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Phenotypic and genetic variation among *Astragalus* species from Turkey

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Abstract

Fatty acid (FAs) and RAPD profiles were used to examine phenotypic and genetic relationships among five Astragalus species, namely A. declinatus, A. microcephalus, A. aduncus, A. xerophilus and A. erinoceus, which were grown as wild in Eastern Anatolia region of Turkey. All of five Astragalus species tested in this study were separated based on the presence and composition of 69 different FAs. Four of Astragalus species including A. declinatus, A. xerophilus, A. erinoceus and A. aduncus, were rich in terms of FA contents containing at least 28-55 different FAs. Six of the twelve decamer primers examined were selected to find out genetic polymorphism in Astragalus species. Total 36 polymorphic bands were observed, ranging in size from 300 bp to 2100 bp. The RAPD results suggested that A. microcephalus, A. erinoceus, and A. aduncus are closely related and completely different than the other species. Three genetically distinct groups were found between Astragalus species. The results of the present study suggest that both RAPD and FA analysis are useful methods, which can be used for differentiation of Astragalus species.

Keywords: *Astragalus* species, Genetic and phenotypic diversity, FAMEs, RAPD

Introduction

Astragalus L. is the largest genus of flowering plants, containing up to 2500 -3000 species [1]. This genus is a member of the legume family (Fabaceae), and traditionally classified in the tribe Galageae. The centre of development seems to be in the arid and semi-arid mountainous parts of the Northern Hemisphere [2]. It is most diverse in the Irano-Turkish region of South-western Asia, the Sino-Himalayan Plateau of south Central Asia, the Central Asian region and the Great Basin and Colorado Plateau of western North America [2].

It is also the largest genus in Turkey where it is represented by 400 species in 62 sections [3]. The delimitations of taxa both at the section and species levels posses' considerable taxonomic problems [4]. For this reason, it is necessary to use taxonomic characters other than morphologic to explain the systematic relationships among *Astragalus* species, some of which are so closely related that it is not possible to delimit them.

Recent advances in the field of molecular biology and gene technology are creating exciting possibilities for the rapid and accurate determination of phenotypic and genetic variations between plant species. Several molecular systematic studies, such as RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic

DNA), analysis of different chloroplast and nuclear ribosomal DNA (ITS) have made earlier phylogenetic relationships within *Astragalus* uncertain. [5-16].Additionally Fatty acid methyl esters (FAMEs) analysis have been utilized for determination of phylogenetic relationship within and among plant species in addition to morphological characters since 1990s [17, 14, 18]. There have been a few attempts to study the phenotypic among species of *Astragalus* [15, 16, 18]. However, no study has investigated the phenotypic and genotypic differences in species of *Astragalus*, including those growing in the Eastern Anatolia region of Turkey. The aim of this study is to use FAME and RAPD amplicons to examine the genetic relationship between five *Astragalus* species, *A. declinatus, A. microcephalus, A. aduncus, A. xerophilus* and *A. erinoceus* wildly grown in Eastern Anatolia region of Turkey.

Material and Methods

Sample Collection

Plant samples of five *Astragalus* species in*cluding A. declinatus, A. microcephalus, A. aduncus, A. xerophilus* and *A. erinoceus* were collected in 2004-2005 at flowering stage from different locations in the vicinity of Erzurum, located in the Eastern Anatolia, Turkey (Table 1).Collected plant materials were dried in the shade, and the leaves of plant were separated from the stem, and ground in a grinder with a 2 mm in diameter mesh. The powdered plant material was then used for DNA extraction. The voucher specimen has been deposited at the Herbarium of the Department of Biology, Atatürk University; Erzurum-Turkey (Table 1).

Extraction and Analysis of FAMEs

Preparation and analysis of FAMEs from whole cell fatty acids from plant samples were performed according to the method described in the manufacturing manual (Sherlock Microbial Identification System version 4.5, MIDI ,inc., Newark, DE). Plant leaves were powdered after lyophilization in liquid nitrogen. Approximately 40 mg of powdered leaf material from each samples was added to 1 ml 1.2 M NaOH in 50% aqueous methanol with 5 glass beads (3 mm dia) in a screw cap tube, then incubated at 100 ^{o}C for 30 min in a water bath. After the saponified samples were cooled at room temperature for 25 min, they were acidified and methylated by adding 2 ml 54% 6 N HCl in 46% aqueous methanol and incubated at 80^{o}C for 10 min in a water bath. After rapid cooling, methylated fatty acids were extracted with 1.25 ml 50% methyl-tert butyl ether (MTBE) in hexane. Each sample was mixed for 10 min and the bottom phase was removed with a Pasteur pipette. The top phase was washed with 3 ml 0.3 M NaOH. After mixing for 5 min then the top phase was removed for analysis. Following the base wash step, the extract (FAMEs) is cleaned in anhydrous sodium sulfate and then transferred into a GC sample vial for analysis.

FAMEs were separated by gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA) with a fused-silica capillary column (25m by 0.2mm) with cross-linked 5% phenyl methyl silicone. The operating parameters for the study were set and controlled automatically by computer program. The chromatograms with peak retention times and areas were produced on the recording integrator and were electronically transferred to the computer for analysis, storage and report generation. Peak naming and column performance was achieved through the use of Eukary calibration standard mix (Microbial ID 1201-A) containing nC9-nC30 saturated and 2&3 hydroxy fatty acids. Cellular fatty acids were identified on the basis of equivalent chain length data. FAME profiles of each plant species tested was identified by comparing the commercial databases (Eukary) with the MIS software package.

Table 1. Name of plant used

OTUs	Herbarium number of the vouchers	Locality	Status	Altitude (m)
A. declinatus	9780	Pasinler, Erzurum	Ir-Tur	1650
A. erinoceus Fich &Mey	9781	Oltu, Erzurum	Ir-Tur	1850
A.microcephalus Willd.	9783	Pasinler, Erzurum	Ir-Tur	1650
A. xerophilus Ledeb.	9793	Oltu, Erzurum	End -Ir-Tur	1780
A. *aduncus* Willd.	9785	Oltu, Erzurum	Ir-Tur	1650

Table 2. Number of amplification products generated with 6 arbitrary oligonucleotide primers in *Astragalus*

Primer	Total Number of RAPD products Per primer	Total Number of polymorphic RAPD products	Number of polymorphic amplification products					Sequence of primer
			A. declinatus	*A. microcephalus*	*A. aduncus*	*A. xerophilus*	*A. erinoceus*	
OPA011	5	5	0	3	1	0	3	5’-CAATCGCCGT-3’
OPC05	6	6	0	3	2	0	4	5’-GATGACCGCC-3’
OPC015	5	5	0	3	2	1	3	5’-GACGGATCAG-3’
OPD03	9	9	1	4	2	0	4	5’-GTCGCCGTCA-3’
OPD07	4	4	0	2	0	0	2	5’-TTGGCACGGG-3’
OPD08	7	7	1	4	2	0	5	5’-GTGTGCCCCA-3’
% polymorphism	36	36	5..55	52.77	25	2.77	58.33	

DNA extraction

Genomic DNA was extracted from powdered plant materials using a modified method described by [19].

Approximately 10-15 mg tissue samples from each plant species were snap frozen in liquid nitrogen in 2 ml Eppendorf tubes. 1000 µl DNA extraction buffer [100mM Tris-HCl (pH 8.0); 50mM EDTA (pH 8.0); 500mM NaCl; 2% SDS (w/v); 2% 2-mercaptoethanol (v/v); 1% PVP (w/v)] was added and mixed well. The tube was placed in a water bath at 65^{o}C and was incubated for 40 min and shaken briefly at 5 minute intervals. The sample was centrifuged at 12 000xg for 15 min at 4^{o}C. The supernatant was transferred and mixed with 1/10 volume 10% CTAB-0.7M NaCl in a new tube. After centrifugation, the supernatant was added with equal volume of chloroform:isoamylalchol (24:1). The DNA was precipitated by the addition of 0.6 volume of freezer-cold isopropanol, left at -20^{0}C for 10min. The DNA was pelleted by centrifugation (12 000xg, 10min) and the isopropanol was poured off; the sample was allowed to air-dry before being dissolved in 100 µl of TE buffer.

RAPDs

Samples were screened for RAPD variation using standard 10-base primers supplied by operon (Operon Technologies Inc., Alameda, CA, USA). Thirty µl of reaction cocktail was prepared as follows: 10x Buffer 3.0 µl, dNTPs (10mM) 1.2 µl, magnesium chloride (25mM) 1.2 µl, primer (5µM) 2.0 µl, taq polymerase (5unit) 0.4 µl, distilled water 19.2 µl sample DNA 3.0 µl (100ng/ µl). Total 10 RAPD primers were tested in this study.

Six of these primers produced amplicons for all of the species of *Astragalus* tested were selected and used further studies based on the preliminary test results (Table 2).

The thermal cycle was: 2 min at 95°C; 2cycles of 30 sec. at 95°C, 1 minute at 37°C, 2 minute at 72°C; 2 cycles of 30 sec. at 95°C, 1 minute at 35°C, 2 minute at 72°C; 41 cycles of 30 sec at 94°C, 1 minute at 35°C, 2 minute at 72°C; followed by a final 5 minute extension at 72°C then brought down to 4^{o}C.

Electrophoresis

The PCR products (27 µl) were mixed with 6xgel loading buffer (3 µl) and loaded onto an agarose (1.5% w/v) gel electrophoresis in 0.5XTBE (Tris-Borate- EDTA) buffer at 70 V for 150 min. Amplification products separated by gel was stained in ethidium bromide solution (2 µl Etbr/100ml 1xTBE buffer) for 40 min. The amplified DNA product was detected by using the Bio Doc Image Analysis System with Uvisoft analysis package (Cambrige, UK).

Data Analysis

PCR products were scored as presence (1) and absence (0) of band for each of the 6 accessions analyzed. Only reproducible bands were scored. For FAME analysis, fatty acids of each plant species were scored as presence (0.1-100%) and absence (0%). Data were used to calculate a Jaccard (1908) similarity.

All of the experiments in this study are repeated at least twice.

Results and Discussion

The species of *Astragalus* tested in this study were separated based on the presence and composition of 69 FAs found in all the species of *Astragalus* species (Table 3). Four species of *Astragalus* including *A. declinatus,* A. *aduncus*, *A. erinoceus*, and *A. xerophilus* were rich in

terms of FA contents containing at least 28-55 FAs. The relative proportions of fatty acid (16:0) were higher (10.16-29.45 %) in all *Astragalus* species (Table 3). The remaining species has limited number of FAs with unique FAMEs profiles. The presence of FAs 8 Dicarboxylic acid, 10:0, 12:0 iso, 12:0 anteiso, 12:0 2-Me, 12:0 ALDE, 12 Primary Alcohol, 13:0 anteiso, 14:0 3OH, 15:0 iso 3OH, 19:0 cyclo c 11-12, 20:0 iso, 13:1: ω6c, 18:1 cis 7 DMA, 18:3:ω6c, 19:1:ω11c, 19:2:ω6c, 20:1:ω8c, 20:5:ω3c and 18.316 ”D”; 18:1:ω9c; 17.678; 12.553 “B”; 22:2:ω6c were only observed in *A. aduncus*, *A. declinatus*, *A. microcephalus, A. xerophylus*, *A. erinoceus* respectively. Especially, the presence of FAs 18:0 cis 9, 10 epoxy, 22:0, 25 N Alcohol, 17.678 were higher in *A. microcephalus*. Results of the present study confirm the previous findings that FAME profiles can be used as phenotypic fingerprint to determine differences between *Astragallus* species closely related [14, 18].

Table 3. Composition of fatty acids in *Astragalus* species.

Fatty acids	*A. declinatus*	*A. microcephalus*	A. *aduncus*	*A. xerophilus*	*A. erinoceus*
8 Dicarboxylic acid	-	-	0.21	-	-
9 Dicarboxylic acid	1.72	-	2.37	0.74	2.41
10 Dicarboxylic acid	-	-	-	-	-
10:0	-	-	0.14	-	-
10:0 iso	-	-	-	-	-
12:0	1.20	-	1.08	0.92	1.15
12:0 2OH	-	-	-	-	-
12:0 iso	-	-	0.26	-	-
12:0 anteiso	-	-	0.35	-	-
12:0 2-Me	-	-	0.18	-	-
12:0 ALDE	-	-	0.38	-	-
12 Primary Alcohol	-	-	0.31	-	-
13:0 anteiso	-	-	0.33	-	-
14:0	1.67	-	0.93	1.26	1.13
14:0 2OH	-	-	-	-	-
14:0 3OH	-	-	0.26	-	-
15:0	0.69	-	0.57	0.46	-
15:0 iso 3OH	-	-	0.18	-	-
16:0	19.55	10.16	19.57	18.50	29.45
16:0 2OH	0.66	-	0.50	0.61	0.51
17:0	0.55	-	0.22	0.49	0.58
18:0	4.40	-	1.94	1.77	5.04
18:0 cis 9,10 epoxy	-	18.06	0.35	0.24	0.42
18 N Alcohol	3.53	-	-	-	4.72
19:0 cyclo c 11-12	-	-	0.76	-	-
20:0	1.37	-	0.58	1.83	4.22
20:0 iso	-	-	0.09	-	-
20 N Alcohol	0.94	-	0.42	0.92	1.22
21:0 iso	-	-	0.35	0.96	-
22:0	1.89	27.88	0.59	2.11	1.80
22 Primary Alcohol	0.59	-	0.46	1.42	2.48
22:0 2OH	1.41	-	0.48	0.79	1.11
23 Primary Alcohol	-	-	-	0.73	-
23:0	-	-	-	0.48	-
24:0	0.73	-	0.47	1.09	1.03
24:0 3OH	21.71	-	5.54	6.31	4.61
25 N Alcohol	0.93	20.61	0.42	0.81	1.25
28:0	-	-	0.48	0.93	1.04
13:1: ω6c	-	-	0.13	-	-
15:1:ω6c	0.53	-	-	0.38	-
16:1:ω6c	-	-	1.40	0.56	1.19
16:1:ω7c	-	-	0.27	0.32	-
16:1:ω7c Alcohol	0.62	-	1.72	0.76	1.25
16:1 cis 7 DMA (ω9)	1.09	-	1.87	1.28	1.22
16:1:ω9c	0.50	-	0.77	0.43	-
16:2:ω6c	-	-	0.91	-	-
17:1 iso G	2.51	-	3.47	3.26	2.63
17:1:ω8c	-	-	-	0.20	0.46

Table 3. Continue

Fatty acids	*A. declinatus*	*A. microcephalus*	*A. aduncus*	*A. xerophylus*	*A. erinoceus*
18:1:ω5c	0.64	-	1.04	-	-
18:1:ω8c	-	7.81	29.58	30.20	12.78
18:1:ω9c	17.35	-	-	-	-
18:1 cis 7 DMA	-	-	0.47	-	-
18:1:ω9c DMA	-	-	1.19	0.59	-
18:1:ω9t Alcohol	3.00	-	4.02	3.87	4.45
18:2:ω6c	4.92	-	7.06	9.96	4.37
18:3:ω6c	-	-	0.49	-	-
19:1:ω6c	3.97	-	-	-	5.98
19:1:ω8t	-	-	1.85	2.66	-
19:1:ω11c	-	-	0.35	-	-
19:2:ω6c	-	-	0.88	-	-
20:1:ω8c	-	-	0.07	-	-
20:5:ω3c	-	-	0.22	-	-
22:2:ω6c	-	-	-	-	0.76
12.553 “B”	-	-	-	0,09	-
15.176	-	-	0.24	0.65	-
17.678	-	15.48	-	-	-
18.316 ”D”	-	-	0.13	-	-
20.343 “D”	-	-	0.75	0.42	-
25.339	1.33	-	0.58	0.99	0.73

Results of RAPD analysis are summarized in Table 2 and Figs 1-3. The size of the amplicons varied from 300 bp to 2100 bp. (Figs 1-3). *A. xerophilus* compared with the other species gave one DNA banding with all six primers tested. (Figs.1-3 and Table 2). Primers OPDO3 and OPDO7 gave the highest and lowest number of RAPD products, respectively (Figs 2,3). The RAPD results showed the presence of three clusters among *Astragalus* species tested. Each cluster is represented by different *Astragalus* species. Except one cluster, divided into two subclusters is including *A. microcephalus, A. erinoceus,* and *A. aduncus* which were the most genetically related species (Fig 4). The RAPD analysis suggested that there were distinct genetic differences between species. All of five *Astragalus* species were clearly distinguished based on a dendrogram constructed using Jaccard UPGMA.

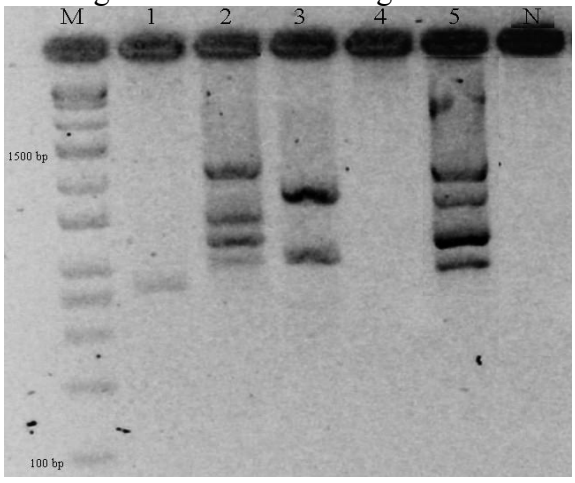

Figure 1. RAPD profiles generated with the primer CO5; Lanes: 1) *A. declinatus*; 2) *A. microcephalus*; 3) *A. aduncus*; 4) *A. xerophilus*; 5) *A. erinoceus*; N; Negative Control M) Molecular Marker (10 kb).

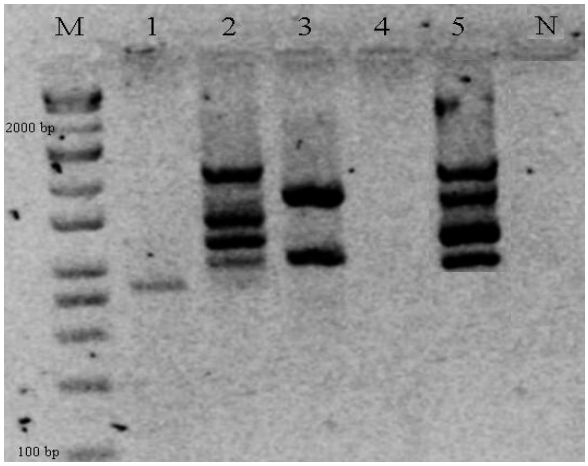

Figure 2. RAPD profiles generated with the primer DO3; Lanes: 1) *A. declinatus*; 2) *A. microcephalus*; 3) *A. aduncus*; 4) *A. xerophilus*; 5) *A. erinoceus*; N; Negative Control M) Molecular Marker (10 kb).

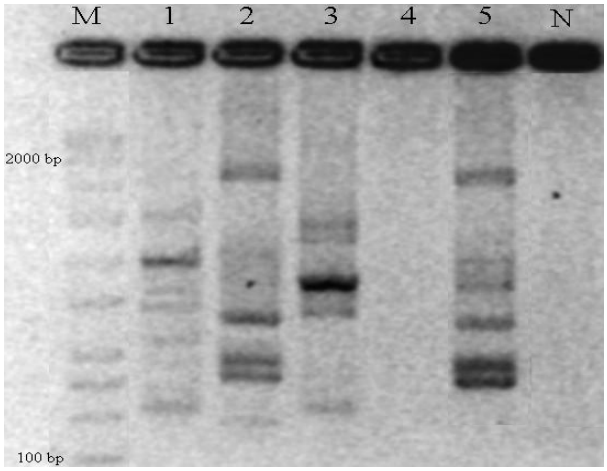

Figure 3. RAPD profiles generated with the primer DO7; Lanes: 1) *A. declinatus*; 2) *A. microcephalus*; 3) *A. aduncus*; 4) *A. xerophilus*; 5) *A. erinoceus*; N; Negative Control M) Molecular Marker (10 kb).

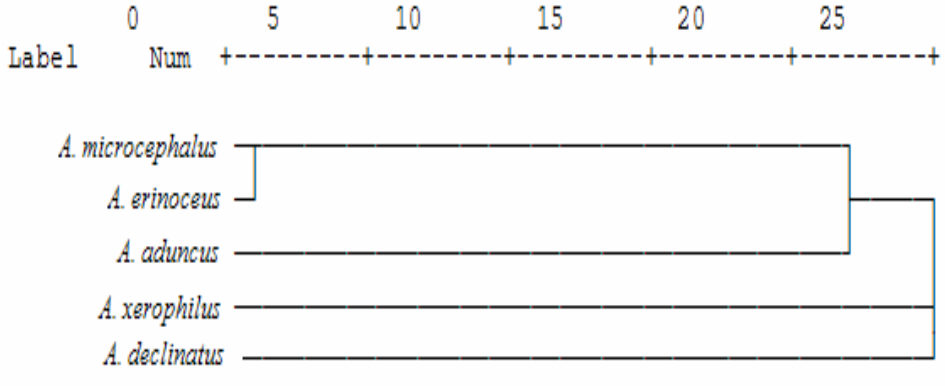

Figure 4. UPGMA dendrogram showing the relationship of *Astragalus*

Both FAME and RAPD profiles have been used to study phenotypic and genetic diversity in many plant species such as accessions of *Triticum*, *Cicer*, *Leucaena*, *Tripsacum, Ixora*, *Hypericum*, *Vicia* and *Astragalus* [20, 17, 14, 21, 22, 10, 18]. RAPD markers have

been used to determine genetic relationship at the species and subspecies level of plants. It is particularly useful for resolving relationship between closely related species and populations of genetically variable species. [23]. RAPD profiles have been also used to assess genetic variation of some *Astragalus* species such as *A. microcephalus, A. membranaceus, A.globosus, A.bicolor, A. mesoleios, A. keyserlingii, A. longistylus* [15, 16, 10], but not the species tested in this study.

In conclusion, the results demonstrated that RAPD and FAME analyses are useful for differentiation and classification of *Astragalus* taxa tested in the present study. Additional phylogenetic studies using chloroplast or mitochondrial gene sequences or appropriate nuclear genes ITS of nr DNA sequences can be helpful to reevaluate the systematic position of Astragalus species. The latter approach may be employed in our future research.

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