetone (%80 acetone + %19 water + %1 HCl) was added. Then samples were homogenized using a homogenizer (2 min) and sonicator (3 min) and kept in a shaker for 3 h. Samples were centrifuged at 10,000 rpm and supernatants were separated carefully. Then acidified ethanol and pure water were applied to residues and the same acidified acetone procedure was repeated for each solvent. Finally all extracts were collected individually and analyzed. All analyses were carried out in triplicate.

Total phenolic contents were calculated based on procedures described by [25]. An aliquot of the extract solution (1 ml) was mixed with Folin-Ciocalteu reagent (4 ml), and sodium carbonate 6% (4 ml). After incubation at room temperature for one hour the absorbance was measured at 600 nm. A regression curve was generated using gallic acid solutions with different concentrations (0,08-0,2 mg ml⁻¹). The total phenolic content of each sample was calculated according to the formula

$y = 6.660x - 0,021$ and $r^2 = 0.999$, and imported as mg of gallic acid equivalents (GAEs) per g of extract.

Flavonoid content of each extract was measured using the method of [26]. Accordingly, an aliquot of the extract solution (1 ml) was mixed with extract solvent (5 ml) and NaNO₂ (300 µl). After incubation for 6 min. at room temperature, 40 µl AlCl₃·6H₂O (% 10) was added and extract solution was allowed to stand for a further 6 min. and stirred. Finally, 2 ml NaOH (1M) was added and after incubation at room temperature for 15 min. the absorbance was measured at 510 nm. The results were expressed as mg of rutin equivalents (QE) per g of extract ($y = 1.105x + 0.046$; $r^2 = 0.997$).

FRAP assay was performed according to the methods of Benzie and Strain [27]. 100 µL of extracted samples were mixed with 3 mL of the FRAP reagent (*ferric reducing ability of plasma*) (FRAP solution: sodium acetate solution (300 mM, PH 3.6), 10 mM TPTZ (2,4,6- Tris (2-pyridyl)-s- triazin) in solution of 40 mM HCL and 20 mM ferric chloride solution.

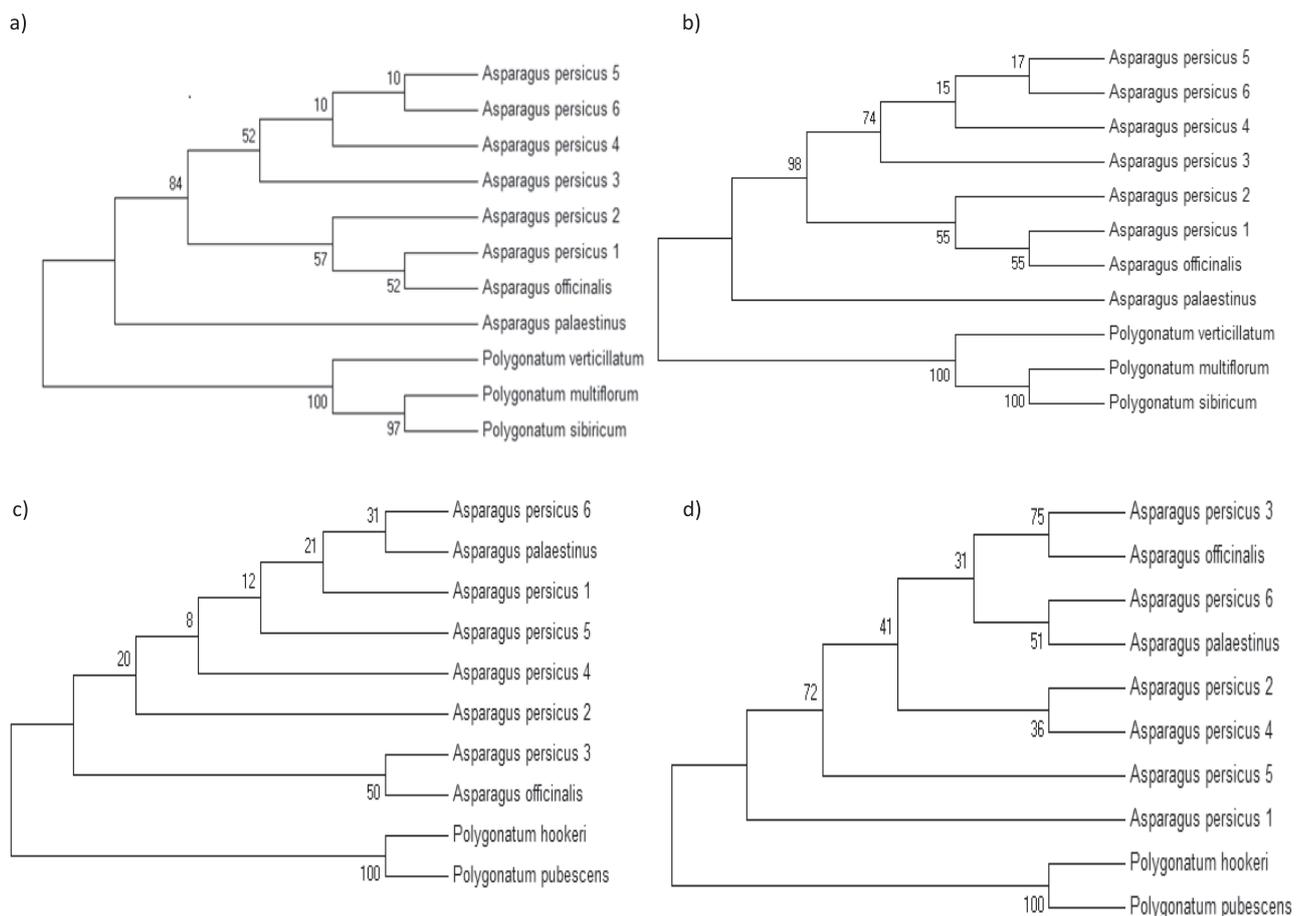


Fig. 1. Genetic relationships between *Asparagus* L. genotypes based on both nrITS4-nrITS5 and Organelle TrnL-f region with diverse phylogenetic analysis methods: a) phylogenetic analysis of asparagus genotypes based on nrITS4-nrITS5 nuclear region with the neighbour-joining method, b) phylogenetic analysis of *Asparagus* genotypes based on nrITS4-nrITS5 nuclear region with the maximum likelihood method, c) phylogenetic analysis of asparagus genotypes based on TrnL-f organelle region with the neighbour-joining method, and d) phylogenetic analysis of *Asparagus* L. genotypes based on TrnL-f organelle region with the maximum likelihood method (numbers on branches show the bootstrap value).

(10:1:1)), measuring the absorbance at 593 nm after incubation at room temperature for 6 min in dark conditions. Blank samples were prepared for acetone, ethanol, and water extracts. Calibration was prepared with ferrous sulfate. The results obtained were presented as $\mu\text{M Fe}^{+2}$ per gram of dried sample using the formula of $y = 0,570x + 0.098$; $r^2 = 0.997$.

Results and Discussion

Phylogenetic Reconstruction Based on Ribosomal DNA ITS and Cpdna TrnI Intron Sequence

Internal transcribed spacer (ITS) regions are used often as molecular markers in plant molecular systematic classification generally [28-29]. ITS has noncoding regions that are characterized by sometimes higher nucleotide substitution rates and have proven useful for inferring phylogenetic relationships at the species and genus levels [24]. Chloroplast DNA (cpDNA) sequence variation is also widely used in evaluating the interspecific relationship between diverse plant families. For investigating molecular classification of the intergenic space, between trnL (UAA) 3'exon and trnF (GAA) gene is much more suitable [24-30]. In this study, a molecular phylogenetic analysis of *Asparagus* L. species that spread in the Van basin was achieved by using nrITS DNA and trnL-F cpDNA sequences. In this study, nrITS sequences ranged from 630 nt to 750 nt among 8 specimens in the study.

trnL-F cpDNA and trnH-psbA sequences are usually used for molecular classification interspecies analyses [31]. In our study, ML and NJ phylogenetic analysis of nrITS4-nrITS5 sequences were shown almost the same profile (Fig. 1a-1b). So, phylogenetic analysis of nrITS4-nrITS5 sequences exhibited compatible results and this genomic region was accepted an an excellent choice for classification of *Asparagus* L. at

the interspecific level because it has a higher number of parsimony-informative sites than trnL-F. ITS sequence based on the NJ and ML tree is comprised of three main clades, while the trnL-F sequence is based on the NJ and ML tree comprised of four main clades (Fig. 1c-d). Bootstrap analysis results also supported the ITS region more efficiently than trnL-F for classifying *Asparagus* L. at an interspecific level. Many phylogenetic and genetic variety studies have been conducted on the ITS region [32-33]. In the current study, ITS and trnL-F analysis indicated some different results of *Asparagus* L. phylogenetic trees. But ITS sequence analysis was found to be more informative based on our analysis. According to studies up to now, ITS repeats in plants, indicating that it contributed to a more informed utilization in phylogenetic analyses and also ITS sequence as a nuclear marker in present plant phylogenetic studies. In many previous studies, it was found that ITS sequence data have and may continue to supply insights into historical introgression, polyploid ancestry, phylogenetic history, genome relationships, and other diverse evolutionary questions [34-35]. However, in a previous study, cpDNA was found to be quite useful and informative for resolving geographically phylogenetic analysis of *Asparagus* L. [3].

Total Phenolics and Flavonoids

Total phenolic and flavonoid contents were calculated based on 1 mg/g concentrations. According to obtained data, TPC (total phenolic content) ranged between 16.890 and 34.768. The highest TPC was found in acetone extract of *A.persicus* sample No. 2, followed by *A. palaestinus*. The other bioactive compound quantified in asparagus samples is flavonoids. Total flavonoid content (TFC) of extracts varied from 0.474 to 0.011. Similar to TPC, the highest value for TFC was quantified for acetone extracts and the lowest value for aqueous extracts.

Table 1. Total phenolics content for different *Asparagus* species.

Species	TP (mg GAE/g DW)		
	Acetone	Ethanol	Aqueous
<i>A. persicus</i> sample #1	26,183±1,36 ^{bc}	22,283±0,65 ^c	9,649±0,85 ^{gh}
<i>A. persicus</i> sample #2	34,768±0,25^a	15,633±1,06 ^{dc}	6,491±0,72 ^{hl}
<i>A. persicus</i> sample #3	22,768±0,03 ^c	11,108±0,32 ^{fg}	3,358±0,69 ^l
<i>A. persicus</i> sample #4	16,890±0,85 ^d	9,574±0,30 ^{gh}	3,458±0,57^l
<i>A. persicus</i> sample #5	25,466±1,09 ^{bc}	12,641±0,97 ^{efg}	6,074±0,49 ^{hi}
<i>A. persicus</i> sample #6	27,583±6,45 ^b	14,999±0,72 ^{def}	7,041±0,14 ^{hi}
<i>A. officinalis</i>	22,508±0,97 ^c	9,866±0,51 ^{gh}	4,158±0,60 ^l
<i>A. palaestinus</i>	27,749±0,42 ^b	9,658±0,16 ^{gh}	4,491±0,71 ^l

TPC – total phenolic content; GAE – gallic acid equivalents; results are expressed as mean ± standart deviation. Levels not connected by the same letters are significantly different.

Table 2. Total flavonoid contents for different *Asparagus* species.

Species	TF (mg RE/g DW)		
	Acetone	Ethanol	Aqueous
<i>A. persicus</i> sample #1	0,460±0,070 ^a	0,234±0,058 ^{cd}	0,088±0,020 ^{efghl}
<i>A. persicus</i> sample #2	0,400±0,041 ^a	0,150±0,064 ^{ef}	0,068±0,025 ^{fghl}
<i>A. persicus</i> sample #3	0,296±0,012 ^{bc}	0,108±0,0 ^{efg}	0,021±0,031 ^{hl}
<i>A. persicus</i> sample #4	0,160±0,005 ^{de}	0,070±0,005 ^{fghl}	0,011±0,002^l
<i>A. persicus</i> sample #5	0,312±0,024 ^{bc}	0,154±0,033 ^{de}	0,056±0,032 ^{ghl}
<i>A. persicus</i> sample #6	0,474±0,020^a	0,140±0,020 ^{ef}	0,038±0,017 ^{ghl}
<i>A. officinalis</i>	0,250±0,013 ^{bc}	0,086±0,012 ^{efghl}	0,021±0,006 ^l
<i>A. palaestinus</i>	0,316±0,023 ^b	0,102±0,005 ^{efgh}	0,010±0,003 ^l

TFC – total flavonoid content; RE – rutin equivalent; results are expressed as mean ± standart deviation. Levels not connected by the same letters are significantly different.

Extraction of phenolic compounds present in the plant material is greatly affected by the type of extraction solvent. Moreover, the polarity of the solvent is of key importance in the solubility of phenolics [19, 36-37]. Among the solvents for extraction, we found that total phenolic content of acetone extracts have higher TPC than those of water and ethanol extracts. As a reason for this situation, it could be considered that acetone has higher polarity than ethanol. Our findings are in agreement with previous studies using acetone, ethanol, and water as solvent [38-39-40], but in congruence with the results reported by Mohammadi and Atik [41]. The differences can be attributed to several factors, such as polarity of the solvents, solvent viscosity, surface tension, and extraction yield.

FRAP

The results (Table 3) showed that FRAP values were higher in acetone extraction than those of ethanol

and aqueous extractions. This indicated that acetone was more efficient in extracting antioxidants in plants compared to ethanol and aqueous. FRAP values differed from 72 to 3 $\mu\text{M g}^{-1}$ sample. Acetone-extracted *A. persicus* sample No. 3 gave the highest FRAP value with 72 $\mu\text{M g}^{-1}$ sample, while the lowest value was found in aqueous extract of *A. persicus* sample No. 3 and *A. palaestinus* (3 $\mu\text{M g}^{-1}$ sample). Some aqueous extracted samples showed negligible activity. There was a significant difference at $p < 0.05$ between the groups. A significant linear relationship between total phenolic content and antioxidant capacity (FRAP) was observed ($r = 0,955$). Similar results have been reported by Ku et al. [42], who compared the effects of different cultivation systems on bioactive content of asparagus. In green asparagus, a strong relationship between ferric-reducing power and total phenolic content indicated that phenolics could be a major factor for both activities [43].

Conclusions

In summary, our results show that the ITS region is more illustrative for a genetic relationship among *Asparagus* species than cpDNA trnL intron sequence. Furthermore, *Asparagus persicus* is a good source of phenolic compounds, together with significant antioxidant capabilities in comparison to *Asparagus palaestinus* and *Asparagus officinalis*. Therefore, this study will be useful for breeding and conserving asparagus germplasm.

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Table 3. FRAP activity of different *Asparagus* species.

Species	FRAP ($\mu\text{M g}^{-1}$ samples (dw))		
	Acetone	Ethanol	Aqueous
<i>A. persicus</i> sample #1	56 ^{ab}	53 ^{bc}	24 ^{ef}
<i>A. persicus</i> sample #2	68 ^a	37 ^{cd}	12 ^{fg}
<i>A. persicus</i> sample #3	72^a	24 ^{ef}	3^g
<i>A. persicus</i> sample #4	50 ^{bc}	23 ^{ef}	4 ^g
<i>A. persicus</i> sample #5	58 ^{ab}	35 ^{de}	14 ^{fg}
<i>A. persicus</i> sample #6	64 ^{ab}	39 ^{cd}	16 ^{fg}
<i>A. officinalis</i>	65 ^{ab}	23 ^{ef}	4 ^g
<i>A. palaestinus</i>	55 ^{ab}	20 ^{ef}	3^g

Results are expressed as mean ± standart deviation. Levels not connected by the same letters are significantly different.

Conflict of Interest

The authors declare no conflict of interest.

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